The Pathophysiology and Management of Columnar-lined lower oesophagus. An *in vivo* study.

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ABSTRACT

Aim

The aetiology of the lower oesophageal columnar metaplasia (Columnar-lined Lower Oesophagus - CLO) is not clearly understood. The aim of this study was to determine the nature and constituents of the gastrointestinal refluxate that induce CLO. In addition, influence of medical acid suppression and surgical control of reflux in CLO is not completely established. A further aim was to assess the therapeutic role of proton pump inhibition (PPI) and anti-reflux surgery in preventing CLO.

Method

Ten *in vivo* CLO models were established by surgical gastro and oesophago intestinal anastomosis in Sprague-Dawley rats to achieve physiological refluxate of gastric, bile, duodenal secretions; and mixed gastric + bile, gastric + duodenal, gastric + pancreatic, duodenogastropancreatic, duodenopancreatic biliary, jejunogastric and jejunoesophageal reflux. PPI elixir was administered to half of each group during 4 months of post-operative period.

In another study 1 model of CLO were established by surgical anastomosis to achieve jejunoesophageal reflux. Half of the group underwent a second operation at 12 weeks to reverse the reflux.

After 4 months the lower oesophagus was examined with H&E stains for length and intensity of columnar change and severity of inflammation by three experienced

pathologist blinded to the experiment. Sections were further stained with diastase PAS, immunostaining (p53, Ki67, TFF2, LHK) and mRNA *in situ* hybridisation for trefoil peptide phenotype (TFF1, TFF2, TFF3) distribution.

Results

Inflammation and columnar metaplasia of the lower oesophagus was seen in all groups. Length of columnar change in gastric reflux (GR) and duodenal reflux (DR) was significantly longer than all other groups [GR (length in cm \pm SEM) (1.3 \pm 0.2) (all p < 0.05), DR (1.17 \pm 0.1) (p < 0.001)]. The severity of inflammation and metaplastic change was higher in the gastric and biliary dominant groups as compared to the other groups. Although, PPI treatment with omeprazole (OMP) did not cause significant change in CLO there was a trend towards longer CLO length in duodenal dominant reflux (DDR) group and shorter CLO length in gastric dominant reflux group (GDR) and biliary dominant reflux group (BDR) compared to untreated group [Columnar mucosa (length in cm \pm SEM) No OMP GDR (1.17 \pm 0.08), DDR (1.05 \pm 0.05) BDR (1.07 \pm 0.17), OMP GDR (1 \pm 0.13), DDR (1.08 \pm 0.15), BDR (0.83 \pm 0.11) (all p > 0.05) (Mann-Whitney U test)].

There was significant weight gain, normalisation of oesophageal pH and decrease in serum & oesophageal bile acid concentration after antireflux procedure [Weight in grams (mean \pm SEM) Group 1 (Non-reversed) (244.58 \pm 9.76) Group 2 (Reversed) (384.86 \pm 6.35), postop pH Group 1 (7.37 \pm 0.08) Group 2 (8.19 \pm 0.05), serum bile acid (µmol/l) Group 1 (120.33 \pm 20.35) Group 2 (38.8 \pm 7.72), oesophageal bile acid (µmol/l) Group 1 (46 \pm 9.49) Group 2 (25.53 \pm 5.10) (all p < 0.03)]. The length of

columnar mucosa, degree of acute inflammation and degree of metaplasia were significantly reduced by anti-reflux surgery (all p < 0.05). The morphology of specialised columnar epithelium and ulcer associated cell lineage tended to revert to native mature epithelium after anti-reflux surgery. The histological changes in these *in vivo* models were similar to human CLO before and after successful anti-reflux surgery.

Conclusion

- 1. Reflux of pure gastric, pure duodenal, pure biliary secretions, mixed refluxates or 'neutral' pH reflux can all produce columnar metaplasia in oesophagus.
- 2. Reflux of gastric or duodenal contents produced metaplasia of much greater intensity and extent compared to other groups.
- Proton pump inhibition showed a trend towards increased columnar metaplasia in duodenal dominant reflux group, and decrease in gastric reflux group.
- 4. Duodenal reflux may be the cause of ineffectiveness of proton pump inhibition in a significant number of patients with CLO.
- 5. Morphology of experimental CLO and its reversal is similar to human.
- 6. Anti-reflux procedure successfully reverts the mitogenic changes of columnar metaplasia induced by chronic gastroesophageal and gastroduodenoesophageal reflux in an *in vivo* model.
- 7. Morphology of CLO after successful anti-reflux surgery resembles mature gastric and intestinal columnar epithelium.

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intestinal phenotype is expressed in other areas

LIST OF ABBREVIATIONS

- CLO Columnar-lined lower oesophagus
- GORD Gastroesophageal reflux disease
- PPI Proton pump inhibitor
- OMP omeprazole
- SEM Standard error of mean
- JGR Jejunogastric reflux
- JOR Jejunoesophageal reflux
- DPBR duodenopancreaticobiliary reflux
- GR gastric reflux
- DGPR duodenogastropancreatic reflux
- DPR duodenopancreatic reflux
- BR biliary reflux
- GBR gastrobiliary reflux
- GDR gastric dominant reflux
- DDR duodenal dominant reflux
- BDR biliary dominant reflux
- µmol/l micromol per litre

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DEDICATION

To my wife and my mother, without their help, devotion and love I would not have

been able to complete this thesis.

ETHICAL CONSIDERATION

The experimental work contained within this thesis was carried out with permission of the Home Office, and contained in the project license number 70/04510, under the Animals (Scientific procedures) act 1986.

Chapter 1. Introduction.

1.1. COLUMNAR-LINED LOWER OESOPHAGUS

1.1.1. Historical perspective

In 1953 Allison and Johnstone first suggested that the Columnar-lined lower oesophagus (CLO) be called Barrett's ulcer (Allison PR et al 1953), even though earlier investigators had accurately described the condition (Lyall A 1937; Tilestone 1906). Prior to 1953, CLO had been variously known as the chronic peptic ulcer of the oesophagus, oesophagus lined by gastric epithelium and congenital short oesophagus (Wright JT 1965; Bosher LH et al 1951). But since 1953, the name of Mr. Norman Barrett became associated with several lower oesophageal pathologies where the squamous epithelium is replaced by columnar cells. Therefore, the columnar-lined oesophagus is referred to as Barrett's oesophagus, the columnar mucosa lining of the oesophagus as Barrett's mucosa, and associated complications: high peptic stricture and adenocarcinoma are called Barrett's stricture and Barrett's adenocarcinoma, respectively. All these pathologies are believed to have similar aetiological factors and pathophysiology related to the gastroesophageal reflux disease, but the exact nature of refluxate, the cell of origin of CLO and preventive measures for progression to adenocarcinoma have not been determined. To determine these factors several experimental in vivo studies have been in progress.



Figure 1.1. Classic Barrett's oesophagus showing columnar metaplasia in lower oesophagus at > 3 cm from muscular gastroesophageal and schematic diagram of epithelium at squamo-columnar mucosal junction.

1.1.2. Definition

The Barrett's oesophagus is traditionally defined as the presence of specialised intestinal-type columnar epithelium with goblet cells, in the lower oesophagus, proximal to 3cm from the oesophagogastric muscular junction (Fig. 1.1). In the last decade, however, virtually all of the traditional concepts of Barrett's oesophagus and CLO have changed resulting in a totally different picture of pathophysiology as compared to the initial descriptions of CLO from 1950's. Most striking changes have been in the epidemiology of CLO and its relationship to gastroesophageal reflux disease and adenocarcinoma of the lower oesophagus.



Figure 1.2. *Incidence of CLO showing binomial peaks, smaller peak in children and larger peak in middle aged people.*

1.2. EPIDEMIOLOGY OF CLO

CLO presents with a bimodal age distribution. A small but significant incidence of CLO is found in children (Hassall et al 1985). The second much larger peak occurs later, in the mainly white Caucasian middle-aged male, 50 - 70 year old (Male 2.3: Female 1), in the Western world. It is found rarely in Afro-Americans and Asians (Bonelli 1993;Cameron et al 1992b). In U. K., data from the United Kingdom Barrett's Registry, shows Male to Female ratio of 1.5, in 2102 CLO cases. The mean age at diagnosis in males, in this group, was 62.0 years (range 53.2 – 66.3) and in females 67.6 years (range 59.3 – 73.4) (Caygill et al 1999). The diagnosis and the prevalence and incidence rates for CLO are most commonly determined by histological examination of biopsy specimen from upper gastrointestinal endoscopy. However, this may not give accurate figures.

Table 1.1. Prevalence and incidence of CLO.

Prevalence of	CLO
Endoscopic series ¹	22.6 cases per 100,000
Autopsy series ¹	376 cases per 100,000
Incidence of (CLO ^a
General population ²	1%
Gastroesophageal reflux disease ²	3 - 5%
Symptomatic reflux ²	10 - 15 %
Institutionalised patients ³	14 - 26 %
Scleroderma ³	37%
Reflux stricture ³	44%

¹(Cameron et al 1990a;Cameron et al 1992b)

^a from endoscopic series

²(Mann et al 1989;Winters et al 1987;Sontag et al 1985;Cameron 1997) ³(Katzka et al 1994;Sarr et al 1985;Bohmer et al 1999)

1.2.1. Changing incidence of CLO

The endoscopic series show increasing incidence of CLO in UK, rising from 1/100,000 to 18/10,000 over a period of 13 years (Prach et al 1997). Similar figures are reported in many Western countries (Sanchez et al 1995) and it has become apparent that, in the last decade, there is a tendency towards increasing prevalence rates of CLO all over the world, including the Far East (Chen 1997).

The reasons for the rise in prevalence and incidence of CLO could be due to dietary changes, rapid growth in the elderly population, obesity etc. (Chen 1997). Another reason could be related to higher rates of endoscopy performed with greater number of biopsies and better histological diagnosis. Also, data collected from endoscopic studies does not give the true prevalence or incidence of CLO, as it is dependent on presentation variables. Furthermore, combined data from prospective autopsy studies suggest that most cases in the population remain undiagnosed, due to disparity between disease severity and symptoms in up to one third of patients and due to self-medication with antacids (Iascone et al 1983;Bremner et al 1997;Stein et al 1993;Cameron 1997;Cameron 1993). There is also a large disparity between endoscopic and pathologic diagnosis of CLO and several studies show that only in 70 – 77.3% cases of suspected CLO is the pathological diagnosis proven (Conio 1993;Kim et al 1994;Andreollo et al 1997). Moreover, classic CLO can contain three subgroups of cells.



Figure 1.3. Intestinal metaplasia (a) H & E stain showing jejunoesophageal junction, (b) Schematic diagram to show distribution of squamous cells, specialised columnar cells, junctional and gastric cells in classic CLO.

1.2.2. Intestinal metaplasia and dysplasia.

The most important of subgroup of cells in the classic CLO is the specialised columnar epithelium with goblet cells; this columnar epithelium consists of intestinaltype cells with non-absorptive brush border – intestinal metaplasia. Studies looking at specialised columnar epithelium or intestinal metaplasia are more important, as it is the premalignant lesion for development into high-grade dysplasia and adenocarcinoma. In a study of 370 patients undergoing gastroscopy, classic CLO was found to in 4.6%, while, specialised columnar epithelium was observed in 13.6% of the cases (de Mas et al 1999). In addition, at repeat endoscopies upto 18% patients who had initial peptic erosions or ulcers healed with a columnar metaplasia suggesting that the suspected sequence of change of 'gastroesophageal reflux – erosive oesophagitis – Columnar metaplasia' is a short time process that may occur as rapidly as the healing of an erosion with squamous epithelium (4 to 6 weeks) (Fontolliet et al 1990; Monnier et al 1995; Ollyo et al 1989; Ollyo et al 1985). Columnar metaplasia can further evolve in three different ways: regression (7.5%), stabilisation (68.3%) and progression (24.2%) (Fontolliet et al 1994; Monnier et al 1987;Ollyo et al 1990). Columnar metaplasia can progress to low-grade and highgrade dysplasia.

1.2.3. High-grade dysplasia in CLO.

High-grade dysplasia is reported in 2% - 24% of CLO in tertiary surgical centres (DeMeester et al 1990;Fennerty et al 1989;Reid et al 1987;Tytgat et al 1989). This figure is dependent on referral patterns and subjective assessment by the pathologists.

Regression of dysplasia has also been reported (Burke et al 1991;Miros et al 1991;Tytgat et al 1989;Dent et al 1991), but this may be reflection of sampling bias. The diagnosis of dysplasia is important because when endoscopic biopsies show highgrade dysplasia, approximately 50% of resected specimens contain evidence of invasive adenocarcinoma (Wright 1997), even when there is no visually recognisable lesion at endoscopy (Atkinson et al 1992;McArdle et al 1992;Reid et al 1988;Rice et al 1993;Rusch et al 1994). Hence, it is important to have correct pathological diagnosis and to know the exact site of these changes, as more strikingly, all the changes of CLO can be found in biopsies within 3 cm of the gastroesophageal junction in the short-segment CLO (Fig. 1.4).



Figure 1.4. Schematic diagram of short-segment CLO where the changes of intestinal metaplasia occur within 3cm of muscular gastroesophageal junction.

1.2.4. Short-segment CLO and dysplasia.

Short-segment CLO has only been recognised in the last decade. The reported prevalence of short-segment CLO, on diagnostic endoscopy, varies from 8% to 32% (Lambert 1997; Donahue et al 1997). The age range and sex ratios in the short segment CLO are similar to the long-segment CLO. Although reflux symptoms may be more common in short-segment CLO (de Mas et al 1999), disturbances in oesophageal motility are less severe and there is less reflux. Furthermore, recognised complications of CLO, such as ulceration, stricture, high-grade dysplasia, and adenocarcinoma, appear to be uncommon in short-segment CLO (Schnell et al 1992; Iqbal et al 1997; Donahue et al 1997). More importantly, intestinal metaplasia in Short Segment CLO is noted in 48% – 61.3% of suspected patients (Johnston et al 1996;Spechler et al 1994;Nandurkar et al 1997;Chalasani et al 1997;Trudgill et al 1997; Weston et al 1996; Polkowski et al 1999), and in one study 17% of shortsegment CLO mucosa had dysplasia, and the length of CLO mucosa in 25% of patient's with early CLO adenocarcinoma measured \leq 3 cm in length (Clark et al 1997). Therefore, patients with short segment CLO must be considered to have a premalignant condition, even though no direct relationship between dysplasia and adenocarcinoma has been proven.

It is now thought that majority of the junctional adenocarcinoma also arise within intestinal metaplasia and dysplasia near the gastroesophageal junction. Therefore, recent reports have focused on redefining the diagnosis of Barrett' oesophagus or CLO (Clark et al 1994;Schnell et al 1992;Spechler et al 1996). Spechler states that 'Barrett's oesophagus' is an imprecise and out dated term and suggests that patients are better classified as having a columnar lined oesophagus with or without the presence of specialised intestinal metaplasia on biopsy (Spechler et al 1996). While this may represent an improved classification of CLO the physiological basis for such a definition is questionable. Furthermore, not all cases of adenocarcinoma are associated with dysplasia and the increase in CLO incidence is not directly related to the phenomenal rise in incidence of adenocarcinoma in the lower oesophagus and gastroesophageal junction.

1.2.5. Adenocarcinoma of the lower oesophagus, junction and cardia of stomach.

During the past 30 years, the incidence of lower oesophageal carcinoma has increased more rapidly than any other solid tumour in the western population, increasing at a rate of 10% per year. In fact, the overall incidence has increased by six-fold in the last 20 years (Haggitt 1992;Blot et al 1991;Pera et al 1993;Bremner et al 1998;Chen 1997;Kruyt et al 1997). If the present trends continue then in 10 years there will upto 14,000 new cases of oesophageal cancer per annum, in UK.

Not only that, the histological patterns of oesophageal cancer are changing with rates of squamous cell carcinomas decreasing and adenocarcinomas increasing rapidly in several western countries and by the early 1990s, adenocarcinoma had become the most common cell type of oesophageal cancer among predominantly young 35 to 55 year old white males, although squamous cell cancers still predominates among other patients (Clark et al 1994;Blot et al 1991;Blot 1994;Oliver et al 1992;Siewert 1989;Moyana et al 1996;Kelsen 1997;DeMeester 1997). The trends are not simply due to gastric cardia cancers now being called oesophageal adenocarcinomas, because

the rates of tumours appearing just below the oesophageal-gastric junction are also increasing. However, there are several problems in assessing the incidence of adenocarcinoma around the gastroesophageal junction.

1.2.6. Confusion with nomenclature of junctional adenocarcinoma.

Part of the problem in assessing the adenocarcinoma of the oeosphagus is that the current International Classification of Diseases by World Health Organisation, for classification of carcinomas of the oesophagus and stomach, causes epidemiological and clinical confusion. When epidemiological and clinical features of each subtype and subsite of adenocarcinomas of the oesophagus and stomach are assessed in studies, the incidence of adenocarcinomas of the lower oesophagus and cardia shows a 3 to 5 fold increase, whereas adenocarcinoma of the subcardia region of the stomach has declined (Dolan et al 1999; Powell et al 1992). Although the incidence of both are greater in men than in women, the proportional rates of increase, particularly for cardia, are very similar in both sexes, indicating a common aetiological factor or factors (Powell et al 1992). Whether, this common aetiological factor is intestinal metaplasia in CLO is not known, but it is suspected that the well-recognised dysplasia-carcinoma sequence may account for the increased incidence of proximal gastric and gastroesophageal junction adenocarcinoma (Schneider et al 1996). Convincing data for this is evident in prospective endoscopic surveillance studies, which show that in patients with proven carcinoma, preceding histological biopsies reveal a progressive worsening of dysplasia (Wright 1997). Though, the exact nature of relationship between dysplasia in CLO and adenocarcinoma of the lower

oesophagus has not been established, there is epidemiological evidence for a relationship.

1.2.7. Relationship between CLO and oesophageal adenocarcinoma.

The causes of increase in incidence of oesophageal adenocarcinoma are not well known but must involve CLO (Blot et al 1999), as the increase in oesophageal adenocarcinoma is seen in parallel with the increase in incidence of CLO in developed nations around the world and 60-100% of these tumours arise in the setting of CLO (Cameron et al 1995; Hamilton et al 1988; Haggitt et al 1978; Clark et al 1996). CLO is associated with a 30- to 125-fold increased risk for adenocarcinoma of the lower oesophagus (Stein et al 1993). This is adenocarcinoma risk is 350 times greater than in the general population (Cortesini et al 1995). Although this metaplastic transformation appears to be a major factor in the changing epidemiology of oesophageal carcinoma, it may not be the sole explanation for this shift from squamous cell carcinoma to adenocarcinoma (Kirby et al 1994). Other important factors - the gastroesophageal reflux disease and reflux oesophagitis, are also known to be associated with the lower oesophageal and junctional adenocarcinoma and the incidence of these diseases has also increased substantially in the preceding decade. Furthermore, gastroesophageal reflux disease is a known precursor for development of CLO, not only in adults but also in children.

1.2.7. CLO in children.

CLO in children is an uncommon entity as compared to adult onset CLO. Though there might be different epidemiology and aetiological basis for CLO in children, it is still found most commonly in children with gastroesophageal reflux. CLO in children is usually recognised after complications of gastroesophageal reflux develop, but it may be relatively silent in childhood and then present with adenocarcinoma in childhood or present in adulthood (Hassall 1993). In children with gastroesophageal reflux disease, CLO is found in 15% - 36% (Bairov et al 1999;Eizaguirre et al 1993;Maksoud et al 1982). Higher incidence is noted in institutionalised children, children operated upon for oesophageal atresia at birth and children with excessive acid exposure or severe duodeno-gastric alkaline reflux (Eizaguirre et al 1993). From these epidemiological studies it is apparent that the CLO and Barrett's adenocarcinoma share similar aetiological factors and as the prevalence and incidence of both is continuing to rise rapidly it is imperative to know these aetiological factors.

1.3. AETIOLOGICAL FACTORS.

A number of aetiological factors have also been identified which may cause CLO.

- Gastroesophageal reflux disease & reflux oesophagitis
- Environmental factors
- Dietary factors
- Helicobacter pylori
- Predisposing medical conditions

The most common aetiological factors associated with CLO is the gastroesophageal reflux disease and reflux oesophagitis, but the exact reflux element causing the change from squamous-lined oesophagitis to columnar-lined CLO is not known and needs to be determined. One way to determine the constituents of the reflux is by iatrogenic surgical anastomosis in the *in vivo* experimental studies, which will also determine the interrelation between different reflux constituents and development of CLO. More commonly, the nature of refluxate is determined by *in vivo* physiological and clinical studies.

Table 1.2. CLO and GORD.

	and the second
	 mechanically defective lower oesophageal sphincter
Characteristics of GORD	• oesophageal motility disorders
in CLO ¹	 Inefficient oesophageal clearance function profound reduction in the contraction
	amplitudes in the distal oesophagusgastric acid hypersecretion
	• excessive reflux of gastric and duodenal contents
	• complications of ulceration, stricture and acute & chronic inflammation

¹ (Kauer et al 1995;Stein et al 1993;Iftikhar et al 1995;Clark et al 1997)

1.3.1. Gastroesophageal reflux disease (GORD) & reflux oesophagitis

Though, gastroesophageal reflux disease is reported by 10% – 44% of the population in the general population questionnaire surveys (Kay et al 1994;Agreus 1998;Anonymous 1988), the prevalence of reflux oesophagitis in Western countries and developed nations is estimated to be only 2% and that of gastroesophageal reflux disease 5% (Yang et al 1988;Chen 1997) because studies of gastroesophageal reflux disease are confounded by the lack of a standardised definition and a diagnostic 'goldstandard' for the disorder. For example, in the Western countries, 20 - 40% of the adult population experience heartburn, which is the cardinal symptom of gastroesophageal reflux disease, but only some 2% of adults have objective evidence of reflux oesophagitis and complications, including oesophageal ulcer, stricture and CLO, are found in up to 20% of patients with verified reflux oesophagitis (Spechler 1992). In fact, gastroesophageal reflux disease in patients with CLO has a more severe character and is more frequently associated with complications as compared with reflux patients without columnar mucosa (Stein et al 1993).

1.3.1.1. Nature of reflux

The reflux of gastrointestinal contents into squamous lined lower oesophagus is particularly damaging and regeneration by columnar metaplasia may be an adaptive mechanism. However, it is not known what components of reflux drive the adaptive metaplasia to complicated CLO. Experimental work on reflux oesophagitis suggests a role for both acidic gastric and alkaline duodenal secretions in causing progression of CLO (Mud et al 1982;Lanas et al 1999;Salo et al 1982;Lillemoe et al 1983).

1.3.1.1.a. Duodenal reflux

It is believed that excessive reflux of alkaline duodenal contents, especially bile – measured as bile acids in oesophageal aspirates and bilirubin in ambulatory Bilitec spectrophotometric analysis, occurs in complicated and longer length of CLO and is believed to be responsible for the development of complications (i.e., stricture, ulcer, and dysplasia) (Caldwell et al 1995;Gillen et al 1988;Jankowski et al 1992;Gotley et al 1991;Stein et al 1993;Johnsson et al 1989).

1.3.1.1.b. Gastric reflux

Other studies show significant increase in peak acid output in complicated CLO suggesting that acid rather than duodenal reflux is the main culprit in CLO (Bremner et al 1997;Sears et al 1995), but one study, in carefully matched controls showed no difference in basal acid output, pentagastrin-stimulated acid output, or pepsin secretion in patients with CLO (Hirschowitz 1996). This conclusion is supported by only a 3% prevalence of CLO reported in 92 patients with Zollinger-Ellison syndrome (Strader et al 1995). It may be that both acid and duodenal reflux contributes to the development of Columnar metaplasia as acid and duodenal contents usually reflux into the esophagus simultaneously (Vaezi et al 1999).

1.3.1.1.c. Gastroduodenal reflux

Clinical studies of combined pH and spectrophotometric monitoring (Bilitec 2000 system) detection of bilirubin, aspirations and chemical analysis of gastirc contents

also show that both acid and bile exposure in the oesophagus are greater in patients with CLO (Caldwell et al 1995;Bechi et al 1993;Kauer et al 1997). The correlation of pH and bilirubin monitoring shows that the majority of oesophageal bilirubin exposure occurred when the pH of the oesophagus was between 4 and 7 (Marshall et al 1997;Stein et al 1994). Numerous experimental studies also favour a synergistic effect of acid and bile reflux in the development and malignant degeneration of CLO (Clark et al 1994;Gillen et al 1988;Pera et al 1993;Johnson et al 1986). However, no studies show direct causal link between concentration of reflux, length of exposure and development of complications. Part of the reason for this is that oesophageal pH monitoring, bilirubin monitoring and pH > 7 are a poor marker for reflux of duodenal contents into the esophagus and false positive results occur if pH is less than 3. Therefore, it may be better in this instance to analyze these interrelations by *in vivo* studies and study the role of various aetiological factors under experimental situation, especially when clinical studies give conflicting results. Other aetiological agents are related to life-style factors.

1.3.2. Life-style factors

Patients with CLO are significantly more likely to be moderate or non-smokers and/or non-drinkers, have higher body mass index, than patients with both severe oesophagitis and adenocarcinoma (p < 0.001) (Gray et al 1993;Korn et al 1997). But as the risk of developing lower oesophageal adenocarcinoma is elevated among heavy smokers (Odds Ratio (OR) = 2.1 - 3.4), heavy alcohol consumption (OR = 1.6 to 9.5), low income (OR = 3.4) and duodenal ulcer (OR=2.2) (Brown et al 1994;Kabat et al 1993;Vaughan et al 1995;Bremner et al 1998), it is possible these factors also influence progression of Columnar metaplasia to dysplasia. However, these factors do not appear to explain the rapid rise in incidence of these tumours and dietary factors may be more important.

1.3.3. Dietary factors

One of the strongest emerging risk factors for CLO and oesophageal adenocarcinoma is obesity. Increases in the prevalence of obesity and the incidence of oesophageal adenocarcinoma are parallel, and several epidemiological studies have shown upwards of threefold excess risk among overweight individuals (Blot et al 1999). Adenocarcinoma of oesophagus and gastric cardia is significantly related to higher intake of dietary calories, total animal fat, red meat and vitamin A, while decreased risk of adenocarcinoma is associated with high ingestion of dietary fibre, increasing consumption of fruits, dark orange vegetables, lutein, niacin, carotene, vitamins B6, C and E, riboflavin, iron, and zinc (Gao et al 1994;Kabat et al 1993;Zhang et al 1997;Ward et al 1997). Part of the reason for increased incidence of adenocarcinoma with higher calorie intake of animal source may be related to cooking methods as meats cooked at high temperatures and for a long duration contain heterocyclic amines & polycyclic aromatic hydrocarbons, which are both mutagens and animal carcinogens (Ward et al 1997).

1.3.4. Helicobacter Pylori

Though a study found that Helicobacter pylori at the squamous-columnar junction was present in 13% of control subjects and in 30% of the patients with gastroesophageal reflux (Csendes et al 1998), and some studies have found positive correlation between Helicobacter pylori and intestinal metaplasia (Goldblum et al 1998;Weston et al 2000), on the whole Helicobacter pylori is not associated with CLO or adenocarcinoma of the lower oesophagus (Hackelsberger et al 1998;Nandurkar et al 1997). Part of the reason for conflicting results is that the studies of Helicobacter pylori are difficult to compare because different groups take biopsies at different sites. In fact, an inverse relationship may exist.

1.3.5. Carcinogen exposure

Some studies have looked at the environmental and dietary carcinogens, especially the N-nitrosamines and suggest that high intake of fat and foods containing precursors of N-nitroso compounds may increase the risk of dysplasia in CLO and development of adenocarcinoma (Lu et al 1986;Bremner et al 1998), but this has not been firmly established.
1.3.6. Predisposing medical conditions

CLO is associated with conditions in which there is prolonged exposure to gastric and duodenal conditions. These include dysmotility syndromes, achalasia, scleroderma, oesophagojejunostomy and hiatus hernia. Other predisposing conditions include caustic injury, chemotherapy. These conditions are also associated with elevated risk of adenocarcinoma (Zhang et al 1996;Van Cutsem et al 1991).

1.3.7. Aetiology of CLO in children

Dysmotility syndromes, iatrogenic causes – surgery for oesophageal atresia, and chronic and severe gastroesophageal reflux disease are the main associated findings. Manometric studies also show disorganised peristalsis with near-absence of propulsive waves and predominance of mass-contractions in children with CLO (Gorostiaga et al 1991). This suggests that motility problems are similar to adults with poor oesophageal clearance mechanism. It may be that the underlying pathophysiology of CLO in children and adults is also similar. It is however difficult to make a diagnosis of CLO in adults and children based on the presentation of GORD or dysmotility syndromes (*see* Table 1.3), as there is little correlation between clinical symptoms and pathology. The diagnosis of CLO is based on other factors.

1.4. DIAGNOSIS OF COLUMNAR-LINED LOWER OESOPHAGUS

A critical issue in the diagnosis of CLO is to determine proximal displacement of the squamo-columnar junction and whether or not there is dissociation between the mucosal and muscle junctions. The squamo-columnar mucosal junction can be identified by several methods (*see* Table 1.4).

1.4.1. Xray studies

In the past, Xray contrast study was the main method of diagnosis but they have low sensitivity for diagnosis of the CLO. This improves when contrast studies show reticular mucosal pattern (Levine et al 1983), focal mural deformity and decreased oesophageal distensibility (Glick 1994), high oesophageal stricture (Chernin et al 1986;Lackey et al 1984), hiatal hernia, and/or oesophageal ulceration (Chen et al 1985;Robbins et al 1977;Bremner et al 1997). Division of diagnostic features into high-risk of CLO for patients with high stricture, ulcer or reticular mucosal pattern; moderate-risk for distal stricture and/or radiological reflux; and low-risk for none of these features, can also increase the diagnostic yield (Bremner et al 1990;Gilchrist et al 1988), but the overall accuracy of contrast studies is suboptimal and the main method of diagnosis nowadays is by endoscopy and biopsy (Bremner et al 1997).

Table 1.3. Presentation of CLO.

	Neutral reflux ²
Asymptomatic ¹	\downarrow sensitivity of CLO mucosa
	Heartburn
GORD ⁴	Regurgitation
	Dysphagia
	Hiatus hernia
Sequelae ⁵	Scleroderma
	Achalasia
	Stricture
Complications ⁶	Ulcer
	Adenocarcinoma

¹ Asymptomatic, minimal symptoms or symptoms disappear and there is very little correlation with the severity of reflux ² 'neutral' or normal pH reflux – mixed acidic gastric and alkaline duodenal reflux

² 'neutral' or normal pH reflux – mixed acidic gastric and alkaline duodenal reflux (Johnson et al 1987)

³ (Bremner et al 1993)

⁴ commonest method of presentation (Bremner et al 1997)

⁵ sequelae of other conditions with increased reflux exposure

⁶ complications of CLO

Table 1.4. Investigations for CLO.

Investigation	Remark	Sensitivity/Specificity
Identij	fication of squamo-columnar mucosal junc	ction
Xrays contrast studies ¹	Only helpful when sequelae develop	Low sensitivity
^{99m} Pertechnetate	Better with extensive disease	High specificity
scintigraphy ²		Low sensitivity ~47%
Transmucosal membrane	Dependent on negative potential	Low sensitivity ~70%
potential difference ³	difference of columnar mucosa	
Manometry and pH	Poor correlation between physiology	Low specificity
studies	and pathology	Low sensitivity
Endoscopy & Biopsy	Inter-observer variations in assessment	High specificity and
	of length and level ⁴	sensitivity
Dyes ⁵	Lugol's 50% Iodine	High specificity and
	Methylene blue & Alcian blue	sensitivity
Identij	fication of muscular oesophagogastric jund	ction
Manometry ⁶	False readings in hiatus hernia or	High specificity
	achalasia	

¹(Bremner et al 1997) ²(Yegelwel et al 1989;Mangla et al 1976) ³(Eckardt et al 1983;Herlihy et al 1984;Orlando 1986)

⁴(Kim et al 1994) ⁵ infrequently used (Canto et al 1996;Nothmann et al 1972)

⁶(Levine et al 1993;Nishimaki et al 1991;Tytgat 1997)

1.4.3. Endoscopy

The proximal migration of squamo-columnar mucosal junction on endoscopy can be seen as upward 'finger-like' projection of velvety salmon-pink mucosa or as a straight circumferential line (McClave et al 1987; Tytgat et al 1989; Tytgat 1997; Tytgat 1989). The level of the mucosal change can be measured with respect to the gastroesophageal junction, which is recognised by the proximal extent of the gastric folds, peristalsis, diaphragmatic movement during respiration and caliber change (McClave et al 1987; Tytgat 1997). But this can be inaccurate in small and large sliding hiatus hernia, and the circumferential asymmetry, lack of accurate level or identification of intestinal metaplasia causes inter-observer differences which complicates assessment of progression and regression of the columnar mucosa. Diagnostic accuracy of the endoscopy and biopsy can be improved by using manometry, which provides a more accurate identification of the muscular junction, but the disadvantage is that if the columnar mucosa is present only on one side, it may be missed by the 'blind' biopsies. Therefore, for accurate assessment of lower oesophageal epithelium four quadrant biopsies every 2cm, with simultaneous localisation of sphincter is required (Levine et al 1993;Nishimaki et al 1991;Tytgat 1997). However, as this procedure is cumbersome some endoscopists take 'blind' four quadrant biopsies at different levels (Tytgat et al 1989), and it is questionable whether precise localisation of the muscular junction is as important as precise histological diagnosis.

Table 1.5. Histological characteris	tics of	CLO.
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Findings	Cell type or character		
Histology			
Fundic, junctional, specialised-type cells ¹	CLO cell		
+ve PAS staining for sulphated mucin ¹	Goblet cells		
Histochemistry			
-ve Gastrin, +ve pepsinogen bands ²	Peptic cells		
\downarrow disaccharide & Beta-galactosidase, \uparrow glucuronidase ³	Type II intestinal cell		
May secrete 5-HT, somatostatin, motilin, pancreatic polypeptide, glucose-dependent insulinotrophic polypeptide, secretin and neurotensin ⁴	Multipotent primordial stem cell		
Immunohistochemistry			
sucrase isomaltase messenger RNA ⁵	intestinal metaplasia		
\downarrow Glutathione & glutathione S-transferase activity ⁶	gastric epithelium, 1 tumour risk		
\downarrow mucosal protein & stress-response protein – Hsp27 ⁷	\downarrow protection to noxious agents		
trefoil peptides – pS2 and human spasmolytic polypeptide ⁸	small intestinal epithelium		
cytoplasmic structural proteins – cytokeratins 7/20 ⁹	long segment CLO epithelium		
Genetic alterations	1		
↑ aneuploidy, microsatellite instability, loss of heterozygosity	molecular changes of unstability ¹⁰		
↑ proto-oncogenes, mutation or deletion of the tumour- suppressor genes ¹⁰ – Apc & p53	Dysplastic cells		
↑ growth regulatory factors ¹¹ – TGF, EGF	Increased proliferation		
↑ tumour proliferation-related factors – Ki67, PCNA, AgNOR	Neoplasia ¹²		
↑ abnormalities of cell adhesion molecule – E Cadherin, α- Catenin	Leads to neoplasia ¹¹		
 ¹ (Paull et al 1976;Haggitt 1994;Abrams et al 1965) ² (Mangla et al 1976;Dalton et al 1977) ³ (Herbst et al 1978) ⁴ (Rindi et al 1987) ⁵ (Wu et al 1993) ⁶ (Peters et al 1993) ⁷ (Soldes et al 1999;Park et al 1994) ⁸ (Hanby et al 1994) ⁹ {Ormsby, Goldblum, et al. 1999 ID: 20315} ¹⁰ (Gleeson et al 1996;Meltzer 1996;Neshat et al 1994;Mor ¹¹ (Jankowski et al 1996) 	ntesano et al 1997)		



Figure 1.5. Pathogenesis of CLO.

1.5. CLO EPITHELIUM

Histological diagnosis from resected specimen or biopsy provides the only definitive diagnosis of CLO. On histological examination, the oesophagogastric junction can be identified by noticing longitudinal fibres, thick muscularis mucosa (Kalish et al 1984) and submucosal glands in the oesophagus (Ireland PE 1933). Paull et al defined three types of columnar epithelium in CLO with distinct zonation (Figure 1.3) (Paull et al 1976). Firstly, an atrophic gastric fundic type of epithelium present distally, with chief and parietal cells. Secondly, a junctional foveolar type of epithelium with cardiac mucous glands in the middle. Thirdly, a proximal specialised columnar epithelium with a non-absorptive villiform surface identical to intestinal metaplasia of the incomplete type (type II or type III) in the stomach, and with mucous glands and goblet cells (Paull et al 1976;Haggitt 1994;Morson et al 1952;Abrams et al 1965). The zonation of the CLO cells is not consistent in all cases (Thompson et al 1983), and when the oesophageal mucosa is mapped using a standardised biopsy protocol, cardiac and gastric fundic-type mucosa is rarely identified in adults above the distal 3 cm of oesophagus (Weinstein et al 1996). Apart from histological staining, the CLO epithelium has also been analysed with histochemistry, immunochemistry and genetic studies (Table 1.5). These studies show that CLO epithelium is metabolically distinct from other gastrointestinal tissue and arises from a multipotent primordial stem cell. The specialised columnar cell reverts to the embryonic form and is genetically unstable, causing mistakes in DNA replication that leads to dysplasia either focally or as field change (Aldulaimi et al 1999). These early premalignant clones produce biological and genetic heterogeneity allowing disease progression under selective

pressure (Jankowski et al 1999). Exploitation of these molecular events can identify suitable genetic markers to help with early diagnosis.

1.5.2. Genetic markers for CLO

Aneuploid cell populations detection by flow cytometric analysis of DNA content is a particularly useful technique that can quantify the percent of cells in various phases of the cell cycle and can be identified in non-neoplastic, dysplastic and malignant biopsies of CLO (Aalykke 1993; Ahnen 1992). Point mutations of the p53 tumor suppressor gene, an early event in adenocarcinogenesis, can also be detected in nondysplastic CLO metaplasia and CLO epithelium adjacent to adenocarcinoma (Casson et al 1991;Stemmermann et al 1994). Techniques of gene amplification such as differential polymerase chain reaction (PCR) have also been used to identify genetic markers for dysplasia (Akiyama et al 1997). Abnormalities of growthregulatory peptides and their receptors are particularly important in the pathogenesis of this condition, as growth-regulatory molecules - Transforming growth factor (TGF) α , its precursor prepro TGF α , Epidermal growth factor and Epidermal growth factor receptor (EGFR) are all increased in CLO when examined by immunohistochemistry, computerised planimeter (Jankowski et al 1992), flow cytometry (Jankowski et al 1992), Ki-67 labelling index (Jankowski et al 1991), proliferating cell nuclear antigen labelling index (Jankowski et al 1992) and by Western-blot analysis (Brito et al 1995). CLO mucosa has potential autocrine growth regulatory mechanisms as there is differential expression of these peptides with increased expression in dysplasia and adenocarcinoma (Brito et al 1995; Jankowski et

al 1991), but immunohistochemical staining shows no associations between staining intensity and degree of EGFR, histology, or tumor stage (al-Kasspooles et al 1993).

The final pathway for dysplasia in CLO may also involve cell adhesion molecules – cadherins and catenins, as there is significant reduction of E-cadherin expression as the CLO metaplasia progresses and these may also prove to be helpful markers of disease progression (Bailey et al 1998;Jankowski et al 1997;Shiozaki et al 1991).

The genetic markers however cannot identify the cell of origin of CLO and experimental studies have to be performed to determine the exact location from which the intestinal metaplasia develops.

1.5.3. The cell of origin

The cell of origin of the CLO is unknown and various concepts have been postulated, including: the adjacent gastric epithelium (Bremner et al 1970), the submucosal glandular epithelium (Gillen et al 1988), the basal cells of the squamous epithelium (Meyer et al 1979), the oesophageal cardiac glands (Feurle et al 1990), pluripotent primordial stem cells (Rindi et al 1987), distinctive epithelial metaplastic cells (Shields et al 1993). Mr. Norman Barrett, himself, initially believed this entity to be congenital (Barrett NR 1950). Occurrence of embryological remnants of columnar-lined oesophagus, as islands in the oesophagus (Taylor AL 1927;Rector LE et al 1941), as microscopic foci (Schride 1904) and occurrence in neonates and young children supported the hypothesis that CLO could well be a congenital lesion (Lyall A 1937;Smithers 1945;Frindlay et al 1931). But the congenital theory for the origin of

the lining has lacked pathological support and the present CLO controversy has, however, changed from the 'congenital or acquired' to the tissue of origin of the columnar cells.

A number of early clinical studies, case reports and experimental studies supported the concept that CLO arises by ascent of any adjacent columnar epithelium as 'creeping' substitution (Naef et al 1972;Goldman et al 1960). Columnar lining of the oesophagus can also develop in patients after total gastrectomy with a loop jejunostomy reconstruction (Meyer et al 1979), after an Ivor-Lewis type of reconstruction following oesophagogastrectomy (Hamilton et al 1977), after oesophagomyotomy for achalasia (Goldblum et al 1994) and in scleroderma of the oesophagus (Recht et al 1988), suggesting that CLO can develop in the absence of a stomach or gastric secretion, and that it can develop in response to the irritation of duodenal contents alone. Columnar epithelium replacement can also occur by both a creeping substitution process and by submucosal mucous gland replacement (Gillen et al 1988).

On the other hand autopsy studies suggest that the oesophageal cardiac glands may be the source of CLO (Adler 1953). While, several reports of development of CLO support the theory of origin from basal layer of squamous epithelium (Meyer et al 1979). Development of a columnar lining following chemotherapy for leukaemia (Dahms et al 1987), following lye ingestion (Spechler et al 1981), and following chemotherapy for breast cancer and testicular cancer (Sartori et al 1991;Peters et al 1993) support this. Experimental studies also show that the proliferating stem cells in basal layer of squamous epithelium have a double differentiation capability with

development of either squamous cancer or adenocarcinoma (Pera et al 1989). Study of CLO using scanning electron microscopy, show a distinctive 'transition zone' cell type having features intermediate between those of squamous and columnar epithelium, and indistinguishable from cells seen in the transformation zone of the uterine cervix (Shields et al 1993). Futhermore, repair of CLO mucosa after ablation therapy with laser and photodynamic therapy, is by squamous re-epithelialisation and possibly by squamous metaplasia within the CLO mucosa itself, implying the existence of pluripotential stem cells within CLO mucosa (Biddlestone et al 1998). Comparison of the inlet patch columnar epithelium seen in upper oesophagus with the columnar lining of CLO patients shows that the specialised columnar epithelium originates from a very immature multipotent stem cell (Feurle et al 1990). As this stem cell can proliferate rapidly and become dysplastic if the stimulus is continued, much effort has been directed towards reversing the main stimulus – the gastrointestinal reflux – by several strategies.

1.6. MANAGEMENT OF CLO.

Medical and surgical efforts to reverse CLO by decreasing gastroesophageal reflux have so far been disappointing (Table 1.7). A potential answer to the problem is removal of the metaplastic epithelium using physical and chemical modalities, but long-term follow up are not conclusive.

1.6.1. Medical management of CLO

Though long-term proton pump inhibition effectively heals patients with oesophagitis (Hetzel et al 1988), control of acid exposure is inadequate for regression of CLO epithelium, as 34 – 58% of patients on long-term acid suppression may develop CLO (Table 1.6) (Wetscher et al 1997;Sharma et al 1997). Furthermore, 'islands' of squamous epithelium may develop in the treated columnar epithelium. Biopsies of these islands show that intestinal metaplasia may still be present in upto a third of patients (Sharma et al 1998). Moreover, as only 12 - 18% of patients with GORD develop CLO and upto 75% of patients destined to develop CLO will never be seen, evaluated, or treated before CLO develops (Bremner et al 1998), it is questionable whether the costs of life-long continuous medical therapy in large numbers of patients is justified and it might be better to identify the subset of patients with CLO who will progress to high-grade dysplasia and adenocarcinoma. The main method of identifying at-risk patients is by endoscopic surveillance.

Table 1.6. Management of CLO.

Management	Authors	Method	Results
Medical	Sharma et al '97 Wetscher et al '97	PPI H2 blockers	34% - 58% develop CLO ¹
Surveillance	Hameetman et al '89	Endoscopic biopsy	Carcinoma developing 1.5 – 4 years in CLO
	Reid et al '92		No carcinoma in 3 –5 yrs, in high-grade CLO
Surgery	Sagar et al '95	Anti-reflux surgery Follow-up 10 years on 56 cases	 24 regression – complete/partial 9 progression to carcinoma 23 (50%) no change
	Bremner & DeMeester '97	Anti-reflux surgery 284 cases since 1974	10 complete regression39 partial regression29 progression (10 carcinoma)203 (71%) unchanged
	Luostarinen '93	Anti-reflux surgery 20 yrs follow-up	2/15 CLO in intact repair 5/6 CLO in incomplete wrap
Medical or Surgical	McCallum et al '91 Oritz et al '96	Fundoplication v's Medical	Partial regression of CLO after surgery in 8/32 Partial regression of CLO after medical treatment in 2/27
Mucosal ablation ¹	Sampliner et al '96 Jackson et al '97 Byrne at al '98 Martin et al '99	Coagulation or laser – Argon, Nd:YAG & KTP, combined with PPI or fundoplication	75 – 100% Reversal of CLO 34 – 58% stricture
	Ackroyd et al '99 Orth et al '99	Photodynamic therapy ²	66 – 80% regression of CLO 4 – 34% complication rate

¹ Intestinal metaplasia present underneath squamous regeneration

² photosensitiser drug with Argon, KTP or gold laser

1.6.2. Surveillance of CLO

Surveillance for CLO is aimed to detect dysplasia – the first step in the neoplastic process, by systemic four-quadrant biopsy protocol (Levine et al 1993). Dysplasia is graded as negative, indefinite, and positive; the latter divided into low-grade and highgrade (Riddell 1996;Axon et al 1994;Riddell 1990). Surveillance intervals for detecting dysplasia are based on the published database of four centres (Miros et al 1991;Reid et al 1992;Robertson et al 1988;Edwards et al 1996;Levine et al 1993;Hameeteman et al 1989;Sagar et al 1995;Peters et al 1994;Brand et al 1980). These database show that carcinoma can develop following low-grade or high-grade dysplasia in 1.5 to 4 years (Hameeteman et al 1989), but in some patients dysplasia may also remain static for 36 to 57 months without degeneration to cancer (Reid et al 1992). Table 1.8 shows the experience of these centres and based on this data, Table 1.9 shows the recommended frequency of surveillance endoscopy (Bremner et al 1998).

If high-grade dysplasia is found then re-biopsy specimens needs to be reviewed by an expert pathologist, followed by resection of the oesophagus provided that the patient is a good surgical candidate. Oesophageal resection is recommended because 40-78% of patients who have had only high-grade dysplasia by endoscopic biopsy have invasive adenocarcinoma in the oesophagectomy specimen (Edwards et al 1996). Such patients who go to resection for high-grade dysplasia typically have an early stage cancer which is often intra-mucosal and has a more favourable prognosis (Altorki et al 1991;Peters et al 1994). However, other authors have recommended using an aggressive biopsy regimen to distinguish cancer from high-grade dysplasia

and correlating flow cytometry and histologic progression with malignancy (Reid et al 1992).

1.6.3. Surgical prevention of CLO

As CLO is associated with end stage GORD and surgical control of reflux is better in the long-term, abolition of reflux by anti-reflux surgery should effectively prevent the development of CLO in patient's who have GORD without CLO and cause regression of CLO in others. The most common anti-reflux procedure is Nissen fundoplication, with good long-term follow up, and laparoscopic fundoplication. This therapeutic option is considered appropriate in younger patients or patients with chronic and severe reflux unresponsive to medical management. There are some reports which describe complete or partial regression of CLO following fundoplication (Brand et al 1980), but unfortunately in most cases there is no change or there is progression of CLO (Table 1.7) (McDonald et al 1996;Sagar et al 1995;Baulieux et al 1999;Ortiz et al 1996;McCallum et al 1991).

Table	1.7.	The	time	interval	between	dysp	lasia	and	develo	pment o	f cancer.

Cancer/Total No.	Percent	Follow-up yrs
3/150	2	3.6 - 10
8/45	18	1.5 - 4.3
21/61	34	0.2 - 4.5
	Cancer/Total No. 3/150 8/45 21/61	Cancer/Total No. Percent 3/150 2 8/45 18 21/61 34

Table 1.8. CLO Dysplasia and Recommended Surveillance.

Dysplaia	Follow-up Endoscopy
None	After two negative, every 3 years
Low-grade	Every 6 months X 2, then every year
High-grade	Expert confirmation and resection for surgical candidates

1.6.4. Comparison of medical and surgical prevention of CLO

There is little evidence which indicates that anti-reflux surgery or medical treatment prevents adenocarcinoma. But evidence does suggest that CLO is better preventable with effective anti-reflux surgery. McCallum et al compared 29 surgically treated patients with CLO without dysplasia to 152 medically treated patients and found that at 5 years of mean follow-up the surgically treated patients were significantly protected from the development of dysplasia and oesophageal cancer (McCallum et al 1991). Ortiz et al (Ortiz et al 1996) randomised 59 patients with CLO to medical or surgical therapy. Partial regression was noted in 8 out of 32 patients after fundoplication compared to 2 out of 27 medically treated patients; however, none of the patients were found to have complete regression. Though one patient from each group developed high-grade dysplasia and adenocarcinoma, the difference did not reach statistical significance. Part of the reason for the discrepancy could be that to prove improvement of CLO is much more difficult than it appears at first sight.

There are several potential reasons that stand in the way of proving that abolition of reflux can confidently predict future freedom from CLO. One reason relates to sampling error. Outside of a prospective study specifically designed to look at this point, it is hard to deduce from published reports when CLO was genuinely absent. Some idea of the scope of the problem comes from an analysis of the VA Cooperative study (Kim et al 1994;Spechler 1992). Analysis of sequential endoscopies in these patients showed that even with the intensive biopsy protocol and frequent meetings of investigators to improve compliance, sampling error was a major problem in classifying the presence or absence of CLO at entry into the study. Of 81 patients free

of CLO at entry, five were found to have Barrett's at 6 weeks follow-up. Of 82 patients with specialised columnar epithelium at entry, five had only oesophagitis at 6 weeks follow up. The discrepancy was even greater for patients diagnosed with columnar lining without specialised epithelium at entry. The second practical obstacle is the fact that proof that the operation fulfilled its mission of reducing reflux is often lacking. Only if an objective measure of reflux established that the operation reduced oesophageal acid exposure to normal can its ability to prevent the development of CLO be assessed. The third problem is that surgery changes endoscopic landmarks leading to erroneous length measurements (Kim et al 1997). A fundoplication and reduction of a hiatal hernia alters the anatomy of the gastro-oesophageal junction, and stretches out the oesophagus. Thus, determining whether there has been a change in the length of the metaplastic segment on post-operative endoscopy can be difficult. Furthermore, it is not easy to biopsy within the fundoplication, particularly when there is a long fundoplication, or when a Collis gastroplasty has been added to the fundoplication. Another factor is the adequacy of the fundoplication. If a patient refluxes through a poorly constructed or failed fundoplication the outcome of this patient will not be an accurate portrayal of the effect of anti-reflux surgery on CLO. Many patients are reluctant to undergo repeat 24-hour pH analysis post-operatively and assessment of the function of the fundoplication is on the basis of the symptoms. A fifth factor is the lag time for resolution of the mucosal cellular and genetic insults that have occurred from the repetitive injury of the gastroesophageal reflux. The development of dysplasia or invasive cancer within 1 year of a fundoplication likely represents an occult process at the time of fundoplication rather than something that has occurred post-operatively. The length of time within which these occult changes can occur as a consequence of pre-fundoplication mucosal injury is unclear.

Very few authors have recorded the presence of CLO metaplasia following surgery when it was absent preoperatively. Luostarinen et al performed endoscopy 20 years after Nissen fundoplication in 21 patients and found CLO in 2 of the 15 patients with intact fundoplication and 5 out 6 patients with defective wraps (Luostarinen et al 1993). Other papers mention healing of oesophagitis, (Johansson et al 1993;Walker et al 1992;Watson et al 1991;Thor et al 1989) but do not report any case of CLO which was not present preoperatively. Thus, despite the limitation of these studies, it appears that the de novo development of CLO is exceedingly rare in patients who have had effective antireflux surgery, but anti-reflux surgery is not effective once CLO develops.

Studies looking at dysplasia or adenocarcinoma after anti-reflux surgery are also inconclusive. At the University of Southern California, out of 34 CLO patients with a mean follow-up of 24 months after surgery, none developed high-grade dysplasia or adenocarcinoma, 9 had complete regression and 4 developed low-grade dysplasia (Bremner et al 1998). McDonald's study from the Mayo clinic (McDonald et al 1996) reported adenocarcinoma developing in three patients following antireflux surgery within 39 months. No patient developed carcinoma beyond this period, despite a median follow-up of 6.5 years, and a maximum follow–up of 18.2 years. The study suggests that surgery prevented malignant initiation in those who were free of the problem prior to surgery but not progression once dysplasia develops. However, review of the English language literature since 1975 reveals a total of 18 patients reported to have developed oesophageal adenocarcinoma within CLO following fundoplication. Although the length of follow-up was not always available, 12

patients (61%) developed cancer within 3.5 years of fundoplication, another two patients (17%) developed cancer within 5 years of fundoplication, and the remaining four patients (22%) developed cancer between 5 and 10 years after fundoplication (Bremner et al 1998). From the literature it is clear that fundoplication does not eliminate the risk of cancer in patients with CLO. This is particularly true in the first several years after fundoplication when an occult cancer may subsequently be found. Furthermore, detecting oesophageal cancer early is the key to improve survival with this disease. Studies are beginning to demonstrate that patients with oesophageal cancer detected within a surveillance program have earlier stage cancers and improved long-term survival. Therefore, surveillance endoscopy after fundoplication in patients with CLO is recommended.

1.6.5. Management of dysplasia

Management of patients with low-grade dysplasia is difficult and controversial. Part of the reason is that it may regress, progress or remain static and it would be prudent not to give the benefit of doubt before considering resection surgery. Current thinking is that low-grade dysplasia should be managed by anti-reflux measures and surveillance. If high-grade dysplasia has been found and confirmed by at least two pathologists and persists in subsequent biopsies during acid-suppressing treatment with proton pump inhibitors, there are various opinions concerning management and treatment (Tytgat 1995). Most surgeons recommend oesophagectomy, as microinvasive adenocarcinoma may be present in the resected specimen in more than half of these patients (Pera et al 1992;Altorki et al 1991;Streitz et al 1993). Several authors have suggested that a very aggressive and frequent biopsy protocol can be established to replace immediate oesophagectomy with expectant management and moving to the former only when cancer is diagnosed (Reid et al 1988;Levine et al 1993). There are some conditions however, which would have to be met, in order to advocate this type of approach.

The first condition which has to be met is that the histological changes of high-grade dysplasia and intramucosal cancer must be reliably differentiated. A study by Reid (Reid et al 1988) points out the difficulty in doing this. They found that even with experienced pathologists there is 14% disagreement over this differentiation. The second important point is that even when high dysplasia is confirmed to be present, approximately 50-78% of these patients may harbour an occult cancer not detected on the surveillance biopsies. The rate of progression from high-grade dysplasia to invasive cancer can be as fast as 10 - 14 months (Blot et al 1993). But, intramucosal cancer is different in terms of biological behaviour from tumours that invade more deeply into the oesophageal wall, as lymph node metastasis and distant spread are uncommon. As a consequence, the 5-year survival for patients with intramucosal tumours differ significantly from submucosal tumours and may be amendable to endoscopic resection (Peters et al 1994; Nishimaki et al 1993). However, this type of approach would require that intramucosal tumours be distinguished from those that are more deeply invasive. Our current ability to do so is questionable. The resolution of the present day endoscopic ultrasound systems is not sufficient to predictably differentiate the fine detail of tumour infiltration in one-fifth of the patients (Peters et al 1994). Newer high frequency systems may improve on this, but at present it must be concluded that there is no reliable way to determine whether a tumour extends beyond the muscularis or not.

1.6.6. Management of adenocarcinoma limited to the mucosa

The development of surveillance programs for patients with CLO has resulted in a dramatic increase in the numbers of patients with tumours limited only to the mucosa. It is in these patients where adequate local therapy of early cancer can be curative. This has led to the application of a variety of new technologies.

The impact which surveillance programs have had on the overall survival in patients with oesophageal cancer was shown in a series by Peters (Peters et al 1994) which studied 52 patients with oesophageal cancer who underwent en bloc resection, 17 of whom were detected while under surveillance programs. Aside from early mortality due to operative complications, no recurrence or mortality was seen in the surveillance population while the majority, about 80%, of those with sporadically detected tumours died of recurrent disease. Of concern with respect to the management of patients with early cancers is the prevalence of lymph node metastasis in these patients. In one series, 186 patients operated on for CLO adenocarcinoma of all stages revealed that 18% of patients with intramucosal lesions had positive lymph nodes, compared to 39% with intramural lesions, and 87% in transmural tumours. Furthermore, 'jump' metastasis can occur in upto 10% of patients (Akiyama et al 1981; Akiyama et al 2000). Consequently, some surgeons perform an en bloc oesophagectomy with mediastinal lymph node dissection, for the treatment of patients with intramucosal tumours, and for patients with CLO and high-grade dysplasia since more than half of these patients will be shown to harbor an undetected intramucosal

tumour at the time for resection, but these patients require a less extensive resection. Unfortunately, results with immunohistochemical studies in some cases reveal micrometastatic disease in patients with histologically normal nodes of ~29% (Akiyama et al 2000).

1.6.7. Ablation of CLO mucosa

A lot of new exciting modalities have been developed to revert the columnar CLO by destroying the mucosa and allowing healing by squamous epithelium. The ablation of CLO mucosa may be thermal, chemical or mechanical. Laser therapy, Electrosurgery and Argon Enhanced Electrosurgery are thermal. Photodynamic therapy is chemical and surgical ultrasound is primarily mechanical. These methods differ only in the means of getting heat into the tissue and causing protein denaturation.

1.6.7.1. Thermal and Chemical Ablation

Carefully controlled ablation to a predetermined tissue depth requires the achievement of temperatures just above the threshold which is known to cause necrosis of 67% of the cells at the ablation boundary, but the depth of damage in thermal ablation is operator technique dependent and therefore variable. In monopolar, bi-polar and argon plasma electrocautery electric current flow causes tissue vapourisation (Kovacs et al 1997). In contact laser ablation, tissue damage is a function of power and exposure time, wavelength and type of laser. The laser can be more specific for CLO if the cells are first primed with porphyrin-based photoactive drugs - sodium porfimer (Photofrin, QLT), 5 aminolevulinic acid (5 ALA). Photodynamic therapy is a treatment in which the photoactive drug is administered 48 hours before the procedure and preferentially absorbed in highly proliferating cells as photosensitiser protoporphyrin, and subsequently activated by dye laser pumped by Argon, KTP or gold vapour lasers (Hinnen et al 1998; van den Boogert et al 1999). When the drug is activated, single oxygen and super oxide radicals are created which destroy cellular organelles thereby killing the tissue. In photodynamic therapy depth of treatment is limited to the penetration of the laser light and is therefore controllable. Unfortunately, due to systemic drug absorption especially in skin, patients must refrain from exposure to sunlight until the drug has been metabolised.

The physical modalities – electrocoagulation and laser are combined with PPI's or fundoplication and can result in reversal of BO in 75 – 100% of cases (Table 1.7). (Brandt et al 1995;Sampliner et al 1993;May et al 1999;Mork et al 1998;Gossner et al 1999;Byrne et al 1998;Dumoulin et al 1997;Barham et al 1997;Fremond et al 1995;Berenson et al 1993;Sampliner et al 1996;Kovacs et al 1997;Martin et al 1999;Jackson et al 1997). Photodynamic therapy also shows squamous regeneration in 66% - 80% and complete tumour destruction in upto 40% of T1 tumours, but little change in T2 tumours (Overholt et al 1999;Barr et al 1996;Regula et al 1995;Orth et al 1999;Savary et al 1998;Messmann et al 1997;Ackroyd et al 1999). Higher rate of reversal using these modalities can be achieved with greater injury to the mucosa, but this can cause stricture in 34 – 58% of cases and complications of stenosis or perforation may develop in 4 – 34% of these patients (Overholt et al 1996). In addition, 30 – 50 % patients have foci of intestinal metaplasia underlying squamous epithelium (Byrne et al 1998;Barham et al 1997). But as first line treatment for lowgrade and high-grade CLO these treatments offer a better survival when compared to 5 - 10% overall mortality with oesophagectomy and in patients with high-risk for surgery these treatment modalities are acceptable alternatives. However, a better understanding and role of these modalities is best understood in the in vivo models.

1.7. EXPERIMENTAL CLO.

Initial experimental work involved establishing models of CLO. In the last decade most of the experimental work has focused on carcinogenesis models, cancer cell lines and therapeutic intervention on these models.

1.7.1. Animal models of CLO

Hennessey and colleagues, in 1968, attempted to induce CLO epithelium in an animal model with no success. The main reason for failure of this model was due to short span of study of 4 weeks. It was only in 1970, that the landmark study of Bremner and colleagues reproduced the specialised columnar epithelium in an experimental canine model (Bremner et al 1970).

1.7.1.a. Canine models

Bremner group performed mucosal excision in lower oesophagus, hiatus hernia and cardioplasty in the canine model to induce CLO. Histamine was given to augment acid production. Their results showed that 'the columnar epithelium can cover part or whole of the animal's distal oesophagus after its mucosal lining has been excised. The extent to which this process develops appears to be intimately related to the presence or absence of gastroesophageal reflux. These experiments support the concept that the columnar-lined distal oesophagus (CLO) may be an acquired condition in which squamous epithelium is replaced, through creeping substitution by columnar cells of gastric or junctional origin' (Bremner et al 1970). In fact, this study demonstrated that

for the development of columnar lined oesophagus you need mucosal damage, incompetent lower oesophageal sphincter with chronic reflux, low gastric content clearance and increased acid production to induce maximal columnarisation of lower oesophagus. The length of study also suggested that at least 8 weeks are required after the mucosal damage for maximal changes. Other experimental models followed, which established beyond doubt that CLO epithelium can be acquired from abnormal and prolonged gastroesophageal reflux (Pollara et al 1983).

To establish the cell of origin and the nature of reflux producing CLO epithelium, Gillen and Hennessey in 1988, induced columnar epithelium in lower oesophagus in another canine model. 'In the presence of gastroesophageal reflux of either acid or a combination of acid and bile, regeneration of mucosal defects in the distal oesophagus was frequently by columnar epithelium. This columnar regeneration was not seen with bile reflux alone. By the use of squamous barriers, it was demonstrated that columnar re-epithelialisation may occur from cells intrinsic to the oesophagus and is not dependent on proximal migration of cardiac columnar epithelium. The cells of origin of this epithelium may be located in the oesophageal gland ducts and is likely to be a multipotent stem cell since the regenerated columnar epithelium may contain goblet cells and parietal cells not normally found in the oesophagus. These findings support the concept that Barrett's epithelium is metaplastic' (Gillen et al 1988). Other studies have reached similar conclusions (Van Nieuwenhove et al 1998). The role of reversing the gastroesophageal reflux was studied by Li and colleagues and they concluded that if both squamous and columnar cell types survive, a mixed pattern of regeneration may occur, but columnar repair will usually predominate because of its

more rapid turnover. If the squamous cells of the mucosa and ducts are destroyed, however, repair will be by columnar epithelium alone (Li et al 1994).

1.7.1.b. Rat models

Unfortunately, the debate on nature of cell of origin of CLO is far from resolved as endoscopic (Naef et al 1972;Goldman et al 1960) and iatrogenic gastrectomy studies (Hamilton et al 1977;Meyer et al 1979) suggest that columnar epithelium can replace denuded lower oesophagus by 'creeping' substitution. Rat models substantiate these theories, as rats do not have any submucosal glands in oesophagus and either squamous or columnar cells can migrate into areas not previously occupied by them (Wong et al 1971). The loss of supporting basement membrane is an important factor in producing the columnar epithelium which subsequently differentiates into a more primitive form, receiving fewer stimuli from the underlying tissue than the normal gastric epithelium. The regeneration of columnar epithelium of the oesophagus is also preceded by reflux oesophagitis and occurs more easily than that of squamous epithelium, suggesting that it is inherently more resistant to acid than squamous epithelium (Seto et al 1993).

1.7.1.c. Rabbit models

Much more work looking at reflux induced oesophagitis has been done in the rabbit with acid and pepsin exposure in acidic pH (Lanas et al 1999;Salo et al 1982) and with trypsin in alkaline pH (Mud et al 1982;Lillemoe et al 1983). It appears that injury to the oesophagus is more dependent on the pH of refluxate (pH<3) than on the

presence of pepsin (Pursnani et al 1998). Another mechanism of the mucosal injury is mediated by free radicals (Katada et al 1999), due to excessive and prolonged reflux of bile with entry and accumulation of bile acids into mucosal cells at acidic pH (Stein et al 1999), and bile salt absorption with disruption of the esophageal mucosal barrier at alkaline pH (Lillemoe et al 1983;Salo et al 1983;Schweitzer et al 1984;Schweitzer et al 1987). Prolonged acid and/or bile exposure leads to the development of adenocarcinoma.

1.7.2. CLO in oesophageal carcinogenesis models

Duodeno-gastroesophageal reflux or unacidified duodenal juice in isolation can induce CLO and oesophageal adenocarcinoma in experimental models (Melo et al 1999;Mirvish 1997). Other models also support a role of reflux of duodenal contents rather than gastric contents in the aetiology of oesophageal adenocarcinoma (Fein et al 1998;Miwa et al 1994;Mirvish et al 1993;Miwa et al 1996) (Chen et al 1999). Duodenal reflux with particularly the pancreaticoduodenal component has also been implicated in the gastric and gastric stump adenocarcinoma (Mason 186; Mason et al 1988). Not only that gastric acid blockade with omeprazole enhances duodenogastric reflux-induced carcinogenesis of the stomach and promotes growth stimulation of the oesophageal mucosa (Wetscher et al 1999). Therefore the presence of gastric juice in refluxed duodenal juice has a protective effect and acid suppression therapy may be detrimental in patients with duodenogastroesophageal reflux (Ireland et al 1996;Mirvish 1997). The mechanism by which duodenal-content reflux stimulates oesophageal carcinogenesis in experimental animals could due to biliopancreatic and

pancreatic reflux as both of these refluxes induce severe oesophagitis and increased oesophageal cell proliferation (Pera et al 1993;Pera et al 1998).

In 1991 Attwood and colleagues, studied the development of oesophageal adenocarcinoma using Nitrosamine to induce neoplasia in male Sprague-Dawley rats. Their results showed that the presence of duodenoesophageal reflux increased the frequency and changed the histological type of oesophageal cancer. This effect is more pronounced with pancreatic juice reflux (P < 0.05) and the combination of pancreatic and bile reflux (P < 0.05) but not with bile reflux alone. It appears that pancreatic juice is the most potent component of the duodenal refluxate (Yamashita et al 1998). This oncogenesis can also be potentiated by carcinogen methyl-n-amylnitrosamine, semipurified high-fat diet(Clark et al 1994), Diethylnitrosamine (Melo et al 1999), or with iron overnutrition (Chen et al 1999). The molecular mechanism of this oncogenesis has been studied using cancer cell lines.

1.7.3. Cancer cell lines

Cell lines from the resected specimens of patients with esophageal cancer have been used to study cell adhesion molecules – E-cadherin and α -catenin (Khare et al 1999), gap junctional intercellular communication and connexin (Garber et al 1997); inflammatory mediators, tumour suppressor genes - p53 gene (Matsubara et al 1999), Fas mediated apoptosis (Hughes et al 1997) and angiogenesis in Barrett's adenocarcinoma (Shimada et al 1992). These studies are helpful in understanding the molecular biology of the CLO epithelium and its progression into adenocarcinoma. Other animal models have been used to study aetiological agents and therapeutic interventions for preventing the progression of CLO into adenocarcinoma.

1.7.4. Carcinogenesis animal models and therapeutic interventions

In vivo models show that iron supplementation (Goldstein et al 1998), endogenous hypergastrinaemia (Karaki et al 1996), alcohol (Mufti et al 1997), N-nitrosononicotine (Gurski et al 1999) and zinc deficiency act as promoters of oesophageal carcinogenesis in the N-nitrosamine rat and mice model (Fong et al 1999;Newberne et al 1997;Newberne et al 1997). While studies with N-Methyl-N-Benzyl nitrosamine show that it is one of the aetiological agent for oesophageal adenocarcinoma (Li et al 1995;Mirvish 1995), with overexpression of cyclin D1 and cyclin E (Wang et al 1996) and disregulation of TGF-alpha and EGFR as important contributory factors (Wang et al 1996). Other studies show the chemopreventive role of the polyphenol fraction of black tea & green tea (Morse et al 1997;Wang et al 1995), garlic-derived diallyl sulfide (Wargovich et al 1993), flavonoids (Yang et al 1997;Tanaka et al 1997), and isothiocyanates (Wilkinson et al 1995;Stoner et al 1998;Stoner et al 1995;Siglin et al 1995).

Ablation of lower oesophageal mucosa by endoscopic cryotherapy device in swine model (Johnston et al 1999), photodynamic therapy with 5-aminolevulinic acid (ALA)-induced porphyrin accumulation in the rat model (van den Boogert et al 1999) and ultrasonic energy applied by laparoscopic Cavitron Ultrasonic Surgical Aspirator (CUSA) in a porcine model (Bremner et al 1998) have also been studied. Apart from

these few studies little experimental work has been done to evaluate therapeutic

intervention in CLO.

Chapter 2. Anatomical consideration of the gastrointestinal tract of rat and rabbit.

<u>Summary</u>

In vivo models of columnar-lined lower oesophagus (CLO) have been established in dog, pig, rabbit, rat, mouse and shrew. The aim of this study was to choose a suitable animal for use as a model of experimental CLO, and choose suitable operative techniques to cause selective reflux of gastrointestinal contents. The gastrointestinal tract and feasibility of operations were compared in cadaveric specimens of the rabbit and rat. The rabbit possessed the largest abdominal cavity (20 cm. Length, 14 cm. Width) compared to the rat (7.5 cm. Length, 5 cm. Width). The anatomy of proximal gastrointestinal tract – oesophagus, stomach, biliary drainage,

and mobility of the jejunum was similar in both animals for ease of dissection and gastrointestinal anastomosis.

The rat was chosen for the preliminary experiments as the forestomach in rat is squamous-lined and affords an easy access for mucosal stripping and gastrointestinal anastomosis.

Introduction

Continuing exposure of lower oesophageal squamous epithelium to mixed duodenal and gastric reflux inevitably leads to changes of columnar metaplasia in a large number of patients who have severe gastroesophageal reflux disease (Caldwell et al 1995). This columnar epithelium is genetically and morphologically unstable, and progresses to neoplasia if the stimulation by mixed refluxates continues (Aldulaimi et al 1999). Hence, various medical, surgical and physical ablation therapies have been used to reverse this progression or eradicate the columnar epithelium before and after irreversible changes develop. The results of these therapies can give conflicting results if the criteria's for diagnosing and staging CLO are not universally accepted or applied.

To establish correct parameters for treating and staging CLO it is necessary to understand the pathophysiology of disease progression and its precipitating reflux. Several studies in human patients have been performed to establish the exact nature of reflux causing CLO (Caldwell et al 1995;Bremner et al 1997). Results from retrospective and prospective studies have been also been used to establish the cell of origin of CLO (Rindi et al 1987;Meyer et al 1979;Shields et al 1993). However, no conclusive results have been demonstrated. This leads to conflicting and dichotomous theories. One of the ways of overcoming these problems is by establishing experimental models which can be universally reproducible.

Animal models of CLO are important to understand the pathophysiology of development of CLO. The importance is explained by the fact that when animal

studies show that a particular type of reflux causes CLO then clinical trials in human patients can be set up to evaluate the medical treatment and surgical procedure in order to reverse the pathology. Therefore, the choice of animal is important.

Previous animal models of CLO have utilised dog, pig, rat, mouse and shrew. Dogs are not used in our laboratories because they are not authorised species on the Certificate of Designation approved by the Home Office. The pig was not chosen as an animal model because it was felt that there would be a high incidence of anaesthetic and post-operative complications with this animal, and the large size of the pig, after 12 weeks of experiments, would make the animal difficult to manage. Mice and shrew were considered too small for surgical intervention. This study compared the anatomy of the gastrointestinal tract of two laboratory animals – the New Zealand White rabbit and the Springer-Dawley rat, in order to choose the most suitable animal for further studies.
<u>Aim</u>

The aim of this study was to choose appropriate animal for use *in vivo* model of CLO and define the most appropriate surgical anastomosis for reflux.

<u>Methods</u>

Four animals, two New Zealand White rabbits (mean weight, 2.8 kg) and two Springer-Dawley rats (mean weight, 350 g) were killed by Schedule 1 methods. The rabbits were killed by anaesthetic overdose of Sodium Pentobarbitone 60 mg/ml (Sagatal) via marginal ear vein. The rats were killed by CO2 inhalation and cervical dislocation.

For each cadaver, a midline abdominal incision was made from xiphoid process to the symphysis pubis to expose the abdominal cavity. The length and width of the abdomen were measured; the gastrointestinal tract of each animal was compared in each animal. The anatomy was identified and mobilisation of oesophagus, stomach, duodenum and jejunum was assessed to find the necessary steps in dissection. The oesophagus, stomach, biliary drainage and mobility of the jejunum were compared in both animals for ease of dissection and appropriate operations. The mobility of organs and feasibility of various gastrointestinal anastomosis was evaluated. The criteria used were: ease of dissection with intact vascular supply, tension free anastomosis with good vascular supply and good operation visibility.

Results

The rabbit possessed the largest abdominal cavity (20 cm. Length, 14 cm. Width) compared to the rat (7.5 cm. Length, 5 cm. Width) (Table 2.1). The anatomy of proximal gastrointestinal tract was similar in both animals (Figure 2.1). However, there were major differences in feasibility of gastrointestinal anastomosis.

Table 2. 1. Comparison of Cadaveric assessment of feasibility for oesophagointestinal anastomosis in rat and rabbit.

Measurements	Rat	Rabbit
Age (weeks)	8	20
Weight (g)	325 - 500	1500 - 3500
Abdominal cavity length (cm)	7.5	20
Width (cm)	5	14
Distance from OGJ to DJJ (cm)	5	10
Common bile duct length (cm)	1	3
Width (mm)	0.7	1.2
Cannula for duct (mm)	0.5	1
Feasibility of	Loop of jejunum very	Mobilisation of jejunum
oesophagojejunostomy	mobile	by dividing mesentery
Length of Roux-en-Y (cm)	2	4

OGJ - oesophagogastric junction

DJJ – duodenojejunal junction



Figure 2. 1. Anatomy of proximal gastrointestinal tract (a) rat cadaver, (b) rat, (c) rabbit.

The required anastomosis would be gastrojeunostomy, oesophagojejunostomy, duodenojejunostomy, choledocojejunostomy and jejunojejunostomy. Gastrectomy would be required in some cases. The jejunum in the rat has a very redundant mesentery allowing mobilisation upto relatively accessible oesophagus and stomach and the vascular supply would not be compromised. In rats there is no gall bladder present and biliary reflux would necessitate cannulation of the bile duct (Figure 2.2).



Figure 2. 2. Cannulation of the bile duct in rat with Portex Nylon 800/200/100® cannula (a) proximal gastrointestinal tract and insertion of cannula into bile duct, (b) cannula draining bile.

In rats the forestomach is squamous-lined therefore, mucosal stripping of the squamocolumnar junction and gastric anastomosis can be performed with greater ease (Figure 2.3). With aid of magnification loupes bile duct cannulation and anastomosis of antegrade oesophagojejunostomy, gastrojejunostomy, duodenojejunostomy and jejunojejunostomy can be performed easily with tension free suturing. In rabbits the squamo-columnar junction is at the oesophago-gastric junction. But in the rabbits larger size affords easy access and mobilisation of the oesophagus and jejunum. However, anterograde oesophagojejunostomy would be under tension and mobilisation of the jejunum is required for tension free anastomosis.



Figure 2. 3. Oesophageal mucosal stripping (a) oesophago-jejunal anastomosis posterior layer, (b) mucosa pulled for mucosectomy.

Discussion

This study was able to compare two animal species, both of which have been used extensively in animal studies. As an experimental model of gastrointestinal reflux and CLO with procedures involving surgery within the abdomen, it is important that the abdominal cavity be large enough to allow easy access to the operative field and safe dissection. This was best achieved with the rabbit.

Several experimental models of reflux oesophagitis have also been established in rabbit (Lanas et al 1999;Salo et al 1982;Lillemoe et al 1983;Salo et al 1983;Schweitzer et al 1984;Schweitzer et al 1987). It is possible to extend these models to develop CLO change by increasing the duration of study. However, the refluxate used in these studies is given orally and this would not imitate the physiological response as would occur in human CLO which develops due to mixed reflux of gastric and duodenal secretions in vast majority of patients (Caldwell et al 1995). The restriction in mobility and dietary habit would also cause difficulty in exaggerated stress response and may introducing selection bias towards more compliant animals. Therefore, only physiological reflux imitating the human pathophysiology should be used. This is best achieved by performing gastrointestinal anastomosis.

The gastrointestinal anastomosis for the gastrointestinal reflux in order to induce CLO should have the following properties: it should be local to the operative field, be of sufficient length to allow easy anastomosis, should be mobilised with an intact neurovascular supply to ensure that the anastomosis functions with normal peristalsis,

the direction of the anastomosis should allow passage of normal food for normal absorption and function of gastrointestinal tract while still inducing reflux. This was best achieved in rat.

In addition, to assess the cell of origin of CLO the squamocolumnar junction needs to be easily identified before and after the surgery. This is necessary to localise precisely the columnar changes, i.e., its progression or regression, with different refluxates. The squamo-columnar junction in rats is best suited for this as it lies in the forestomach. The squamous lined forestomach also requires little dissection and mobilisation for surgical anastomosis and mucosal stripping. This would substantially decrease the period for operation and anaesthetic time, thus, minimising postoperative and anaesthetic complications which may introduce bias.

In view of the results, the rat was chosen for preliminary experiments. After assessing results of the preliminary experiments various gastrointestinal anastomosis would be performed to induce gastrointestinal reflux, oesophagitis and CLO, in order to define the exact nature of reflux causing CLO, identify underlying cell of origin and study therapeutic manipulation with medical acid suppression and surgical anti-reflux surgery.

A further advantage of choosing rat is that the anatomy of rat is particularly suited to assessing the cell of origin. As there are no columnar lined glands in the rat oesophagus any columnar metaplasia must result from either encroachment from adjacent columnar epithelium or from stem cells in the basal layer of squamous epithelium. This metaplastic epithelium can therefore, be further analysed for

expression of squamous cytokeratin antibody and trefoil peptide to determine the exact cell of origin. The trefoil peptides are not expressed in normal oesophagus while cytokeratin antibodies are specific for squamous stem cell derived epithelium and are not found in normal columnar epithelium. In addition, the distribution of different trefoil peptides could be used to assess the changes of columnar metaplasia before and after acid suppression medication and anti-reflux surgery. The trefoil peptides are differentially expressed in various intestinal columnar epithelium (Hanby et al 1994) and therefore, exact morphology of experimental CLO epithelium can be compared to human CLO epithelium and to other intestinal epithelium.

Conclusion

When comparing the rabbit and the rat, both animals possessed features suitable for use as animal model of gastrointestinal reflux and CLO. When comparing the various anastomosis, access for oesophageal mucosal stripping and ease of operation both animals were found suitable. Anatomy of rat, however, affords easy access to the squamo-columnar junction and therefore, preliminary experiments were to be performed on it.

Chapter 3. Jejunogastric reflux and columnar metaplasia.

Summary

Aim. A suitable model of CLO must closely imitate the pathology of severe gastroesophageal reflux disease and reflux oesophagitis causing CLO. This study aims to develop CLO by reflux of mixed gastric and duodenal secretions by anastomosing first part of jejunum to gastrotomy and mucosal stripping of squamouslined forestomach to create iatrogenic ulcer.

Method. Two groups of animals were studied. Group 1 (Control) underwent sham laparotomy. Group 2 (Jejunogastric reflux – JGR) underwent gastrotomy, squamous mucosal stripping and gastrojejunostomy. Physiological assessment of preoperative and postoperative weight, pH, blood and oesophageal bile acid concentration was performed for each animal. After conclusion of the study, gastrojejunostomy anastomosis was examined by an experienced pathologist, blinded to the experiment, with Haematoxylin and Eosin (H & E) staining for columnar metaplasia.

Results. Fourteen specimens were collected during the study period of 6 months [Length of study in days (mean \pm SEM) Group 1 (198 \pm 13.70), Group 2 (189 \pm 18.19)]. There was no significant difference for preoperative and postoperative pH, serum bile and gastric bile concentration in Group 1 (Control) (all p > 0.05). In Group 2 (JGR) there was no difference between preoperative and postoperative pH values and gastric bile concentration [preoperative pH (8.16 \pm 0.23), postoperative pH (8.4 \pm

1.55) preoperative gastric bile acid (μ mol/l) (31.25 ± 9.32), postoperative gastric bile (44.5 ± 8.66), but the postoperative serum bile acid concentration was increased [preoperative serum bile acid (μ mol/l) (58 ± 35.04), postoperative serum bile acid (508.33 ± 202.50)].

Histological assessment with H & E staining in Group 1 revealed normal squamous mucosa in all cases. In Group 2 columnar metaplasia of squamous mucosa with marked acute and chronic inflammation was seen in all animals. The metaplastic epithelium was of gastric fundic type.

Conclusion.

- 1. This model closely imitates the pathophysiology of severe gastroesophageal reflux disease and regeneration by columnar metaplasia at squamo-columnar junction.
- 2. In the presence of chronic mixed jejunogastric reflux columnar metaplasia can replace squamous epithelium at the squamo-columnar junction in all cases.
- 3. Mixed reflux of gastric and duodenal contents can produce 'neutral' pH reflux. Asymptomatic 'neutral' pH reflux can cause columnar metaplasia.
- 4. Cells for columnar metaplasia can arise by encroachment from adjacent gastric columnar epithelium.

Introduction

The pathophysiology of the CLO is not clearly understood. The exact nature of reflux causing the columnarisation of squamous oesophageal epithelium in humans is not clearly defined, but is believed to involve reflux of mixed duodenal and gastric contents (Kauer et al 1995;Stein et al 1993;Iftikhar et al 1995;Clark et al 1997). Clinical and experimental studies strongly support the role of duodenal (bile and pancreatic) secretions in the development of complications of gastroesophageal reflux disease and neoplastic progression of columnar metaplasia (Mud et al 1982;Lanas et al 1999;Salo et al 1982;Lillemoe et al 1983). But, the role of mixed reflux in inducing changes from reflux oesophagitis to columnar metaplasia is little studied.

Furthermore, various theories have been proposed for the cell of origin of columnar metaplasia (Bremner et al 1997). It is believed to arise from multipotent stem cell in squamous or columnar epithelium or as replacement columnar epithelium from any adjacent columnar epithelium. There is evidence to support these various theories in clinical and experimental studies. The most convincing candidate is multipotent stem cell because the columnar metaplasia in humans can exhibit characteristics of secretory neuroendocrine and intestinal type epithelium (Rindi et al 1987). The phenotype and flow cytometric analysis shows that the columnar metaplastic cell is immature primitive cell which is more resistant to acid but genetically unstable and in presence of continued stimulus becomes dysplastic (Aldulaimi et al 1999). The origin of this cell lineage is not clearly established. As there are no wild type models of CLO several groups have developed animal models using various techniques in different animals to study the nature of reflux and cell of origin of columnar metaplasia. Gillen et al and Bremner et al developed canine model of CLO by mucosal stripping and cardioplasty (Bremner et al 1970;Gillen et al 1988). Seto & Kobori obtained CLO in rats by repeated mucosal injury (Seto et al 1993). While Wong & Finckh induced heterotopic and ectopic islands of squamous or columnar epithelium, around squamo-columnar junction in the rat, by mucosal incisions (Wong et al 1971). Columnar metaplasia was induced in variable number of animals in these studies. Therefore, therapeutic interventions is difficult to evaluate and a more suitable model of CLO is required that can induce columnar metaplasia in all cases. It is possible to combine these various models to induce CLO in all the animals.

After preliminary cadaveric assessment Springer-Dawley rat was found to be most suitable animal for inducing columnar metaplasia at the squamo-columnar junction and was chosen for initial study in order to cause reflux of mixed jejunogastric reflux, severe peptic ulcer disease and healing by columnar metaplasia. The squamocolumnar junction in the rat lies at the junction of forestomach and distal glandular stomach and therefore it provides excellent opportunity to induce and assess changes at the mucosal squamo-columnar junction (Richardson et al 1994).

Aim

The aim of this study was to set up an experimental model of CLO in rat and define the most appropriate surgical anastomosis for reflux of gastrointestinal contents. Therapeutic intervention with Proton pump inhibitor omeprazole was also studied to evaluate role of decreasing the reflux in this model.

Methods

Twenty Springer-Dawley male rats were selected for the study. Group 1 (Control – 10 animals) underwent sham Laparotmy. Group 2 (JGR – 10 animals) were operated on to produce jejunogastric reflux and columnar metaplasia of squamous-lined forestomach.

Physiological assessment

After acclimatisation period of one week, physiological assessment of intraoesophageal pH, oesophageal aspirate for bile acid measurement and venous blood sample for serum bile acid estimation was performed for each animal. Animals were first anaesthetised in induction chamber using Halothane. Degree of anaesthesia was assessed by depth and rate of breathing pattern and web space pinching. After induction, anaesthesia was maintained by mask and physiological measurements performed. Intraoesophageal pH was measured using MicroDigitrapper ambulatory pH monitoring system (Synectics Medical) using monocrystant antimony paediatric pH catheter (Synectics Multi-use pH Catheter). The probe was calibrated using standard Synectics pH 7.01 and pH1.01 buffer solutions. The reference electrode was placed over shaved area of the abdomen using electrode gel. The pH catheter was then inserted transorally into oesophagus and pH measurement taken. Intraoesophageal aspiration for measurement of intraoesophageal bile acid was performed by injecting 5 ml Normal Saline enterally by gavage and aspirating the

solution. Venous blood for estimation was obtained from rat-tail vein and centrifuged at 1000rpm for three minutes to obtain serum. Oesophageal and serum bile acid concentration were measured using the Enzabile test kit (*see* Appendix 2).

Operative technique

One to two weeks after the physiological assessment both groups had surgery. Solid food was withdrawn for 24 hours prior to the procedure.

Each animal was anaesthetised in the induction chamber using Halothane and oxygen. After induction the abdominal hair were shaved and skin prepared with betadene aqueous solution. Halothane & oxygen anaesthesia was maintained using mask. In Group 1 (Control) upper midline laparotomy was performed and closure was in two layers using PDS 4.0 suture. In Group 2 (JGR) upper midline laparotomy was performed to mobilise stomach. Gastrotomy was performed at the level of squamocolumnar junction. The gastric contents were partially evacuated. The squamous mucosa at the junction was then stripped (3 mm by 3 mm). Proximal jejunal loop was then mobilised to close the gastrotomy by gastrojejunostomy using prolene 6.0 sutures. The anastomosis was assessed for patency and integrity and laparotomy was closed in two layer using PDS 4.0 suture.

Post operative management

During recovery all the animals were given Temgesic 0.5 ml subcutaneously (s/c) and kept in Recovery room with stable temperature of 28° C. Water was provided *ab libitum*. After overnight recovery the animal was inspected for distress. Signs of surgical distress were assessed by posture, movement and withdrawl pattern of the animal. If the animal exhibited signs of surgical distress Temgesic 0.5 ml s/c was administered. The suture line and abdominal girth were inspected for integrity and postoperative problems.

Solid food was then administered if the animal showed favourable recovery and transferred to ordinary room. Omeprazole elixir (1.4 mg/kg/d) was administered by enteral feed to half of the animals in each group. The study was terminated at 4 months. If the animal died before completion of the study a post-mortem examination was performed to establish the likely cause of death.

Post-operative physiological assessment

At the end of study each animal underwent physiological assessment for weight, pH, intraoesophageal and serum bile concentration prior to termination by Schedule 1 method.

Histopathological preparation

At autopsy, lower 2 cm of the oeosphagus and proximal stomach with anastomosis was removed en bloc and paraffin wax blocks and slides were produced (*see* Appendix 3). The sections were then stained using the Haematoxylin and Eosin method (*see* Appendix 3). An experienced pathologist blinded to the experiment examined the slides. Microscopic evidence of oesophagitis, degree of acute & chronic inflammation and length of columnar metaplasia was scored according to a grading system (Appendix 3).

Statistical Analysis

The results were analysed using SRS and ARCUS statistical packages. The mean changes in values from pre-operative to end of study for comparison between different groups were compared, for pH data, weight measurements, blood bile acid concentration, oesophageal aspirate bile acid concentration, using the single-tailed Student t-test. Values were expressed as means \pm SEM. Microscopic histopathology was compared using the Mann-Whitney U test. Statistical significance was designated at the 0.05 level.

Results

Fourteen specimens were collected over a period of six months [Length of study (days \pm SEM) Group 1(198 \pm 9.80 days) Group 2 (189 \pm 18.19 days)].

There was significant weight gain in all animals (all p > 0.05) (Table 3.1)(Figure 3.1). In Group 1 (Control) there was no significant difference between preoperative and postoperative values for pH, blood bile or oesophageal bile (all p > 0.05) (Table 3.1) (Figure 3.1 & 3.2). In Group 2 (JGR) pH values and oesophageal bile acid concentration were not significantly altered (p > 0.05), but serum bile acid concentration decreased after surgery (Table 3.1).

Comparison between PPI administered subgroup and other was not made, as the number of surviving animals was very small.

Table 3. 1. Physiological Assessment for Group 1 (Control) and Group 2 (Jejuno-gastric reflux).

Group1

	Weight (gms.)	рН	Serum Bile (µmol/1)	Gastric Bile (µmol/l)
Preop	357.8 ± 20.38	7.95 ± 0.13	359.2 ± 114.42	27.4 ± 5.19
Postop	659.6 ± 14.93	7.83 ± 0.40	328 ± 59.31	40 ± 8.74
p value	< 0.05	> 0.05	> 0.05	> 0.05

Group 2

	Weight (gms.)	рН	Serum Bile (µmol/l)	Gastric Bile (µmol/l)
Preop	425.67 ± 30.68	8.3 ± 0.16	401.25 ± 135.28	31.25 ± 9.32
Postop	652.75 ± 25.87	8.2 ± 0.66	62.25 ± 14.92	44.5 ± 8.66
p value	< 0.05	> 0.05	< 0.05	> 0.05





Figure 3. 1. Weight and pH changes in Group1 (Control) and Group2 (Jejunogastric reflux – JGR) before and after surgery.





Figure 3. 2. Serum bile acid and gastric bile acid concentration before and after surgery in Group1 (Control) and Group2 (JGR).

Histopathology

In Group 1 (Control) all animals had normal squamous mucosa with no signs of acute or chronic inflammation. In Group 2 (JGR) all animals had columnar metaplasia of squamous mucosa associated with acute and chronic inflammatory infiltrates. In one specimen > 50% of length of specimen had columnar metaplasia, in others < 50% of length had columnar changes. The columnar metaplastic epithelium was of gastric fundic type.

Morbidity and Mortality

In Group 2 (JGR) six animals died due to various anaesthetic and post surgical problems. The causes of death were: anaesthetic induction, wound breakdown, anastomotic leak, anastomotic dehiscence, aspiration and obstruction. Scybalous impaction of solid material at the anastomotic site was the most common reason for the post surgical deaths. Nearly all deaths occurred within the first 48 hours. One animal survived for 14 days. Post mortem examination revealed anastomotic dehiscence with pneumoperitoneum.

As the first five deaths were all attributed to the obstruction at the site of anastomosis it was felt that decreased alimentation prior to the main operations would be helpful. After discussing with a named Veterinary Surgeon, it was decided to administer pineapple juice for five days preoperatively to help with digestion of hair collagen. Liquid diet consisting of Complan feed would be instituted for five days preoperatively. Animals would be starved for 24 hours before operation. Complan

liquid feed would continue into the post operative period for 2 weeks when a mixed diet of solid and liquid feed would be given. One week after the mixed diet solid food would be recommenced. To decrease corpophagia animals would be kept on grid cage for 48 hours preoperatively and continued for 5 post-operative days.

Validation of result

Animals in Group 1 (Control) were used to establish a baseline for comparison of changes in the animals with gastrointestinal reflux. They show that there is a very wide variation in the physiological parameters between individual animals. This might prejudice assessment of results between various groups. The changes in the physiological parameters were observed between preoperative results and postoperative results. These did not reach statistical significance. However, they do suggest that any surgical intervention may alter the physiology of the animal; this is probably related to surgical and environmental stress. Other reason for these changes is due to the fact that older animals may have different physiology or that sampling errors may become more pronounced if physiological evaluation is performed too quickly. Other reason for the wide variation in physiological parameters is related to technical and operator dependent problems. Overuse of pH catheter can cause false readings. The calibration errors become more pronounced with older buffer solutions.

The reason for wide variation in the blood bile estimation is related to the fact that if blood serum is contaminated with haemolysed blood then a very high false value for blood bile concentration may be observed. To decrease this possibility serum was separated from the blood on the day of the procedure. As the quantity of serum and aspirate for measurement of bile acid concentration was very little it was felt that any error due to concentration would be universal and would not be statistically apparent.

Discussion

Clinical studies of CLO are subject to patient presentation bias, subjective bias of the researchers and lack of universally applicable diagnostic criterias for assessing changes in length and metaplasia of replaced columnar epithelium (Narbona-Arnau et al 1994). The cause-effect relationship between degree and type of reflux and development of columnar metaplasia is also poorly defined. Part of the reason for this is that it is difficult to obtain reliable data on this cause-effect relationship from clinical studies that must not only be longitudinal and prospective but also cover a long period of time and involve a great many patients and data (Skinner 1990). This requires multicenter efforts that tend to accumulate subjective variables, thus affecting the significance of the results obtained (Giuli et al 1988).

Experimental research could overcome several of these problems. But it is difficult to extrapolate experimental results to humans because experimental techniques create artificial milieu far removed from presumed pathophysiology that leads to columnar metaplasia in human (Narbona-Arnau et al 1994). For this reason many experimental studies have only variable number of animals that develop columnar metaplasia. To be able to closely imitate the presumed human aetiology of CLO experimental studies must reliably and consistently induce columnar metaplasia in all cases using gastric and duodenal secretion. The only way to rectify this problem is by using *in vivo* techniques to cause reflux of physiological secretions.

The model developed in this study overcomes aforementioned problems and uses physiological secretions foreign to squamous epithelium to induce columnar

metaplasia in all cases. This preliminary study shows that iatrogenic ulcer in squamous epithelium at squamo-columnar junction, in the presence of chronic gastrointestinal reflux, heals by acute & chronic inflammation and columnar metaplasia. Therefore, this model imitates human *in vivo* situation of severe gastroesophageal reflux disease, peptic ulceration, reflux oesophagitis and development of CLO in the presence of chronic mixed reflux.

Furthermore, with mixed gastrointestinal reflux there is increased bile reflux into the oesophagus and systemic absorption of bile acid into circulation. But the pH may not significantly altered. This is found in clinical studies of CLO patients. This is due to the fact that gastrointestinal refluxate contains a mixture of acidic gastric and alkaline duodenal, bile and pancreatic secretions and therefore a mixed may only produce 'neutral' pH reflux (Caldwell et al 1995;Hirschowitz 1996;Bechi et al 1993;Kauer et al 1997). The 'neutral' pH reflux is asymptomatic and may lead to delay in diagnosis of CLO in majority of patients (Johnson et al 1987;Bremner et al 1993;Bremner et al 1993). In this model 'neutral' reflux produces columnar metaplasia. Hence, a large proportion of patients with CLO may have continuing damage due to chronic 'neutral' pH reflux that continues to exert stimulus on metaplastic columnar epithelium leading to neoplastic changes, yet remain asymptomatic or have minimal symptoms. 'Neutral' pH reflux could be reason for late presentation of some lower oesophageal adenocarcinoma and causes difficulty with diagnosis.

Use of symptom index for diagnosing and screening for adenocarcinoma of lower oesophagus or CLO is of little value where 'neutral' pH reflux is present (Johnson et al 1987;Bremner et al 1993;Bremner et al 1993). Diagnosis and screening for CLO

should rely on oesophagoscopy and definite histological diagnosis of not only columnar metaplasia but also specialised columnar epithelium.

Columnar epithelium of lower oesophagus can be found in many normal individuals, as islands or as circumferential or finger like projections extending from squamocolumnar junction – the Z line. Columnar lining of the oesophagus can also develop in patients after total gastrectomy with a loop jejunostomy reconstruction (Meyer et al 1979), after an Ivor-Lewis type of reconstruction following oesophagogastrectomy (Hamilton et al 1977). Columnar epithelium replacement can also occur by submucosal mucous gland replacement (Gillen et al 1988). Furthermore, several longitudinal studies of columnar epithelium in oesophageal strictures document gradual encroachment and advancement of columnar epithelium to proximal oesophagus (Naef et al 1972;Goldman et al 1960). This suggests that any columnar epithelium can replace the squamous epithelium and this advancing edge is capable of continuous advancement in presence of chronic reflux. Our study supports this hypothesis.

Other experimental and clinical studies suggest that the columnar epithelium arises from squamous epithelium. Comparison of the inlet patch columnar epithelium seen in upper oesophagus with the columnar lining of CLO patients shows that the specialised columnar epithelium originates from a very immature multipotent stem cell (Feurle et al 1990), and it is believed that the process leading to Barrett's esophagus does not imply 'creeping substitution from the cardio-fundus mucosa' ascending to cover a mucosectomized area, but rather metaplasia of superficial cells of oesophageal glands (Gillen et al 1988). Our results disagree with these findings and

suggest that columnar epithelium at the squamo-columnar junction arises by encroachment.

One of the reasons for different results with experimental studies is related to the fact that many of these do not imitate human oesophageal injury secondary to chronic reflux but use augmented acid secretion or administration of oral acid and/or alkali to achieve columnar epithelium. This situation does not reproduce the presumed aetiology and pathology of reflux oesophagitis or CLO and therefore does not produce columnar metaplasia in all cases studied. Our model overcomes these problems by initially imitating severe gastroesophageal reflux disease, ulceration and peptic oesophagitis, and in this model columnar metaplasia is found universally. Therefore, analysis of columnar epithelium in this model and its morphology is more reflective of human CLO before dysplasia develops.

As there are no columnar-lined oesophageal glands in rat oesophagus and the regenerative columnar epithelium is of gastric type and squamous mucosa underwent mucosectomy it is unlikely that the gastric type columnar metaplasia at the squamo-columnar junction of rat arises from either basal cell layer of squamous epithelium, columnar cell-lined glands or from stem cell arising from squamous epithelium. On the other hand our results support the findings from early clinical studies which show ascent of squamo-columnar junction in presence of chronic reflux (Naef et al 1972;Goldman et al 1960), and experimental findings of Wong & Finckh which show that either squamous or columnar epithelium is capable of becoming heterotopic or ectopic (Wong et al 1971). Furthermore, our results agree with findings of Richardson et al that the replacement epithelium is of gastric type near the rat squamo-columnar

junction (Richardson et al 1994). Also study of classic Barrett's epithelium by Paul et al suggests that the specialised columnar epithelium is found as a leading edge of columnar metaplastic epithelium and has gastric fundic epithelium near the gastric epithelium (Paull et al 1976). Our study also shows that the metaplastic epithelium is of gastric type and agree with other experimental studies that suggest that the regenerative epithelium is related to type of epithelium surviving after injury and columnar epithelium is predominant because of its high turnover (Li et al 1994). Hence, CLO with specialised columnar metaplastic epithelium is reflective of increased proliferation in presence of continuing stimulus. It is hoped that if the stimulus is removed then proliferation of columnar epithelium should revert to more stable, mature phenotypes of columnar epithelium. Further experimental studies have been performed to assess role of reversing the chronic reflux on CLO.

Chapter 4. Animal model of Columnar-lined lower oesophagus and role of proton pump inhibitor in reversing the reflux.

Summary

Aim. In order to induce Columnar-lined lower oesophagus (CLO) in the squamouslined lower oeosphagus mucosal stripping can be performed to create iatrogenic ulcer and reflux of gastric, duodenal, biliary and pancreatic contents into the lower oesophagus can be achieved by anastomosing the first part of jejunum to oeosphagus end-to-side. Thus, combination of reflux and mucosal stripping would imitate *in vivo* situation of severe peptic ulcer disease, severe reflux oesophagitis and CLO. This model can be used to study role of proton pump inhibitor (PPI) omeprazole in reversing reflux, inflammation and CLO for different refluxates.

Method. *In vivo* CLO models were established surgically in Sprague-Dawley rats by physiological refluxate of gastric, bile, duodenal secretions; and mixed gastric + bile, gastric + duodenal, gastric + pancreatic, duodenopancreaticobiliary, duodenogastroesophageal and jejunoesophageal reflux. Half of the animals in each reflux group were administered oral PPI during the whole of postoperative period. After 4 months the lower oesophagus was examined with H&E stain, diastase PAS and immunostaining for length and intensity of columnar change and severity of inflammation by three experienced pathologists blinded for the procedure.

Results. Inflammation and columnar metaplasia of the lower oesophagus was seen in all groups. The columnar epithelium had columnar cells with pseudo-absorptive brush border and mucin positive goblet cells. Ectopic columnar and squamous islands, misplaced glands, mixed squamocolumnar glands, ulcer associated cell lineage buds, acini and glands were seen at the regenerating squamo-columnar junction. Length of columnar change in gastric reflux (GR) and duodenal reflux (DR) was significantly longer than all other groups [GR (length in cm \pm SEM) (1.3 \pm 0.2) (all p < 0.05), DR (1.17 \pm 0.1) (p < 0.001)]. The severity of inflammation and metaplastic change was higher in the gastric and biliary dominant groups as compared to the other groups. Although, PPI treatment with omeprazole (OMP) did not cause significant change in CLO there was a trend towards longer CLO length in duodenal dominant reflux (DDR) group and shorter CLO length in gastric dominant reflux group (GDR) and biliary dominant reflux group (BDR) compared to untreated group [Columnar mucosa (length in cm \pm SEM) No OMP GDR (1.17 \pm 0.05), DDR (1.05 \pm 0.05) BDR (1.07 \pm 0.17), OMP GDR (1 \pm 0.13), DDR (1.08 \pm 0.15), BDR (0.83 \pm 0.11) (all p > 0.05)].

Conclusion.

- Reflux of pure gastric, pure duodenal, pure biliary secretions or mixed refluxates can produce columnar metaplasia in oesophagus.
- 2. Reflux of gastric or duodenal contents produced metaplasia of much greater intensity and extent compared to other groups.
- 3. PPI Omeprazole increased columnar metaplasia in duodenal dominant reflux group, and decreased in gastric dominant and biliary dominant reflux group.
- Duodenal reflux may be the cause of ineffectiveness of PPI -Omeprazole in a significant number of CLO patients.

Introduction

The study in previous chapter showed that columnar epithelium can replace squamous epithelium in presence of jejunogastric reflux. The metaplastic columnar epithelium was of gastric fundic type suggesting that healing at squamo-columnar junction can occur by encroachment of columnar epithelium from adjacent columnar epithelium, but the exact phenotype of this epithelium was not evaluated. Furthermore, Jejunogastric reflux model did not imitate the human *in vivo* situation of developing columnar metaplasia proximal to gastroesophageal muscular junction. Hence, therapeutic interventions to prevent development of columnar metaplasia in lower oesophagus cannot be studied adequately using that model. Therefore, CLO model needs to induce columnar metaplasia proximal to gastroesophageal junction by imitating the presumed pathophysiology of human CLO.

Oral administration of acid/alkali or increased acid production combined with iatrogenic damage to gastroesophageal sphincter has been used to induce CLO in animals, but these models do not replicate pathophysiology of gastroesophageal reflux disease (Bremner et al 1970;Gillen et al 1988). Furthermore, pathological CLO is preceded by oesophagitis. Hence, it would be better to induce oesophagitis first. Oesophagitis models have been developed by Levrat et al using reflux of physiological gastrointestinal secretions by surgical gastrointestinal anastomosis (Levrat et al 1962). Similar model was created to study gastric adenocarcinoma and gastric stump adenocarcinoma by Mason et al (Mason 1986; Mason et al 1988). Hence, Levrat model of oesophagitis and Mason model of gastric adenocarcinoma can

be adapted to induce CLO using reflux of physiological secretions and study therapeutic intervention to prevent development and progression of CLO.

The therapeutic intervention in CLO patients most commonly involves reversing or suppressing the reflux using histamine H-2 blockers and proton pump inhibitors. But some of these patients have a reduced sensitivity to acid reflux and disappearance of symptoms may not correlate with efficient control of acid reflux (Ortiz et al 1999). Though long-term proton pump inhibition effectively heals patients with oesophagitis (Hetzel et al 1988;Bate et al 1990;Bardhan 2000;Bardhan 1995), control of acid exposure is inadequate for regression of CLO epithelium, as 34 – 58% of patients on long-term acid suppression may develop CLO (Wetscher et al 1997;Sharma et al 1997). Medical efforts to reverse CLO by decreasing gastroesophageal reflux have so far been disappointing and give mixed results. Part of the reason for this is related to decreased acid clearance in CLO patients (Fiorucci et al 1989).

Regression or non progression of CLO has been reported in some studies using proton pump inhibitor, but macroscopic squamous islands are commonly found in columnar lined lower oesophagus in these patients (Cooper et al 1998;Malesci et al 1996). Biopsies of these islands show that intestinal metaplasia may still be present in upto a third of patients and has the potential to progress (Sharma et al 1998)(Garewal et al 1999). Role of proton pump inhibitor in development of these 'squamous islands' with different refluxates and morphology of regenerated squamous epithelium is little studied under experimental conditions.

<u>Aim</u>

The aim of this study was to induce Columnar-lined lower oesophagus in rat animal model by surgically causing selective or combined reflux of upper gastrointestinal tract secretions – the gastric, biliary, pancreatic and duodenal secretions. Therapeutic intervention with proton pump inhibitor omeprazole will be studied to evaluate role of decreasing the gastric component of gastrointestinal reflux.

Methods

One hundred Springer-Dawley male rats were selected for the study and divided into 10 groups of ten animals each.

Physiological assessment

After acclimatisation period of one week, physiological assessment of intraoesophageal pH, oesophageal aspirate for bile acid measurement and venous blood sample for serum bile acid estimation was performed for each animal. Animals were first anaesthetised in induction chamber using Halothane, anaesthesia maintained by mask and physiological measurements performed. Intraoesophageal pH was measured using MicroDigitrapper ambulatory pH monitoring system with monocrystant antimony paediatric pH catheter (Synectics Medical). The probe was calibrated using standard Synectics pH 7.01 and pH1.01 buffer solutions. The reference electrode was placed over shaved area of the abdomen using electrode gel. The pH catheter was then inserted transorally into oesophagus and pH measurement taken.

Intraoesophageal aspiration for measurement of intraoesophageal bile acid was performed by injecting 5 ml Normal Saline enterally by gavage and aspirating the solution. Venous blood for estimation was obtained from rat-tail vein and centrifuged at 1000rpm for three minutes to obtain serum. Oesophageal and serum bile acid concentration were measured using the Enzabile test kit (*see* Appendix 1).

Preoperative preparation

Alimentation of gastrointestinal tract was reduced by administering liquid Complan feed for five preoperative days. To help digestion of hair collagen pineapple juice was mixed with Complan. To decrease coprophagia animals were maintained on the grid cage for 48 hours and food withdrawn for 24 hours prior to the operation.

Induction and maintenance of anaesthesia

Each animal was anaesthetised in the induction chamber using Halothane and oxygen and anaesthesia maintained using mask and Halothane & oxygen mixture.

Operative technique

An upper midline laparotomy was performed in all animals. The oesophagus was mobilised, heavy tied (Vicryl 2.0) placed at the oesophagogastric junction and transected above the tie. The lower posterior mucosa of the oesophagus was stripped in 2mm X 2mm square shape. Different oesophago-intestinal anastomosis was performed for each group (*see below*). Vagus nerve was preserved in each case. The anastomosis was checked for integrity and patency and laparotomy closed in twolayer with PDS 4.0 suture.

Group 1 (Control)

Ten animals in this group had simple laparotomy performed.

<u>Group 3 (Jejunoesophageal reflux – JOR or duodenogastroesophageal reflux –</u> <u>DGOR) (Figure 4.1a)</u>

Ten animals in this group had end-to-side interrupted single layer (Ethilon 6.0 suture) oesophagojejunostomy anastomosis performed by mobilising the 1st part of jejunum.

<u>Group 4 (duodenal, bile and pancreatic reflux – DPBR) (Figure 4.1b)</u>

Gastrectomy and end-to-side oesophagojejunostomy was performed in this group.

<u>Group 5 (gastric reflux – GR)</u> (Figure 4.1c)

All ten animals in this group underwent end-to-side gastrojejunostomy, end-to-side oesophagojejunostomy, and duodenal diversion by side-to-side jejunojejunostomy.
<u>Group 6 (gastroduodenal reflux – DGR) (Figure 4.1d)</u>

The stomach and first part of duodenum was isolated by placing a heavy tie (Vicryl 1.0) at the first part of duodenum. The ten animals then had end-to-side duodenojejunostomy, en-to-side oesophagojejunostomy and pancreatico-biliary diversion performed by side-to-side jejunojejunostomy.

<u>Group 7 (duodenal reflux – DR) (Figure 4.1e)</u>

Gastrectomy and isolation of first part of duodenum by placing Vicryl 1.0 tie, end-toside duodenojejunostomy, end-to-side oesophago-jejunostomy and side-to-side jejunojejunostomy was performed to cause reflux of pure duodenal contents.

<u>Group 8 (gastroduodenopancreatic reflux – DGPR) (Figure 4.2b)</u>

In this group of ten animals end-to-side oesophagojejunostomy with a distal biliary diversion was performed. The biliary diversion was achieved by cannulating the bile duct with *Portex Nylon 800/200/100*® cannula and performing distal choledocojejunal anastomosis.

<u>Group 9 (duodenopancreatic reflux – DPR) (Figure 4.2c)</u>

All ten animals in this group underwent oesophagojejunostomy, gastrectomy and distal biliary diversion by choledocojejunostomy.

<u>Group 10 (biliary reflux – BR)</u> (Figure 4.2d)

Pure bile reflux was achieved in ten animals by cannulation of the bile duct with *Portex Nylon*® cannula, choledocojejunostomy and oesophagojejunostomy. Distal jejunojejunostomy was used to divert gastric, duodenal and pancreatic secretions.

<u>Group 11 (gastric biliary reflux – GBR) (Figure 4.2e)</u>

Gastric and biliary reflux was achieved by end-to-side gastrojejunostomy, cannulating the bile duct with *Portex Nylon*® cannula, end-to-side choledocojejunostomy and oesophagojejunostomy. Distal jejunal diversion required side-to-side jejunojejunostomy.

Post operative management

During recovery all the animals were given Temgesic 0.5 ml subcutaneously (s/c) and kept in Recovery room with stable temperature of 28° C. Water was provided ad libitum. After overnight recovery the animal was inspected and Temgesic 0.5 ml s/c administered if distressed. The suture line and abdominal girth were inspected for integrity and postoperative problems. Solid food was then administered if the animal showed favourable recovery and transferred to ordinary room. Omeprazole elixir (1.4 mg/kg/d) was administered by enteral feed to half of the animals in each group. The study was terminated at 4 months. If the animal died before completion of the study a post-mortem examination was performed to establish the likely cause of death.



Figure 4.1. Schematic diagrams of surgical anastomosis for (a) JOR – Jejunoesophageal reflux, (b) DPBR – Duodenopancreaticobiliary reflux, (c) GR – Gastric reflux, (d) DGR – Gastroduodenal reflux, (e) DR – Duodenal reflux.



Figure 4. 2. Schematic diagrams of surgical anastomosis for (a) Bile duct cannulation, (b) DGPR – Duodenogastropancreatic reflux, (c) DPR – Duodenopancreatic reflux, (d) BR – Biliary reflux, (e) GBR – Gastrobiliary reflux.

Post-operative physiological assessment

At the end of study each animal was assessed for weight, pH, intraoesophageal and serum bile concentration prior to termination by Schedule 1 method.

Histopathological preparation

At autopsy, lower 2 cm of the oeosphagus and proximal stomach with anastomosis was removed en bloc and paraffin wax blocks and slides were produced (*see* Appendix 3). The sections were then stained using the Haematoxylin and Eosin method (*see* Appendix 3). The slides were examined by an experienced pathologist blinded to the experiment. Microscopic evidence of oesophagitis, degree of acute & chronic inflammation and length of columnar metaplasia was scored according to the grading system in Table 6.2.

The specimens showing columnar metaplasia were then cut further to stain with PAS Alcian blue diastase to assess glandular architecture and confirm columnarisation. Further histological assessment of selective slides was made using *in situ* technique and immunocytohistochemistry (*see* Appendix 3). These included proliferation analysis using p53 and Ki67, cytokeratin & trefoil peptide analysis using Streptividin – Peroxidase (SABC) technique with monoclonal antibodies – LHK antibody and Human Spasmolytic Polypeptide Mouse monoclonal IgM antibody respectively.

Statistical Analysis

The results were analysed using MS Excel (Microsoft MS Office 2000) and ARCUS (Arcus Pro-II Version 2.15a Statistical Analysis Software, 1993) statistical packages. The mean changes in values from pre-operative to end of study for comparison between different groups were compared, for pH data, weight measurements, blood bile acid concentration, oesophageal aspirate bile acid concentration, using the single-tailed Student t-test. Values were expressed as means \pm SEM.

Significance between different groups was calculated if there were > 6 cases. To achieve this several subgroup results were combined which were:

1. Gastric dominant reflux group (GDR)

JOR + GOR + DGPR = GDR

2. Duodenal dominant reflux (DDR)

DR + DGR + DPR = DDR

3. Biliary dominant reflux (BDR)

DPBR + BR + GBR = BDR.

Microscopic histopathology was compared using the Mann-Whitney U test. Statistical significance was designated at the 0.05 level.

Results

Two hundred and five specimens were collected from 56 animals over a period of 12 months [study days for each group (mean \pm SEM) (114.72 \pm 1.89)] (Table 4.1).

Table 4. 1. Number of animals surviving and study period.

Groups	Number of animals	Days of Study (days)
Control	10	211
JOR	7	125.46 ± 1.38
DPBR	7	132.23 ± 1.84
GR	5	139.65 ± 0.39
DGR	6	119.40 ± 1.55
DR	6	104.17 ± 1.01
DGPR	5	99.62 ± 0.13
DPR	4	94.27 ± 0.80
BR	3	84.71 ± 3.38
GBR	3	90 ± 1.39

JOR – Jejuno-oesophageal reflux.

DPBR – Duodeno-pancreatico-biliary reflux.

GR – Gastric reflux.

DGR – Duodeno-gastric reflux.

DR – Duodenal reflux.

DGPR - Duodeno-gastro-pancreatic reflux.

DPR - Duodeno-pancreatic reflux.

BR – Biliary reflux.

 $GBR-Gastro-biliary\ reflux.$

Physiological Measurements

There was weight gain during the study in Control, JOR, DPBR, GR, DGR, DGPR and DPR groups (Figure 4.3). Largest weight gain was in the control group [preop weight (g) (358.61 ± 15.06) postop weight (g) (657.67 ± 16.56) (p < 0.05)] (Figure 4.3). Two groups (BR and GBR) did not have weight change between preop and postoperative weight (Figure 4.3).

The postoperative pH values were decreased in JOR, GR, DGPR, BR and GBR groups in comparison to control and significantly increased in DR group (Figure 4.3) [pH (mean \pm SEM) control (8.93 \pm 0.27), JOR (6.96 \pm 0.40), GR (7.04 \pm 0.52), DGPR (5.6 \pm 0.66), BR (7.06 \pm 0.82), GBR (7.43 \pm 0.19) DR (9.22 \pm 0.13) (p < 0.05)]. Control, DPBR and DGR groups did not have significant change between preoperative and postoperative pH values (p > 0.05).

Postoperative serum bile acid concentration (Figure 4.4) was decreased in JOR, DPBR, DGR and DGPR groups [serum bile acid concentration (μ mol/l) (mean ± SEM) control (320 ± 59.06), JOR (143.43 ± 57.16), DPBR (159.71 ± 55.96), DGR 130 ± 26.95), DGPR (101.33 ± 31.82) (p < 0.05)] and did not change in GR, DR, BR and GBR groups when compared with preoperative values.

The postoperative intra oesophageal bile acid concentration was increased in JOR, DPBR, GR, DR, BR and GBR groups when compared to control values (all p < 0.005) and remained unchanged in DGR, DGPR and DPR groups (all p > 0.05) (Figure 4.4).





b

Groups

- 1 Control (n = 10).
- 3 JOR (n = 7) Jejuno-oesophageal reflux.
- 4 DPBR (n = 7) Duodeno-pancreatico-biliary reflux.
- 5 GR (n = 5) Gastric reflux.
- 6 DGR (n = 6) Duodeno-gastric reflux.
- 7 DR (n = 6) Duodenal reflux.
- 8 DGPR (n = 5) Duodeno-gastro-pancreatic reflux.
- 9 DPR (n = 4) Duodeno-pancreatic reflux.
- 10 BR (n = 3) Biliary reflux.
- 11 GBR (n = 3) Gastro-biliary reflux.





a



b

Groups

- 1 Control (n = 10).
- 3 JOR (n = 7) Jejuno-oesophageal reflux.
- 4 DPBR (n = 7) Duodeno-pancreatico-biliary reflux.
- 5 GR (n = 5) Gastric reflux.
- 6 DGR (n = 6) Duodeno-gastric reflux.
- 7 DR (n = 6) Duodenal reflux.
- 8 DGPR (n = 5) Duodeno-gastro-pancreatic reflux.
- 9 DPR (n = 4) Duodeno-pancreatic reflux.
- 10 BR (n = 3) Biliary reflux.
- 11 GBR (n = 3) Gastro-biliary reflux.



Histological changes

Columnar metaplasia of the lower oesophagus was seen in all cases. Length of columnar metaplasia was not related to change in weight (correlation coefficient r = 0.38) or pH values (r = -0.38). Length of columnar change in duodenal reflux (DR) groups was longer than all other groups [DR (length in cm ± SEM) (1.17 ± 0.11) (p < 0.001)] (Figure 4.7).

The severity of acute inflammation was higher in the biliary dominant groups and lower in gastric and duodenal dominant groups (Figure 4.6). The BM score was highest in biliary dominant groups – JOR, DPBR and BR when compared to other groups [JOR (BM score \pm SEM) (10.14 \pm 0.76) (all p <0.05), DPBR (9.43 \pm 0.30) (p < 0.05), BR (7.33 \pm 1.33) (p < 0.05)] (Figure 4.11)(Table 4.6, 4.8). The BM score was lowest in GR, DGR and DGPR groups when compared to other groups [GR (4.83 \pm 1.28) (p < 0.05), DGR (4.83 \pm 0.60) (p < 0.05), DGPR (5 \pm 1.08) (p < 0.05)].



Degree of inflammation

- 1 Mild inflammation
- 2 moderate inflammation
- 3 severe inflammation
- 4 ulceration

Groups

- 1 Control (n = 10).
- 3 JOR (n = 7) Jejuno-oesophageal reflux.
- 4 DPBR (n = 7) Duodeno-pancreatico-biliary reflux.
- 5 GR (n = 5) Gastric reflux.
- 6 DGR (n = 6) Duodeno-gastric reflux.
- 7 DR (n = 6) Duodenal reflux.
- 8 DGPR (n = 5) Duodeno-gastro-pancreatic reflux.
- 9 DPR (n = 4) Duodeno-pancreatic reflux.
- 10 BR (n = 3) Biliary reflux.
- 11 GBR (n = 3) Gastro-biliary reflux.









b

Groups

- 1 Control (n = 10).
- 3 JOR (n = 7) Jejuno-oesophageal reflux.
- 4 DPBR (n = 7) Duodeno-pancreatico-biliary reflux.
- 5 GR (n = 5) Gastric reflux.
- 6 DGR (n = 6) Duodeno-gastric reflux.
- 7 DR (n = 6) Duodenal reflux.
- 8 DGPR (n = 5) Duodeno-gastro-pancreatic reflux.
- 9 DPR (n = 4) Duodeno-pancreatic reflux.
- 10 BR (n = 3) Biliary reflux.
- 11 GBR (n = 3) Gastro-biliary reflux.

Figure 4. 6. (a) Columnar length changes and (b) Barrett's metaplasia score with different refluxates.

Mucosal Stripping (mucosectomy – MS)

There was no significant difference between mucosectomy (MS) and no mucosectomy (No MS) group in length of columnar metaplasia, degree of acute & chronic inflammation and Barrett's metaplasia score [columnar metaplasia length (MS) (1.52 ± 0.07) (No MS) (1.57 ± 0.08) , acute inflammation (MS) (1.42 ± 0.18) (No MS) (1.35 ± 0.15) , chronic inflammation (MS) (1.39 ± 0.07) (No MS) (1.44 ± 0.06) , Barrett's metaplasia score (MS) (4.78 ± 0.30) (No MS) (4.94 ± 0.27) (all p > 0.05) (Figure 4.8).



Figure 4. 7. *Histological changes of columnarisation, acute & chronic inflammation and Barrett's metaplasia score with mucosal stripping.*

PPI - Omeprazole (OMP) and changes with different refluxates

PPI treatment did not result in significant change in weight, serum bile acid or oesophageal bile acid concentration in Gastric dominant reflux group (GDR) [JOR + GOR + DGPR = GDR], Duodenal dominant reflux (DDR) [DR + DGR + DPR = DDR] or Biliary dominant reflux (BDR) [DPBR + BR + GBR = BDR] when compared to untreated group (all p > 0.05) (Figure 4.9). PPI treatment did not result in change in postoperative pH values in GDR, DDR and BDR groups when compared to untreated groups (all p > 0.05) (Figure 4.10). Use of PPI did not significantly decrease the degree of acute inflammation in any group when compared to untreated group (all p > 0.05) (Mann-Whitney U test) (Figure 4.10).

PPI treatment caused decreased columnar change in GDR and BDR groups compared to untreated group but this was not statistically significant [Columnar mucosa (length in cm \pm SEM) No OMP GDR (1.17 \pm 0.08), DDR (1.05 \pm 0.05) BDR (1.07 \pm 0.17), OMP GDR (1 \pm 0.13), DDR (1.08 \pm 0.15), BDR (0.83 \pm 0.11) (all p > 0.05) (Mann-Whitney U test)] (Figure 4.11). Treatment with PPI did not significantly alter the Barrett's metaplasia score in any group (all p > 0.05) (Mann-Whitney U test)] (Figure 4.11).







Groups

OMP - PPI OmeprazoleNo OMP - no PPI OmeprazoleGDR - Gastric dominant reflux (n = 8 in OMP, n = 9 in No OMP)DDR - Duodenal dominant reflux (n = 6 in OMP, n = 10 in No OMP)BDR = Bile dominant reflux (n = 6 in OMP, n = 7 in No OMP)

Figure 4. 8. *Changes in (a) weight, (b) serum bile and (c) oesophageal bile acid, with PPI omeprazole in different reflux groups.*



a



b

Groups

OMP - PPI OmeprazoleNo OMP - no PPI OmeprazoleGDR - Gastric dominant reflux (n = 8 in OMP, n = 9 in No OMP)DDR - Duodenal dominant reflux (n = 6 in OMP, n = 10 in No OMP)BDR = Bile dominant reflux (n = 6 in OMP, n = 7 in No OMP)

Figure 4. 9. *Changes in (a) pH and (b) acute inflammation with omeprazole in different reflux groups.*







b

Groups

OMP - PPI OmeprazoleNo OMP - no PPI Omeprazole GDR - Gastric dominant reflux (n = 8 in OMP, n = 9 in No OMP) DDR - Duodenal dominant reflux (n = 6 in OMP, n = 10 in No OMP) BDR = Bile dominant reflux (n = 6 in OMP, n = 7 in No OMP)

Figure 4. 10. *Changes in (a) columnar mucosal length and (b) Barrett' metaplasia score with omeprazole in different reflux groups.*

H & E and Alcian blue and neutral pH diastase PAS staining (Figure 4.16 – 4.18)

There was widespread and marked acute (neutrophil) and chronic (lymphocyte and macrophage) inflammatory infiltrate present with regenerative squamous and columnar epithelium in all specimens, especially at the squamo-columnar junction. Lesser degree of inflammation and regeneration was seen away from the junction. Ulceration was noted in several specimens and tended to have columnar regeneration on distal and squamous regeneration on proximal edges.

Glandular regeneration with mucinous cystic changes and multiple budding pattern was prevalent at the squamocolumnar junction in some slides. Displaced and mixed squamo-columnar glands were also noted at the junction. Architecture near the squamocolumnar junction was disorganised and reverted to more organised squamous or intestinal columnar epithelium distal from the junction.

The metaplastic columnar epithelium near the junction had cells with pseudoabsorptive features of brush border. Mucin secreting goblet cells were present in this epithelium. Some of the columnar epithelium was extending underneath regenerative squamous epithelium.

Islands of regenerating columnar epithelium with glandular regeneration were occasionally noted underneath muscularis mucosa. Ectopic islands of columnar and squamous epithelium were seen in several slides. Epithelial pearls were occasionally buried underneath regenerating columnar epithelium. Palisading of epithelial cells was also noted. There was no dysplasia noted in any of the specimen.



Figure 4. 11. *High power photomicrograph (20X) of squamo-columnar junction, (a)* H &E, (b) DAB/PAS, (c) anti-TFF2 antibody (magnification 20X).



Figure 4. 12. Glandular regeneration with mucinous cysts and multiple budding pattern seen in columnar lined glands (magnification 10X).

Immunohistochemistry and in situ antibody staining (Figure 4.16 (c), 4.17 (c))

P53 and Ki67 proliferation related antigens staining showed regenerative squamous and columnar epithelium. Cytokeratin antibody labelling (LHK antibody) did not stain the columnar metaplastic epithelium.

Monoclonal antibody *in situ* hybridisation with antibody for trefoil peptides TFF1, TFF2, TFF3 using 35S-labelled riboprobes [mpS2, rSP, rITF respectively, rBAC, DAB/HSP (diaminobenzene HSP peptide), anti TFF2] showed UACL (ulcer associated cell lineage) buds and acini at the squamo-columnar junction and on the distal edge of ulcers, similar to intestinal-type II columnar epithelium. The columnar epithelium distal from the squamocolumnar junction was intestinal with villous crypt architecture, Paneth cells and UACL buds at the base.

Morbidity and Mortality

Thirty animals did not complete the study period of 60 days. The causes of mortality were related to early postoperative complications of anaesthetic induction, wound breakdown, anastomotic leak, anastomotic dehiscence, aspiration and obstruction. Scybalous impaction of solid material at the anastomotic site was the most common reason for the post surgical deaths. Nearly all deaths occurred within the first 48

Discussion

One of the ways of overcoming difficulty of studying prevention of lower oesophageal columnar metaplasia (CLO) is by *in vivo* models, which reproduce CLO successfully and consistently. Chronic exposure of denuded lower oesophageal squamous epithelium to gastrointestinal secretions has been used to cause columnar metaplasia with variable results (Gillen et al 1988;Bremner 1982;Hennessy et al 1968;Pollara et al 1983;Martin et al 1992). Other CLO models using different approaches in different animals also produce inconsistent grades of CLO and columnar metaplasia is generated in unpredictable manner. A revision of the approach to inducing columnar metaplasia is helpful where the CLO model initially develops reflux oesophagitis and columnar metaplasia will be generated as a secondary consequence of healing of reflux oesophagitis in the presence of unremitting gastrointestinal reflux.

By combining two different models – CLO model using mucosectomy (Bremner et al 1970) and Oesophagitis model using reflux of physiological secretions (Levrat et al 1962), nine different models of CLO have been successfully created that reproduce columnar metaplasia in all cases. These models develop columnar metaplasia using physiological gastric, duodenal, bile, and mixed duodenopancreatic, duodenogastric, gastro biliary, duodenopancreatic biliary, duodenogastric and duodenogastroesophageal (jejunal) refluxate. The columnar metaplasia produced is phenotypically similar to specialised columnar epithelium with goblet cells as in duodenoesophageal models and in human CLO (Goldstein et al 1997). Studies of secretory trefoil peptide phenotype indicate that cell lineage of columnar metaplasia

in these models is consistent with Ulcer Associated Cell Lineage – UACL (Wright 1996), but does not arise from cardiac esophageal glands as rats do not have these glands. Analysis of cellular architecture with cytokeratin antibody reveals that the cell lineage is not derived from squamous epithelium but is dependent on columnar cells originating from adjacent intestinal epithelium. The morphology of this columnar metaplastic epithelium is predominantly glandular regenerative at advancing edge with specialised pseudo absorptive columnar cells, goblet cells and consistently high proliferation index as indicated by p53 and Ki67 antigens. The UACL buds and acini are also preferably clustered at this edge indicating that the changes of UACL and specialised columnar epithelium are a function of rapid and consistent proliferation in presence of continuing stimulus.

There is a graduated and almost linear change in trefoil phenotype expression and decrease in proliferation activity away from the advancing edge to distal mature intestinal epithelium in this model. The intervening epithelium exhibits temperance in proliferation and inflammation suggesting that this epithelium is not subject to same stimulus as the edge and therefore its phenotype is characteristic of mature intestinal epithelium but with occasional acini and glandular elements persisting. The relative lack of neutrophils, UACL glands and specialised columnar epithelium in the intervening epithelium despite same reflux influence as epithelium at the edge, indicates that autocrine mediated and neutrophil & macrophage derived inflammatory modulators are predominantly active in initiating and coordinating activity at the advancing edge. This is where there is lack of cellular contact and changes of adhesion molecules in cells. Changes in cell adhesion molecules catenins and e-cadherins, combined with increased proliferative stimulus from inflammatory

modulators such leukotrienes and interleukin family of molecules, and proliferation peptides – endothelial derived growth related factor & tumour growth factor – work synergistically to drive cellular proliferation. This may be similar to chronic injury activity at the Marjolin's ulcer, chronic osteomyelitis and chronic colitis where rapid and constant turnover of cells with free radical mediated damage results in imbalance of genetic mistakes and repair mechanisms. Though, molecular mechanisms of metaplasia in these models were not studied this model would be suitable for investigating evolution of the molecular pathophysiology of CLO. Depending on length of study these models can be utilised as models of gastroesophageal reflux disease (GORD), reflux oesophagitis or CLO. Longitudinal studies of similar model have been successfully used as UACL model (Hanby et al 1994) and for Barrett's associated lower oesophageal adenocarcinoma model (Chen et al 1999). Hence, these models provide a framework for understanding molecular events in generation of metaplasia and dysplasia at various stages.

It is known from experimental studies that metaplasia and neoplastic progression in the CLO associated adenocarcinoma models is driven by chronic reflux of carcinogenic mixed reflux of gastric and duodenal secretions (Melo et al 1999), with duodenal component exerting the most influence (Attwood et al 1992;Miwa et al 1994). Pancreatic secretion in duodenal reflux is found to be the most important carcinogen with bile exerting only a cocarcinogenic effect (Pera et al 1993), whereas gastric secretion has a protective effect in some models and does not induce CLO in other models (Attwood et al 1992;Ireland et al 1996).

Our results contradict these findings and support early studies of CLO where columnar change was induced by augmented gastric secretions (Bremner et al 1970), and agree with models of reflux oesophagitis where all types of reflux combinations produce oesophagitis with pure bile, pure gastric and pure duodenal secretions causing most changes (Lanas et al 1999;Kivilaakso et al 1980;Evander et al 1987;Levrat et al 1962;Lanas et al 1999). Our study demonstrates that reflux oesophagitis and CLO can be produced by reflux of any gastrointestinal secretions, with gastric and duodenal secretions causing greatest increase in length of columnar metaplasia, and biliary reflux and mixed biliary reflux causing greatest increase in acute and chronic inflammation and degree of CLO metaplasia while pancreatic secretions causes lesser degree of these changes.

The development of columnar epithelium of the oesophagus is preceded by reflux oesophagitis (Seto et al 1993) and it may be that additional molecular events, such as oxygen radicals and nitrogen species are initiated with mucosal stripping in presence of acidic gastric (pepsin and conjugated bile salts) and alkaline pancreatic (trypsin) and/or bile (deconjugated bile salts) reflux which digests the superficial layers of highly resistant keratinised oesophageal epithelium leading to severe esophagitis and healing by dominant columnar repair because of its more rapid turnover (Goldstein et al 1998;Li et al 1994;Aldazabal et al 1990;Estevao-Costa et al 1994;Lanas et al 1999;Kivilaakso et al 1980;Lillemoe et al 1983). Increase in total body mass index and dietary fat is believed to enhance this columnar metaplastic transformation in presence of gastroduodenal juice in clinical and experimental studies (Clark et al 1994). However, results in our study demonstrate that there is no correlation between weight and degree or length of metaplasia. This is similar to a weak correlation

noticed between BMI and CLO in clinical studies. Furthermore, there was no significant increase in columnar metaplasia or inflammation established with squamous mucosal damage and there was no linear correlation between pH and length of columnar change. This would indicate that additional molecular events mediated by autocrine or inflammatory modulators play prominent role in establishment of metaplasia and dysplasia. The initiation of these molecular events is most certainly dependent on reflux, ulceration and mucosal damage but propagation of these events is most likely dependent on inherent genetic susceptibility of the individual.

Excessive use of acid suppression is also believed to contribute to development of CLO due to unopposed duodenal, pancreatic and biliary reflux (Mirvish 1997).

Though some long-term studies of high dose proton pump inhibitor omeprazole show significantly reduced percentage of time in which the pH was < 4.0 in oesophagus and reduced length of CLO epithelium at 6 and 12 months (Malesci et al 1996;Marshall et al 1998), it is known that use of proton pump inhibitor omeprazole does not effectively reverse the changes of reflux oesophagitis and columnar metaplasia in all cases (Kouzu et al 1998;Narbona-Arnau et al 1994). In fact, in one study treatment of CLO with omeprazole 20 mg daily for periods of up to 6 years did not cause any regression in length of CLO segment (Cooper et al 1998). Use of powerful acid-reducing regimens also fails to heal a significant proportion of peptic ulcer of oesophagus (Barrett's ulcer) (Lee et al 1988).

Our results confirm ineffectiveness of omeprazole in mixed gastrointestinal reflux and also show that in duodenal dominant reflux use of omeprazole showed a trend

towards increased columnar change. Part of the failure of acid suppression in reducing CLO is related to significantly lower oesophageal sphincter pressure, increased percentage of time with oesophageal pH below 4, increased number of reflux episodes lasting more than 5 minutes, higher percentage exposure to acid reflux in upright or supine positions and markedly reduced acid clearing capacity in CLO patients (Fiorucci et al 1989).

As antireflux therapy using acid suppression has generally failed to induce regression of CLO epithelium, columnar metaplastic mucosal ablation using electrocautery or laser energy combined with gastric acid suppression with histamine H-2 blockers and proton pump inhibitors has been used to reverse CLO. Partial or complete reepithelialisation with squamous tissue has been reported after both mucosal ablation & acid suppression or omeprazole treatment alone (Berenson et al 1993;Kochman 1999; Marshall et al 1998). The regenerated squamous epithelium presents as large macroscopic squamous islands or microscopic squamous islands within abnormal CLO mucosa or as squamous encroachment of CLO mucosa at the squamo-columnar junction (Gore et al 1993), but columnar derived glandular tissue still persists beneath squamous epithelium. The nature and potential for progression of this buried glandular tissue is not clearly established. One study which used biomarkers for proliferation characteristic (Ki-67, p53), immunohistochemical methods with two antibodies, DO-1 and DO-7, and ornithine decarboxylase activity to assess for cancer risk in CLO patients with squamous islands found that completely reversed squamous epithelium is biologically similar to normal squamous epithelium and is of low cancer risk (Garewal et al 1999). In contrast, partial reversal, manifest as squamous islands, is accompanied by biomarker abnormalities (Garewal et al 1999), suggesting that

potential for progression of columnar cells to neoplasia is still present. In our study we used proliferation markers Ki 67, p53 and immunostaining with trefoil peptide phenotype TFF2 antibody to reach similar conclusions.

Hence it may be better to do antireflux surgery in mixed and duodenal dominant reflux as anti-reflux surgery can cause regression of CLO in up to 50% of these patients (Kouzu et al 1998;Attwood 1993;Pera et al 1993). Healing in experimental model where reflux is reversed by surgery is dependent on depth of injury and cell types surviving (Li et al 1994). Repair by columnar epithelium may predominate because of its rapid turnover, but this columnar epithelium may exhibit mature phenotypes. Further studies are under way to assess the nature of this surviving columnar epithelium.

Conclusion

Columnar-lined lower oesophagus in the rat animal model can be induced by any gastrointestinal refluxate. Reflux of physiological pure gastric, duodenal, pancreatic or biliary secretions or any combination of these can cause healing of ulcer or oesophagitis in the lower oesophagus by multipotent columnar cells. The most prominent changes are with pure duodenal and gastric secretions.

Columnar metaplasia is associated with marked acute & chronic inflammation of reflux oesophagitis and does not require prior ulceration of squamous mucosa. The regenerating columnar epithelium is similar to specialised intestinal-type II metaplasia and has ulcer associated cell lineage.

Omeprazole administration shows a trend towards increase in length of columnar metaplasia in duodenal dominant reflux groups, and decrease in gastric dominant reflux group. Duodenal reflux may be the cause of ineffectiveness of Omeprazole in a significant number of CLO patients.

Omeprazole can be used as treatment in reflux oesophagitis and CLO caused by pure gastric reflux patients but anti-reflux surgery may be more effective in other groups.

Chapter 5. Morphological changes associated with experimental Columnar-lined lower oesophagus after successful anti-reflux surgery.

<u>Summary</u>

Aim. Chronic acid and bile reflux into lower oesophagus induces development of specialised columnar metaplasia and progression to dysplasia. The aim of this study was to determine role of complete reversal of this reflux on metaplastic columnar epithelium in *in vivo* setting and study the morphology of changing columnar epithelium after anti-reflux surgery.

Method. Vagus preserving oesophagojejunostomy operation with lower oesophageal mucosectomy was performed on Springer-Dawley rats to achieve mixed reflux into lower oesophagus. Reversal of reflux by modified Roux-en-Y operation was performed on half of the animals after 3 months. Histological examination of lower oesophagus for columnar length changes, degree of acute & chronic inflammation and metaplasia using H & E, diastase Alcian blue PAS stains was performed by three experienced pathologists blinded to the experiment. Immunostaining for proliferation antigen activity (p53, Ki67), cytokeratin (LHK), trefoil peptide (TFF2) and *in situ* hybridisation for trefoil peptide mRNAs with 35S-labelled riboprobe was used to determine morphological changes.

Results. Fifty-four specimens were collected from 17 animals over study period of 6 months. There was significant weight gain, normalisation of oesophageal pH, decrease in serum and oesophageal bile acid concentration after antireflux procedure [Weight in grams (mean \pm SEM) Group 1 (Non-reversed) (244.58 \pm 9.76) Group 2 (Reversed) (384.86 \pm 6.35), postop pH Group 1 (7.37 \pm 0.08) Group 2 (8.19 \pm 0.05), serum bile acid (µmol/l) Group 1 (120.33 \pm 20.35) Group 2 (38.8 \pm 7.72), oesophageal bile acid (µmol/l) Group 1 (46 \pm 9.49) Group 2 (25.53 \pm 5.10) (all p < 0.05)]. Columnar epithelium with goblet cells was noted in all specimens. The length of columnar mucosa, degree of acute inflammation and degree of metaplasia were significantly reduced by anti-reflux surgery (all p < 0.05). The morphology of specialised columnar epithelium and ulcer associated cell lineage tended to revert to native mature epithelium after anti-reflux surgery. The histological changes of trefoil peptide phenotype and proliferation antigen activity in the *in vivo* models were similar to human CLO before and after successful anti-reflux surgery.

Conclusion.

- Successful anti-reflux surgery in CLO results in abolishing of reflux and return of body weight, oesophageal pH, serum and oesophageal bile towards normal value.
- 2. Morphology of experimental CLO and its reversal is similar to human.
- Anti-reflux procedure effectively reverts the mitogenic changes of columnar metaplasia induced by chronic oesophageal reflux.

Introduction

Columnar-lined lower oesophagus (CLO) is the result of a triad of environmental factors and chronic reflux of gastroduodenal contents causing inflammatory infiltrate to modulate healing in genetically predisposed population. The chronic reflux of gastroduodenal secretions results in replacement of squamous-lined lower oesophagus by columnar epithelium which is further modified by duodenal components – bile and pancreatic juice – to intestinal type metaplasia and dysplasia (Pera et al 1993). This change to intestinal type columnar metaplasia – CLO – is initiated by oxidative damage on DNA (Chen et al 2000), and its extent is dependent on degree and duration of oesophageal acid and bilirubin exposure (Oberg et al 1999).

The oxidative damage in CLO can be studied using phenotypic markers for proliferation antigens (p53, Ki-67), growth factors (epidermal growth factor (EGF), cerbB2, and transforming growth factor (TGF- α)), cell adhesion molecules (Ecadherin, catenins) and DNA aneuploidy (Mueller et al 2000; Younes et al 2000). Flow cytometric measurements (ploidy, proliferative index) are most frequently used and are the most promising markers that correlate with degree of dysplasia in CLO (Gimenez et al 2000). The evolution of oxidative damage and natural history of dysplasia is currently used to monitor patients by screening endoscopy before carcinoma develops but the oxidative damage is difficult to predict accurately using currently available markers and the best marker which presently exists to identify high-risk lesions in CLO is the histologic identification of dysplasia in endoscopic biopsies (Mueller et al 2000;Rudolph et al 2000). Diagnosis of dysplasia is important because when endoscopic biopsies show high-grade dysplasia, approximately 50% of resected specimens contain evidence of invasive adenocarcinoma (Wright 1997), even when there is no visually recognisable lesion at endoscopy (Atkinson et al 1992;McArdle et al 1992;Reid et al 1988;Rice et al 1993;Rusch et al 1994). Once high-grade dysplasia is detected patient is considered for oesophagectomy. But, as oesophagectomy can be associated with high morbidity and mortality, medical and surgical therapy to reduce gastroduodenal reflux are being considered to halt evolution of CLO metaplasia to high grade dysplasia.

Methods to reduce damage to oesophageal mucosa by eliminating exposure to harmful gastroduodenal secretions have yielded inconsistent results. Histamine H2blockers and proton pump inhibitor mediated acid suppression is highly effective and safe for reducing duration and episodes of oesophageal acid exposure, and control of peptic reflux oesophagitis (Klinkenberg-Knol et al 2000;Marshall et al 1998;Sontag et al 1997). But, acid suppression alone does not result in complete reversal of length of metaplastic columnar mucosa and nearly half of patients on medical acid suppression regimens develop further CLO (Peters et al 1999;Sharma et al 1997;Wetscher et al 1997). Though partial re-epithelialisation by squamous islands using high dose proton pump inhibitor has been reported in some CLO patients (Cooper et al 1998), biopsies of these islands show that intestinal metaplasia may still be present in upto a third of these patients and the metaplasia has high proliferation antigen activity indicating potential for neoplasia (Garewal et al 1999;Sharma et al 1998).

Part of the reason for failure of medical acid suppression is that complete symptom eradication does not guarantee normalization of intraoesophageal pH profile in CLO patients (Ouatu-Lascar et al 1999). Furthermore, acid suppression needs to be given in

massive doses to be effective in gastric hypersecretion and in mixed reflux may be more harmful (Ireland et al 1996). Moreover, as < 20% of patients with GORD develop CLO and upto 75% of patients destined to develop CLO will never be seen, evaluated, or treated before CLO develops (Bremner et al 1998), life-long continuous medical therapy in large numbers of GORD patients is unjustified. Antireflux surgery may be a more cost effective management and induce regression or halt progression of intestinal metaplasia in large number of patients (DeMeester et al 2000).

Antireflux surgery is superior to all forms of medical acid suppression, and mixed gastrointestinal reflux into lower oesophagus can be completely suppressed by open or laparoscopic Nissen fundoplication (Stein et al 1998). But regression of CLO occurs in unpredictable manner after antireflux procedure (Baulieux et al 1999;Attwood 1993). The regression is dependent on depth of injury and cell types surviving (Li et al 1994). If columnar cells survive the repair by columnar epithelium may predominate because of its rapid turnover, but it is not known if this columnar epithelium will exhibit mature epithelial phenotypes as the stimulus for progression to neoplasia is removed.

Role of reversing chronic gastroduodenal reflux and its effect on regression of CLO can be more accurately evaluated using *in vivo* models. The morphology of reversed and non-reversed columnar epithelium can also be assessed more precisely in models. Linear and temporal progression of CLO can also be gauzed more concretely using models. The present study is designed to assess histological changes, proliferation activity and trefoil peptides distribution in CLO after complete reversal of chronic gastroduodenal reflux.

Materials and Method

Twenty 10-week-old Springer-Dawley male rats (300 - 350 g) were selected for the study and divided into 2 groups of ten animals each. They were housed 4 to a cage and kept under standard laboratory conditions. Pellet diet and water *ab libitum* was provided.

Physiological assessment of intraoesophageal pH, oesophageal aspirate for bile acid measurement and venous blood sample for serum bile acid estimation was performed for each animal under Halothane anaesthesia preoperatively and postoperatively. Intraoesophageal pH was measured using MicroDigitrapper ambulatory pH monitoring system with monocrystant antimony pediatric pH catheter (Synectics Medical®). Intraoesophageal aspirate was obtained by injecting 5 ml Normal Saline by gavage and aspirating the solution. Venous blood from tail vein was obtained to measure serum bile acid. Oesophageal and serum bile acid concentration were measured using commercially available Enzabile test kit (Bio Stat Diagnostic Systems®).

Preoperative alimentation of gastrointestinal tract was reduced by administering liquid Complan ® feed for five preoperative days and continued for two weeks postoperatively. To help digestion of hair collagen pineapple juice was mixed with Complan. To decrease coprophagia animals were maintained on the grid cage for 48 hours and food withdrawn for 24 hours prior to the operation.
Halothane and oxygen anaesthesia was used to perform upper midline laparotomy in all animals. The oesophagus was mobilised, heavy tie (Vicryl 2.0) placed at the oesophagogastric junction and transected above the tie. The lower posterior mucosa of the oesophagus was stripped in 2mm X 2mm square shape. All twenty animals had end-to-side interrupted single layer (Ethilon 6.0 suture) oesophagojejunostomy anastomosis performed by mobilizing the 1st part of jejunum (Figure 4.1a). Ten animals in Group 1 (Non-reversed) animals did not have any further procedure. Ten animals in Group 2 (reversed) animals had further operation performed at 3 months to reverse the reflux by modified Roux-en-Y operation. Vagus nerve was preserved in each case. The anastomosis was checked for integrity and patency and laparotomy closed in two-layer with PDS 4.0 suture. Subcutaneous Temgesic® was used for postoperative pain control and animals had access to food and water 12 hours after the operation.

At the end of study lower oeosphagus was removed en bloc, fixed in 10% neutral buffered formalin and paraffin wax block sections produced. Serial sections on slides were stained using the Haematoxylin and Eosin (H & E) and for diastase Periodicacid Schiff/Alcian blue (diastase/dPAS/AB).

Immunostaining using Streptavidin-biotin complex – Peroxidase (SABC) technique was used for staining p53 and Ki67, cytokeratin LHK and trefoil peptide HSP (TFF2) antibodies (Hanby et al 1997).

In situ hybridisation technique for trefoil peptides mRNA pS2 (TFF1), SP (TFF2), ITF (TFF3) using ³⁵S-labelled antisense riboprobes was used to study distribution of mRNAs for each of three TFF genes (Hanby et al 1997).

Three experienced pathologists blinded to the experiment examined the slides. Microscopic evidence of oesophagitis, degree of acute & chronic inflammation and length of columnar metaplasia was scored according to a grading system (Table 5).

Statistical Analysis

The results were analysed using ARCUS (Arcus Pro-II Version 2.15a Statistical Analysis Software, 1993) statistical package. The mean changes in values between different groups were compared, for pH data, weight measurements, serum bile acid concentration, oesophageal aspirate bile acid concentration, using the single-tailed Student t-test. Values were expressed as means \pm SEM. Microscopic histopathology was compared using the Mann-Whitney U test. Statistical significance was designated at the 0.05 level.

Results

Fifty-four specimens were collected from animals over a study period of 8 months (Table 5.1 & 5.2) [Study period Group 1(Non-reversed) in days (mean \pm SEM) (106 \pm 0.42) study period Group 2 (Reversed) (123 \pm 1.54)]. The period of reversal of reflux (Group 2) was 29.67 \pm 0.23 days.

The postoperative weight, pH value were significantly increased in Group 2 (reversed) compared to Group 1 (Non – reversed) (Figure 5.1) [weight in grams (mean \pm SEM) Group 1 (244.58 \pm 9.76) Group 2 (384.87 \pm 6.35), pH Group 1 (7.37 \pm 0.08) Group 2 (8.19 \pm 0.05) (all p < 0.05) (Student T- test)]. Serum bile acid concentration and oesophageal bile acid concentration were significantly decreased in the reversed group (Figure 5.2) [serum bile acid concentration (µmol/l) Group 1 (120.33 \pm 20.35) Group 2 (38.8 \pm 7.72), oesophageal bile acid concentration (µmol/l) Group 1 (46 \pm 9.49) Group 2 (25.53 \pm 5.10) (all p < 0.05) (Student t- test)].

Histological examination showed that the columnar mucosal length was significantly decreased but still present in lower oesophagus (Figure 5.3) [(Columnar mucosal length in cm. Group 1 (1 ± 0.10) Group 2 (0.52 ± 0.05) (p < 0.05) (Student t-test)]. The Barrett's metaplasia score (BMS) was significantly decreased (Figure 5.3) [BMS Group 1 (7.21 ± 0.51) Group 2 3.43 ± 0.36) (p < 0.05) (Mann-Whitney U test)].

Table 5. 1. Number of animals and specimens collected at the end of study.

Groups	Number of animals	Number of specimens	
Non reversed*	8	24	
Reversed+	9	30	

*Non- reversed – group with continued reflux of gastrointestinal contents + Reversed – group with reversal of reflux after 2 months

Table 5. 2. Period of study, reflux days and period with no reflux.

Groups	Study Period (days)	Period Reflux (days)	Period no Reflux (days)
Non-Reversed	106 ± 0.42	106 ± 0.42	0
Reversed	123.47 ± 1.54	93.8 ± 1.59	29.67 ± 0.23



a



b

Groups

- 1 Non reversed (n = 8)
- 2 Reversed (n = 9)





Oesophageal bile



1

2

3

0 0

b

Groups

- 1 Non reversed (n = 8)
- 2 Reversed (n = 9)



149

a



a



b

Groups

- 1 Non reversed (n = 8)
- 2 Reversed (n = 9)



<u>Histology of columnar epithelium</u> (Figure 5.11, 5.12)

Intestinal type columnar metaplasia with goblet cells was present in all animals. Ulceration of lower oesophageal mucosa was present in 13 of Group 1 (non-reversed) specimens and in 1 of Group 2 (reversed) specimens. There was widespread and marked acute (neutrophil) and chronic (lymphocyte and macrophage) inflammatory infiltrate present with regenerative squamous and columnar epithelium in all Group 1 specimens, especially at the squamo-columnar junction. Lesser degrees of inflammation and regeneration were seen away from the junction with intestinal type columnar epithelium present distally and squamous epithelium present proximally. The columnar change furthest from the advancing edge was of small bowel morphology (crypt villi, Paneth cells, Brunner glands). Glandular regeneration with mucinous cystic changes and multiple budding pattern was prevalent at the squamocolumnar junction, reminiscent of UACL glands. Displaced and mixed squamo-columnar glands were also noted at the junction. Architecture near the squamocolumnar junction was disorganised and reverted to more organised squamous or intestinal columnar epithelium distal from the junction. The metaplastic columnar epithelium near the junction had cells with pseudo-absorptive features of brush border with scattered mucin secreting goblet cells. Some of the columnar epithelium was extending underneath regenerative squamous epithelium. Islands of regenerating columnar epithelium with glandular regeneration was occasionally noted underneath muscularis mucosa. Ectopic islands of columnar and heterotopic squamous islands were seen in several slides. Epithelial pearls were occasionally seen buried underneath regenerating columnar epithelium. Palisading of epithelial cells was also noted. In group1 there was a trend towards columnar and glandular regeneration with columnar

islands and mixed squamo-columnar glands predominating. In Group 2 intestinal-type columnar epithelium was noted at distal part of squamo-columnar junction and squamous epithelium at proximal part of the junction. There was lesser degrees of acute & chronic inflammatory infiltrate, regenerative epithelium and glandular regeneration present. In group 2 there was a trend towards squamous regeneration with squamous islands predominating. Some of the group 2 specimens did not show columnar epithelium. There was no dysplasia noted in any of Group 1 or Group 2 specimens.



Figure 5. 4. Diastase PAS staining of squamo columnar junction to show distribution of goblet cells in columnar metaplastic epithelium at (a) low (5X), (b) medium (100X) and (c) high (200X) power of magnification.



Figure 5. 5. Diastase PAS stain for neutral and acid mucin (a) low power (50X), (b) medium power (100X), to show metaplastic columnar epithelium and mucin secreting goblet cells, and p53 antibody staining using immunoperoxidase technique (c) low power (50X), (d) medium power (100X), to show proliferation activity in squamous and columnar metaplasia at the squamocolumnar junction.

Immunohistochemistry and in situ antibody staining (Figure 5.13, 5.14)

P53 and Ki67 proliferation related antigens staining was detected in regenerative squamous epithelium and at bases of crypts in columnar epithelium. Cytokeratin antibody labelling (LHK antibody) selectively stained regions of reactive squamous epithelium and did not stain the columnar metaplastic epithelium. Immunohistochemistry for trefoil peptide HSP (TFF2) showed UACL (ulcer associated cell lineage) buds and acini at the squamo-columnar junction and on the distal edge of ulcers, similar to intestinal-type II columnar epithelium. The columnar epithelium distal from the squamocolumnar junction was intestinal with villous crypt architecture, Paneth cells and UACL buds at the base.

In situ hybridisation for trefoil peptides using ³⁵S-labelled riboprobes revealed expression of mRNAs for each of three TFF genes in metaplastic columnar epithelium. No trefoil peptide or mRNA was seen in native oesophageal squamous epithelium. UACL lineage trefoil peptide phenotype was seen in 11 of Group 1 (Nonreversed) and one of Group 2 (reversed) specimens with TFF1 mRNA in upper portions of lineage, TFF2 peptide and its mRNA in deeper glands and TFF3 mRNA localised patchily throughout the UACL.

One specimen showed trefoil peptide phenotype characteristic of gastric cardiac type epithelium with TFF1 and TFF2 mRNA localising to superficial compartment and TFF2 mRNA in deeper glands. Other specimens showed characteristics of colonic and small bowel epithelium morphology. TTF2 mRNA was demonstrable in surface epithelium of incomplete- but not complete-type intestinal (specialised) metaplasia.

Gradual sequential linear change in trefoil peptide and three mRNAs expression away from the squamo-columnar junction was noted in both groups with predominant UACL phenotype with incomplete intestinal type phenotype near the junction in Group 1 changing to complete small bowel phenotype. In Group 2 gastric cardiac and colonic phenotype expression intervened between scarce UACL and incomplete intestinal phenotype at the junction and small bowel phenotype.



Figure 5. 6. In situ hybridization of (a) PS2, (b) HSP, (c) rITF mRNA to show trefoil peptide phenotype TFF1, TFF2, TFF3 expression respectively in columnar epithelium. Aberrant expression of antibodies is present in superior part of each photograph suggesting trefoil peptide phenotype of gastric epithelium. Small intestinal phenotype is expressed in other areas.



Figure 5. 7. In situ hybridization with (a) PS2, (b) HSP, (c) rITF mRNA to show trefoil peptide phenotype TFF1, TFF2, TFF3 expression respectively in columnar epithelium after reversal of gastrointestinal reflux. The phenotype expressed is of mature small intestinal epithelium.

Discussion

There is a paucity of suitable models to study therapeutic intervention of Columnarlined lower oesophagus (CLO), partly due to difficulty in reproducing specialised intestinal type columnar epithelium and/or Classical Barrett's using pathology comparable to human gastroesophageal reflux disease (GORD). This has resulted in reliance on human in vivo analysis of pathophysiology and clinical trials of medical and surgical intervention to prevent evolution of CLO to adenocarcinoma. But these trials may give conflicting results due to differences in methodology, selection bias and inconsistent end points (Narbona-Arnau et al 1994). One of the ways of overcoming this difficulty of studying prevention of lower oesophageal columnar metaplasia (CLO) is by *in vivo* models which reproduce CLO successfully and consistently. Chronic exposure of denuded lower oesophageal squamous epithelium to gastrointestinal secretions has been used to cause columnar metaplasia in animal models with variable results (Gillen et al 1988;Bremner 1982;Hennessy et al 1968;Pollara et al 1983;Martin et al 1992). Though most studies achieve columnar metaplasias in ~60% of case, in some studies less than 50% of animals had columnar change. This inconsistency in inducing columnar metaplasia results in difficulty in evaluating effects of therapeutic procedures on CLO in animal models. For this reason effect of medical and surgical anti-reflux therapy in models has been difficult to extrapolate to patients (Narbona-Arnau et al 1994). In addition, some of these studies achieve partial healing and reversal of CLO.

In one study 6 out of 10 animals underwent an antireflux procedure to suppress acid secretion after initial mucosectomy, cardioplasty and pentagastrin augmented acid reflux. Healing of the mucosal defect was predominantly by columnar epithelium before and after anti-reflux surgery but squamous epithelial islands were noted after reflux suppression (Li et al 1994). This suggests that regeneration of mucosal defect is dependent on type of cell surviving (Li et al 1994). However, it is difficult to extrapolate findings from this study to *in vivo* clinical setting as it did not induce columnar epithelium in all cases. Furthermore, it does not imitate the situation of mixed refluxate which is most commonly associated with human CLO. In our study we exposed oesophageal mucosal defect to physiological refluxate of gastric & proximal duodenal secretions and proximal jejunal secretions and managed to attain intestinal type columnar epithelium in all cases.

Studies of human CLO pathophysiology also show that there is an exponential increase in oesophageal acid and bile exposure from GORD patients without oesophagits to those with erosive oesophagitis and benign CLO to CLO associated adenocarcinoma (Caldwell et al 1995;Gillen et al 1988;Jankowski et al 1992;Bremner et al 1997;Stein et al 1993;Vaezi et al 1999;Sears et al 1995). This situation is replicated in our model and therefore, it most closely mimics the presumed pathophysiology of severe GORD with peptic ulceration of lower oesophagus, reflux oesophagits and CLO due to oesophageal acid and bile exposure.

The oesoplageal acid and bile exposure can be completely suppressed by antireflux surgery (Peters et al 1999;Sharma et al 1997;Stein et al 1998;Wetscher et al 1997).

However, anti-reflux surgery is considerably difficult to study in models unless the changes of GORD and CLO are known to be present before surgery. The model must also effectively show complete cessation of reflux activity and return towards normal parameters for PH, serum & oesophageal bile and weight of animal. Complete suppression of reflux as measured by pH changes, serum bile and oesophageal bile acid concentration was achieved in our model and all animals benefited by statistically significant weight gain. In addition, this model can be further expanded to study histological changes after anti-reflux surgery in GORD, peptic (acidic) and biliary (alkaline) reflux oesophagitis, CLO, UACL, columnar dysplasia and adenocarcinoma. One further advantage with this model is that it most closely imitates *in vivo* histological changes of reversing CLO by open anti-reflux surgery.

Open antireflux surgery is a well-established surgical procedure for the successful treatment of GORD with long-term success rates of > 85% and operative mortality of less than 1% (Rossetti et al 1977). Studies examining the postoperative outcomes after open antireflux surgery have reported slightly worse results in patients in whom CLO was diagnosed preoperatively. Attwood et al reported that in 19 CLO patients symptoms recurred or persisted in 21% of cases after surgery (Attwood et al 1992). Ortiz et al demonstrated in 59 BO patients that surgery had better outcome than medical treatment (Ortiz et al 1996). However, these studies have used symptom index as their endpoint and failure rates of 54% at 88 months in non-complicated CLO, 64% failure rate for complicated CLO at 106 months have been reported with open Nissen fundoplication and Collis gastroplasty (Csendes et al 1998), and therefore it is not surprising that no or very little regression of abnormal intestinal metaplastic mucosa may be noted (Chen et al 1999). Open antireflux surgery in

patients with CLO results in a high percentage of failures at very late follow-up because it cannot completely avoid acid and duodenal reflux into the oesophagus (Csendes et al 1998). For this reason laparoscopic approach has been much investigated to reverse CLO.

Excellent long term results have been reported for Laparoscopic Nissen fundoplication with an average mortality of 0.1% and recurrence rate of 1 - 8%(Watson et al 1998;Dallemagne et al 1998;Watson et al 1996;Dallemagne et al 1996). Patients diagnosed preoperatively as having CLO have been included as part of these studies, with the incidence of CLO reported to vary between 10% and 15% in many large series (Watson et al 1997). Studies specifically targeting the outcomes within this cohort of patients for CLO have found that regression of CLO is possible but infrequent and unpredictable after antireflux procedure (Baulieux et al 1999). In one study 56 patients 24 had partial or complete regression of CLO, 23 remain unchanged, 9 showed progression after operation with carcinoma developing one patient (Sagar et al 1995). In another study no increase in length of CLO was seen and 7 patients (27%) had complete or partial regression (Baulieux et al 1999). In another study complete disappearance of short segment Barrett's esophagus (< 3cm) was noted in 2 out 14 patients and 10 additional patients had squamous re-epithelialisation. No patients in this study showed progression of dysplastic change, and four patients demonstrated complete disappearance of low-grade dysplasia suggesting that successful antireflux surgery can produce squamous re-epithelialisation and stabilization or apparent improvement in dysplasia of CLO patients (Low et al 1999). Overall, antireflux surgery can cause regression of CLO in up to 50% of patients (Kouzu et al 1998). Our results agree with the results of laparoscopic anti-reflux series and show that anti-

reflux surgery can cause partial or complete regression of CLO. The specialised columnar epithelium after elimination of reflux reverts to mature gastric, colonic or intestinal type. Majority of ulceration in the specialised columnar epithelium heals with predominately squamous regeneration or with islands of squamous epithelium in more mature columnar epithelium. Hence, it is possible that complete regression of columnar epithelium in lower oesophagus can be achieved by excising or ablating the columnar mucosa and decreasing harmful refluxates.

In studies of acid suppression with omeprazole after laser photoablation, a relatively common finding is of residual glandular mucosa beneath squamous epithelium (Biddlestone et al 1998). This situation was rarely encountered in our model. While, bipolar electrocoagulation after antireflux operations is more effective in promoting regression of CLO (Montes et al 1999), and regenerated esophageal epithelium arising after endoscopic Nd-YAG laser ablation CLO epithelium in reflux-free environment after surgery is of squamous type (Salo et al 1998). Our findings agree with these clinical studies which show clear benefits of anti-reflux surgery after mucosal ablation. This benefit can also be analysed histologically using trefoil peptide distribution.

Trefoil peptide analysis and RT-PCR (recombinant polymerase chain reaction) analysis in human CLO metaplasia shows expression of TFF1 and TFF3 phenotypes in all cases and decreased TFF2 expression (Labouvie et al 1999). This is comparable to findings in our model of CLO in rats. Histological changes observed in the mucosa of rats in region of oesophagojejunostomy in ulcer-associated cell lineage (UACL) model also show that TFF3 mRNA localised patchily throughout UACL, whereas

TFF1 mRNA is found in the upper portions of the lineage and TFF2 mRNA and its product in the acini (Hanby et al 1997). Analysis of Group 1 (non-reversed) specimens in our study show similar morphological features, proliferation activity and trefoil peptide gene expression attributes of UACL model (Hanby et al 1997), and morphological features consistent with human pyloric glands/UACL (Hanby et al 1994). Furthermore, specialised columnar epithelium was observed in all of Group 1 (non-reversed) specimens with TFF2 peptide and its mRNA expression in surface epithelium of columnar epithelium similar to incomplete-type specialised intestinal metaplasia in humans (Hanby et al 1994).

The distribution of the trefoil peptides TFF1 and TFF2 and their mRNAs in humans show that cardiac-type epithelium resembles true gastric antral epithelium, with TFF1 and TFF2 mRNA localisation to the superficial/foveolar compartment and TFF2 mRNA alone in acini (Hanby et al 1994). In one specimen of Group 2 (reversed) analogous phenotype distribution was noted. In addition, TFF1 mRNA distribution in both Group 1 and 2 shows columnar epithelium away from squamo-columnar junction morphologically identical to true small intestinal surface epithelium (Hanby et al 1994). This epithelium exhibited characteristics of mature, non-metaplastic epithelium with absorptive surface villi present on surface cells, Brunner glands in crypts with UACL activity and proliferation zone confined to crypts and glands. This indicates that cellular mechanisms for proliferation adopt native epithelial properties with removal of mitogenic stimulus. The nature of mitogenic stimulus was not determined in this model but histological changes before reversal of reflux would indicate that once proliferation of metaplastic epithelium is initiated then propagation of metaplasia can be by any reflux, i.e., normal physiological acidic gastric reflux,

alkaline duodenal reflux individually or synergistically as mixed 'neutral' pH reflux (Johnson et al 1987).

It is not known at what stage will the reversal of reflux by effective anti-reflux surgery will cease to be of benefit, but it must be performed before neoplasia develops. Molecular pathology of CLO suggests that disruption of cell adhesion molecules E-cadherin and catenin complex is important in CLO cell achieving neoplastic potential (Washington et al 1998). This disruption of cell adhesion is associated with loss of contact inhibition of proliferation and allow escape from growth contact signals (Aldulaimi et al 1999). In addition, changes in E-cadherin expression are an important step in development and progression of malignancy in CLO. Reduced and disordered expression of E-cadherin appears to be related to transcriptional and post-translational events respectively, and both appear to represent altered cell adhesion associated with invasion and metastasis in CLO neoplasms (Bongiorno et al 1995). This effect is potentiated by endothelial derived growth factor and its receptor and tumour growth factor (Aldulaimi et al 1999). The factors responsible for loss of cell adhesion and cell achieving immortality are incompletely evaluated and it is not known whether the loss of E-cadherin-mediated cell adhesion is a prerequisite for tumour progression or is a consequence of dedifferentiation during tumour progression. E-cadherin inactivation does lead to formation of adenomas and forced expression of E-cadherin suppresses proliferation, while alteration in cytoplasmic β -catenin is involved with modification of cell signals (Jankowski et al 1997), but it is not known whether reversal of reflux in CLO is beneficial after E-cadherin inactivation and cell signal disruption. As this effect is poorly studied in early CLO, this model would be helpful in evaluating interaction

between cell-adhesion complex molecules, oncogenes, tumour suppressor genes and proliferation peptides before and after surgery. Furthermore, relationship between flow cytometry, aneuploidy, DNA damage, field changes and reversal of these changes can be analysed using this model and results can be extrapolated to patients.

Conclusion

Localisation of trefoil peptide phenotypes and proliferation activity in our model has facilitated definition of the disparate epithelium types seen before and after anti-reflux procedures in both native oesophageal and jejunal epithelia and more accurately defined the changing columnar species. In absence of mitogenic gastroduodenal reflux specialised columnar epithelium reverts to less proliferative and more mature native epithelium in all cases. It is possible that this process can be accelerated by mucosal excision or mucosal ablation, but this possibility was not investigated in this model.

Chapter 6. Discussion and Contribution to Surgical Practice.

Columnar metaplasia of lower oesophagus with specialised columnar cells and goblet cells (Columnar-lined lower oesophagus – CLO) is a known precursor for the adenocarcinoma of lower oesophagus (>50 % of oesophageal adenocarcinoma specimens have high grade CLO) and is closely associated with junctional adenocarcinoma (Clark et al 1996;Spechler et al 1996;Wright 1997). Animal models using carcinogens and iatrogenic reflux of gastrointestinal contents confirm this association (Chen et al 1999;Goldstein et al 1998).

CLO is also always preceded by reflux oesophagitis and accompanied by acute and chronic inflammatory infiltrate (Seto et al 1993;Spechler 1992). But, the underlying pathophysiological changes, the reflux constituents and molecular precipitants causing transformation from reflux oesophagitis to CLO are incompletely understood. Animal models have been used to study these changes and establish the relationship between different reflux types, individual fractions of reflux and development of reflux oesophagitis, CLO and oesophageal adenocarcinoma (Gillen et al 1988;Bremner 1982;Hennessy et al 1968;Pollara et al 1983;Martin et al 1992). These models have also been used to evaluate role of dietary components, iron supplementation and nitrosamine induced changes (Clark et al 1994;Li et al 1995;Pera et al 1998;Mirvish 1995). Yet, these models suffer from several drawbacks. They do not emulate the pathophysiological mechanisms causing CLO or generate specialised columnar epithelium or cause columnar metaplasia in all cases. This can introduce experimental bias, lag and lead time errors and leads to erroneous conclusions. Furthermore, it is difficult to reliably assess therapeutic medical and surgical intervention which can be accurately extrapolated to human *in vivo* situation (Skinner 1990;Giuli et al 1988). One way to overcome these problems is to create a model of CLO that can effectively and consistently produce CLO in all cases. The columnar metaplastic epithelium in the model must also exhibit characteristics associated with specialised epithelium as this epithelium is most frequently associated with neoplastic transformation in humans (Schneider et al 1996). These models must also be reproducible in different species because there are anatomical similarities and differences between them which could cause problems with extrapolation of results to *in vivo*.

Animal models of reflux oesophagitis and CLO have been studied on several animals. Most frequently studied animal – the Springer-Dawley rat and New Zealand white rabbit – were assessed for their suitability in developing a more consistent model of CLO. By comparing size of abdominal cavity, access for operation and feasibility of gastrointestinal anastomosis it was established that both animals had features suitable for developing CLO model, but the squamous lined forestomach in rat requires little dissection and mobilisation for surgical anastomosis & mucosal stripping. Therefore, rat was chosen to develop model of columnar metaplasia by using combined mucosal stripping and jejunogastric reflux (JGR).

The JGR model successfully generated columnar metaplasia in all the animals. This model also closely replicates human *in vivo* situation of severe gastroesophageal reflux disease, peptic ulceration, oesophagitis and development of CLO in the presence of chronic reflux. This model also demonstrated that in gastrointestinal reflux there is increased bile reflux into the oesophagus with increased systemic

absorption of bile acid, though intraoesophageal pH may not be significantly altered. Therefore, 'neutral' pH reflux can cause columnar metaplasia. This is noted in many clinical studies of CLO and is due to the fact that gastrointestinal refluxate contains a mixture of gastric, duodenal, bile and pancreatic secretions and mixed reflux may only produce 'neutral' pH reflux (Johnson et al 1987;Stein et al 1993;Stein et al 1999;Clark et al 1997).

As JGR model successfully produces columnar metaplasia in all cases the relationship between different reflux constituents and development of CLO can be studied by performing various gastrointestinal anastomosis to achieve selective reflux of pure gastric, duodenal, bile and various mixed reflux combinations. This is best achieved in lower oesophagus proximal to the gastroesophageal sphincter similar to *in vivo* CLO by oesophageal mucosal stripping and oesophagointestinal anastomosis (Bremner et al 1970;Levrat et al 1962). The therapeutic manipulation of proton pump inhibitor in preventing CLO in various refluxates can also be adequately studied in this model by administering oral PPI omeprazole to the group during postoperative period.

Nine models of CLO using different refluxates were successfully produced with columnar metaplasia in all cases. This columnar metaplasia had specialised columnar epithelium with goblet cells and secretory trefoil peptide phenotype expression similar to human CLO and Ulcer Associated Cell Lineage glands (Hanby et al 1994;Wright 1996). The columnar epithelium generated in these models had increased proliferation activity as indicated by p53 and Ki67 antigen expression. The activity of the antigens was highest in rapidly dividing basal layer of squamous epithelium, crypts of

intestinal epithelium and the advancing columnar metaplastic epithelium. The expression of UACL glands trefoil peptide phenotype distribution was likewise increased in the crypts and at the edge indicating that expression of particular phenotypes can be found at sites of rapid turnover in proliferation zones. Thus, advancing edge at the squamo-columnar junction exhibits phenotype expression akin to crypts of intestinal epithelium. Furthermore, glandular element is predominant in the lower parts of advancing columnar edge. This is identical to glandular activity at crypt related glands and Brunner glands. Therefore, the advancing edge shows healing of ulcers in presence of chronic reflux with cellular activity similar to replacement epithelium as part of normal cell cycle and turnover of normal intestinal epithelium. The resulting columnar epithelium shows trefoil peptide phenotype expression and presence of goblet cells which relates to adjacent mature epithelium. This suggests that CLO cell originates from any columnar epithelium which remains after damage of squamous mucosa by chronic reflux of gastrointestinal secretions (Li et al 1994). Thus, at the oesophagogastric mucosal junction damage to mucosa may heal by gastric type epithelium, at the oesophagojejunostomy healing at the junction is by intestinal type epithelium and in human CLO it is from either from gastric epithelium or cells of columnar lined oesophageal glands. Only at the advancing edge is immature highly proliferative specialised columnar epithelium found and this reverts to more established phenotype once stimulus for rapid turnover of cells is removed. Thus, CLO represents a healing process in chronic injury akin to chronic inflammatory bowel disease, Marjolin's ulcer, chronic osteomyelitis, cervical metaplasia, etc., but this healing involves replacement of squamous epithelium by genetically unstable columnar epithelium that has a propensity to malignant

transformation once normal cell adhesion complex (E-cadherin) and cell signal mechanisms (β -catenin) are disrupted (Aldulaimi et al 1999).

The mechanisms for cellular transformation and signal disruption can be initiated by any reflux. Therefore, in mixed acidic gastric and alkaline duodenal reflux the patient may not be experiencing any symptoms due to 'neutral' pH reflux yet cellular proliferation is continually being fueled by asymptomatic but noxious stimulus (Johnson et al 1987). This would account for relatively asymptomatic presentation of CLO in large proportion of population. It is reasonable to presume that in mixed reflux there will be activation of several brush border and pancreatic enzymes that may cause initial squamous mucosal injury, initiating oxygen free radical injury thus triggering metaplasia. As the mechanisms for columnar metaplasia can be triggered by any pure or mixed reflux, it is possible that once metaplastic and neoplastic transformation is initiated even normal physiological reflux could also stimulate the aberrant mucosa leading eventually to relatively silent asymptomatic neoplasia that present late.

A model where interaction of various enzyme activation, oxygen radical species and refluxates can be studied will be very be helpful to elucidate the initial triggering mechanisms responsible for neoplasia. For this a model of CLO is required where columnar metaplasia can be consistently produced and quantified. Our study clearly establishes that CLO with intestinal-type epithelium can be consistently and easily produced using these models. Furthermore, an efficient and reproducible Barrett's metaplasia scoring system has been developed that can be used to compare therapeutic modalities. In addition, by varying the time factor sequential changes from

gastroesophageal reflux disease, reflux oesophagitis, CLO metaplasia to CLO associated adenocarcinoma can be studied in this model (Chen et al 1999;Hanby et al 1994).

Results from our models of CLO show that reflux oesophagitis precedes CLO and reflux oesophagitis can also be produced by mixed reflux or any fractions of the mixed reflux. Gastric and duodenal secretions bring about greatest increase in length of columnar metaplasia, but biliary reflux and mixed biliary reflux cause greatest increase in acute inflammation while pancreatic secretions cause lesser degree of these changes. These changes might potentiate CLO in patients with acid suppression (Mirvish 1997).

In fact, duodenal reflux may be the cause of ineffectiveness of proton pump inhibitor Omeprazole in a significant number of CLO patients as its use does not effectively reverse the changes of reflux oesophagitis and columnar metaplasia in majority of *in vivo* cases (Kouzu et al 1998;Narbona-Arnau et al 1994). Omeprazole use in models of CLO caused by duodenal dominant reflux confirms this.

Hence it may be better to do antireflux surgery in mixed and duodenal dominant reflux as anti-reflux surgery can cause regression of CLO in up to 50% of patients (Kouzu et al 1998;Attwood 1993;Pera et al 1993). Whether use of combined acid suppression and oesophageal mucosal coating agents is helpful in suppressing columnar changes is not known. Use of partial mucosectomy, coagulation with electrocautery, mucosal ablation with laser and photodynamic therapy to treat CLO give mixed and conflicting results. The conflict between suitability of these

therapeutic interventions can be resolved using animal models in comparative studies. Our models of CLO will be very extremely helpful to study and evaluate these newer treatment modalities quantitatively and qualitatively.

Appendix 1. Enzabile method for estimating bile acid concentration.

Bile acid estimation with Enzabile kit

Enzabile ® (Nycomed Pharma As, PO CLOx 5012 Majorstua, N-0301 Oslo, Norway Tel. +47 22 96 36 36) is a commercially available test kit for the enzymatic, colourimetric determination of total 3-alpha hydroxy bile acids in serum, intestinal juice. Bile acids are not normally found in stomach or oesophagus, and are not altered by intragastric acid. Measurements of total bile acids in gastric aspirate is therefore considered to be a valuable marker for gastrointestinal bile reflux, and may give important information about the animals with iatrogenic bile reflux.

Test principle

The 3-alpha-hydroxy bile acid in the sample reacts with NAD and enzyme 3-alpha-HSDH to produce 3-keto bile acid, H+ and NADH. NADH and Hydrogen ion H+ react with NBT and enzyme diaphorase to produce NAD+ and formazan. Formazan has a stable blue colour with absorption maximum at 540 nm. Colourimetric estimation of the final product, formazan, can be used to determine the concentration of 3-alpha-hydroxy bile acid (Nycomed 1997). The Enzabile method shows a linear relation between absorbance at 540 nm and 3-alpha-hydroxy bile acid concentration upto 200 µmol/l. the standard curve is obtained by using bovine serum based standards containing glycochenodeoxycholic acid. When aqueous standards are used, reduced sensitivity is obtained, indicating that are components in the serum matrix that enhance the absorbtivity of the formazan dye.

The enzyme 3-alpha-HSDH is isolated from Pseudomonas testosteroni. The highly purified enzyme preparation is free from interfering dehydrogenases such as 7-alpha-HSDH, 12-alpha-HSDH and alcohol dehydrogenase (Nycomed 1997). 3-alphahydroxysteroids other than bile acids are present in serum conjugated to either sulfate or glucoronide through the 3-alpha-hydroxyl group and will therefore not interfere in this assay. Heparin and haemoglobin cause decreased recovery of total bile acids and therefore heparinized plasma or haemolysed specimens can't be used.

The kit contains the following reagents:

- Sample reagent. The composition after reconstitution is 3-alpha hydroxysteroid dehygrogenase >20 U/l, Diaphorase 125 U/l, NAD 1.0 mmol/l, Nitrobluetetrazolium salt (NBT) 0.3 mmol/l, Sodium phosphate buffer 65 mmol/l, Oxamic acid 45 mmol/l, Sodium azide 0.02% and Stabilisers.
- 2. Blank reagent. Contains all the constituents of the Sample reagent except for the initiating enzyme, 3-alpha hydroxysteroid dehydrogenase.
- 3. Reconstituting buffer. Sodium phosphate buffer at pH 7.0 65 mmol/l.
- Stop reagent. Hydrochloric acid (HCl) 200 mmol/l, Surfactant. The stop reagent is used to clear up the reaction mixture and to increase the stability of the colour developed.
- Enzabile Standards. Contain glycochenodeoxycholic acid (GCDC) lyophilised in a CLOvine serum matrix, in concentrations of 5, 25 and 100 μmol/l.

Procedure for estimating the 3-alpha-hydroxy bile acids

- Reconstitution of reagents. One vial of the Sample reagent and one vial of Blank reagent are reconstituted with exactly 10.0 ml of Reconstituting buffer. This is left for ten minutes.
- 2. Analysis. Pipette into test plate.

	Sample tube	Blank tube		
Serum (S), standard (ST) or water (RB)	200 µl	200 µl		
Sample reagent	500 µl	-		
Blank reagent	-	500 μl		
Mix and incubate for 20 minutes at room temperature $(20^{\circ} - 25^{\circ} \text{ C})$				
Stop reagent	500 µl	500 μl		
Mix thoroughly and read the absorbance of sample tube (A sample) and blank tube (A				
blank) at 540 nm against the respective reagent blanks (RB).				

For serum samples giving an absorbance exceeding the upper range of linearity dilution with NaCl 0.9 mol/l was done.

- 3. Measuring conditions. Wavelenght 540 nm, Absorbance range 0-2 A, Curvette pathlength 1.0 cm, zero adjustment with distilled water.
- 4. The net absorbance values for standards (Delta A ST) and serum samples (Delta A
 - S) were calculated by the following formula.

Delta A ST = A ST Sample - A ST Blank

Delta A S = A S Sample – A S Blank

The standard curve is prepared by plotting the net absorbance (Delta A ST) against the concentration of the Enzabile Standard solutions. The 3-alpha-hydorxy bile acid concentration in the serum samples, C (umol/l) is read from the standard curve. Adjustments are made for any diluted serum.

Appendix 2. Specimen preparation and Histological staining.

Paraffin wax blocks and slide preparation

At autopsy, the lower 2 cm of the oeosphagus, and proximal stomach or anastomosis was removed en bloc. The specimens were opened and then dissected out. The lower oeosphagus and the anastomosis were pinned on a cork sheet and put in 10% neutral buffered formaline saline. The specimen was then fixed longitudinally for 48 hours and embedded in paraffin wax using automatic processing machine. A routine automatic process over 24 hours was used (Drury et al 1967). After being embedded in paraffin wax, the specimens were cut on a microtome to produce three longitudinal sections of the gastro-oesophageal or gastro-jejunal junction, 3 µm thick, from each specimen. The sections were gently lowered onto the surface of water 5-10 degree C below the melting point of the wax (melting point 60 degree C), and mounted onto glass slides coated with glycerine jelly. They were then dried in 37 degree C for 24 hours. Before staining, the sections were taken to water: paraffin was removed from the tissue by immersion for 1-2 minutes first in xylene, intermediate xylene, and then an alcohol-xylene mixture. The xylene was then removed by immersion for one minute in each of two changes of absolute alcohol. The sections were then treated for 1-2 minutes with 90% alcohol, 70% alcohol, and then immersed in distilled water.
Haematoxin and Eosin

The sections were then stained using the Haematoxylin and Eosin method (Drury et al 1967). This technique consisted of staining with Haematoxylin for 20 minutes in a jar. The sections were washed in running tap water for 10 minutes, and returned to the jar only if there was insufficient degree of staining. Excess stain was removed by decolourization with 0.5% hydrochloric acid in 70% alcohol for 15 seconds. After washing in running alkaline tap water for 10 minutes to regain the blue colour, the sections were stained in 1% aqueous eosin for 3 minutes. Surplus stain was washed off in water, and after dehydration in alcohol and clearing in xylene, the sections were mounted in a synthetic resin medium.

PAS Diastase (Alcian blue)

Method

- 1. Paraffin sections to water.
- 2. Filtered 1% Alcian blue in 3% acetic acid (pH 2.5) <u>5 minutes</u>.
- 3. Wash in water.
- 4. Counterstain in filtered 1% neutral red <u>1 minute</u>.
- 5. Blot dry differentiate in 100% alcohol, then clear in xylene and mount.

<u>Results</u>

Most acid mucins except some of the strongly sulphated group - blue, nuclei - red.

Combined Alcian blue/PAS

Method

- 1. Paraffin sections to water.
- 2. Filtered 1% Alcian blue in 3% acetic acid (pH2.5) <u>5 minutes</u>.
- 3. Wash in water.
- 4. 1% periodic acid 5 minutes.
- 5. Wash in water.
- 6. Schiff's reagent 8 minutes. (This may depend upon the batch of reagent)
- 7. Wash in distilled water.
- 8. Harris's haematoxylin 30 seconds.
- 9. Simultaneously "blue and pink" in tap water.
- 10. D.C.M.

<u>Results</u>

- blue
- red
- purple
- blue

Diastase (Amylase) 0.1% for 15 minutes.

Streptividin - Peroxidase (SABC) technique

For mouse monoclonal antibodies

- 1. Dewax sections and take down to 100% alcohol.
- 2. Block endogenous peroxidase (A), 10 minutes. Rehydrate in alcohols to water.
- 3. Trypsinise (B) or microwave (C) as necessary. Wash in water and rinse in PBS.
- 4. Incubate sections in normal rabbit serum (1:25), for 10 minutes (optional). Drain.
- 5. Incubate for 35 minutes in primary antibody at optimal dilution.
- 6. Wash in two changes of PBS, 5 minutes each.
- 7. Incubate for 35 minutes in biotinylated rabbit anti-mouse C69 (Dako E354) 1:300.
- 8. Wash in two changes of PBS, 5 minutes each.
- 9. Incubate for 30 minutes in Streptividin-Peroxidase C188 (Dako P 397) 1:500.
- 10. Wash in two changes of PBS, 5 minutes each.
- 11. Prepare peroxidase substrate (DAB) 5mg DAB, Sigma (D5637) 10 mls of PBS plus 20 microl 30% Hydrogen Peroxide. Incubate in DAB for 2 minutes.
- 12. Wash in water and counterstain in Haematoxylin for 2 minutes. Wash, differentiate and blue.
- 13. Dehydrate, clear and mount in DPX-type mountant.

For rabbit polyclonal antibodies

Steps 1–3 as above.

- 4. Normal swine serum 1:25 for 15 minutes. Drain.
- 5. Incubate for 35 minutes in primary antibody at optimal dilution.
- 6. Wash in two changes of PBS, 5 minutes each.
- 7. Incubate for 35 minutes in biotinylated swine anti-rabbit (Dako E353) C70. 1:500.

Steps 8 – 13 as above.

A. Blocking of endogenous Peroxidase

2.4 ml of 30% Hydrogen Peroxide in 400 ml Methanol.

B. Trypsin

100 mg Trypsin (From beef pancreas: BDH 39041)
100 mg Calcium Chloride
100 ml Distilled water
Adjust pH to 7.8 using N/10 NaOH. Incubate at 37C.

C. Microwaving

Microwave in 0.01M citrate buffer pH 6 for 10 minutes at 700W. Preheat buffer. 2.94g Tri-sodium Citrate in 1000ml distilled water. Adjust pH with M Acetic Acid.

For frozen sections

- 1. Dry sections at room temperature for 15 minutes.
- 2. Fix in cold (-20) Acetone for 15 minutes. Drain and rinse in PBS.
- 3. Block in 2.4 ml H2O2 in 400 ml PBS for 5 minutes* Rinse in PBS.

Steps 4 - 13 as aBOve.

- * Block in a) 0.6% Hydrogen Peroxide in PBS for 5 minutes, or
 - b) 0.1% Diphenyl Hydrazine for 5 minutes, or
 - c) 0.1% Periodic acid for 5 minutes.

Human Spasmolytic Polypeptide Mouse monoclonal

Indirect staining protocol

Paraffin sections

- 1. Dewax sections and take down to 100% alcohol.
- 2. Block endogenous peroxidase (2.4 ml of 30% Hydrogen Peroxide in 400 ml Methanol), for 10 minutes.
- 3. Wash in running tap water, then rinse in PBS.
- 4. Incubate for 35 minutes in hSP antibody, neat.
- 5. Wash in two changes of PBS, 5 minutes each.
- 6. Incubate for 35 minutes in goat anti-mouse IgM, 1:100 (Sigma A 8786 or equivalent).
- 7. Wash in two changes of PBS, 5 minutes each.
- 8. Prepare peroxidase substrate (DAB) 5mg DAB, 10 mls of PBS plus 20 microl of 30% Hydrogen Peroxide. Incubate in DAB for 2 minutes.
- 9. Wash in water and counterstain in Haematoxylin for 2 minutes. Wash, differentiate and blue.
- 10. Dehydrate clear and mount in DPX-tupe mountant.

Frozen sections

- 1. Let sections dry at room temperature for at least 20 minutes.
- 2. Fix in Formalin (NBF), for 5 minutes and wash in water.
- 3. Block endogenous peroxidase (2.4 ml of 30% Hydrogen peroxide in 400 ml
- PBS). Wash in water and then PBS.

Proceed as in 4. above.

NB. Antibody to human Spasmolytic Polypeptide is an IgM.

Appendix 3. Histological grading score for degree of

metaplasia.

Col. Length (cm.)	(L) Score	*Acute Infl.	(A) Score	⁺ Chronic Infl.	(C) Score	Metpl.	(M) Score	Grading Score L+A+ C+M	Degree of Metpl.
0	0	0	0	0	0	0	0	0	0
0.5	1	Mild	1	Minimal	1	Islands	1	1 - 4	Minimal
1	2	Mod.	2	Mild	2	Sq reg.	2	5 – 8	Mild
1.5	3	Severe	3	Moderate	3	gl reg.	3	9 – 12	Moderate
2	4	Ulcer	4	Severe	4	Sq + gl reg.	4	> 12	Severe

Col. – Columnar

cm. – centimetres

Infl. – inflammation

Mod. - moderate

Sq – squamous

gl-glandular

reg. – regeneration

Metpl. – metaplasia

(L) Score – Columnar Length score

(A) score – acute inflammation score

(C) score – chronic inflammation score

(M) score - metaplasia score

*Acute inflammation – semi-quantitative assessment of neutrophil infiltrate in epithelium and stroma:

mild – few cells, moderate – clusters of neutrophils, severe – clustered and widespread, ulcer – clustered and widespread neutrophils with ulceration.

⁺**Chronic inflammation** – semi-quantitative assessment of lympho-plasmacytic infiltrate in epithelium and stroma:

minimal – very occasional single cells, mild – diffuse scanty, moderate – clustered, severe – clustered and widespread.

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