

Barrett's oesophagus and oesophageal adenocarcinoma: how does acid interfere with cell proliferation and differentiation?

R C Fitzgerald

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Acid, a principal component of gastro-oesophageal refluxate, may contribute to the development and malignant progression of Barrett's oesophagus. Oesophageal pH monitoring studies have demonstrated that patients with Barrett's oesophagus have severe and chronic acid reflux. However, there is overlap between the amount of acid exposure in patients with oesophagitis compared with patients with Barrett's oesophagus. This suggests that factors other than acid may be important in the aberrant oesophageal cell differentiation process that leads to the development of the metaplastic Barrett's mucosa. The other factors important in the aetiology of Barrett's oesophagus are poorly understood but probably include both genetic and environmental factors.

Barrett's oesophagus is a disorder characterised by abnormal differentiation and proliferation. The development of the metaplastic Barrett's mucosa occurs when there is a switch from one differentiated epithelium to another cell lineage not normally found in the oesophagus. Specifically, the normal stratified squamous mucosa is replaced by glandular mucosa, which is usually a mosaic of gastric and intestinal phenotypes. Many investigators restrict the definition of Barrett's oesophagus to mucosa containing the intestinal subtype, termed intestinal metaplasia. This is characterised by a columnar epithelium with a brush border and goblet cells, accompanied by the expression of intestine specific genes.¹ Although the metaplastic Barrett's mucosa resembles the native gastric or intestinal subtypes it is abnormally differentiated (fig 1).

The Barrett's epithelium has abnormal proliferation indices compared with non-metaplastic epithelium found elsewhere in the gastrointestinal tract. For example, the number of proliferating cells in Barrett's oesophagus is increased compared with squamous oesophagus and duodenum²⁻³ and there is associated dysregulation of cell cycling.⁴ Furthermore, the proliferative compartment extends beyond the glands and the lower crypts towards the surface⁵⁻⁶ (fig 2). When Barrett's oesophagus progresses towards cancer, the epithelial cells increasingly subvert the intrinsic mechanisms that limit the proliferative capacity of normal cells. Furthermore, as pro-

liferation increases, the degree of cellular differentiation decreases. This highly proliferative epithelium with altered differentiation is characterised morphologically by dysplasia and may ultimately evolve to invasive cancer (fig 1).

The transition from squamous epithelium to Barrett's oesophagus and the subsequent dysplasia-carcinoma sequence occurs in the context of exposure to gastro-duodenal refluxate. However, the degree to which the components of refluxate have a causal role in the pathogenesis of these phenotypic changes is not fully understood. In this review the discussion will be restricted to acid, a key component of refluxate. However, it should be remembered that the other constituents of refluxate, such as bile, may also play an important role. This review focuses on the role of acid in the differentiation and proliferation of the Barrett's epithelium with reference to laboratory and clinical studies. The relevance of these observations to clinical practice are then discussed and the areas of uncertainty that warrant further research are highlighted.

ABNORMAL ACID EXPOSURE IN BARRETT'S OESOPHAGUS

There is good evidence to suggest that the components of gastro-oesophageal refluxate are an important aetiological factor in Barrett's oesophagus.⁷⁻⁸ 24 h ambulatory pH monitoring studies have shown that patients with Barrett's oesophagus have more oesophageal acid exposure than healthy controls or patients with mild heartburn, but a degree of exposure similar to patients with severe oesophagitis. The greater acid exposure of Barrett's oesophagus results from longer periods of acid reflux (greater than 5 min), rather than from a greater number of reflux episodes. Barrett's oesophagus patients may be predisposed to more severe acid reflux because of two principal pathological mechanisms. Firstly, mechanical dysfunction of the lower oesophageal sphincter; and secondly, the decreased amplitude of distal oesophageal contractions in Barrett's oesophagus patients compared with healthy controls or patients with oesophagitis. This impairment in oesophageal peristaltic activity reduces the ability to clear refluxate from the oesophagus.⁹⁻¹⁰ It is also

Abbreviations: COX-2, cyclo-oxygenase 2; MAPK, mitogen activated protein kinase; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; PPI, proton pump inhibitor; PGE₂, prostaglandin

Correspondence to:
Dr R C Fitzgerald, Cancer
Cell Unit, Hutchison-MRC
Research Centre,
Cambridge CB2 2XZ, UK;
rcf@hutchison-mrc.cam.
ac.uk

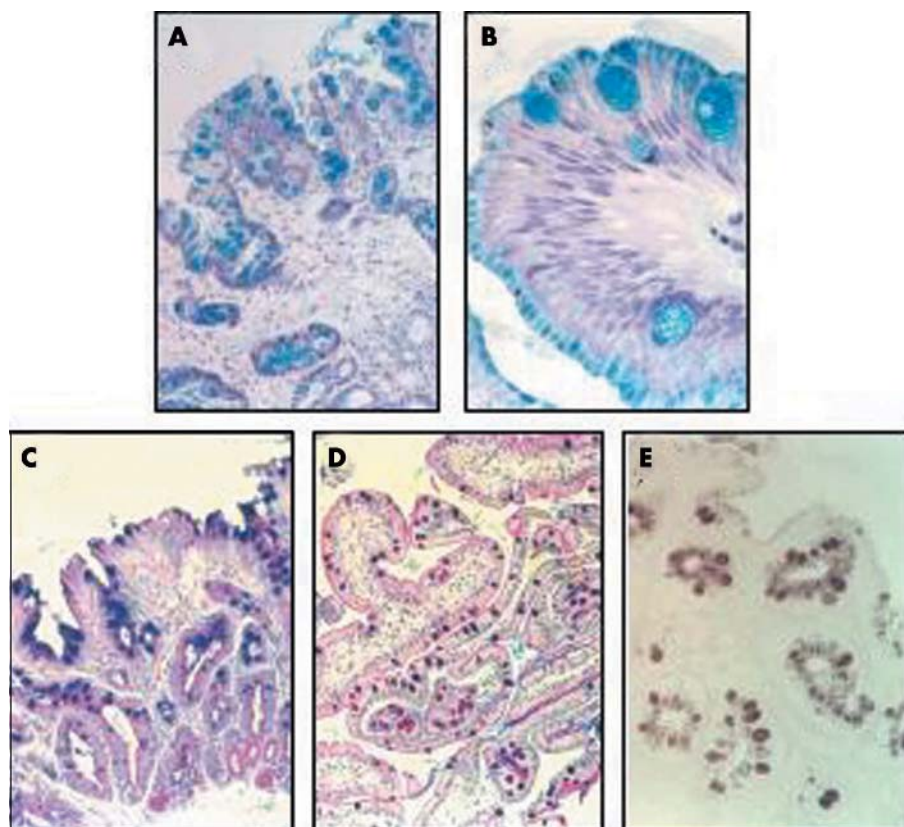


Figure 1 Abnormal differentiation status of the Barrett's epithelium. (A) and (B) low and high power fields, respectively, of alcian blue staining in Barrett's oesophageal sections showing the blue acidic and neutral mucins in goblet and non-goblet columnar cells. (C) PAS-alcian blue staining for neutral mucins (magenta) and N-acetylated sialo-mucins (blue) found in type IIB intestinal metaplasia. (D) the same PAS-alcian blue staining in duodenal section, note that mucins here are mainly neutral (magenta). (E) a high iron diamine-alcian blue staining showing dark brown sulphomucins characteristic of type IIB incomplete IM.

interesting to note that in studies on the role of bile salts in Barrett's oesophagus the pH of the refluxate is a determinant of cellular damage. For example, *in vitro* experiments on rabbit oesophageal mucosa demonstrated that taurine conjugated bile salts cause mucosal damage at pH 2, whereas unconjugated bile salts and trypsin are more harmful at pH 5–8.¹¹ Furthermore, recent oesophageal aspiration studies have shown increased levels of taurine conjugated bile salts in Barrett's oesophagus that are temporally associated with acid reflux.¹²

DEVELOPMENT OF BARRETT'S METAPLASIA

The phenomenon of metaplasia may occur anywhere throughout the gastrointestinal tract and is thought to be a reparative response to injury. There is increasing evidence that pluripotential stem cells may be capable of differentiation along a lineage quite distinct from the parent organ.¹³ In the context of Barrett's oesophagus, it is likely that the pluripotential stem cells in the squamous oesophagus or neighbouring gastric epithelium are triggered to differentiate according to an intestinal type of cell lineage. These intestinal cells will then be able to undergo clonal expansion and hence the abnormal mucosa will be maintained.

Three possibilities for the tissue of origin for Barrett's metaplasia have been hypothesised.¹ Firstly, the *de novo* metaplasia theory proposes that pluripotential stem cells of inflamed squamous mucosa in the exposed papillae are damaged and abnormally differentiate producing Barrett's stem cells. However, recent human studies suggest, based on $\beta 1$ integrin expression, that the squamous oesophageal stem cells are located in the interpapillary zone.¹⁴ Secondly, the transitional zone metaplasia theory suggests that pluripotential stem cells at the gastro-oesophageal junction (transitional zone) colonise the gastric cardia or distal oesophagus in response to noxious luminal agents. Thirdly, the duct cell metaplasia theory suggests that stem cells located in the glandular neck region of oesophageal

ducts are thought to selectively colonise the oesophagus in response to squamous mucosal damage. The basis for this mechanism is the ulcer associated cell lineage.¹ All of the proposed theories for the origin of metaplasia require a noxious luminal agent, such as acid.

Apart from observational data there is little direct evidence to show that exposure to refluxate has a causal role in the development of Barrett's metaplasia in humans. The development of Barrett's metaplasia is rarely observed *in vivo* and hence it is thought that the columnar lined mucosa probably develops to its full length over a period of weeks.¹⁵ Furthermore, to date it has not been possible to induce oesophageal metaplasia *in vitro* through exposure of cell or organ cultures to acid or other components of refluxate.

It is interesting to note that patients with Barrett's oesophagus undergoing endoscopic ablative therapies seem to require a high degree of acid suppression in order to permit regeneration of squamous epithelium, although the evidence is somewhat contradictory.^{16, 17}

There have been a number of animal models that have demonstrated that severe and chronic exposure of the distal oesophageal mucosa to refluxate can induce metaplasia. These models are usually in the context of a prior mucosal injury or with the addition of a carcinogen or an oxidising agent such as iron.^{18, 19} In a recent rat model for oesophageal adenocarcinoma Buttar *et al* induced Barrett's oesophagus with a jejuno-oesophageal loop without an additional defect in the lower oesophageal mucosa.²⁰ However, the Barrett's segment was limited to <2 mm from the gastro-oesophageal junction.²⁰

Recently an increased understanding of the genetic factors underlying the determination of stem cell fate in embryogenesis has opened up new avenues. Studies on genetic susceptibility to Barrett's oesophagus are still in their infancy and the likely candidate genes are speculative. However, certain homeobox transcription factors are likely to be involved. Homeobox genes determine cell fate and general

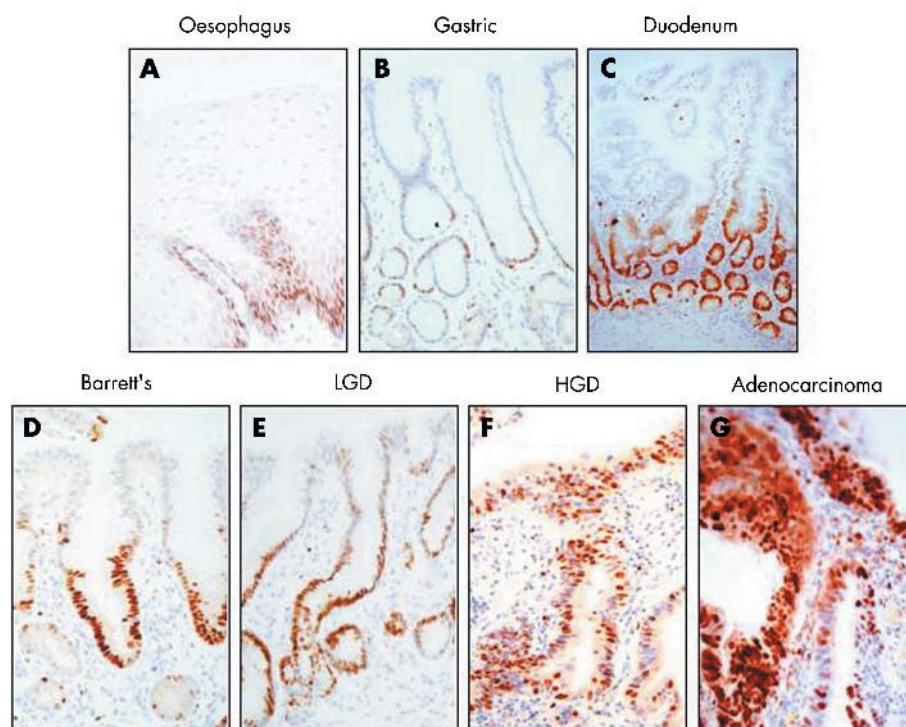


Figure 2 Abnormal proliferation compartments of Barrett's oesophagus and associated dysplasia. Expression of a proliferation marker mini-chromosome maintenance protein (Mcm2) is shown by immunohistochemistry. Whereas the proliferative compartment is confined to the basal layers (normal squamous oesophagus, (A) and in the crypts and the glands (normal stomach and duodenum, (B) and (C)), in Barrett's metaplasia proliferation extends towards the surface (D). With increasing dysplasia the number of proliferating cells increases with expansion of the proliferative compartment (E-G).

pattern formation in many tissues, particularly in regard to cephalo-caudal patterning. The homeobox containing proteins *cdx1* and *cdx2* appear to regulate epithelial differentiation. For example, ectopic expression of the intestine specific transcription factor *cdx2*, belonging to the caudal related homeobox gene family, has been shown to induce intestinal metaplasia of the stomach in transgenic mice.²¹

The difficulty in inducing oesophageal metaplasia in laboratory models probably reflects the multifactorial pathogenesis. Hence, in humans, although exposure to reflux almost certainly plays a role in the development of Barrett's oesophagus, it has to be seen in the context of genetic susceptibility and the oesophageal microenvironment. For example, Barrett's oesophageal biopsies have higher levels of anti-inflammatory Th2 cytokines compared with oesophageal biopsies from patients with oesophagitis and endoscopically negative reflux.²²⁻²³ The complex gene-gene and gene-environmental interactions may explain why only a small proportion of people with reflux (approximately 10%) develop Barrett's oesophagus.²⁴⁻²⁵

Hopefully, with more robust animal models combined with a molecular epidemiological approach, the contribution of genetic and environmental factors in the development of Barrett's metaplasia can be clarified.

ROLE OF ACID IN BARRETT'S CARCINOGENESIS

Epidemiology

The striking increase in the incidence of oesophageal adenocarcinoma over the past two to three decades suggests that environmental factors are important. People have suffered from heartburn and indigestion symptoms for many years. Hence, if acid exposure is a risk factor for oesophageal adenocarcinoma,²⁶ it is not clear why there should have been such a dramatic increase in incidence in recent years.

Accurate epidemiological data on the incidence of heartburn, dyspepsia, and Barrett's oesophagus is difficult to come by due to the wide variation in the diagnostic classification

and the availability of over the counter medications. The American data suggest a dramatic reduction in hospitalisation rates for duodenal ulcer from 1975 to 1995, presumably secondary to the eradication of *Helicobacter pylori*. However, over the same time period hospitalisation rates for heartburn increased.²⁷ There are also data to suggest that the increasing incidence of Barrett's oesophagus has outstripped the increasing utilisation of endoscopy over the past 20 years in the UK. Again, these data may also be confounded by increased general practitioner referral rates for endoscopy and an increased awareness of Barrett's oesophagus by endoscopists.²⁸⁻²⁹

Several reasons have been proposed to explain the recent increasing incidence of reflux disease. Firstly, the population in the West have been getting fatter, which will tend to lead to the development of a hiatus hernia and to a disruption of the anatomy of the gastro-oesophageal junction. Secondly, there has been an increase in the use of drugs that reduce the lower oesophageal sphincter pressure. Thirdly, eradication of *H pylori* will tend to restore the acid secreting capacity of the stomach and lead to an increase in the acid content of refluxate. Fourthly, it has been suggested that the increased ingestion of nitrate containing foods in the white middle classes and the widespread use of nitrate based fertilisers since the second world war may have resulted in increased nitrate concentrations in the stomach, which may in turn increase the likelihood of reflux occurring.³⁰⁻³¹ However, these plausible explanations remain hypotheses that require substantiating by epidemiological studies.

Hyperproliferative response to acid

The question therefore arises as to whether continued exposure to acid in patients with established Barrett's oesophagus might contribute to carcinogenesis. Using an ex vivo culture technique of human biopsies, it has been demonstrated that acid and bile can alter the Barrett's cell phenotype. Because it would be unrealistic to expect cell changes amounting to

dysplasia to occur following short term exposure to components of refluxate, cell proliferation, and differentiation were used as surrogate markers for the dysplastic potential. A more differentiated cell with a low proliferation status would not be expected to harbour malignant potential and vice versa. These experiments demonstrated that the pattern of exposure to an acid stimulus is an important determinant of the resulting phenotype. Hence, continuous exposure to pH 3.5 over a 24 h period resulted in a more differentiated epithelial cell phenotype with a more mature brush border. In contrast, pulsatile acid or bile exposure (1 h pH 3.5 followed by pH 7.4 over a 24 h period) led to an increase in cell proliferation.³ This hyperproliferative effect of acid on Barrett's epithelial cells has now been confirmed in cell lines and *in vivo* at endoscopy.³² Interestingly, pulsatile exposure to bile has also been shown to have a similar hyperproliferative effect using similar culture models.³³

The mechanisms underlying the hyperproliferative response of the Barrett's mucosa have still not been fully elucidated but include activation of the sodium-hydrogen exchanger, which is known to directly affect progression of cells through the cell cycle.³⁴⁻³⁵ Cellular acid exposure may also cause alterations in cell signalling and hence activation of transcription factors leading to cell proliferation. For example, activation of mitogen activated protein kinase (MAPK) signalling pathways have been demonstrated by acid exposure. These experiments involved acid perfusion of patients' distal oesophagus at endoscopy as well as *in vitro* experiments of human Barrett's adenocarcinoma cell lines.³²

In order to more fully elucidate the effects of acid exposure on gene expression we have used a microarray approach. Using an Affymetrix (High Wycombe, UK) array with 22 000 genes, changes in expression were identified for 130 genes. Analysis of gene function identified immediate (0.5 to 2 h) down regulation of genes associated with programmed cell death (apoptosis) and early (4 to 10 h) up regulation of genes associated with proliferation. The gene expression profile suggested that the increase in proliferation may involve enhanced MAPK pathways secondary to a decrease in the expression of negative regulators. Suppression of apoptosis, confirmed by western blot and ELISA assays, may occur via p53 dependant mechanisms. This approach has confirmed the previous work on proliferation effects via alterations in MAPK and also provides candidate genes and signalling pathways for further analysis.³⁶

RELATIONSHIP BETWEEN ACID, COX-2 EXPRESSION, AND INCREASED CELL PROLIFERATION

Cyclo-oxygenase 2 (COX-2) is a membrane bound glycoprotein that functions as a rate limiting enzyme in the generation of prostanoids from arachidonic acid. COX-2 has a role in carcinogenesis via effects on cell proliferation, apoptosis, and angiogenesis. Recently, data have shown that COX-2 expression is increased in Barrett's oesophagus.³⁷ *Ex vivo* experiments using endoscopic biopsies of Barrett's oesophagus have shown that acid and bile can up regulate COX-2 expression³⁸ and lead to enhanced prostaglandin (PGE₂) release.³⁹ On the other hand, addition of a selective COX-2 inhibitor (NS-398) or inhibition of protein kinase C (PKC) (using bisindolylmaleimide BIM), led to a dramatic decrease in PGE₂ and a reduction in the proliferation of Barrett's epithelial cells as well as oesophageal adenocarcinoma cells *ex vivo*.⁴⁰⁻⁴¹ These findings have led to the development of a hypothetical model in which acid results in an early activation of PKC ϵ , followed by up regulation of COX-2 expression, enhanced PGE₂ production, and thus enhanced cell proliferation. Further work will determine whether there is a direct relationship between PGE₂, COX-2

up regulation, MAPK signalling, and cell proliferation or apoptosis status. The most definitive evidence for the role of COX-2 in the context of reflux comes from an animal model of reflux induced adenocarcinoma (Lerut's model). Using this model rats that were fed MF-tricyclic (a selective COX-2 inhibitor) or sulindac (a non-selective COX inhibitor) in the chow had reduced relative risk of development of oesophageal carcinoma by 55% compared with placebo. This reduction correlated with reduced levels of PGE₂. This is quite compelling evidence for a role for COX in this inflammation driven model, although how well this animal model correlates with carcinogenesis in humans is not clear.²⁰

RELATIONSHIP BETWEEN HYPERPROLIFERATION AND DYSPLASIA

Although it has been clearly demonstrated that there is a hyperproliferative response following pulsatile acid and bile exposure, it has not been proven that this contributes to the development of dysplasia. It is possible that the hyperproliferative response of Barrett's epithelial cells may lead to an accumulation of genetic abnormalities through a vicious cycle effect (fig 3). This may explain the degree of variation in somatic mutations seen between individuals. For example, a study of evolutionary relationships of somatic mutations suggests that mutations occur in no obligate order and clonal expansion of genetic instability leads to cancer over a process of months to years.⁴² Alternatively, key somatic mutations may be the primary event leading to altered cellular proliferation independent of acid exposure, which could be an epiphenomenon. These issues need further investigation.

CLINICAL RAMIFICATIONS OF RESEARCH

In the light of the laboratory findings that pulsatile acid exposure increases Barrett's epithelial cell proliferation and that increased COX-2 expression is associated with carcinogenesis, there has been an interest in using acid suppressants and COX-2 inhibitors as cancer chemoprevention agents.

A preliminary clinical study has shown that after 6 months' of complete acid suppression on a proton pump inhibitor (PPI), villin expression increased and proliferating cell nuclear antigen (PCNA) expression decreased ($p < 0.001$), ($n = 24$ patients). In contrast, after 6 months' of persistent acid reflux on a PPI there was no change in villin or PCNA expression ($n = 15$ patients).⁴³ These findings suggest that complete acid suppression may be important in order to increase cell differentiation and reduce cell proliferation. However, the sample size and the follow up period was not sufficient to determine whether the incidence of dysplasia would be different between the two groups.

Another immunohistochemical study examined the effect of acid suppression on cell cycle stage. Again, it was found that patients on more powerful acid suppressants (PPIs as opposed to H₂ receptor antagonists or gaviscon) had a relative increase in the expression of cyclin dependent kinase inhibitors p16 and p21 and a relative increase in the cell cycle oncogenes cyclin D1 and cyclin E. These results would suggest that complete acid suppression may be important to ensure appropriate progression through the cell cycle.⁴⁴

Antireflux surgery is an alternative to pharmacological acid suppression, which should also control the reflux of bile and other potentially important constituents of refluxate. Several investigators have analysed the incidence of oesophageal adenocarcinoma in patients with Barrett's oesophagus who have been treated with either acid suppressants or surgical antireflux procedures. The outcomes of these studies are conflicting (less incident adenocarcinomas in the surgical group,⁴⁵⁻⁴⁶ (less incidence carcinomas in the PPI group⁴⁷⁻⁴⁸). The largest of these studies was the cohort study by Ye *et al*⁴⁸ in which data were available from over 6000 patients in the surgical group.

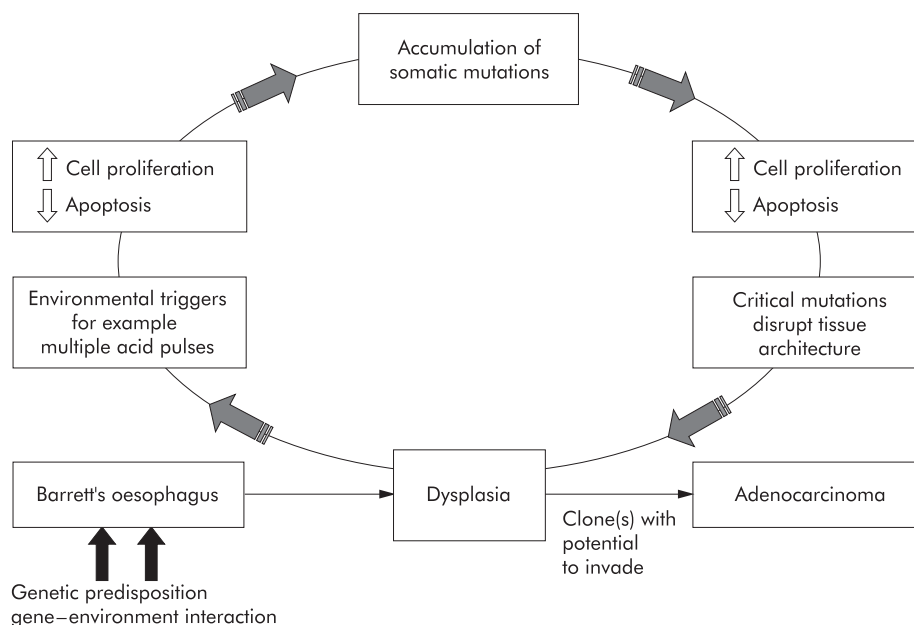


Figure 3 Acid and other luminal constituents may lead to a hyperproliferative and anti-apoptotic response of Barrett's epithelial cells. This in turn may lead to an accumulation of genetic abnormalities through a vicious cycle effect. The somatic mutations may be accumulated in a non-predictable order. Cancer will occur once the mutation(s) provides the cells with the capacity to invade.

Interestingly, although the incidence of oesophageal adenocarcinoma was 14-fold higher following antireflux surgery, there was also a 6-fold increase in patients on medical treatment for reflux compared with the control group.

There are several problems with these studies. Firstly, we do not know whether the patients had complete or incomplete acid suppression on medical treatment or following anti-reflux surgery. Secondly, over the time period that these studies were conducted both the acid suppressant drugs evolved as well as the types of antireflux surgery.

PRACTICAL CONSIDERATIONS

With the advent of powerful PPIs and the development of drugs to inhibit transient lower oesophageal relaxations, there is the potential to achieve virtual complete acid suppression in patients with Barrett's oesophagus. However, it should be remembered that symptom relief does not necessarily ensure that sufficient control of oesophageal acid exposure has been achieved. Furthermore, "normalisation" of intra-oesophageal acid exposure using standard criteria does not necessarily imply complete intra-oesophageal anacidity during the 24 h study and significant variability exists among different reports.^{49–50} In a small study, 30 patients with Barrett's oesophagus were all treated with lansoprazole 15–30 mg daily, until they were asymptomatic. 12/30 patients had abnormal pH studies due to ineffective PPI induced intragastric acid suppression. Using 24 h pH monitoring as a guide to treatment, it seems possible that complete intra-oesophageal acid suppression may be achieved.⁵¹

For patients with persistent acid reflux higher doses of PPI drugs may be required in combination with an H₂ antagonist at night.⁵² In these circumstances oesophageal pH measurements can be used to determine the minimum dose to eliminate symptoms due to acid reflux. The large variability in individual responses to PPIs may be as a result of individual variations in: the oral bioavailability of PPIs; the acid secreting potential of the gastric mucosa; and polymorphisms in the cytochrome P450 2C19 enzyme. In addition, the timing of PPI therapy in relation to meals, and hence alterations in parietal cell activity table, may also affect PPI efficacy.⁵³

There have been some concerns raised about the potential detrimental effects of complete acid suppression, such as the effects of bacterial overgrowth and long term hypergastrinaemia, which may in itself increase the risk of cancer development. A recent paper has added credence to this hypothesis by demonstrating that the Barrett's epithelium expressed cholecystikinin receptors and gastrin can induce proliferation via these receptors *in vitro*.⁵⁴ However, to date the clinical risks appear to be hypothetical and there is no evidence to contradict the widely held view that long term PPI treatment appears to be extremely safe. However, in my view, until it is shown that complete acid suppression has a role in cancer prevention then complete acid suppression cannot be advocated. Furthermore, without the evidence base for this practice complete acid suppression may pose unnecessary side effects on the patients with a significant cost attached.

COX-2 AND PPI IN COMBINATION

Epidemiologic studies have shown that aspirin and non-steroidal anti-inflammatory drugs that non-selectively inhibit COX are associated with a lower risk of oesophageal adenocarcinoma.^{55–56} Furthermore, a recent study by the Stanford group showed a striking reduction in cell proliferation characteristics following 10 days' treatment of patients with a high dose PPI and a COX-2 inhibitor.⁵⁷

It has been recognised for some time that a large, long term randomised controlled trial is required in order to test the role for acid suppressants and COX-2 inhibitors as chemopreventive agents.

CONCLUSIONS

In summary, Barrett's oesophagus is an epithelium characterised by abnormal differentiation and proliferation. The significance of acid on the development and progression of Barrett's oesophagus is still not fully understood. It is increasingly apparent that acid exposure has to be seen in the context of other environmental and molecular factors.

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