The role of acid and bile reflux in oesophagitis and Barrett’s metaplasia

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Abstract

The precise mechanisms whereby gastro-oesophageal reflux disease causes reflux oesophagitis and Barrett’s oesophagus are not clear, even though these diseases have been known to be linked for many years. Recent studies indicate a role for the reflux-induced inflammatory response of oesophageal squamous epithelial cells and the immune cells in the pathogenesis of reflux oesophagitis. Although reflux oesophagitis commonly heals with oesophageal squamous cell regeneration, in some individuals the oesophagus heals through the process of metaplasia, a condition termed Barrett’s oesophagus. Recent studies indicate that individual differences in the reflux-mediated response of oesophageal squamous epithelial cells in the type of immune response and/or in signalling pathways that regulate cell proliferation or cell phenotype may determine whether the oesophagus heals with the regeneration of squamous cells or through Barrett’s metaplasia.

Introduction

The prevailing concept of reflux oesophagitis pathogenesis is essentially a chemical burn model of injury. It is assumed that refluxed gastric acid and pepsin cause caustic cell injury and cell death, with progression from the luminal surface to the submucosa. More recent data from our group suggest that reflux oesophagitis develops as an immune-mediated injury which begins as a lymphocytic infiltrate in the submucosa that progresses toward the luminal surface, a process which may be initiated by the release of cytokines by reflux-exposed oesophageal squamous cells. In most individuals, reflux oesophagitis heals with squamous cell regeneration. In some, however, reflux oesophagitis heals through the process of metaplasia. This condition, Barrett’s oesophagus, predisposes to the development of oesophageal adenocarcinoma. It is not clear why only a minority of individuals with reflux oesophagitis develop Barrett’s metaplasia. There are both clinical and experimental data to suggest that the oesophageal squamous epithelium of patients with Barrett’s oesophagus is predisposed to developing metaplasia in response to reflux injury. Taken together, these studies suggest that reflux-mediated differences in the type of immune response and/or in signalling pathways that regulate cell proliferation or cell phenotype may determine whether the oesophagus heals with the regeneration of squamous cells or through Barrett’s metaplasia.

Reflux oesophagitis develops as an immune-mediated injury, rather than a caustic injury

For more than 50 years, the prevailing concept has been that reflux oesophagitis results from a caustic chemical injury that starts at the luminal surface and progresses to the deeper layers of the tissue. It has been thought that the reflux of gastric acid and pepsin into the oesophagus damages the tight junctions between the epithelial cells causing the intercellular spaces to dilate and H⁺ ions to enter into the epithelium [1–3]. Continued injury from an acute acid-induced chemical burn and death of the surface oesophageal epithelial cells has been assumed to recruit neutrophils to the epithelium. As the injury progresses into the deeper layers of the epithelium and the surface epithelial cells continue to die, a proliferative response has been presumed to ensue, leading to basal cell and papillary hyperplasia to replace the refluxed-damaged surface cells [4–6].

Our laboratory recently began using a rat model of reflux oesophagitis in which the oesophagus is surgically connected to the duodenum with the stomach remaining in place [7]. That oesophagoduodenostomy results in the free flow of gastric and duodenal contents into the oesophagus, causing severe reflux oesophagitis. However, other investigators using this model have noted that oesophagitis can take weeks to appear after the operation (N. Buttar, personal communication). Such a protracted time course to observe the oesophageal injury seems counterintuitive, because reflux oesophagitis is assumed to be initiated by the release of cytokines by reflux-exposed oesophageal squamous cells [4–6].

After performing an oesophagoduodenostomy in the rat, our group conducted a systematic study of the early histological events in the development of reflux oesophagitis [7]. We
found that at day 3 following oesophagoduodenostomy, there was no apparent damage to the surface epithelial cells and oesophageal inflammation was most prominent in the submucosal layer of the tissue [7]. This early inflammatory infiltrate comprised T-lymphocytes, determined by positive immunostaining for CD3, which is a marker of T-cells, and negative immunostaining for CD20, a marker of B-cells [7]. The inflammation, predominantly comprising T-lymphocytes, increased to reach significantly elevated levels in the lamina propria and epithelium by weeks 1 and 3 respectively [7]. Neutrophils were not detected in any layer of the oesophageal tissue until 7 days after the operation [7]; eosinophils were rarely detected over this same time period (R.F. Souza, unpublished work). Moreover, basal cell hyperplasia was apparent by week 1, but erosions of the surface epithelial cells were not found until week 4 [7]. These findings are exactly the opposite of those expected if reflux oesophagitis developed from a caustic chemical injury. As discussed above, an acid burn model would be expected to progress from the surface epithelial cells to the submucosa, and to start with infiltration of neutrophils. In contrast, reflux oesophagitis in the animal model started as a lymphocytic infiltration of the submucosa that progressed to the mucosal surface and neutrophils were seen after the T-lymphocytes [7]. Moreover, basal cell hyperplasia was observed weeks before surface erosions were noted, suggesting that it is not the loss of surface epithelial cells that triggers basal cell hyperplasia in this animal model [7]. Therefore our systematic study of the development of reflux oesophagitis in the rat oesophagus after oesophagoduodenostomy does not support the prevailing concept of reflux oesophagitis developing as the result of a caustic chemical (acid) burn model of injury beginning at the luminal surface.

Rather, in this animal model, the initial event appears to be immune cell infiltration, suggesting that gastro-oesophageal reflux might cause oesophageal squamous cells to produce cytokines. Exposure of telomerase-immortalized normal oesophageal squamous epithelial cell lines to a combination of acid and bile salts significantly increased secretion of the cytokines IL (interleukin)-8 and IL-1β after 2 and 4 days respectively [7]. In addition, the conditioned medium from those cells caused a significant increase in the migration rates of T-cells and neutrophils [7]. The addition of an IL-8-blocking antibody to the conditioned medium prevented the migration rate of neutrophils, but not that of T-cells, suggesting that IL-8 may play a central role in recruiting neutrophils to the epithelium in reflux oesophagitis [7]. Using immunohistochemical staining on the rat oesophagus, we also observed increased expression of IL-8 by the epithelial cells within 2 weeks of oesophagoduodenostomy [7]. A number of other investigators have also demonstrated the secretion of pro-inflammatory cytokines by oesophageal squamous cells in reflux oesophagitis, but in most of those studies, it was not clear whether cytokine production was a cause or an effect of the oesophagitis [7]. In one study by Yamaguchi et al. [8], for example, the oesophageal mucosal was found to express inflammatory cytokines within 3 h of the surgical induction of reflux using a rat model of oesophagitis, and that the administration of anti-neutrophil serum prevented the development of reflux oesophagitis. Overall, these findings support a new concept for the development of reflux oesophagitis in which gastro-oesophageal reflux causes oesophageal squamous epithelial cells to secrete cytokines that attract immune cells, and it is the immune cells, not acid, that ultimately damage the oesophageal mucosa.

**GORD (gastro-oesophageal reflux disease), reflux oesophagitis and Barrett’s oesophagus**

In addition to causing reflux oesophagitis, GORD is also a primary risk factor for Barrett’s oesophagus [9]. Barrett’s oesophagus develops through metaplasia, the process in which one adult cell type replaces another. In the case of Barrett’s metaplasia, the normal stratified squamous epithelium is replaced by a specialized intestinal type of columnar epithelium. In most individuals, the reflux-damaged lining of the oesophagus heals with the regeneration of oesophageal squamous cells. However, in a minority of individuals, and for reasons that remain unclear, the reflux-damaged oesophagus heals with the replacement of oesophageal squamous cells by metaplastic specialized intestinal-like columnar cells. Some data suggest that the oesophageal squamous epithelium of patients with Barrett’s oesophagus is predisposed to develop metaplastic changes in response to peptic injury. For example, in patients who have oesophagectomy with oesophago-gastric anastomosis, some studies report the development of columnar metaplasia in the oesophageal remnant significantly more often in patients who had Barrett’s oesophagus pre-operatively than in those without Barrett’s oesophagus, despite the presence of a similar degree of post-operative reflux oesophagitis [10,11]. There are also data to suggest that the oesophageal squamous epithelium of patients with Barrett’s oesophagus is exposed to greater amounts of gastric reflux, which might also predispose to healing through metaplasia [12]. Regardless of the reason for the metaplastic predisposition, it is conceivable that in oesophageal squamous epithelium, individual differences in the responses of molecular signalling pathways to gastric reflux may facilitate the healing of reflux-damaged squamous cells through metaplasia rather than through squamous cell regeneration.

Regeneration refers to the replacement of damaged epithelium by new cells and relies on proliferation and differentiation. This is the primary way in which the oesophageal lining is repaired following reflux-induced injury [13,14]. It is well established that chronic GORD increases proliferation in oesophageal squamous epithelium. For example, in oesophageal epithelium from an animal model of reflux oesophagitis, cells in the basal zone (proliferative zone of the oesophagus) of the oesophageal epithelium demonstrated increased proliferation rates compared with cells in the basal zone of non-inflamed oesophageal squamous epithelium [15]. Likewise, in biopsy specimens of oesophageal squamous mucosa from patients...
with severe ulcerative reflux oesophagitis, cells in the basal zone demonstrated increased rates of proliferation compared with those from patients with no or only mild reflux oesophagitis [16]. It therefore appears that gastric reflux normally increases proliferation in oesophageal squamous epithelium, and it is possible that this increase in proliferation facilitates regeneration and repair of injured mucosa.

Cell proliferation can be regulated by a number of signalling pathways, including the MAPK (mitogen-activated protein kinase) pathway. Growth factors, mitogens and acidic pH have been found to activate the MAPK kinase MEK1/2 [MAPK/ERK (extracellular-signal-regulated kinase) kinase 1/2], which in turn phosphorylates and activates ERK1/2 [17]. ERK1/2 in turn transmits mitogenic signals to the nucleus, leading to cell proliferation and differentiation [18]. We have found that acid perfusion of the oesophageal squamous epithelium in vivo activates the pro-proliferative kinase ERK1/2 in patients who have GORD without Barrett’s oesophagus, but not in those with Barrett’s oesophagus [19,20]. Moreover, oesophageal squamous biopsy specimens from patients with Barrett’s oesophagus demonstrate expression of an inhibitory phosphorylated form of MEK1/2, whereas no expression of this inhibitory phosphoprotein was detected in oesophageal squamous biopsy specimens from GORD patients without Barrett’s oesophagus [20]. Using microarray technology, increased levels of expression of Dkk-1 (where Dkk is Dickkopf) and Dkk-4 genes, which regulate proliferation and apoptosis, have been found in the oesophageal squamous epithelium of GORD patients without Barrett’s oesophagus compared with those with Barrett’s oesophagus [21]. These data suggest that, in oesophageal squamous epithelium, differences at baseline and in reflux-mediated induction of signal transduction pathways that regulate cell proliferation and apoptosis may determine whether the oesophagus heals through squamous cell regeneration or through metaplasia.

**Barrett’s oesophagus: a metaplastic response to gastro-oesophageal reflux**

**Metaplasia arising from stem cells**

The major components of gastric refluxate are acid and bile salts, therefore the following discussion will focus on the role of acid and bile salts in the formation of oesophageal metaplasia. As discussed above, Barrett’s oesophagus is the condition in which the normal oesophageal squamous epithelium is replaced by a metaplastic specialized intestinal-like epithelium. This metaplastic process could happen by changing the differentiation pattern of stem cells or by changing already fully differentiated cells [22]. Conceivably, the reflux of acid and bile salts could interfere with this process by causing a change in the differentiation pattern of either the stem cells or the differentiated cells, resulting in metaplasia.

In general, the stem cells which give rise to the oesophageal epithelium are thought to reside within the oesophageal tissue itself. It has been demonstrated that injuries in a number of organs may heal not only through the proliferation and differentiation of tissue resident stem cells, but also through the proliferation and differentiation of stem cells derived from the bone marrow [23]. Our laboratory has investigated the contribution of bone marrow stem cells to the development of Barrett’s oesophagus using a rat model. For this study, the bone marrow of female rats was destroyed by irradiation and then reconstituted with bone marrow from male donors [24]. The female rats then underwent an oesophagojenoctomy, which results in severe ulcerative oesophagitis and in intestinal metaplasia [24,25]. In both squamous cells and metaplastic columnar cells of the oesophagus, nuclear staining for the Y chromosome was found in female rats that had received bone marrow transplants from male donors [24]. In contrast, no nuclear staining for the Y chromosome was observed in control female rats after oesophagojenoctomy that had not received bone marrow transplants [24]. These observations suggest that bone-marrow-derived stem cells may contribute to oesophageal regeneration and metaplasia in this rat model of reflux oesophagitis and Barrett’s oesophagus, and a stem cell origin might explain the predisposition of Barrett’s metaplasia to cancer formation.

As discussed above, the oesophageal squamous epithelial cells in vivo in the rat model of reflux oesophagitis and in vitro demonstrated the expression and secretion of inflammatory cytokines including IL-8 following exposure to acid and bile salts [7]. Cytokines such as IL-8 have been found to regulate the mobilization of stem cells out of the bone marrow and into the general circulation where they become available to home to sites of tissue injury [26]. More recent data have found that IL-8 is also chemotactic for bone marrow mesenchymal stem cells, the non-haemopoetic stem cell population [27]. So perhaps the type of immune response elicited by reflux-exposed oesophageal squamous cells may predispose to metaplasia formation by recruiting bone-marrow-derived stem cells to the injured oesophagus. In support of such a hypothesis, Moons et al. [28] have found that reflux patients with Barrett’s oesophagus are more likely to have a pro-inflammatory genotype and less likely to have an anti-inflammatory genotype than those patients without Barrett’s oesophagus, suggesting that patients who develop Barrett’s oesophagus may be genetically predisposed to mounting a more severe inflammatory response in the setting of reflux oesophagitis. Thus the severity of the immune response as well as the type of immune response may predispose some individuals with GORD to developing Barrett’s oesophagus.

**Metaplasia arising from differentiated cells**

Metaplasia may also arise by changing the differentiation pattern of fully differentiated cells, a process termed trans-differentiation. In general, such metaplasias arise between neighbouring tissue types during embryological development [22]. Initially, the cells lining the oesophagus are of a columnar phenotype owing to the expression of certain genes induced by high levels of morphogenic stimuli present early on during
in utero development. As development proceeds, there is a progressive decline in the levels of the morphogenetic stimuli and a progressive replacement of the columnar lining of the oesophagus by a stratified squamous one [29,30]. Therefore, by altering a particular pattern of gene expression, it is possible for the oesophageal epithelium to change between a squamous and a columnar phenotype. In support of this notion, Milano et al. [31] exposed oesophageal squamous cells in vitro to BMP4 (bone morphogenetic protein 4) and found that the cells changed from a squamous to a columnar phenotype [31].

It is conceivable that the components of gastric refluxate may alter gene expression patterns in oesophageal squamous cells such that metaplasia forms. The genes controlling cell phenotype are often regulated by transcription factors. One family of transcription factors implicated in murine and human intestinal development is the CDXs (Caudal-related homeoboxes), including CDX1 and CDX2 [32]. The CDXs are members of the homeobox family of transcription factors, and they are known to mediate the differentiation of intestinal epithelial cells. Animal studies have found that epithelial cells in the small and large intestine, but not those in the normal oesophagus or stomach, express CDX1 and CDX2 [33]. Intestinal metaplasia in the stomachs of mice can be induced by forcing the gastric epithelial cells to express either Cdx1 or Cdx2, suggesting that these genes trigger intestinal-like differentiation [33–37].

In a rat model of surgically induced reflux oesophagitis and Barrett’s oesophagus, Cdx2 expression was detected in the cells of the basal layer of the squamous oesophagus before the formation of specialized columnar epithelium, suggesting that Cdx2 expression in squamous cells may precede the development of Barrett’s oesophagus [38]. CDX2 expression has been detected by immunostaining in 100% of biopsy specimens of specialized intestinal metaplasia, but not in any of the biopsies of normal oesophageal squamous epithelium [39]. In contrast with normal non-inflamed squamous epithelium, CDX2 expression has been detected in inflamed oesophageal squamous epithelium, and its expression precedes that of other types of intestinal markers such as MUC2 (mucin 2), SI (sucrase-isomaltase), DEFA5 (defensin-5) and ALPI (alkaline phosphatase) [40]. Cdx2 mRNA expression has also been found in the oesophageal squamous epithelium in six of 19 patients with Barrett’s oesophagus, supporting the notion that CDX2 expression in oesophageal squamous cells may precede the development of Barrett’s oesophagus [38,41].

Finally, data in vitro have begun to explore mechanisms whereby acid and bile salts can regulate expression of CDX2. In HET-1A immortalized human oesophageal squamous cells, the bile salts deoxycholic acid and chenodeoxycholic acid have been found to increase CDX2 mRNA expression, and, when bile salts are combined with acid, demethylation of the CDX2 promoter can be detected [42,43]. Moreover, the increase in CDX2 expression in the HET-1A cells was followed morphologically by the formation of crypt-like structures and the up-regulation of intestinal genes such as villin, sucrase-isomaltase and mucin 2 [43,44]. In addition to promoter demethylation, data suggest that acid and bile salts can stimulate CDX2 promoter activity. In mouse and rat oesophageal keratinocytes cultured in vitro, Cdx2 promoter activity was increased following exposure to acid or certain bile salts (dehydroxycholic acid and cholic acid) respectively [45,46]. Moreover, in human adenocarcinoma cells from the gastro-oesophageal junction, transcriptional activity was increased by acid- and bile-salt-mediated binding of p50, a stimulatory subunit of NF-κB (nuclear factor κB), to its promoter-binding site within the CDX2 promoter [47]. Taken together, these data suggest that exposure to acid and bile salts, the components of gastric refluxate, can increase transcription of CDX2 in oesophageal squamous cells, thereby initiating metaplastic transformation. Therefore it is possible that, in oesophageal squamous epithelium, differences in reflux-mediated expression of genes such as CDX2 that regulate cell phenotype may predispose to Barrett’s metaplasia.

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