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The Role of Acid and Bile Reflux in Esophagitis and Barrett's Metaplasia

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Abstract

The precise mechanisms whereby gastroesophageal reflux disease causes reflux esophagitis and Barrett's esophagus 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 esophageal squamous epithelial cells and the immune cells in the pathogenesis of reflux esophagitis. Although reflux esophagitis commonly heals with esophageal squamous cell regeneration, in some individuals the esophagus heals through the process of metaplasia, a condition termed Barrett's esophagus. Recent studies indicate that individual differences in the reflux-mediated response of esophageal squamous epithelial cells in the type of immune response and/or in signaling pathways that regulate cell proliferation or cell phenotype may determine whether the esophagus heals with the regeneration of squamous cells or through Barrett's metaplasia.

Introduction

The prevailing concept of reflux esophagitis 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 esophagitis develops as an immune-mediated injury which begins as a lymphocytic infiltrate in the submucosa that progress toward the luminal surface, a process which may be initiated by the release of cytokines by reflux-exposed esophageal squamous cells. In most individuals, reflux esophagitis heals with squamous cell regeneration. In some, however, reflux esophagitis heals through the process of metaplasia. This condition, Barrett's esophagus, predisposes to the development of esophageal adenocarcinoma. It is not clear why only a minority of individuals with reflux esophagitis develop Barrett's metaplasia. There are both clinical and experimental data to suggest that the esophageal squamous epithelium of patients with Barrett's esophagus 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 signaling pathways that regulate cell proliferation or cell phenotype may determine whether the esophagus heals with the regeneration of squamous cells or through Barrett's metaplasia.

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

For more than 50 years, the prevailing concept has been that reflux esophagitis 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 esophagus damages the tight junctions between the epithelial cells causing the intercellular spaces to dilate and hydrogen ions to enter into the epithelium [1–3]. Continued injury from an acute, acid-induced chemical burn and death of the surface esophageal 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](REF).

Our laboratory recently began using a rat model of reflux esophagitis in which the esophagus is surgically connected to the duodenum with the stomach remaining in place [7]. That esophagoduodenostomy results in the free flow of gastric and duodenal contents into the esophagus causing severe, reflux esophagitis. However, other investigators using this model have noted that esophagitis can take weeks to appear after the operation (personal communication, Navtej Buttar, MD, Mayo Clinic, Rochester, MN). Such a protracted time course to observe the esophageal injury seems counterintuitive, because reflux esophagitis has been assumed to result from a chemical, acid-induced burn and caustic chemical injuries develop rapidly. After performing an esophagoduodenostomy in the rat, our group conducted a systematic study of the early histologic events in the development of reflux esophagitis [7]. We found that at day 3 following esophagoduodenostomy, there was no apparent damage to the surface epithelial cells and esophageal inflammation was most prominent in the submucosal layer of the tissue [7]. This early inflammatory infiltrate was comprised of 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 esophageal tissue until 7 days after the operation [7]; eosinophils were rarely detected over this same time period (unpublished data, R.F. Souza). 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 opposite of those expected if reflux esophagitis 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 esophagitis 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 esophagitis in the rat esophagus after esophagoduodenostomy does not support the prevailing concept of reflux esophagitis 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 gastroesophageal reflux might cause esophageal squamous cells to produce cytokines. Exposure of telomerase immortalized normal esophageal squamous epithelial cell lines to a combination of acid and bile salts significantly increased secretion of the cytokines interleukin (IL)-8 and IL-1β after 2 and 4 days, respectively [7]. In addition, the conditioned media 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 media 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 esophagitis [7]. Using immunohistochemical staining on the rat esophagus, we also observed increased expression of IL-8 by the epithelial cells within 2 weeks following esophagoduodenostomy [7]. A number of other investigators have also demonstrated the secretion of pro-inflammatory cytokines by esophageal squamous cells in reflux esophagitis, but in most of those studies it was not clear whether cytokine production was a cause or an effect of the esophagitis [7]. In one study by Yamaguchi et al., for example, the esophageal mucosal was found to express inflammatory cytokines within 3 hours after the surgical induction of reflux using a rat model of esophagitis, and that the administration of anti-neutrophil serum prevented the development of reflux esophagitis [8]. Overall, these findings support a new concept for the development of reflux esophagitis in which gastroesophageal reflux causes esophageal squamous epithelial cells to secrete cytokines that attract immune cells, and it is the immune cells, not acid, that ultimately damage the esophageal mucosa.

Gastroesophageal Reflux Disease (GERD), Reflux Esophagitis, and Barrett's Esophagus

In addition to causing reflux esophagitis, GERD is also a primary risk factor for Barrett's esophagus [9]. Barrett's esophagus 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 esophagus heals with the regeneration of esophageal squamous cells. However in a minority of individuals, and for reasons that remain unclear, the reflux-damaged esophagus heals with the replacement of esophageal squamous cells by metaplastic, specialized-intestinal like columnar cells. Some data suggest that the esophageal squamous epithelium of patients with Barrett's esophagus is predisposed to develop metaplastic changes in response to peptic injury. For example, in patients who have esophagectomy with esophago-gastric anastomosis, some studies report the development of columnar metaplasia in the esophageal remnant significantly more often in patients who had Barrett's esophagus preoperatively than in those without Barrett's esophagus, despite the presence of a similar degree of postoperative reflux esophagitis [10;11]. There are also data to suggest that the esophageal squamous epithelium of patients with Barrett's esophagus 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 esophageal squamous epithelium, individual differences in response of molecular signaling 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 esophageal lining is repaired following reflux-induced injury [13;14]. It is well established that chronic GERD increases proliferation in esophageal squamous epithelium. For example, in esophageal epithelium from an animal model of reflux esophagitis, cells in the basal zone (proliferative zone of the esophagus) of the esophageal epithelium demonstrated increased proliferation rates compared to cells in the basal zone of non-inflammed esophageal squamous epithelium[15]. Likewise, in biopsy specimens of esophageal squamous mucosa from patients with severe, ulcerative reflux esophagitis, cells in the basal zone demonstrated increased rates of proliferation compared to those from patients with no or only mild reflux esophagitis [16]. Therefore, it appears that gastric reflux normally increases proliferation in

esophageal 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 signaling pathways including the mitogen-activated protein kinase (MAPK) pathway. Growth factors, mitogens, and acidic pH have been found to activate the MAP kinase kinase MEK1/2 which in turn phosphorylates and activates the extracellular signal-regulated protein kinases (ERK) 1 and 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 esophageal squamous epithelium *in vivo* activates the pro-proliferative kinase ERK1/2 in patients who have GERD without Barrett's esophagus, but not in those with Barrett's esophagus [19;20]. Moreover, esophageal squamous biopsy specimens from patients with Barrett's esophagus demonstrate expression of an inhibitory phosphorylated form of MEK1/2 whereas no expression of this inhibitory phospho-protein was detected in esophageal squamous biopsy specimens from GERD patients without Barrett's esophagus [20]. Using microarray technology, increased levels of expression of Dickkopf (Dkk)-1 and Dkk-4 genes, which regulate proliferation and apoptosis, have been found in the esophageal squamous epithelium of GERD patients without Barrett's esophagus compared to those with Barrett's esophagus [21]. These data suggest that in esophageal squamous epithelium, differences at baseline and in reflux-mediated induction of signal transduction pathways that regulate cell proliferation and apoptosis may determine whether the esophagus heals through squamous cell regeneration or through metaplasia.

Barrett's Esophagus: a metaplastic response to gastroesophgeal 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 esophageal metaplasia. As discussed earlier, Barrett's esophagus is the condition in which the normal esophageal 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]. Conceivable, 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 esophageal epithelium are thought to reside within the esophageal tissue itself. Recent studies have 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 esophagus using a rat model. For this study, the bone marrow of female rats was destroyed by irradiation and then reconstituted with bone marrow with that of male donors [24]. The female rats then underwent an esophagojejunostomy, which results in severe, ulcerative esophagitis and in intestinal metaplasia [24;25]. In both squamous cells and metaplastic columnar cells of the esophagus, nuclear staining for Y chromosome was found in female rats that had received bone marrow transplants from male donors [24]. In contrast, no nuclear staining for Y chromosome was observed in control female rats after esophagojejunostomy that had not received bone marrow transplants [24]. These observations suggest that bone marrow derives stem cells may contribute to esophageal regeneration and metaplasia in this rat model of reflux esophagitis and Barrett's esophagus, and a stem cell origin might explain the predisposition of Barrett's metaplasia to cancer formation.

As discussed above, the esophageal squamous epithelial cells *in vivo* in the rat model of reflux esophagitis 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 chemotaxtic for bone marrow mesenchymal stem cells, the non-hematopoetic stem cell population [27]. So perhaps, the type of immune response elicited by reflux-exposed esophageal squamous cells may predispose to metaplasia formation by recruiting bone marrow derived stem cells to the injured esophagus. In support of such a hypothesis, Moons et al. has found that reflux patients with Barrett's esophagus are more likely to have a pro-inflammatory genotype and less likely to have an anti-inflammatory genotype than those patients without Barrett's esophagus suggesting that patients who develop Barrett's esophagus may be genetically predisposed to mounting a more severe inflammatory response in the setting of reflux esophagitis [28]. Thus, the severity of the immune response as well as the type of immune response may predispose some individuals with GERD to developing Barrett's esophagus.

Metaplasia arising from differentiated cells

Metaplasia may also arise by changing the differentiation pattern of fully differentiated cells, a process termed transdifferentiation. In general, such metaplasias arise between neighboring tissue types during embryological development [22]. Initially, the cells lining the esophagus are of a columnar phenotype due 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 morphogenic stimuli and a progressive replacement of the columnar lining of the esophagus by a stratified squamous one[29;30]. Therefore, by altering a particular pattern of gene expression, it is possible for the esophageal epithelium to change between a squamous and a columnar phenotype. In support of this notion, Milano *et al.* exposed esophageal squamous cells *in vitro* to bone morphogenic protein 4 (BMP4) 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 esophageal 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 caudal related homologues, including CDX1 and CDX2 [32]. The CDXs are members of the homeobox gene 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 esophagus 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 esophagitis and Barrett's esophagus, Cdx2 expression was detected in the cells of the basal layer of the squamous esophagus prior to the formation of specialized columnar epithelium suggesting that Cdx2 expression in squamous cells may precede the development of Barrett's esophagus [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 esophageal squamous epithelium [39]. In contrast to normal, uninflamed squamous epithelium, CDX2 expression has been detected in inflamed esophageal squamous epithelium, and its expression precedes that of other types of intestinal markers such as MUC2, sucrase-isomaltase, defensin-5 and alkaline phosphatase [40]. Cdx2 mRNA expression has also been found in the esophageal squamous

epithelium in 6 of 19 patients with Barrett's esophagus supporting the notion that CDX2 expression in esophageal squamous cells may precede the development of Barrett's esophagus [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 esophageal 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 upregulation of intestinal genes such as villin, sucrase-isomaltase, and MUC2 [43;44] In addition to promoter demethylation, data suggest that acid and bile salts can stimulate CDX2 promoter activity. In mouse and rat esophageal 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 gastroesophageal junction, transcriptional activity was increased by acid and bile salt-mediated binding of p50, a stimulatory subunit of NF-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 esophageal squamous cells thereby initiating metaplastic transformation. Therefore, it is possible that in esophageal squamous epithelium, differences in reflux-mediated expression of genes that regulate cell phenotype like CDX2 may predispose to Barrett's metaplasia.

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