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From Reflux Esophagitis to Esophageal Adenocarcinoma

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Abstract

Reflux esophagitis causes Barrett's metaplasia, an abnormal esophageal mucosa predisposed to adenocarcinoma. Medical therapy for reflux esophagitis focuses on decreasing gastric acid production with proton pump inhibitors. We have reported that reflux esophagitis in a rat model develops from a cytokine-mediated inflammatory injury, not from a caustic chemical (acid) injury. In this model, refluxed acid and bile stimulate the release of inflammatory cytokines from esophageal squamous cells, recruiting lymphocytes first to the submucosa and later to the luminal surface. Emerging studies on acute reflux esophagitis in humans support this new concept, suggesting that reflux-induced cytokine release may be a future target for medical therapies. Sometimes, reflux esophagitis heals with Barrett's metaplasia, a process facilitated by reflux-related nitric oxide (NO) production and Sonic Hedgehog secretion by squamous cells. We have shown that NO reduces expression of genes that promote a squamous cell phenotype, while Hedgehog signaling induces genes that mediate the development of the columnar cell phenotypes of Barrett's metaplasia. Agents targeting esophageal NO production or Hedgehog signaling conceivably could prevent the development of Barrett's esophagus. Persistent reflux promotes cancer in Barrett's metaplasia. We have reported that acid and bile salts induce DNA damage in Barrett's cells. Bile salts also cause NF- κ B activation in Barrett's cells, enabling them to resist apoptosis in the setting of DNA damage, and likely contributing to carcinogenesis. Oral treatment with ursodeoxycholic acid prevents the esophageal DNA damage and NF- κ B activation induced by toxic bile acids. Altering bile acid composition might be another approach to cancer prevention.

Keywords

Barrett's metaplasia; nitric oxide; cytokine; sonic hedgehog; ursodeoxycholic acid

Gastroesophageal reflux disease (GERD) causes reflux esophagitis, reflux esophagitis causes Barrett's esophagus, and the metaplasia of Barrett's esophagus predisposes to esophageal adenocarcinoma [1]. Barrett's esophagus is the condition in which the normal squamous lining of the esophagus is replaced by a metaplastic, intestinal type lining as a

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result of chronic injury due to GERD [1]. Although Barrett's metaplasia seems to guard against symptom generation due to GERD, it harbors a predisposition to esophageal adenocarcinoma formation [1]. In 2015, our primary therapeutic strategy for GERD and its complications is to target gastric H⁺K⁺ ATP-ase with proton pump inhibitors (PPIs) to reduce acid reflux [2]. However, PPIs have number of shortcomings for the treatment of GERD. PPIs are very effective at healing reflux esophagitis, but they are not as good at relieving GERD symptoms such as heartburn [3]. For example, in a large, multicenter, randomized, double blind, parallel group trial, the two PPIs, esomeprazole and lansoprazole, had healing rates for erosive esophagitis that were greater than 85% at 8 weeks [3]. However, these agents did not perform quite as well with GERD symptoms. At 4 weeks, only about 60% of patients reported complete resolution of their GERD symptoms with PPIs whereas approximately 40% of patients still complained of persistent symptoms [3]. A subsequent, large, retrospective study confirmed these findings [4]. Moreover, it is not clear if PPIs prevent cancer in Barrett's metaplasia because the evidence is all indirect and not proven in controlled trials (Reviewed in [5]). Because of the shortcomings of PPI therapy, novel therapeutic targets, other than gastric acid production, are needed for treating reflux esophagitis and its complications such as Barrett's esophagus.

Therapeutic Strategies for Treating Reflux Esophagitis

Pathogenesis of Reflux Esophagitis: Acid Burn or Cytokine Sizzle

For nearly 100 years, the traditional concept has been that reflux esophagitis results from a caustic, chemical injury. When esophageal squamous epithelium is exposed to reflux, acid and pepsin are thought to damage the junctions between cells, making the epithelium permeable, and allowing acid to enter and attack the epithelial cells. This acid burn causes cell death, which triggers the infiltration of granulocytes like neutrophils and eosinophils. The death of surface cells is also assumed to induce a proliferative response that results in basal cell and papillary hyperplasia to repair the injured epithelium [6]. Based on this model of reflux esophagitis, it is not surprising that, in 2015, our therapeutic strategies are designed to prevent caustic injury from acid by using agents like PPIs.

In 2009, our group published an alternative concept for the pathogenesis of reflux esophagitis in which reflux esophagitis begins as a cytokine-mediated injury rather than a caustic chemical injury [7]. Using an animal model in which reflux esophagitis was induced by creating a surgical esophagoduodenostomy, we studied the early histologic events of reflux esophagitis. On post-operative day 3, we found that the esophageal mucosa was intact, and lymphocytes were the first inflammatory cells observed within the submucosa [7]. We were surprised that, in this animal model, inflammation did not start in the mucosa, and the first inflammatory cells were lymphocytes, not granulocytes. We then determined whether the lymphocytes were T or B cells using immunostaining for CD3, a T cell marker and CD20, a B cell marker. We found that the infiltrating lymphocytes were CD3⁺ and CD20⁻, demonstrating that they were T cells [7]. By post-operative week 3, we observed profound basal cell and papillary hyperplasia, but the surface cells were still intact, so this hyperplasia was not due to the death of surface cells [7]. In accompanying experiments, we found that cultures of esophageal squamous cells derived from patients with GERD secreted

interleukin (IL)-8, a potent pro-inflammatory cytokine, when they were exposed to acidic bile salts, and that secretion of IL-8 induced the migration of lymphocytes and neutrophils [7]. Similar to our squamous cells in culture, we found expression of IL-8 by reflux-stimulated esophageal squamous cells in our animal model *in vivo* [7]. Based on these findings, we proposed that reflux esophagitis develops as a cytokine-mediated-inflammatory injury. In this model, the reflux of acid and bile doesn't destroy epithelial cells directly, but rather induces them to secrete pro-inflammatory cytokines. These cytokines attract lymphocytes first, not neutrophils or eosinophils, and they induce the basal cell and papillary proliferation characteristic of GERD. We postulate that ultimately, it is inflammatory cells that mediate the epithelial injury, not the direct caustic effects of refluxed gastric acid (Figure 1). So perhaps the pathogenesis of reflux esophagitis is more like a cytokine “sizzle” than an acid “burn”.

This alternative concept is based on rat studies, and it is not clear if this model of the pathogenesis of reflux esophagitis is applicable to humans. However, some recent circumstantial evidence in humans suggests that this model is valid. *Kondo et al.* enrolled 14 healthy, male volunteers and randomized them to receive 30 minutes of either saline or acid perfusion through a tube with its tip positioned in the distal esophagus [8]. Distal esophageal biopsy specimens were obtained before and after the perfusion, and levels of prostaglandin E2 (PGE2), a pro-inflammatory cytokine, were measured. Acid, but not saline perfusion, significantly increased PGE2 levels in the esophageal mucosa without inducing surface erosions or immune cell infiltration, suggesting that acid exposure causes cytokine secretion prior to any effects on epithelial cell viability or immune cell migration in humans [8].

Pathogenesis of Heartburn: Cytokine Sizzle?

It is intriguing to speculate that perhaps targeting reflux-stimulated cytokine secretion by esophageal squamous cells might produce a better symptomatic response than that of the currently used PPIs. It is thought that the sensation of heartburn is conveyed by the visceral sensory neurons that reside within the deep layers of the esophageal mucosa [9]. These visceral sensory neurons respond to hydrogen ions and other inflammatory mediators via the expression of chemosensitive nociceptors such as the acid-sensing ion channel 3 (ASIC3) and the transient receptor potential vanilloid receptor 1 (TRPV1), which are thought to trigger the generation of heartburn symptoms [10]. Elevated levels of IL-8 have been demonstrated in biopsy specimens from patients with erosive and non-erosive reflux disease (NERD) [11,12]. Furthermore, high levels of IL-8 in biopsy tissues of NERD patients are a positive predictor of symptomatic recurrence [13]. These findings suggest that reflux-induced symptoms may also result from the cytokine-sizzle, and that future therapies should be targeted at preventing cytokine-mediated inflammation and not just at gastric acid control.

Therapeutic Strategies for Preventing Barrett's Esophagus

Barrett's esophagus develops through metaplasia, the process in which one type of tissue replaces another type of tissue [14]. The pathogenesis of Barrett's metaplasia remains poorly understood, but we have seen some progress in the last few years. The process starts with the

reflux of acid and bile from the stomach that damages the squamous mucosa of the distal esophagus. This mucosal damage can heal either through the regeneration of more squamous epithelium, or through columnar metaplasia (i.e. specialized intestinal metaplasia) in which columnar cells replace the damaged squamous cells.

Origin of Barrett's Metaplasia

The progenitor cells for this columnar metaplasia are not known, but we have a number of candidates. Columnar metaplasia might develop if chronic GERD causes mature esophageal squamous cells to change into columnar cells. This is called transdifferentiation, a process that involves individual, fully differentiated cells, and it seems unlikely that transdifferentiation could produce and then maintain the mixture of gastric and intestinal cell phenotypes found in Barrett's metaplasia (Figure 2) [1,15]. It seems more likely that a complex tissue like Barrett's metaplasia would arise from abnormal differentiation of an immature progenitor cell that has the capacity to produce and maintain multiple different cell types. There are several possible sources for these immature progenitor cells. They could be native to the esophagus. For example, they might be basal cells of the squamous epithelium, or immature cells that line the ducts of esophageal submucosal glands [16,17]. The immature progenitor cells might reside in the proximal stomach. Recent studies in mice have suggested that progenitor cells from the gastric cardia, or a peculiar population of embryonic-type cells at the gastroesophageal junction might migrate up the esophagus to repair the damaged squamous mucosa [18,19]. In a rat model of reflux esophagitis, Barrett's metaplasia developed from circulating stem cells from the bone marrow that were transported through the blood to the damaged tissue where they differentiated into columnar cells [20]. Intestinal cells are not found normally in the esophagus or stomach, and so, whichever of these is the cell of origin, the development of Barrett's metaplasia must involve some reflux-related reprogramming of the expression of key developmental transcription factors, a process termed transcommitment (Figure 2). The studies discussed below support a reflux-related reprogramming pathogenesis for Barrett's esophagus involving the Hedgehog (Hh) pathway and sex determining region Y-box 2 (SOX2), a transcription factor that promotes stratified squamous epithelia development [21-23].

Hedgehog Pathway Inhibition Might Prevent Barrett's Metaplasia

Hedgehog pathway signaling is activated by Hh ligand binding to its transmembrane receptor called "patched" (PTCH). In the absence of ligand binding, PTCH inhibits smoothened (Smo), a signal transducer protein. Following ligand binding, Smo is released from PTCH inhibition and activates Gli transcription factors to regulate downstream target genes [22]. *Wang et al.* demonstrated that esophageal squamous cell lines and squamous tissues exposed to acid and bile salts *in vitro* or to gastroesophageal reflux *in vivo* exhibit epithelial-mesenchymal Hh signaling, leading to the expression of the columnar transcription factors SOX9 and forkhead box protein A2 (FOXA2), which in turn caused the squamous cells to express genes that influence columnar and goblet cell differentiation such as cytokeratin 8 and mucin 2 (MUC2) [21,22]. Therefore, if reflux-related reprogramming of progenitors in the esophagus is initiated by Hedgehog signaling, then perhaps agents that inhibit the Hedgehog pathway might prevent the development of Barrett's esophagus. In a surgical rat model of reflux esophagitis, Barrett's metaplasia and esophageal

adenocarcinoma, treatment with a Smo inhibitor for 18 weeks postoperatively significantly reduced the incidence of Barrett's metaplasia and esophageal adenocarcinoma compared to untreated controls, supporting a role of Hedgehog pathway inhibition to prevent Barrett's metaplasia in patients with GERD [24].

Reduction of Esophageal Nitric Oxide (NO) Production Might Prevent Barrett's Metaplasia

Most studies on molecular events underlying the metaplasia of Barrett's esophagus have focused primarily on acid and bile salts and the upregulation of genes involved in columnar differentiation such as SOX9 and Caudal type homeobox 2 (CDX2) [21,25]. However, it seems equally plausible that this squamous-to-columnar metaplasia also involves noxious components of gastroesophageal reflux other than acid and bile salts, like nitric oxide (NO), and involves the downregulation of genes that regulate squamous differentiation such as SOX2 and the isoforms of tumor protein p63 (p63) [26,27]. During episodes of gastroesophageal reflux, high concentrations of NO can be generated in the esophageal lumen from dietary nitrate found in green, leafy vegetables and other foods [28]. This NO can react with oxygen to form highly toxic reactive nitrogen species that lead to tissue damage [29]. NO generated from the diet has been shown to reach genotoxic concentrations at the gastroesophageal junction in GERD patients with and without Barrett's esophagus [30]. *Endo et al.* demonstrated that dietary nitrate accelerates the development of metaplasia in a rat model of reflux esophagitis [31].

Until recently, little had been known regarding the mechanisms whereby NO generated in the esophagus from dietary nitrate might contribute to the development of Barrett's metaplasia. *Asanuma et al.* found that NO exposure caused S-nitrosylation of protein kinase B (PKB or Akt), which blocks its phosphorylation to an active state, and that interfering with Akt signaling leads to reductions in esophageal squamous cell expression of SOX2, a transcription factor that promotes stratified squamous epithelia development [23,26]. Using tissue specimens from rats with surgically-induced reflux esophagitis fed a diet supplemented with NO, the investigators found that the squamous-lined distal esophagus exhibited diminished staining for SOX2 when compared with that from animals with surgically-induced reflux esophagitis that were not fed a diet supplemented with NO [23]. Moreover, NO decreased the expression of the TA and NP isoforms of p63, another transcription factor that promotes stratified squamous epithelia, and increased the expression of CDX2, a transcription factor that promotes intestinal differentiation [27,32]. The combination of these events might lead to the development of the intestinal metaplasia of Barrett's esophagus, suggesting that perhaps therapies that reduce esophageal NO production might prevent Barrett's metaplasia in patients with GERD.

Therapeutic Strategies for Preventing Esophageal Adenocarcinoma

We have just discussed some potential novel therapeutic targets for GERD-induced reflux esophagitis, and Barrett's esophagus. Now let us turn our attention to GERD-induced adenocarcinoma formation in Barrett's esophagus. In a number of organs, chronic inflammation is linked with cancer development. Acid and bile salt reflux is well known to

cause chronic reflux esophagitis, but there are also direct effects of acid or bile salts on epithelial cells, independent of inflammation effects, that are discussed below.

Inhibition of Gastric Acid Secretion

Acid reflux causes reflux esophagitis, and PPIs are very effective at healing the inflammation caused by acid reflux [2]. However, acid exposure also exerts direct effects, independent of effects on inflammation, on Barrett's epithelial cells. In earlier studies, we found that acid exposure caused non-neoplastic Barrett's epithelial (BAR-T) cell lines to produce reactive oxygen species (ROS), which then damaged DNA [33]. Among the types of DNA damage observed were DNA double strand breaks (DSBs), the most deleterious of all forms of DNA damage [33]. In fact, agents that cause DSBs can be considered carcinogens, and so acid may be a carcinogen in Barrett's esophagus [34]. This *in vitro* finding was explored in a translational study in which endoscopic biopsies of Barrett's metaplasia were obtained before and after esophageal perfusion with 0.1N hydrochloric acid in 6 patients with Barrett's esophagus [33]. In all six patients, acid perfusion caused DNA damage, as detected by increases in phospho-H2AX levels by Western blotting, in biopsies of Barrett's metaplasia [33]. Persistent DNA damage can lead to genomic instability and promote cancer formation. Consequently, it certainly makes sense to control acid reflux for patients with Barrett's esophagus, and PPIs are the most effective medications for controlling acid reflux [2]. However, despite the use of PPIs, the incidence of esophageal adenocarcinoma has continued to rise [35]. Since PPIs are so effective at controlling gastric acid secretion, there might be another noxious component of gastroesophageal reflux contributing to the development of this cancer. Data from a number of studies suggest that bile acids are prime candidates for that other noxious component (Reviewed in [36]).

Altering the Bile Acid Composition of Gastric Refluxate

Patients with Barrett's esophagus have significantly more esophageal exposure to bile acids and higher esophageal luminal concentrations of bile acids than GERD patients without Barrett's esophagus [37,38]. Some bile acids are more toxic than others. Deoxycholic acid (DCA) is an especially toxic, hydrophobic bile acid. Gut bacteria metabolize cholic acid produced by the liver into DCA [36]. In Barrett's patients on PPIs, the decrease in gastric acid allows bacteria to grow in the stomach, and these bacteria can metabolize cholic acid into DCA and can deconjugate DCA. Thus, it is not surprising that unconjugated DCA is found in relatively high concentrations in the gastric refluxate of Barrett's patients who are taking PPIs [37,39].

In non-neoplastic BAR-T cells, exposure to DCA generates ROS that cause DNA damage with DSBs, suggesting that DCA is a carcinogen in Barrett's esophagus [40]. In addition to inducing DNA damage in BAR-T cells, DCA also activates the NF- κ B pathway, which prevents apoptosis [41]. This activation of the NF- κ B pathway enables cells with potentially carcinogenic DNA damage to survive and potentially become malignant. Thus, in Barrett's metaplasia, DCA causes both DNA damage and NF- κ B activation, a combination that might predispose to cancer development. Might we prevent cancer in Barrett's esophagus by altering the composition of bile reflux?

All bile acids are not equally toxic. Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid that is not genotoxic, and in fact might even protect against genotoxicity (Reviewed in [36]). UDCA has been shown to protect against inflammatory bowel disease-associated colon cancer in patients with primary sclerosing cholangitis [42]. Recently, our group has reported that, in Barrett's cells exposed to acid and bile salts, pretreatment with UDCA decreases oxidative stress, DNA damage and NF- κ B activation, whereas simply mixing UDCA with DCA had no effect [40]. These findings suggested that UDCA does not protect from oxidative stress directly, but rather induces the production of some protective factor. We explored whether pretreatment with UDCA might increase the expression of anti-oxidant enzymes, including glutathione peroxidase1 (GPX1), catalase, SOD1, and SOD2. Pretreatment with UDCA increased the expression of GPX1 and catalase (but had no effect on the expression of SOD1 and SOD2) by activating NF-E2-related factor 2 (Nrf2) signaling [40].

Our *in vitro* studies showed that treatment with UDCA can prevent the DNA damage and NF- κ B activation caused by toxic bile acids in Barrett's epithelial cells. Those observations suggested that UDCA could be a chemopreventive agent for patients with Barrett's esophagus, and we designed a translational study to explore that possibility. Endoscopic biopsies of Barrett's metaplasia were obtained before and after esophageal perfusion with either 250 μ M DCA, 250 μ M UDCA, or 250 μ M DCA after 8 weeks of treatment with oral UDCA (10 mg/kg) [40]. A total of 21 patients completed this protocol, and we found that perfusion of the esophagus with DCA, but not UDCA, resulted in a significant increase in DNA damage and NF- κ B activation [40]. After the patients were treated with oral UDCA for 8 weeks, DCA perfusion of the esophagus did not cause DNA damage or NF- κ B activation [40]. Moreover, in the group of 21 patients, 8 weeks of UDCA treatment significantly increased GPX1 and catalase expression levels [40]. These findings demonstrate that oral UDCA treatment significantly increases antioxidant levels and protects against oxidative damage induced by GERD in Barrett's esophagus. Future therapies that target bile acid composition could be chemopreventive for patients with Barrett's esophagus.

In conclusion, data from animal models of reflux esophagitis suggest a role for cytokine-mediated inflammatory injury as the inciting event in the pathogenesis of reflux esophagitis, rather than a caustic chemical injury. This mechanism underlying the pathogenesis of reflux esophagitis requires confirmation in human studies which are currently ongoing in our laboratory. Nevertheless, these findings suggest that future GERD therapies should be targeted at preventing cytokine-mediated inflammation and not just at gastric acid control. The past 5 years have seen an explosion of research into the origin of Barrett's esophagus, and controversy currently exists as to whether GERD-induced transdifferentiation and/or transcommitment is involved. Regardless, insights gained into the underlying molecular mechanisms for the development of Barrett's esophagus have uncovered the role of developmental pathways like Hedgehog and transcription factors like SOX2 that perhaps may become future targets for therapeutic agents. The primary strategy for cancer prevention in patients with Barrett's esophagus has been to treat GERD with PPIs, and to perform endoscopic surveillance for dysplasia [1]. Unfortunately, this strategy has not been effective, as the frequency of esophageal adenocarcinoma continues to rise. Acid and bile salts cause carcinogenic DNA damage, and therefore therapies designed to prevent damage from both

acid and bile salts might be chemopreventive for patients with Barrett's esophagus. Thus, new insights into understanding the pathogenesis of reflux esophagitis, Barrett's metaplasia, and its progression to esophageal adenocarcinoma have highlighted molecular pathways and molecules for targeted therapies in 2015 and beyond.

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Figure 1.

Reflux Esophagitis: A Cytokine-Mediated Inflammatory Injury. Based on our published findings using an animal model of reflux esophagitis, we proposed that reflux esophagitis develops as a cytokine-mediated inflammatory injury. In this model, the reflux of acid and bile salts doesn't destroy epithelial cells directly, but rather induces them to secrete pro-inflammatory cytokines. These cytokines attract T lymphocytes first, and they induce the basal cell proliferation characteristic of GERD-induced injury. Ultimately, it is inflammatory cells that mediate the epithelial injury, not the direct caustic effects of refluxed gastric acid, which is then followed by the recruitment of neutrophils to the site of injury.

Figure 2.

Conceptual Overview of Potential Origins of Barrett's Metaplasia. Potential pathways for origin include (A) Transdifferentiation which is the process in which individual, fully differentiated cells (i.e. squamous) change directly into another type of fully differentiated cells (i.e. intestinal-type columnar cell) in the setting of GERD-induced inflammation. (B) Transcommitment which is the process in which immature progenitor cells are reprogrammed in the setting of GERD-induced inflammation to give rise to the multiple cell types that comprise intestinal-type metaplasia characteristic of Barrett's esophagus. Progenitor cells from the esophageal squamous or submucosal gland ducts, gastric cardia or gastroesophageal junction (GEJ) or circulating bone marrow derived cells have been proposed as the cell of origin.