How Good Is the Neosquamous Epithelium?

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Key Words

Neosquamous epithelium · Barrett's esophagus ·
Claudin-4 · Tight junction

Abstract

Background/Aims: Endoscopic radiofrequency ablation of dysplastic Barrett's esophagus (BE) combined with proton pump inhibitor therapy is commonly utilized for preventing progression of dysplastic BE to esophageal adenocarcinoma. Fundamental to the success of this and all ablative approaches is the healing of the ablated areas of BE with a stratified squamous epithelium referred to as 'neosquamous epithelium' (NSE). Although NSE appears 'normal' endoscopically, the reemergence of BE over time in the previously ablated segments raises the question of the health and integrity of NSE. **Methods:** The health of NSE was recently investigated in endoscopic biopsies in vitro in a group of patients after ablation while on proton pump inhibitors. Biopsies of NSE were compared to upper squamous epithelium (USE) from the same patients morphologically (light microscopy) and with respect to barrier function by measuring electrical resistance and fluorescein flux in mini-Ussing chambers. **Results:** Compared to USE, NSE exhibited dilated intercellular spaces and inflammation and defective barrier function by low electrical resistance and high fluorescein flux. Moreover, NSE exhibited downregulation of claudin-4, a highly expressed protein in squamous tight junctions. **Conclusion:** NSE has defective

barrier function in part due to downregulation of claudin-4. Since downregulation of claudin-4 increases paracellular permeability to cations, e.g. hydrogen ions, NSE is more vulnerable to attack and damage by acidic and weakly acidic refluxates – a phenomenon that may contribute in part to the re-emergence of BE.

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Background

Barrett's esophagus (BE) denotes the presence of specialized columnar epithelium lining the distal esophagus [1]. It arises as a form of repair following damage to esophageal stratified squamous epithelium by reflux of acidic gastric contents into the esophagus. BE is present in some 10–20% of patients with gastroesophageal reflux disease (GERD), and although it has properties that make it more acid resistant than native esophageal squamous epithelium, it is a premalignant lesion with an estimated risk of progressing to esophageal adenocarcinoma of 0.12–0.5% per year [2, 3]. Currently, 8,000 cases of esophageal adenocarcinoma occur per year in the USA and these have a dismal 5-year survival rate of approximately 15%. Moreover, esophageal adenocarcinoma frequency has increased sixfold in the USA within 30 years, making it the number one cancer in terms of rate of rise [4–6]. With the rapid rate of rise and high mortality of esopha-

geal adenocarcinoma, its forerunner, BE, has been aggressively sought out on endoscopy in GERD patients; and when identified targeted for therapy.

One popular strategy for treating BE and thus preventing its progression to esophageal adenocarcinoma is through endoscopic ablation using modalities such as multipolar electrocoagulation, photodynamic therapy, laser therapy, cryotherapy, mucosal resection, argon plasma coagulation, and radiofrequency ablation [7–12]. Ablation of BE combined with acid suppression by proton pump inhibitor therapy has been shown to achieve complete reepithelialization of the wounded areas with neosquamous epithelium (NSE) [11, 13–16]. The more recent availability of radiofrequency ablation has led to a resurgence of interest and enthusiasm for the ablative approach because of its reported efficacy, safety, and ease of performance [17–19]. For instance, one report of a large (127 patients) multicenter, prospective, sham-controlled randomized trial of radiofrequency ablation for dysplastic (low-/high-grade) BE [20] showed successful ablation and reepithelialization by NSE in 77%, with a 2% serious complication rate and no deaths. This and earlier favorable reports explain the growing popularity of radiofrequency ablation and its expanding utilization from high- to low-grade dysplasia and, more recently, to Barrett's metaplasia [8, 21–23].

Neosquamous Epithelium

NSE is not only a byproduct of the ablation strategy, but also an essential element for success in preventing esophageal adenocarcinoma. This it does in the short term by covering the wound with an epithelial barrier that is known to lack the malignant potential of its predecessor [11, 16, 24, 25]. However, in the near and long term, this barrier must be durable in its capacity to protect the esophagus against injury, especially from exposure to noxious contents in gastroesophageal refluxates. When ineffective, the result is tissue inflammation and reduced viability, the consequences of which can include reemergence of BE and instability in BE buried beneath the NSE. Indeed, after ablation by argon plasma coagulation and replacement by NSE, reemergence of BE has been reported to occur in up to 66% of patients, with a median time to recurrence of 15.5 months [26–28].

Many reports attribute this to the failure of proton pump inhibitor therapy to control esophageal acidity [29–31]. Indeed, Kahaleh et al. [32] observed that following Barrett's ablation by argon plasma coagulation, pH

monitoring was abnormal in 69% of patients (i.e. pH <4.0 for >4.2% of a 24-hour period) despite taking 40 mg of omeprazole daily, and that the recurrence rate of BE in this group was 83% (15/18 patients) compared to a recurrence rate of only 13% (1/8 patients) in those with normal pH monitoring. The implications here are that despite proton pump inhibitor therapy, acid reflux is common and capable of destroying NSE, the consequence of which is reemergence of BE – the latter presumably restored as a form of adaptive protection because of greater acid resistance [33–35]. Also, in support of this sequence is the observation that even when asymptomatic, a high percentage of BE patients on proton pump inhibitors continue to have pathologic acid reflux [36].

In addition to reemergence of BE, reflux damage to NSE can also destabilize any buried Barrett's glands beneath it. This occurs in two ways: (1) loss of barrier function by NSE allows greater access of luminal contents to buried Barrett's glands [11, 37, 38], and (2) reflux-induced injury to NSE produces inflammation whose by-products, prostaglandins, oxy-free radicals, and cytokines, expose buried Barrett's glands to known promoters of cell turnover and malignancy [39–43]. In keeping with this concern is the fact that esophageal adenocarcinoma is known to occur in buried Barrett's glands [44–46] and buried Barrett's glands have been found in up to 44% of patients following Barrett's ablation by argon plasma coagulation [26, 27, 29, 47]. Notably, the prevalence of buried BE following radiofrequency ablation is reported to be far lower, i.e. 5% in one recent report [20]. Nonetheless, when high-resolution three-dimensional optical coherence tomography was applied to image candidates undergoing radiofrequency ablation treatment, subsquamous intestinal metaplasia was found in 63% of patients after complete eradication of BE [48]. This indicates that buried Barrett's glands are a fact of life for all ablative methods and their reported prevalence is almost always going to be an underestimate because of the inherent sampling error associated with detection of buried Barrett's glands by random blind biopsy.

Barrier Function

The gastroenterology community of physicians and scientists is well versed in the importance of epithelial barrier function for maintenance of the health and integrity of the organ and its host. This is because, when defective, it is known to play a pathogenetic role in the signs and symptoms of such common disorders as inflamma-

tory bowel disease, alcoholic liver disease, and GERD [49–51]. Indeed, GERD with its associated damage to native esophageal squamous epithelium has considerable relevance for NSE. For instance, it has been shown experimentally that acid damage to the (rabbit) esophageal epithelium is initiated by a break in the junctional barrier, a break that increases paracellular permeability to hydrogen ions and uncharged molecules, and which results in a morphologic change referred to as ‘dilated intercellular spaces’ within the esophageal epithelium [52–54]. Dilated intercellular spaces in the esophageal epithelium were then shown by us and others to be a histopathologic feature of GERD and a strong correlate of heartburn [55–59]. Further, the break in the junctional barrier leads to cell necrosis by allowing acid access to the basolateral membrane of squamous cells which contains an acid-absorbing sodium-independent, chloride-bicarbonate exchanger [60–62]. Excess acid absorption, in turn, lowers cytosolic pH, raises cytosolic calcium, and initiates cell edema and cell necrosis [62, 63] – promoting at the microscopic level, and later macroscopic level, inflammatory and erosive lesions within the esophageal epithelium. The inflammatory reaction is usually evident by infiltration of the esophageal epithelium by eosinophils and polymorphonuclear leucocytes [64]. In effect, reflux-induced destruction of esophageal epithelium results from a series of pathologic events that begin with a break in the junctional barrier. In other words, a break in the junctional barrier of the esophageal epithelium, particularly if exposed to acid reflux, is a prescription for trouble – trouble that jeopardizes the health and integrity of both the epithelium and organ. Viewed through this prism, a break in the junctional barrier of NSE, particularly if exposed to acid reflux, would by jeopardizing its health and integrity also promote the reemergence of BE and instability of buried Barrett’s glands beneath it. NSE had been reported to have a ‘normal appearance’ on endoscopy and light microscopy [11], though its barrier function had not been rigorously explored until a report described below by Jovov et al. [65].

Methods

To examine the barrier function of NSE, Jovov et al. [65] performed endoscopic (jumbo) biopsies of NSE (taken from areas previously covered by BE) and upper squamous epithelium (USE) from the proximal one third of the esophagus (and at least 5 cm above proximal margin of BE) of the same patient at varying intervals after ablation and at a time when the ablated area was completely replaced by NSE. All patients were adult (18–75 years of

age), male, Caucasian, and asymptomatic while taking proton pump inhibitors (esomeprazole 40 mg) once or twice a day. Biopsies used in the analysis were performed at 8 ± 2.5 months (range: 3–22) following the last ablation procedure, and all but 3 patients (2 argon plasma coagulation, 1 photodynamic therapy) had ablation performed by radiofrequency ablation [65].

Results

Dilated intercellular spaces were apparent on light microscopy in 13 of 13 patients (100%) with NSE while dilated intercellular spaces was only present in the paired USE in 2 of 13 patients (15%) ($p < 0.05$). In addition, NSE had a modest degree of eosinophilia (<15 high-power field) in 7 of 13 patients (54%), while 0 of 13 patients (0%) had eosinophilia in USE ($p < 0.05$; fig. 1). Both abnormalities in NSE are compatible with GERD, with dilated intercellular spaces suggesting a break in the junctional barrier and eosinophilia suggesting active inflammation. In 9 patients, paired biopsies of NSE and USE were mounted in mini-Ussing chambers for measurement of electrical resistance and fluorescein (molecular weight: 300) flux. As shown in figure 2, mean electrical resistance was significantly lower for NSE than USE and mean fluxes of fluorescein were higher for NSE than USE, though the latter did not reach statistical significance. Further, figure 3 which utilizes data of all patients for whom a value for electrical resistance was available after ablation showed that there was no relationship between electrical resistance and time after ablation of BE – an observation indicating that the defect in barrier function was unrelated to postprocedure wound healing and persisting despite the fact that all patients were on proton pump inhibitors and asymptomatic (no heartburn). These results support the concept that NSE, independent of time after ablation, has impaired barrier function and that the defect is characterized by low electrical resistance, high fluorescein flux, and dilated intercellular spaces, which reflects an increase in paracellular permeability to both ions (low electrical resistance) and uncharged molecules (fluorescein flux). To explore the basis for the defective barrier in NSE, using qRT-PCR in NSE and USE, we compared the expression profiles of 21 claudins [65]. As shown in figure 4 using ZO-1 as reference, the claudin profile for NSE and USE was similar except for significant reductions in claudin-4 and claudin-10. Further, reduced claudin-4 expression for NSE and USE was paralleled by reduced claudin-4 protein expression on Western blot, while distribution by immunohistochemistry remained similar (data not shown).

Fig. 1. A light photomicrograph of a hematoxylin-eosin-stained section of native esophageal USE (left panel) and neosquamous epithelium from the lower esophagus (right panel). Note that the neosquamous epithelium shows dilated intercellular spaces (white arrows) and has a prominent infiltrate of eosinophils (black arrows) when compared with the normal-appearing 'native' USE. Original magnification $\times 60$. The **inset** in the right panel is an electron photomicrograph to better illustrate the dilated intercellular spaces in NSE. Original magnification $\times 3,000$. Reprinted from Jovov et al. [65].

Fig. 2. The transepithelial electrical resistance (R_T) (**a**) and fluorescein (FITC) flux (**b**) are illustrated for NSE and native esophageal USE from the same patients, and healthy native distal esophageal squamous epithelium from subjects without esophageal disease (controls). Note that NSE has significantly lower transepithelial electrical resistance and higher fluorescein flux values than both USE and healthy controls, indicating higher paracellular permeability to ions and uncharged molecules, respectively. * $p < 0.05$ compared with healthy controls. Reprinted from Jovov et al. [65].

Summary

NSE has defective barrier function characterized by dilated intercellular spaces, low electrical resistance, and high fluorescein flux; in these respects it mirrors the abnormalities previously reported in the distal esophageal epithelium from patients with GERD [52, 66]. Since NSE and GERD share a common environment, i.e. exposure to refluxed gastric contents, the changes are likely to have a common pathogenesis. NSE was also shown to express low levels of claudin-4 in its tight junction and low levels of claudin-4 may be one reason for its vulnerability to refluxed gastric acid. This concept receives support from the literature showing that reductions in claudin-4 in MDCK II cells are accompanied by lower electrical resistance and increased paracellular permeability, specifically to cations such as sodium ions [67]. Given that hydro-

Fig. 3. A plot of the transepithelial electrical resistance (R_T in ohms $\cdot$ cm²) in NSE vs. the time (months) from the last performance of radiofrequency ablation of BE. Note that there is no trend for transepithelial electrical resistance to improve with time for NSE (linear regression $R^2 = 0.008$, $p = 0.7$). Reprinted from Jovov et al. [65].

Fig. 4. Claudin gene expression profile for NSE and for native esophageal USE. Claudin expression levels are referenced to expression levels for ZO-1, which is set at 1.0. Note that expression of claudin-4 and claudin-10 were significantly lower in NSE than

in USE, while there was no difference in expression of the most prominently expressed claudin, i.e. claudin-1. Error bars = $((2^{\%CV})/100) \times (\text{relative expression})$. * $p < 0.05$ compared with USE. NT = Not tested. Reprinted from Jovov et al. [65].

gen ions are smaller cations than sodium, it is plausible that the documented increase in sodium ion permeability would be paralleled by increased paracellular permeability to hydrogen ions.

Jovov et al. [68] indirectly support this possibility when, in collaboration with Dr. James Anderson's laboratory, claudin-18 was transfected into MDCK II cells. In this study electrical resistance increased and paracellular permeability decreased to cations, with the decrease being reflected in reduction in permeability to both sodium and hydrogen ions. Based on these observations, Jovov and colleagues hypothesized that low claudin-4 in NSE increases paracellular permeability to cations, such that in the presence of luminal acidity, there is diffusion of hydrogen ions into the intercellular space in sufficient quantities to damage and further impair the tissue's barrier function. Notably, based on current knowledge of the claudins and barrier function, low claudin-4 can account for low electrical resistance and increased paracellular permeability to hydrogen ions, but may not account for

the increase in fluorescein flux since overexpression of claudins can alter ion permeability without affecting permeability of uncharged molecules [69]. Therefore, it is possible that an initial increase in hydrogen ion flux through the defective tight junction leads to further acid damage to the junction, allowing for the increase in flux of uncharged molecules such as fluorescein.

This hypothesis readily fits the junctional damage observed for acid-exposed NSE, but is to some extent problematic clinically since virtually all patients are prescribed once- or twice-a-day proton pump inhibitors after ablation. Yet, this apparent contradiction can be explained in the following two ways: (1) even when asymptomatic on proton pump inhibitors, a majority of the patients with BE have been shown to continue to have pathologic acid reflux [36], and (2) the presence of low claudin-4 expression would increase acid penetration into NSE over a wide range of luminal pHs, making it more vulnerable to damage even when exposed to the 'weakly acidic' refluxates created in patients taking proton pump inhibitors.

Indeed weakly acidic refluxates have been shown to account for persistent heartburn in some patients with GERD when taking proton pump inhibitors [70, 71]. In effect, the downregulation of claudin-4 in the tight junctions of NSE makes the tissue more vulnerable to attack and damage upon exposure to acidic and weakly acidic refluxates – and by so doing can account in part for its destruction and reemergence of BE.

Disclosure Statement

The author declares that no financial or other conflict of interest exists in relation to the content of the article.

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