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Conversion of metaplastic Barrett's epithelium into post-mitotic goblet cells by γ-secretase inhibition

Vivianda Menke[1], Johan H. van Es[2,3], Wim de Lau[2,3], Maaike van den Born[2,3], Ernst J. Kuipers[1], Peter D. Siersema[1,5], Ron W. F. de Bruin[4], Johannes G. Kusters[1,6] and Hans Clevers[2,3,*]

SUMMARY

Barrett's esophagus (BE) affects approximately 2% of the Western population and progresses to esophageal adenocarcinoma (EAC) in 0.5% of these patients each year. In BE, the stratified epithelium is replaced by an intestinal-type epithelium owing to chronic gastroduodenal reflux. Since self-renewal of intestinal crypts is driven by Notch signaling, we investigated whether this pathway was active in the proliferative crypts of BE. Immunohistochemistry confirmed the presence of an intact and activated Notch signaling pathway in metaplastic BE epithelium, but not in the normal human esophagus. Similar observations were made in two well-known human Barrett's-derived EAC cell lines, OE33 and SKGT-5. We then sought to investigate the effects of Notch inhibition by systemic treatment with a γ-secretase inhibitor in a well-validated rodent model for BE. As we have shown previously in normal intestinal epithelium, Notch inhibition converted the proliferative Barrett's epithelial cells into terminally differentiated goblet cells, whereas the squamous epithelium remained intact. These data imply that local application of γ-secretase inhibitors may present a simple therapeutic strategy for this increasingly common pre-malignant condition.

INTRODUCTION

Barrett's esophagus (BE) affects approximately 2% of the Western population and progresses to esophageal adenocarcinoma (EAC) in 0.5% of these patients each year (Lagergren, 2005; Ronkainen et al., 2005; van Soest et al., 2005). In BE, the multilayered epithelium near the stomach is replaced by an intestinal-type epithelium owing to chronic gastroduodenal reflux.

In an attempt to improve adenocarcinoma prognosis with an early diagnosis, the American College of Gastroenterology recommends that BE patients are enrolled in endoscopic surveillance programs (Wang and Sampliner, 2008). Therapy, however, is currently not available for BE patients.

The presence of Barrett's dysplasia, particularly high-grade dysplasia, is one of the risk factors for adenocarcinoma (Reid et al., 1988; Schmidt et al., 1985; Smith et al., 1984). An unsuspected adenocarcinoma is identified in approximately 30-40% of esophagi that are resected for high-grade dysplasia (Falk et al., 1999; Gilbert and Jobe, 2009; Hameeteman et al., 1989; Hamilton and Smith, 1987; Lee, 1985). Nevertheless, the intra- and inter-observer variation in the diagnosis of dysplasia leaves a lacuna in the management of patients with Barrett's-related dysplasia (Montgomery et al., 2001). Although the management of high-grade dysplasia is controversial, most institutes consider esophagectomy if the diagnosis is confirmed by pathology (Gilbert and Jobe, 2009; Schnell et al., 2001).

In the intestine, self-renewal of the epithelium is driven by intense proliferation of progenitor cells that reside in crypt compartments. Genetic disruption of Notch signaling in this tissue results in the rapid conversion of all proliferative cells into differentiated goblet cells (van Es et al., 2005). The activation of Notch signaling is critically dependent on an intramembrane protease complex termed γ-secretase (Baron, 2003; De Strooper et al., 1999; Mumm and Kopan, 2000). This protease complex is also implicated in the pathogenic processing of the amyloid precursor protein in Alzheimer's disease (Kopan and Goate, 2000). For this reason, multiple γ-secretase inhibitors have been developed as potential Alzheimer's drugs. Somewhat fortuitously, these inhibitors are efficient Notch inhibitors. Not surprisingly, administration of these inhibitors to rodents induces changes in the intestine that resemble the effects that occur upon genetic loss of Notch signaling (Milano et al., 2004; Searfoss et al., 2003; van Es et al., 2005; Wong et al., 2004), while (pre-)clinical studies have revealed a single major side effect of γ-secretase inhibitors: the induction of goblet cells in the intestine (Lundkvist and Naslund, 2007).

Multiple Notch pathway components are expressed in intestinal crypts and, together, constitute a functional signaling pathway (Sander and Powell, 2004; Schroder and Gossler, 2002; van Es et al., 2005). As with the intestinal epithelium, the Barrett's epithelium contains proliferative crypt-like compartments. To investigate whether Notch signaling was active in the proliferative cells of BE, we studied histology in human biopsy specimens, analyzed Barrett's-derived EAC cell lines and performed Notch inhibition on a well-validated rat model for BE (Fein et al., 1998; Levrat et al., 1962; Sato et al., 2002; van den Boogert et al., 1999).

RESULTS

Notch signaling in human biopsy specimens

To study several parameters of Notch signaling, we used immunohistochemistry on serial sections of normal human colon

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[1] Department of Gastroenterology and Hepatology and [4] Department of Surgery, Erasmus MC, University Medical Centre Rotterdam, 3015 CE Rotterdam, The Netherlands
[2] Hubrecht Institute for Developmental Biology and Stem Cell Research, 3584 CT Utrecht, The Netherlands
[3] University Medical Centre Utrecht, 3584 CX Utrecht, The Netherlands
[5] Present address: Department of Gastroenterology and Hepatology, University Medical Centre, Utrecht, 3584 CX Utrecht, The Netherlands
[6] Present address: Department of Microbiology, Tergooiziekenhuizen, 1201 DA Hilversum, The Netherlands
*Author for correspondence (h.clevers@hubrecht.eu)

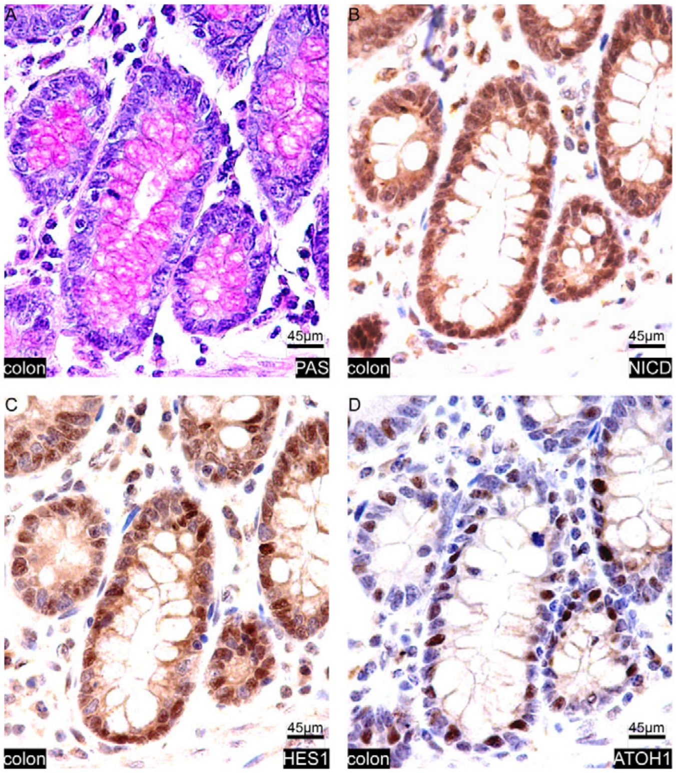

Fig. 1. Notch pathway components in serial sections of the human colon. (A) PAS staining for goblet cells (pink) in crypt structures of the colon. (B) NICD staining (brown) occurs in virtually all epithelial nuclei, indicative of active Notch signaling. Note the negative (blue) nuclei of stromal cells. (C) HES1 staining (brown) occurs in the nuclei of most cells in the colon, indicative of active Notch signaling. (D) ATOH1 staining (brown) reveals that a minority of differentiated cells express this goblet cell marker in the colon. Note that ATOH1 is repressed by active Notch signaling.

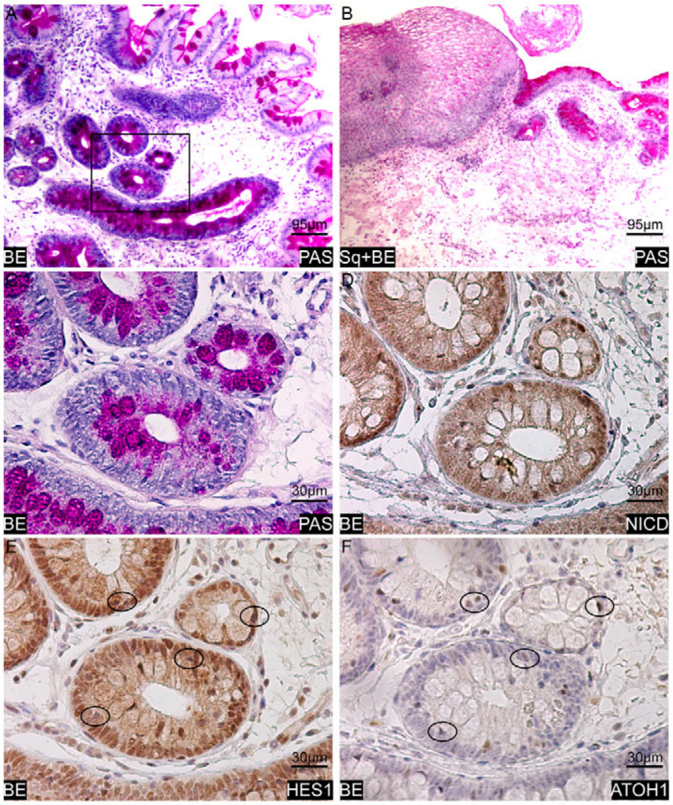

Fig. 2. Notch pathway components in BE. (A) PAS staining for goblet cells (pink) in a biopsy specimen from BE. (B) PAS staining for goblet cells (pink) in the same biopsy specimen shown in A, from the squamous epithelium next to BE. (C-F) Serial sections of BE from the same patient specimen shown in A and B. (C) PAS staining for goblet cells (pink) in crypt structures of BE. (D) NICD staining (brown) occurs in virtually all epithelial nuclei, indicative of active Notch signaling. Note the negative (blue) nuclei of stromal cells. (E) HES1 staining (brown) occurs in the nuclei of most cells in BE, indicative of active Notch signaling. (F) ATOH1 staining (brown) reveals that a minority of differentiated cells express this goblet cell marker in BE (black ovals). Note that ATOH1 is repressed by active Notch signaling.

(Fig. 1A-D) and Barrett's epithelium (Fig. 2A-F). Fig. 1A and Fig. 2A-C utilize a periodic acid-Schiff (PAS) stain for goblet cells to demonstrate the similarity in epithelial architecture between the two tissues. The hallmark of active Notch signaling is the nuclear localization of the cleaved Notch intracellular domain (NICD). An antibody that is specific for the N-terminal sequence of NICD revealed that the nuclei of colon crypts, as well as of BE cells, contained readily detectable NICD in their nuclei (Fig. 1B; Fig. 2D). The Hairy/Enhancer of Split (HES) transcriptional repressors are encoded by genes that are direct targets of Notch (Heitzler et al., 1996; Oellers et al., 1994). The prototype human *HES* gene, *HES1*, is controlled by Notch signaling in the intestine (van Es et al., 2005). Immunohistochemical analysis revealed that HES1 was indeed strongly expressed in BE cells, similar to in colon epithelial cells (Fig. 1C; Fig. 2E). In the intestine (van Es et al., 2005; Yang et al., 2001), as in other tissues (Zheng et al., 2000), Notch signaling represses the *ATOH1* gene through HES1. In turn, ATOH1 drives intestinal epithelial cells into the secretory lineage to become goblet cells. Similar to in the intestine, ATOH1 was also expressed in the differentiated goblet cells of the Barrett's lesions (Fig. 1D; Fig. 2F).

Active Notch pathway in the Barrett's-derived EAC cell lines, OE33 and SKGT-5

To confirm the presence of an active Notch pathway, we analyzed two well-known human Barrett's-derived EAC cell lines, OE33 and SKGT-5 (Altorki et al., 1993). Cells were grown under standard conditions. RNA was isolated and subjected to northern analysis for the expression of *NOTCH1-4* and for the five ligands Jagged 1 and 2 (*JAG1*, *JAG2*) and Delta-like 1, 3 and 4 (*DLL1*, *DLL3*, *DLL4*). Both cell lines expressed *NOTCH1-3* (Fig. 3A) but not *NOTCH4* (not shown). Of the five ligands, we only detected expression of *JAG1* (Fig. 3A; and data not shown). *HES1* mRNA was readily detectable, implying the presence of an active Notch signaling pathway. Treatment with the γ-secretase inhibitor dibenzazepine (DBZ), a potent inhibitor of the Notch pathway in cell culture and in vivo (Milano et al., 2004; van Es et al., 2005), readily reduced *HES1* mRNA levels (Fig. 3B).

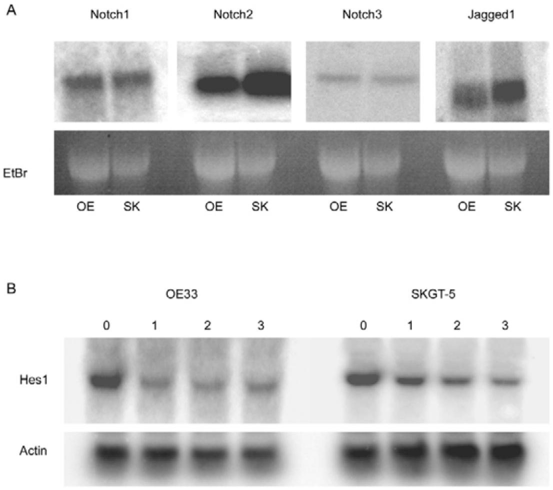

Fig. 3. Northern blot analysis of Notch pathway components in Barrett's-derived EAC cell lines. (A) Both OE33 (OE) and SKGT-5 (SK) cells express *NOTCH1* (7.7 kb), *NOTCH2* (11.2 kb), *NOTCH3* (8.0 kb) and *JAG1* (5.9 kb). Bottom row: ethidium bromide (EtBr) mRNA was used as a loading control. (B) Cells were cultured for the indicated number of days (top) in DBZ at 200 nM. *HES1* mRNA (1.5 kb) is rapidly reduced (top). Bottom row: actin mRNA was used as a loading control.

Notch signaling in a BE rat model

We then sought to investigate the effects of γ-secretase inhibitor treatment in a well-validated rat model for BE (Fein et al., 1998; Levrat et al., 1962; Sato et al., 2002; van den Boogert et al., 1999) in which the esophagus and the jejunum are surgically joined to create chronic reflux. After 4-6 months, these rats consistently develop columnar metaplasia with goblet cells in the distal esophageal epithelium, closely mimicking BE in humans (Fig. 4).

As in the human samples, the Notch signaling pathway was not activated in the healthy squamous epithelium of the rat (not shown). This contrasted with the BE segment that had developed in the distal esophagus of rats with surgically induced BE (Fig. 4E,F). We observed the presence of NICD in the nuclei of epithelial cells by immunohistochemistry (Fig. 4E). Nuclear ATOH1 staining, although clearly present, was only observed in a few scattered cells (Fig. 4F).

Notch inhibition in a BE rat model converts proliferative cells of Barrett's epithelium

Dose-finding studies revealed that intraperitoneal injection of the γ-secretase inhibitor DBZ (Milano et al., 2004) caused efficient goblet cell conversion in the small intestine of rats after five daily injections at 30 μmol/kg (data not shown). Six months after the surgical procedure, the rats were subjected to a 5-day treatment regimen and sacrificed for histological analyses of the small intestine, colon and the esophagus (Figs 5 and 6; and data not shown). For comparison, the same histological analyses were performed on control rats carrying the same surgical anastomosis, but not treated with DBZ (Fig. 4A-D), and healthy control rats that had squamous epithelium lining the normal esophagus (data not shown).

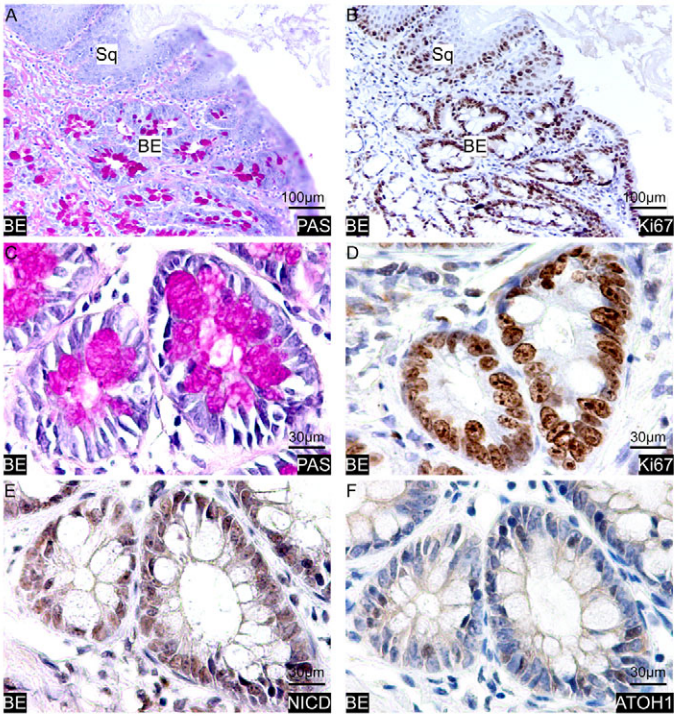

Fig. 4. Barrett's epithelium deriving adjacent to squamous epithelium has an active Notch signaling pathway. (A,B) Serial sections of the boundary of normal squamous epithelium and BE epithelium after the induction of BE by surgical esophagojejunal anastomosis. (A) The PAS stain (pink) illustrates the aberrant presence of goblet cells in crypt-like structures. (B) A Ki67 stain (brown) for the presence of proliferative cells in the basal layer of the squamous esophageal epithelium, as well as throughout the BE epithelium. (C-F) Serial sections of an untreated BE rat. (C) Magnification of the PAS staining (pink). The morphology and histology of the columnar epithelium and goblet cells mimic BE in humans. (D) Magnification of the Ki67 stain (brown). Note the proliferation in all nuclei of columnar BE cells. (E) NICD (brown) reveals intranuclear staining in the rat BE, indicative of active Notch signaling. (F) ATOH1 staining (brown), which controls the goblet cell fate. Note that ATOH1 is repressed by active Notch signaling. Sq, squamous epithelium; BE, Barrett's esophagus epithelium.

In all rats, DBZ treatment led to near-complete conversion of intestinal epithelial cells of the gut into goblet cells (data not shown), as published previously (van Es et al., 2005), indicating that effective systemic DBZ levels were reached. The DBZ treatment had a dramatic effect on the BE crypts in all surgically treated rats when compared with the control rats. Immunohistochemical analyses of serial sections of untreated rats and DBZ-treated Barrett's epithelium rats are presented in Figs 4 and 5, respectively. The Barrett's crypts displayed intense PAS staining, indicative of goblet cell conversion and a massive secretion of mucous (Fig. 4C; Fig. 5E), whereas cell cycling, as shown by Ki67 staining, was severely diminished (Fig. 4D; Fig. 5F). As expected, Notch inhibition occurred effectively, as shown by the absence of nuclear NICD staining in Barrett's nuclei (Fig. 4E; Fig. 5G), and a strong reduction in nuclear HES1 staining was also observed (data not shown). ATOH1, in turn, was dramatically de-repressed since essentially all Barrett's nuclei now contained this protein (Fig. 4F; Fig. 5H). Although DBZ treatment induced cell-cycle arrest in BE cells, the adjacent normal squamous epithelium of the esophagus remained unaffected (compare Fig. 4A,B with Fig. 5A-D). In some areas, the

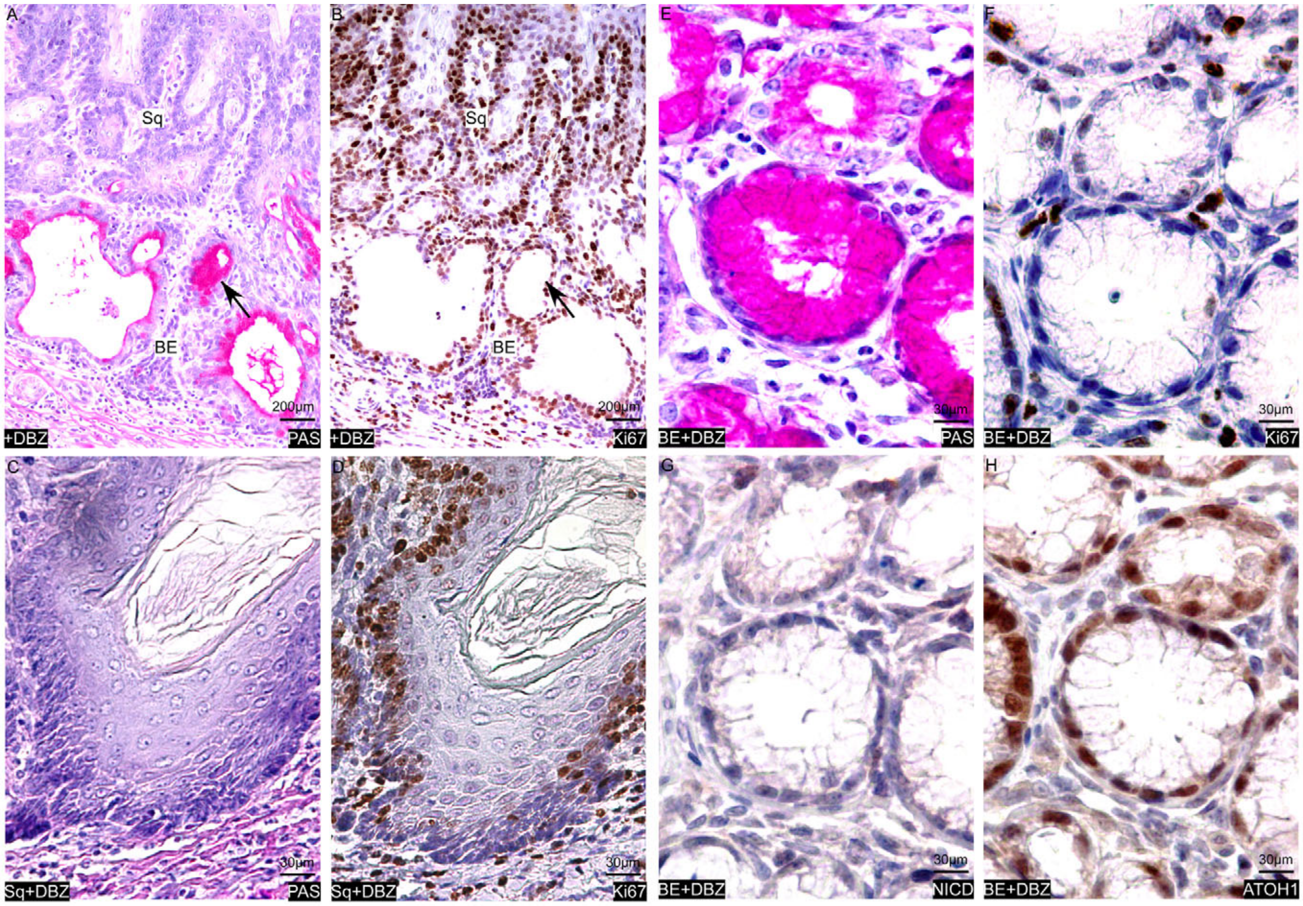

Fig. 5. Notch inhibition by the γ-secretase inhibitor DBZ does not affect the esophageal epithelium yet converts BE epithelial cells into terminally differentiated goblet cells. (A,B) Serial sections of a region containing squamous epithelium and early submucosal BE lesions in a DBZ-treated BE rat. (A) PAS staining (pink) identifies the BE islands. (B) Ki67 staining (brown) reveals normal proliferation in the squamous epithelium and the virtual absence of proliferation in the adjacent BE islands. (C) Magnification of the PAS staining (pink) in the squamous epithelium at the site of BE development (esophagitis). Squamous epithelium is not affected by DBZ treatment and no goblet cells are present. (D) Magnification of the Ki67 staining (brown) in the squamous epithelium at the site of BE development (esophagitis). Note the proliferation at the basal layer of the squamous epithelium. (E) Magnification of the PAS staining (pink). Note the almost complete replacement of columnar morphology by mature goblet cells with flat basal nuclei. (F) Magnification of the Ki67 staining (brown). Note the almost complete loss of proliferation upon DBZ treatment. (G) NICD (brown) reveals intranuclear staining in the rat BE, whereas the staining is virtually absent in the DBZ-treated rat, indicative of effective inhibition of Notch signaling. (H) ATOH1 staining (brown) after DBZ treatment reveals a virtually complete de-repression of *ATOH1* gene expression, which controls the goblet cell fate. Sq, squamous epithelium; BE, Barrett's esophagus epithelium.

effect of DBZ resulted in the effective exfoliation of the entire BE epithelium as a mucous mass, as exemplified in Fig. 6, essentially leaving a bare yet undamaged submucosa.

DISCUSSION

The golden standard for diagnosing BE is the histology of columnar epithelium with goblet cells (Sharma et al., 2004). The stage of the disease is determined by the following grades, which predict an increasing chance for the development of EAC: BE without dysplasia, BE with low-grade dysplasia, BE with high-grade dysplasia, and EAC. The grade of dysplasia determines the appropriate surveillance interval (Wang and Sampliner, 2008). Surgical resection of the esophagus takes place when patients are at the high-grade dysplasia or EAC stage of disease (Gilbert and Jobe, 2009). There is currently no curative therapy for BE; endoscopy combined with histology-based surveillance for early detection of EAC remains the only tool to offer patients (Wang and Sampliner, 2008).

The resemblance of metaplastic BE epithelium to colon epithelium prompted us to apply insights gained in intestinal biology to BE. The Notch pathway plays a dominant role in the self-renewal of normal colonic epithelium. When blocked, all proliferative epithelial cells instantaneously convert into goblet cells. The same phenomenon occurs in adenomas of the intestine upon inhibition of the Notch signaling pathway (van Es et al., 2005).

In the current study, we confirm the notion that the Notch pathway is active in BE by histological analysis of biopsies and by biochemical studies in two BE cell lines, OE33 and SKGT-5. Treatment of these cell lines with the γ-secretase inhibitor DBZ, shown to be a potent inhibitor of the Notch pathway in cell culture (Milano et al., 2004; van Es et al., 2005), readily reduced mRNA levels of the Notch target gene *HES1*, which is indicative of Notch

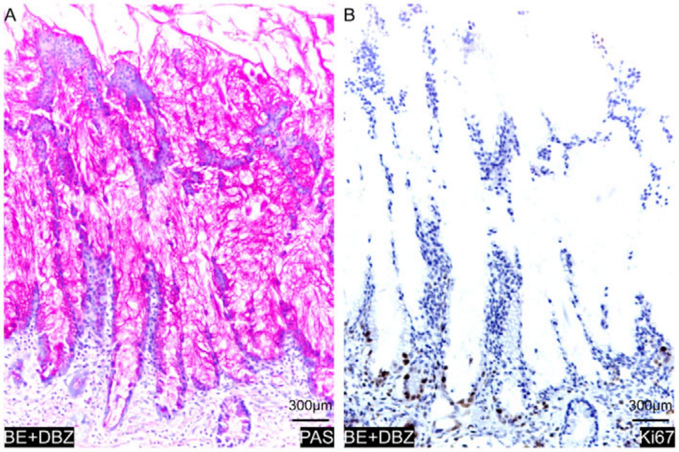

Fig. 6. DBZ treatment can induce virtually complete exfoliation of BE epithelium. (A,B) Serial sections of a BE epithelial region showing the extensive effects of DBZ treatment. PAS staining (pink) (A) and Ki67 staining (brown) (B) reveal that post-mitotic goblet cells have dissolved into a mucous mass, effectively demonstrating chemical ablation of the metaplastic epithelium by DBZ. Note the apparent absence of effects on the histology of the submucosa.

pathway inhibition. When applied to a surgical rat model of BE in vivo, we subsequently document that Notch inhibition converts the proliferative Barrett's cells into terminally differentiated goblet cells, whereas the squamous epithelium remains apparently unaffected. As with all animal models, there must be caution with regards to extrapolation of the results from the animal model to humans. For example, rats do not have submucosal glands in the esophagus, which may contribute to the establishment of BE in humans. Yet, this particular model appears to mimic the development of BE and EAC (Fein et al., 1998; Levrat et al., 1962; Sato et al., 2002; van den Boogert et al., 1999).

This study indicates that Notch inhibition by DBZ in BE mirrors the effects on the normal absorptive epithelium of the intestine (van Es et al., 2005) in that Notch inhibitors can completely remove proliferative cells from the BE segment. Although the effect of Notch inhibitors on the BE segment is dramatic, we currently do not know what esophageal lining will develop after the conversion of the epithelium, since we could not observe animals for longer time periods after the systemic Notch inhibition owing to the deleterious effects in the intestine.

The effect of systemic delivery of Notch inhibitors on the intestine complicates their use as therapeutic agents in Alzheimer's disease (Lundkvist and Naslund, 2007). Phase II studies have already taken place to test the safety, tolerability and response to γ-secretase inhibitors (Fleisher et al., 2008; Lannfelt et al., 2008; Wilcock et al., 2008). Since the lesions in BE reside in a tissue environment that essentially appears to be refractory to the principal side effect of γ-secretase inhibitors, local delivery of these compounds by supramucosal application, or by submucosal injection during endoscopy of the esophagus, may circumvent these complications. After injection, the multilayered squamous epithelium of the healthy esophagus is predicted to stay intact, whereas metaplastic BE cells are forced to differentiate. Such local γ-secretase inhibitor treatment may be applicable to Barrett's patients of all stages. Taken together, our data imply that local application of Notch inhibitors may present a simple therapeutic strategy for this increasingly common pre-malignant condition.

METHODS

Histology

All histology was performed as described elsewhere (van Es et al., 2005).

Antibodies

For immunohistochemistry, serial sections of 4 μm were blocked for endogenous peroxidase with 1% H_2O_2 in 100% methanol for 30 minutes. Antigen retrieval was performed with 10 mM monocitric acid (pH 6.0) at 100°C for 15 minutes. The slides were blocked with non-immune serum for 20 minutes at room temperature. The sections were stained using primary antibodies against goblet cells (PAS), proliferative cells (anti-Ki67, 1:500; BD Pharmingen, San Diego, CA), Notch cell-cycle factor (anti-Hes1, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), Notch transcription factor [anti-ATOH1, 1:3000; provided by A. Helms and J. Johnson (Helms and Johnson, 1998)] and cleaved Notch1 receptor (anti-Notch1, 1:75; Cell Signaling Technology, Boston, MA). Binding of the primary antibody was visualized by the addition of Envision (HRP-labeled mouse antibody, undiluted; DAKO, The Netherlands). Normal, healthy squamous epithelium and human colon were used as controls. Three independent observers (V.M., M.v.d.B. and H.C.) evaluated the sections for the immunohistochemical stainings.

Tissue culture and DBZ treatment

To inhibit γ-secretase activity in the human Barrett's-derived EAC cell lines OE33 (European Collection of Cell Cultures, Salisbury, UK) and SKGT-5 (Altorki et al., 1993), cultures were incubated for the indicated number of days in 200 nM of DBZ. DBZ was custom synthesized to more than 99.9% purity (Syncom Pharmaceuticals) and diluted in dimethyl sulfoxide (DMSO).

Northern blotting

mRNA was run on a 1.5% agarose gel and blotted to Zeta-Probe membranes (Bio-Rad Laboratories, Hercules, CA). Hybridization with radioactive probes was performed at 68°C in the presence of ExpressHyb (BD Biosciences, Clontech, Palo Alto, CA) solution. The RadPrime DNA labeling system (Invitrogen, Carlsbad, CA) was used to label probes with ^{32}P-dCTP. The following IMAGE clone fragments were used to produce probe DNA: *NOTCH1*, *Not*I-*Eco*RI fragment of ID 3066192; *NOTCH2*, *Not*I-*Sal*I fragment of ID 6055379; *NOTCH3*, *Eco*RI-*Hin*dIII fragment of ID 6184018; *NOTCH4*, *Not*I-*Sal*I fragment of ID 4779663; *JAG1*, *Xho*I-*Eco*RI fragment of ID 5212818; *JAG2*, *Pst*I fragments of ID 6459190; *DLL1*, *Not*I-*Eco*RI fragment of ID 5224361; *DLL3*, *Eco*RI-*Xho*I fragment of ID 3508262; *DLL4*, *Not*I-*Eco*RI fragment of ID 5722973; *HES1*, *Hin*dIII-*Sac*I fragment of ID 4749611.

Animal treatments

Surgery

Eight-week-old male Wistar rats were obtained from Harlan, England and housed under standard pathogen-free conditions with a maximum of three animals per cage. Experienced technicians

TRANSLATIONAL IMPACT

Clinical issue

Barrett's esophagus (BE) affects approximately 2% of the Western population and progresses to esophageal adenocarcinoma in 0.5% of these patients each year. Cancer of the esophagus is almost invariably lethal, and its incidence has increased dramatically in recent years. BE is believed to be caused by chronic reflux from the acidic contents of the stomach and bile, which converts the squamous epithelium lining the esophagus into columnar epithelium resembling that of the lower intestine. Subsequent mutations then lead to adenocarcinoma. Currently, there is no cure for BE once it is established. Patients are routinely monitored by endoscopy, while the reflux is treated to prevent progression to more advanced disease. Eventually, endoscopic surgical intervention may be necessary to remove affected tissue. Basic research into esophageal adenocarcinoma has focused on determining the molecular events required for the initial squamous-columnar transition, the genes required for progression, and possible methods of inhibiting or reversing the pre-cancerous and cancerous changes. It is possible that, as the mutated columnar epithelium is similar to colonic epithelium, the Notch pathway, which is a signaling cascade that is central to both normal and neoplastic colonic development, may be involved.

Results

The authors previously found that the Notch pathway controls the vigorous cell division in the lining of the normal gut. Here, they show using biopsy samples that the Notch pathway is not active in the normal squamous lining of the esophagus but that it is highly active in the areas of the esophagus that have changed into columnar Barrett's epithelium. To determine whether inhibition of the Notch pathway could revert or destroy Barrett's epithelium, dibenzazepine (DBZ), a known inhibitor of the Notch pathway, was used to treat rats with surgically induced Barrett's epithelium. As shown previously in normal colonic epithelium, Notch inhibition converted the proliferative Barrett's cells into arrested terminally differentiated goblet cells, whereas the normal squamous epithelium was unaffected. In some cases, the Barrett's epithelium was entirely exfoliated, leaving bare submucosal tissue.

Implications and future directions

These data imply that local application of Notch inhibitors may present a simple therapeutic strategy for BE. However, further studies are required to optimise a method of delivery and, importantly, to determine the nature of any epithelial regrowth following treatment.

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carried out all of the animal handling. After an acclimatization period of 1 week, the animals were operated on. BE was induced in twelve rats by gastrectomy with esophagojejunostomy, as reported previously (Levrat et al., 1962; Sato et al., 2002; van den Boogert et al., 1999). Three rats were treated with DBZ. No incisions were made in the three control rats. The rats were sacrificed at 6 months after the induction of BE. The esophagus was removed, fixed in 10% neutral buffered formalin for 24 hours, and embedded in paraffin. General health status and weight were monitored at least twice per week; weight loss of more than 20% of the pre-operative body weight, severe regurgitation, aspiration that the animal did not recover from within 24 hours, or apathetic behavior prompted us to exclude the animal from the study. The experimental study protocol was approved by the local animal experimental committee.

DBZ treatment

Six months after the surgical procedure, three of the operated rats and three control rats were subjected to a 5-day treatment regimen with intraperitoneal DBZ at 30 μmol/kg, and sacrificed at day 6 for histological analyses of the small intestine, colon and the esophagus. The general health status of the rats was not affected and their weight was not diminished.

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COMPETING INTERESTS

H.C. and J.v.E. are inventors on patent applications that claim the use of Notch inhibitors as a treatment for intestinal diseases.

AUTHOR CONTRIBUTIONS

H.C., J.v.E., P.D.S., J.G.K., R.W.F.d.B. and E.J.K. conceived and designed the experiments; V.M., W.d.L. and M.v.d.B. performed the experiments; J.v.E., V.M., W.d.L. and M.v.d.B. analyzed the data; V.M. and H.C. wrote the paper.

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