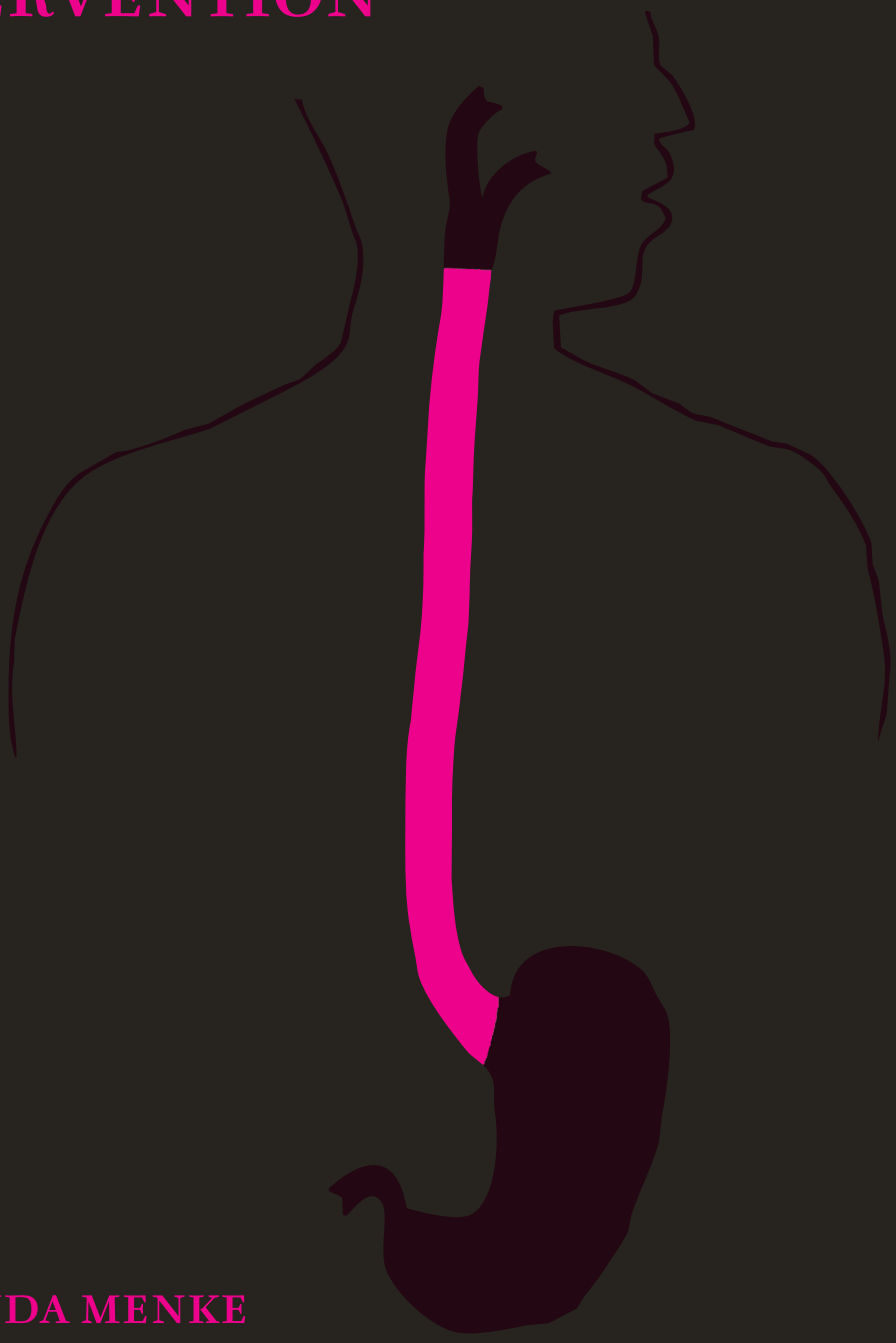
BARRETT'S ESOPHAGUS: ORIGIN, DEVELOPMENT AND OPTIONS FOR THERAPEUTIC INTERVENTION



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Barrett's esophagus:

Origin, development and options for therapeutic intervention

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The past is behind me.
The future don't exist.

- Lissie, This much I know, Catching A Tiger

Voor mijn ouders

Contents

CHAPTER I

INTRODUCTION ON BARRETT'S ESOPHAGUS

Introduction on Barrett's esophagus

Barrett's esophagus (BE) consists of a metaplasia in which the normal squamous cells lining the esophagus are replaced by a mixture of gastric and intestinal lining cells. The intestinal-type lining cells are often referred to as specialized columnar cells and include e.g. goblet cells. For a number of years, some scientists thought that there were two types of BE; one in which the normal lining was replaced with gastric type cells only, and the second in which intestinal cells were present. Presently, intestinal metaplasia is required for the diagnosis of BE because intestinal metaplasia is the only type of esophageal columnar epithelium that clearly predisposes to malignancy [1, 2].

BE is a complication of chronic gastroesophageal reflux disease (GERD), primarily in white men. GERD is a disease in which there is reflux of acidic fluid from the stomach and bile contents from the duodenum into the esophagus. It most commonly causes heartburn, but many patients do not have any symptoms.

A reason for limited understanding of the development of BE is, that all patients undergoing medical examinations when GERD symptoms are present, are either suffering from reflux esophagitis or are already suffering from BE. Only a subpopulation of patients with reflux esophagitis develop BE, thus regular examination on patients with reflux esophagitis has not delivered a large population with BE. Unfortunately, most patients are diagnosed with BE at first clinical presentation. Consequently only limited (if any) patients are available for testing putative hypotheses on the early onset and progression of this disease. Basic research into BE 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.

Although BE is a premalignant condition, it is the main risk factor for the development of esophageal adenocarcinoma (EAC). EAC is the fastest rising malignancy in Western countries over the last three decades, the EAC treatment options are poor and it has a high mortality rate [3]. GERD is associated with an increased risk of both BE and EAC development [4]. Persistent gastroesophageal reflux activates several pathways leading to BE and eventually EAC. However, the underlying mechanisms leading to the GERD-BE-EAC cascade remain unknown.

The adaptive immune response in reflux esophagitis differs from that in BE, while the composition of the reflux during the GERD-BE-EAC cascade remains con-

stant. Reflux esophagitis in GERD is characterized by a cell-mediated immune response, while a humoral immune response predominates in BE [5]. Moreover, use of anti-inflammatory drugs such as non-steroid anti-inflammatory drugs (NSAIDs) and aspirin have been shown to diminish mucosal inflammation and establish a lower incidence of BE and EAC in both humans and animals [6]. In addition, depressed cellular immunity is both associated with BE development [7], and with decreased immune surveillance leading to cancer [8].

As it is almost impossible to establish the transition from reflux esophagitis into BE in humans, we used an established animal model with surgically induced reflux esophagitis [9, 10] to study the role of the immune response in the development of reflux esophagitis, and the conversion of squamous epithelium into BE. The development of BE was established in all rats 6 months after surgically induced chronic reflux. The conversion from squamous into columnar epithelium mimicked the human BE histology. Parallel with the development of intestinal metaplasia, a transition of the immune response pattern was noted. In reflux esophagitis, the submucosal layer showed a cell-mediated Th1-like inflammation profile, while in BE, the submucosal layer contained a predominant humoral Th2-like inflammation profile (**Chapter 2**). The role of the immune response was studied in various rat strains that had a predisposition for the Th1 and/or Th2 immune response. Th1-prone Lewis rats showed Th1 effector cells at an early onset of the hyperplasia-esophagitis-BE cascade, as well as the longest esophageal segment with reflux esophagitis compared to the Th2-prone BN and intermediate Wistar rat strain. Moreover, Lewis rats activated Th2 effector cells earlier after surgically induced reflux and developed the longest BE segment compared to Th2-prone BN and intermediate Wistar rats. We concluded that a Th1-predominant immune status may predispose to the development of BE following reflux esophagitis (**Chapter 3**).

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine involved in the Th1 immune response [11]. Esophageal TNF expression is responsive to local concentrations of reflux components [12] and inflammatory cytokines [13], but is also controlled on a genetic level. We assessed the association of the TNF-β (*NcoI*) polymorphism, and the local effect on TNF production in the esophagitis-BE-EAC cascade. The TNF-β *Nco*I A/A genotype showed a significantly higher frequency and TNF was locally expressed in BE and EAC. These data indicate that TNF plays a role in the development of BE and EAC (**Chapter 4**).

The adaptive immune response against toxic substances is related with the nu-

clear pregnane X receptor (PXR) [14, 15]. PXR plays a key role in the regulation of hepatobiliary transport systems and of enzymes that confers a protective role against toxic bile acids [16]. PXR was expressed in BE and EAC tissue, and nuclear localization was seen in EAC tissue. Upon stimulation with lithocholic acid, PXR translocated to the nuclei of EAC cells. The PXR polymorphism was associated with BE. Therefore, PXR may play a role in the prediction of BE disease and possibly in the treatment of esophageal disease to prevent EAC (**Chapter 5**).

The stratified squamous epithelium of the healthy esophagus possesses a variety of intrinsic defenses that enable it to resist gastric and bile reflux [17, 18]. Growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are associated with epithelial defense mechanisms [19]. EGF plays an important role in the protection and repair of mucosal damage, and non-physiologic levels are associated with gastrointestinal tumors. We found that genetic variation of EGF was associated with reduced EGF expression and increased risk for reflux esophagitis, BE, and EAC development. Over-expression of the EGF-receptor in esophageal adenocarcinoma is associated with advanced pathologic tumor classification and lymph node metastasis, however, not with an increased risk for tumor development or survival. We hypothesized from our results that the decreased EGF activity in the esophagus could trigger the promotion of a tumor environment, thereby either directly or indirectly affecting the risk for EAC development. This indicates that reduced mucosal protection resulting from genetically decreased EGF expression enhances esophageal tumor development (**Chapter 6**).

Myo9B is a protein involved in epithelial cell cytoskeletal organization and predominantly expressed in the immune system [20]. A defect in the intestinal barrier function by genetic variation of the Myo9B gene could be a factor for the genesis of intestinal inflammation in celiac disease and inflammatory bowel disease [21, 22]. Increased mucosal permeability is recognized as one of the earliest histological changes in GERD [23]. Genetic variation of Myo9B was associated with an increased risk for BE and EAC development. Cytoplasmic Myo9B expression was determined in reflux esophagitis, BE, and EAC, but most prominent in epithelial cells of BE and EAC. Myo9B might play a role in the etiology of BE and EAC by disturbing the cytoskeletal and immunological organization, therefore increasing the permeability of the epithelial barrier (**Chapter 7**).

As with the intestinal epithelium, the Barrett's epithelium contains proliferative crypt-like compartments. In the intestine, self-renewal of the epithelium is driven

by Notch signaling. Notch inhibition results in rapid conversion of all proliferative cells into differentiated goblet cells [24]. The activation of Notch signaling is critically dependent on an intramembrane protease complex termed γ-secretase. This protease complex is also implicated in the pathogenic processing of the amyloid precursor protein in Alzheimer's disease [25]. 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 [24, 26], while (pre-) clinical studies have revealed a single major side effect of γ-secretase inhibitors: the induction of goblet cells in the intestine [27]. In BE, 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. These data imply that local application of Notch inhibitors may present a simple therapeutic strategy for BE conversion (**Chapter 8**).

The research presented herein identified histological and immunohistochemical characteristics in the development of GERD and BE, we evaluated biomarkers and genetic factors involved in the GERD-BE-EAC cascade, and eventually introduced a possible option for therapeutic intervention with Notch inhibitors. With increasing availability of compelling information on various biomarkers, e.g. TNF, PXR, EGF and Myo9B, there has been an interest in evaluation of panels combining clinical features, endoscopic criteria, and molecular biomarkers as tools of risk stratification in BE. In future, we hope to identify the patients at risk. While GERD may be considered an immune-mediated disease, this concept opens ways to develop new treatment leading to the prevention of carcinogenesis in BE. Our research might be a small step forward in the identification of biomarkers and immunological targets in the GERD-BE-EAC cascade, however, many of the fields are still open and future efforts are necessary.

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CHAPTER 2

IMMUNE RESPONSE IN THE DEVELOPMENT OF ESOPHAGITIS AND BARRETT'S ESOPHAGUS

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Abstract

Background: Barrett's esophagus (BE) is a pre-malignant condition caused by gastro-esophageal reflux disease (GERD) and predisposes to esophageal adenocarcinoma. The conversion of GERD into BE occurs in a subsection of patients and is hardly ever observed, while BE is diagnosed at clinical presentation. Therefore, the underlying pathogenic mechanisms of BE development remain unclear and need to be studied in a GERD-BE disease model. In a well-validated rodent model for BE, the immunohistochemical processes involved in the initiation and chronic persistence of BE were determined.

Materials and methods: BE was surgically induced in Wistar rats by gastrectomy with esophagojejunostomy. At 0, 3, and 6 months the presence of histological inflammation and metaplasia was analyzed. Proliferative cells (Ki67), intestinal metaplasia (CDX2), goblet cells (WE9), and local inflammatory markers for monocytes (ED1), macrophages (ED2), T cells (CD3), cytotoxic T cells (CD8), B cells (CD45RA), and plasma cells (IgG) were determined.

Results: Esophageal lesions at 0-3 months after surgery were characterized by esophagitis and increased numbers of the Th1 effector cells macrophages and cytotoxic T cells without significant metaplasia, and at 3-6 months after surgery characterized by BE as specialized intestinal metaplasia with CDX2 and WE9 expression, and a marked influx of the Th2 effector cells eosinophils, B cells, and plasma cells. In addition, lymphoid aggregations were formed over time, which correlated in size with an increasing inflammatory response (r=0.68).

Conclusions: GERD results in an early cell-mediated response in esophagitis while the later humoral response occurs in human BE-like. This suggests that the development of intestinal metaplasia is accompanied by a gradual shift in immune response. Therefore the immune response might be involved in the induction of BE.

Introduction

Barrett's esophagus (BE) is a pre-malignant condition, observed exclusively in a proportion of patients with gastro-esophageal reflux disease (GERD). BE is the most prominent risk factor for the development of esophageal adenocarcinoma (EAC), to which it has an annual progression rate of 0.5 -1% [1, 2]. EAC has a high mortality rate and is currently the fastest rising malignancy in the Western world [3, 4]. There is currently no curative therapy for BE; endoscopy combined with histology-based surveillance for early detection of EAC and symptom relief are the only tools to offer patients. A better understanding of the pathologic mechanisms underlying this disease cascade is a prerequisite for the development of therapeutic options. [5, 6].

The individual alterations required for malignant progression in the GERD-BE-EAC cascade are poorly understood. A prospective patient-based evaluation would require a large cohort and considerable time span as GERD only converts to BE in a small percentage of cases. The diagnosis of BE occurs at first clinical presentation and BE patients already display a predominantly humoral immune response. As it is almost impossible to establish the spatial and temporal relationship of BE development in humans, the aid of an established animal model with surgically induced reflux esophagitis [27, 28] to study the changes in the immune response parallel with the histological development of intestinal metaplasia is inevitable.

Persistent gastro-esophageal reflux is associated with an increased risk of BE and EAC development [7, 8]. The focus on the composition and the role of reflux, including gastric and bile acids [9-11], suggested that in particular bile acids are important in BE development [12]. Bile acids can increase transcription of CDX2 in esophageal squamous cells, thereby initiating metaplastic transformation [13, 14].

It has been thought that chronic reflux into the distal esophagus damages the tight junctions between the epithelial cells causing the intercellular spaces to dilate and H^+ ions to enter into the epithelium [15-17]. Acute reflux-induced chemical injury and death of the surface esophageal epithelial cells has been assumed to recruit neutrophils to the epithelium. Damage to the deeper layers of the epithelium may elicit a proliferative response, leading to basal cell and papillary hyperplasia [18-20]. However, a recent study suggested an alternative concept in which reflux does not cause direct chemical injury, but stimulates esophageal epithelial cells to secrete chemokines which mediate damage of esophageal tissue through an immune response [21].

The inflammatory immune response has been associated with the transition from GERD to BE [22]. Recent studies showed that the inflammatory process in GERD differs from that in BE, while the composition of the reflux during the GERD-BE-EAC cascade remains constant. GERD is characterized by a cell-mediated immune response, while a humoral immune response predominates in BE [23]. In addition, depressed cellular immunity is both associated with BE development [24], and with decreased immune surveillance leading to cancer [25, 26].

In current study, an animal model was used in order to obtain more information on the initial steps of BE development. The obtained data support our hypothesis that the development of BE occurs in surgically induced GERD. Parallel with the BE development, a shift in the immune response from a cell-mediated immune response in GERD to a predominately Th2 immune response in BE is determined. These data suggest that the immune response might play a role in BE development.

Materials and methods

Animals

Six-week-old male Wistar rats were obtained from Harlan, England and housed under standard pathogen-free conditions with a maximum of 3 animals per cage. Experienced technicians carried out all animal handling. The animals were fed a commercially available natural diet for rats (SDS, England) and had free access to tap water. After an acclimatization period of 1 week, the animals were randomly divided into three groups in a time course design of t = 0, 3, and 6 months. General health status and weight was monitored at least twice per week; weight loss of more than 20% of the preoperative body weight, severe regurgitation, aspiration not recovering within 24h, or apathetic behavior prompted us to exclude the animal from the study. The local experimental animal committee approved the experimental study protocol.

Surgical procedure

Several rat models were tested in our preliminary study (data not shown); first a model based on esophagoduodenal anastomosis, second a model based on esophagojejunostomy with gastrectomy, and third a model based on esophagojejunostomy with ligation and preservation of the stomach [27, 28]. The esophagoduodenal model had a 7mm large anastomosis that closed spontaneously in 3 out of 5 rats, and the other 2 rats did not show reflux esophagitis after 2 months. The esophagojejunostomy model with gastrectomy (GEJ) was performed in 30 rats to determine the development of SIM over time. Each month 2 rats post-GEJ and 1 SHAM rat were sacrificed on a timeline from 0 to 6 months. Ten rats died prior to reaching their study endpoint. The result of this preliminary study revealed that no macroscopic or microscopic differences were observed between the native rats and the SHAM operated rats in any of the arms of the experiment. However, when the esophageal samples of the GEJ rats were studied, two typical stages of GEJ-induced changes could be distinguished; an initial phase with esophagitis and a late phase with formation of intestinal metaplasia. These defects were most prominent at respectively 3 months (initial phase) and 6 months (late phase) post-GEJ. The rats with a esophagojejunostomy and the ligated stomach in place, showed the same histology as the rats with esophagojejunostomy with gastrectomy, but more animals (50%) died due to necrosis of the stomach. We measured the pH of

the reflux on paper test strips (AlkaLife, Eindhoven, The Netherlands) at the surgical anastomosis and more proximal in the esophagus; at all times the pH showed a value around 6.0. We did not find an acidic variety in the reflux of the three different rat models. Based on the survival and the histological findings, in the present study, SIM was induced by gastrectomy with esophagojejunostomy (GEJ). The technique was performed as previously reported [27, 29, 30] (Figure 1). SHAM rats only had abdominal laparotomy, with palpitation of the esophagus and the jejunum. No incisions were made in six control rats. The rats were randomly sacrificed at 0, 3, and 6 months post-GEJ. The esophagus was then removed, and Swiss rolls were prepared with the luminal side at the inside and the distal end of the esophagus in the centre. Each Swiss roll was fixed in 10% neutral buffered formalin for 24 hours, and embedded in paraffin. The embedded samples were stored at RT until used for histology and immunohistochemistry.

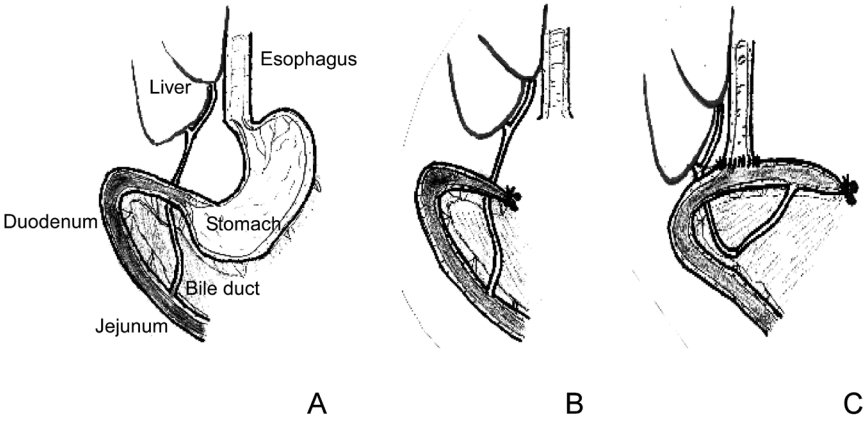

Figure 1.
Schematic representation of the gastrectomy with esophagojejunostomy (GEJ); the normal rat anatomy (A), status after gastrectomy (B), and an end-to-side esophagojejunal anastomosis at 2cm distal from the insertion of the bile duct with a blind-ending duodenum (C).

Pathological analysis

Paraffin samples were cut at 4μm, deparaffinized in xylene, hydrated using a graded series of alcohol, and stained with hematoxylin-eosin (H&E). Six levels of the Swiss rolls were stained for histopathological analysis. A specialized gastrointestinal pathologist (HvD) assessed all slides following international standard criteria

for the presence of hyperplasia, hyperkeratinization, papillomatosis, ulceration, focal metaplasia, SIM, and carcinoma with subtype. Ulceration was defined as large amounts of inflammatory cells that replace the epithelial layer. Focal metaplasia was defined as the presence of ducts with mixed, squamous and columnar epithelium at distance from the intestinal segment around the anastomosis. SIM was defined as intestinal columnar epithelium with goblet cells above the anastomositic site marked by the black monocryl suture. Parts of squamous epithelium within SIM and a continuous basal membrane of SIM with the esophagus also differentiated the jejunum from SIM at the site of the anastomosis. The rats with a small segment of dysplasia (1/21) and esophageal cancer (3/21) were primarily excluded, but that did not have influence on the mean mucosal thickness, the SIM segment length nor on the histological and immunohistochemical data. Therefore, these rats were not excluded from our data.

Immunohistochemical analysis

For immunohistochemistry, sections of 4μm were blocked for endogenous peroxidase activity with 1% H_2O_2 in 100% methanol for 30 minutes. Antigen retrieval was performed with 10mM monocitric acid (pH 6.0) at 100°C for 15 minutes, except for OX8 staining, that does not require antigen retrieval. The slides were blocked with non-immune serum for 20 minutes at room temperature. The sections were stained using primary antibodies against intestinal epithelium (anti-CDX2 clone AMT 28; 1:100 dilution; Novocastra Laboratories Ltd, Newcastle, UK), goblet cells (anti-WE9; 1:200; gift from Dr. D.K. Podolsky, Gastrointestinal Unit, Massachusetts General Hospital, Boston, USA), proliferative cells (anti-Ki67; 1:500; BD Pharmingen, San Diego, USA), monocytes and macrophages (anti-ED1; 1:100; Serotec, Oxford, UK), tissue macrophages (anti-ED2; 1:100; Serotec, Oxford, UK), T cells (anti-CD3; 1:100; BD Pharmingen, San Diego, USA), $CD8^+$ T cells (anti-OX8; 1:500; Serotec, Oxford, UK), B cells (anti-OX33 {CD45RA}; 1:300; Serotec, Oxford, UK), and anti-IgG (Goat-anti-rat IgG; 1:100; Jackson). Binding of the primary antibody was visualized by the addition of Envision. Normal rat esophagus, rat colon, rat spleen and rat mesenterial lymph nodes were used as a control.

Two independent observers (VM, JGK) evaluated the sections for the immunohistochemical stainings and the mean error between observers was always <5%. To determine the grade of inflammation, the number of immune cells (i.e. ED1, OX8, OX33, IgG) were scored in 5 randomly selected microscopic fields at 3 months

post-GEJ, and at 6 months post-GEJ in the proximal non-SIM esophagus (called 6 months prox) as well as in the distal esophagus with SIM. The slides were assessed using a Zeiss microscope (Axioskop 20, Carl Zeiss, Sliedrecht, The Netherlands) with a standard magnification (200x).

The mean length of the SIM segment, the mucosal thickness at set distances from the anastomosis, and the area of the lymphoid aggregations in the tissues were determined on images, recorded with a Nikon camera (DS-5M-U1) and analyzed with Nikon Eclipse Net 2000 software (Nikon, Badhoevedorp, The Netherlands) that were calibrated with our Zeiss Axioskop 20 microscope.

Statistical analysis

The study was powered for the known 30% mortality rate of this model to yield a total of 20 animals in each arm after a 6-months follow-up. One-sample variables were analyzed by the Student's t-test and expressed as the mean of at least 3 animals with a standard error of the mean (SEM). Statistical relevance of correlations was determined with Pearson's correlation test. Nominal variables were analyzed with the $\chi 2$-test. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted with the SPSS software package v11.1 (SPSS, Chicago, IL).

Results

In total, 60 rats were included in this study, among which 6 untreated rats, 3 SHAM rats at 3 months, 3 GEJ rats at 3 months, 27 SHAM rats at 6 months, and 21 GEJ rats at 6 months after surgery (Table 1).

Table 1.

	T=0 (n=6) / SHAM (n=30)	T=3months (n=3)	T=6months (n=21)
MACROSCOPIC VIEW			
Proximal esophagus	Normal	Normal	Pathological thickening
Distal esophagus	Normal	Pathological thickening	Pathological thickening
H&E			
Proximal esophagus	Squamous	Hyperplasia	Hyperplasia, Ulcers, Lymphoid aggregations Patchy BE submucosal
Distal esophagus	Squamous	Hyperplasia, Ulcers, Lymphoid aggregations, Patchy BE submucosal	Lymphoid aggregations, BE
MARKERS			
Proximal esophagus			
Monocytes/macrophages (ED1)	+	+	++
CD3 T cells	+	+	++
CD8 T cells	-	+	++
B cells (OX33)	-	-	+
Plasma cells (IgG)	-	-	+
Proliferation (Ki67)	Basal membrane	-	Submucosa
BE (CDX2)/Goblet cells (WE9)	-	-	Submucosa
Distal esophagus			
Monocytes/macrophages (ED1)	+	++	++
CD3 T cells	+	++	++
CD8 T cells	+	++	++
B cells (OX33)	-	+	++
Plasma cells (IgG)	-	+	++
Proliferation (Ki67)	Basal membrane	Submucosa	BE mucosa
BE (CDX2)/Goblet cells (WE9)	-	Submucosa	BE mucosa

Macroscopic analysis

At 3 months post-GEJ

Macroscopic analysis of the SHAM esophagus revealed no obvious pathology; closer inspection showed an esophagus of standard thickness with a continuous white mucosal layer. At 3 months post-GEJ, all rats presented a pathological, thickened tissue at the distal esophagus. The lumen contained red superficial ulcers located in the white mucosal layer of both the proximal and distal esophagus.

At 6 months post-GEJ

Macroscopic analysis of the SHAM operated rats after 6 months still revealed no obvious abnormalities; the esophagus was of standard thickness with a continuous white mucosal layer. The GEJ rats at 6 months all presented a pathological, thickened, solid distal esophagus while the lumen and the anastomosis were still accessible. Whitish nodular patches were prominent in the lumen of the distal esophagus. Red superficial ulcers were randomly spread in the white mucosal layer of the proximal esophagus.

Histological analysis

At 3 months post-GEJ

Microscopic analysis of the SHAM esophagus in H&E stainings showed a regular pattern of the multi-layered squamous epithelium without inflammation at the surface or in the deeper layers (Figure 2a and 2d). In contrast, the esophagus of all GEJ rats showed an irregular mucosal pattern with hyperplasia, hyperkeratosis, papillomatosis, and ulceration of the distal esophagus. Lymphoid aggregations had developed at the level of the basal membrane (Figure 2b, 2e, and Figure 3). Therefore, the mucosal thickness was determined at four steady distances from the surgical anastomosis, and the SHAM and the GEJ groups were separately analyzed. The course of the mucosal thickness from the proximal to the distal esophagus was plotted in a graph, and the slope of the mucosal thickness was significantly steeper at 3 months post-GEJ compared to SHAM rats ($p<0.001$) (Figure 4).

Closer analysis of the H&E stained specimen demonstrated the development of small focal areas of SIM with goblet cells in 30% of the GEJ rats at 3 months. Patchy sites with columnar epithelium and goblet cells were observed in the deeper mu-

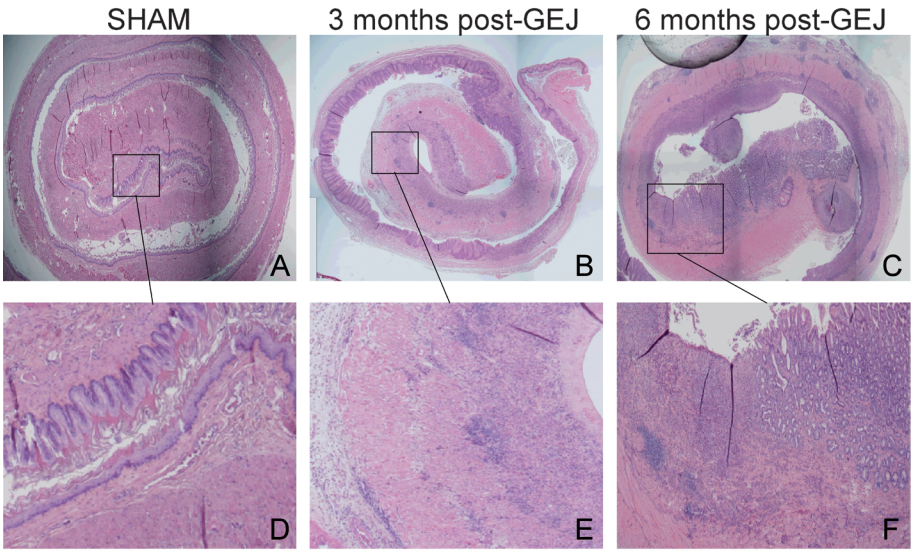

Figure 2.
Hematoxylin and eosin staining (H&E) of rat esophagus at 0 (SHAM), 3, and 6 months post-GEJ. Figures 2A-C (Magnification 1.5x) are Swiss rolls of the complete esophagus with the distal esophagus in the centre and the proximal esophagus as end tail. Figures 2D-F are magnified at the indicated sites (Magnification 12.5x). SHAM rat esophagus with a standard mucosa (A and D). At 3 months post-GEJ, the presence of a distally increased mucosal thickness, an absent keratin layer, small lymphoid aggregations (dark purple grouped cells) and focal SIM at the basal layer (B and E). At 6 months post-GEJ, the presence of an overall increased mucosal thickness, proximal esophagitis with ulcerations and focal SIM at the basal layer, medially ulcerative tissue with small lymphoid aggregations (dark purple grouped cells) and SIM at the basal layer, distally complete SIM (21/21 rats with BE) with large lymphoid aggregations (la) (C and F).

cosal layers at the basal membrane of the distal esophagus. CDX2 and WE9 staining confirmed the presence of columnar epithelium and goblet cells in this initial phase (Figure 5a and 5c). SHAM rats did not show CDX2 or WE9 expression (data not shown).

At 6 months post-GEJ

Comparing the esophagus of SHAM rats with that of rats at 6 months post-GEJ revealed marked macroscopic differences. All GEJ rats presented a pathological, irregular mucosal pattern and the proximal esophagus was always characterized by hyperplasia, hyperkeratosis, papillomatosis, and ulceration (Figure 3). Microscopic analysis of the H&E stained specimens revealed that all rats at 6 months post-GEJ had developed SIM in the distal esophagus above the anastomotic site (Figure 2f), while the esophagus of the SHAM group animals did not show such abnormali-

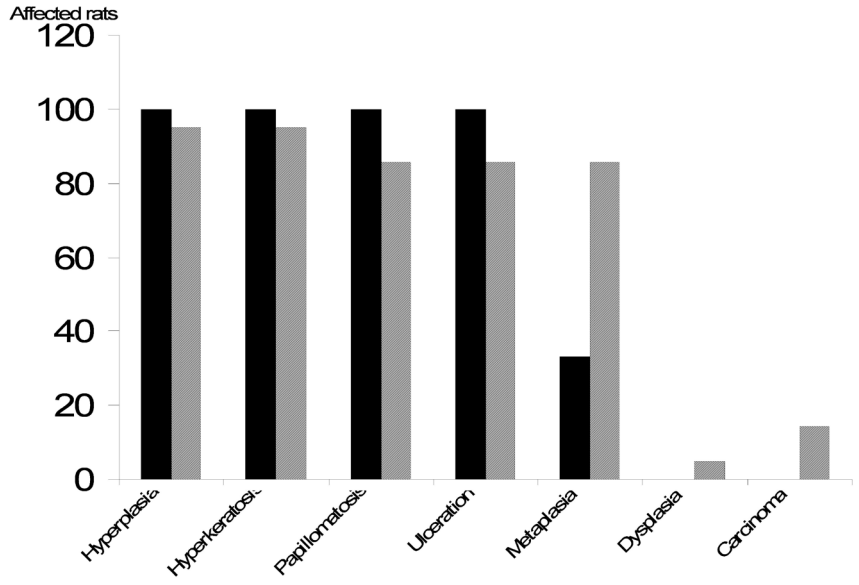

Figure 3.
Presence of hyperplasia, hyperkeratosis, papillomatosis, ulceration, and metaplasia in the esophageal mucosa of the distal esophagus at 3 months (solid bars, n=3) and both the proximal and distal esophagus 6 months (hatched bars, n=21) post-GEJ.

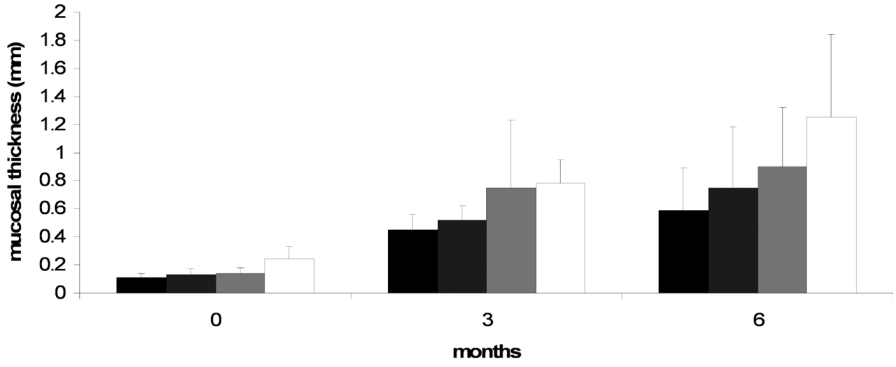

Figure 4.
Mean mucosal thickness, measured at 4 steady distances from the anastomosis. The value of the first bar (black) represents the measurement at 40mm proximally from the anastomosis, the last bar (white) represents the measurement at the anastomosis, and the two bars in between are at 10mm and 20mm proximally from the anastomosis, respectively. The mucosal thickness differs significantly between 0 and 3 months ($p<0.001$), between 0 and 6 months ($p<0.001$), and between 3 and 6 months post-GEJ ($p<0.001$).

ties (Figure 2c). Among the 21 GEJ rats, three developed esophageal cancer at 6 months post-GEJ: 1 EAC, 1 mixed adenosquamous cell carcinoma, and 1 squamous

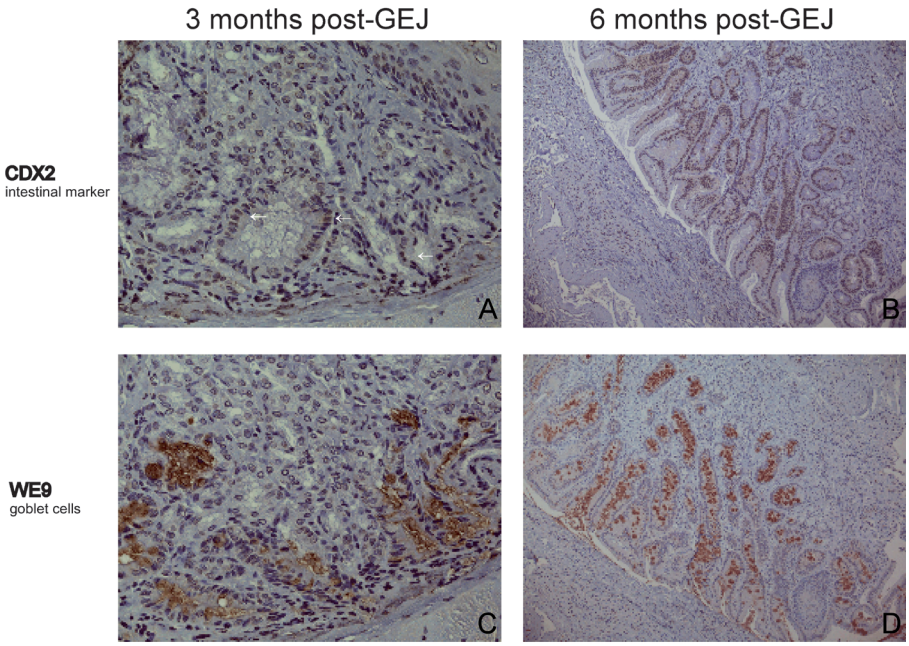

Figure 5.
SIM in the rat esophagus. CDX2 staining indicates the focal presence of columnar epithelial cells in the esophagus at 3 months post-GEJ (A) while clearly present at 6 months (B). WE9 staining for mucin 2 indicates the focal presence of goblet cells in the esophagus at 3 months post-GEJ (A) while clearly present at 6 months (B).

cell carcinoma. The course of the mucosal thickness from the proximal to the distal esophagus was plotted in a graph, and the slope of the mucosal thickness was significantly steeper at 6 months post-GEJ compared to the SHAM rats ($p<0.001$) and the GEJ rats at 3 months ($p<0.001$) (Figure 4).

CDX2 and WE9 staining confirmed the presence of columnar epithelium and goblet cells (Figure 5b and 5d). In addition, specific expression in focal areas at the level of the basal layer in the medial esophagus was observed, defining small islands of SIM in the medial rat esophagus. Closer analysis of the stained specimen demonstrated the development of several small foci of SIM with goblet cells in the proximal and medial esophagus of all GEJ rats at 6 months. PAS staining revealed the presence of separated focal metaplasia at several height levels in the esophagus (Figure 6). Because of the patchy, inhomogenous SIM design, it was not possible to isolate BE mucosal thickness and differentiate the mucosal thickness between the various histological outcomes, like esophagitis, SIM, dysplasia and carcinoma.

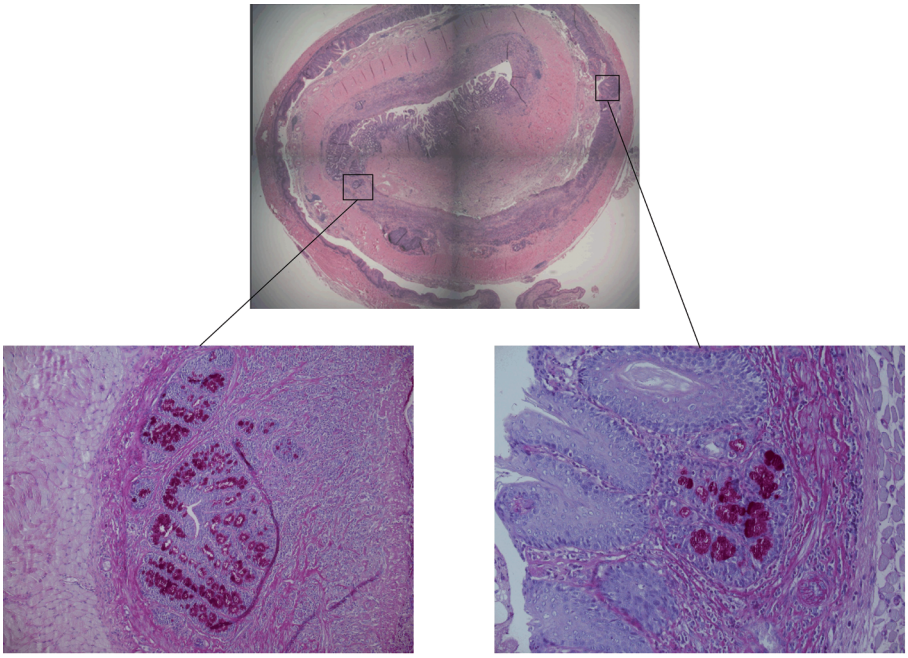

Figure 6.
H&E staining of a Swiss roll with the complete esophagus at 6 months post-GEJ shows an irregular mucosa from the proximal to the distal end. SIM known as columnar metaplasia with goblet cells in a patchy design in the rat esophagus. Focal columnar epithelium with goblet cells is magnified 40x at 10 and 20mm from the distal, anastomotic side. PAS staining of the enlarged sites indicates focal goblet cells at both levels of the medial esophagus.

Immunohistochemical analysis

At 3 months post-GEJ

The cellular composition of the mucosal inflammation was defined with markers for monocytes and macrophages (ED1), T cells (CD3), cytotoxic T cells (CD8), B cells (OX33), and plasma cells (IgG) (Figure 7). Macrophages and cytotoxic T cells are Th1-derived effector cells as these cells play an important role in the cell-mediated immunity. Immune cells related to the humoral immune response such as B cells and plasma cells are Th2-derived effector cells.

The proximal esophagus at 3 months post-GEJ contained a hyperplastic mucosa with a low number of inflammatory cells, mainly consisting of monocytes, macrophages, and T cells located between the squamous epithelial cells. Almost no B cells and plasma cells were observed (data not shown). In the distal esophagus, the total number of inflammatory cells per microscopic field was 5-fold higher com-

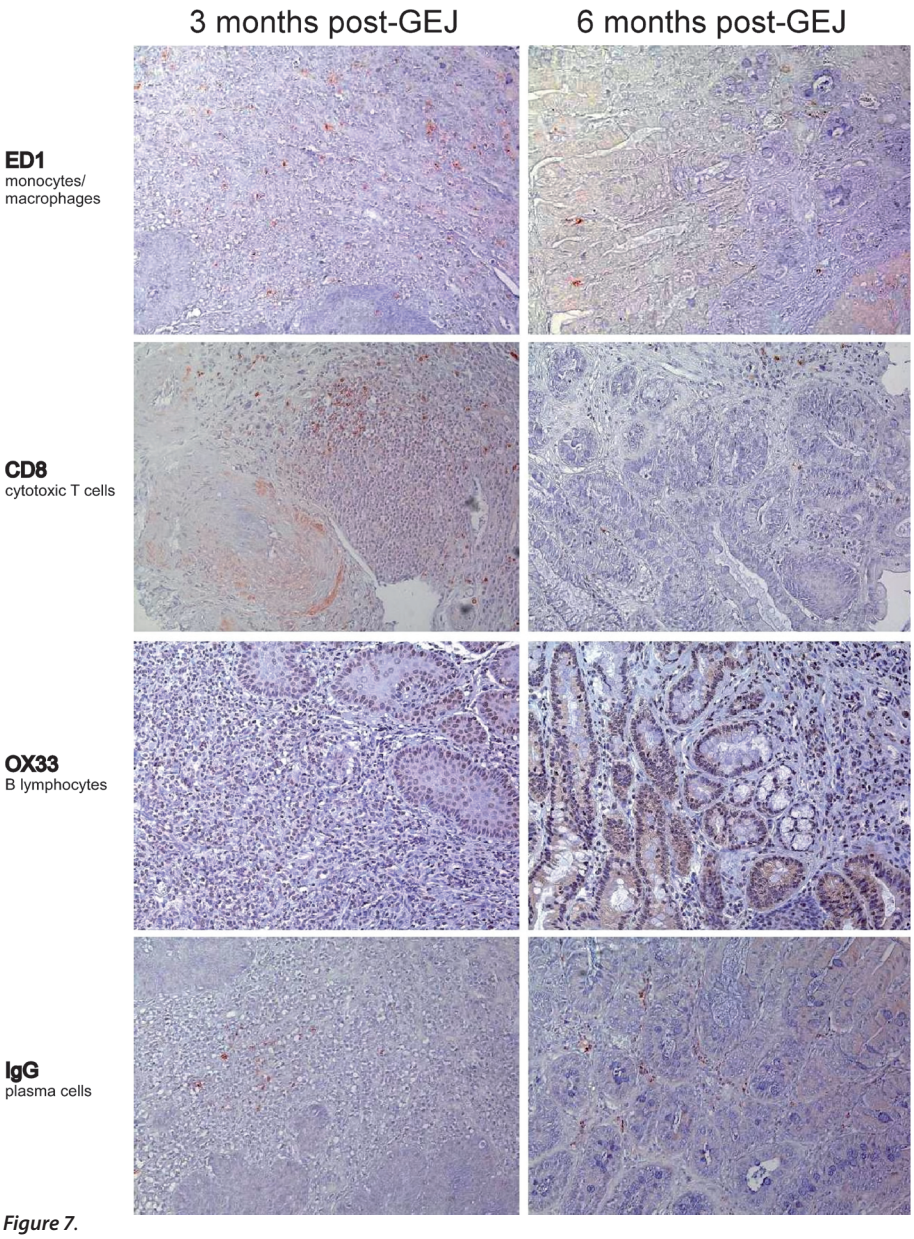

Figure 7.
Typical examples of the immunohistochemical staining of rat esophagus at 3 and 6 months post-GEJ. ED1 staining for monocytes and macrophages (red staining) at 3 and 6 months. CD8+ T cells (red) at 3 months and 6 months. OX33 staining for B cells (red), a large proportion of these cells expressed IgG (red) at 3 months and 6 months.

pared to both the SHAM controls, as well as the proximal esophagus of the same GEJ rats (data not shown). The distal esophagus demonstrated a high number of

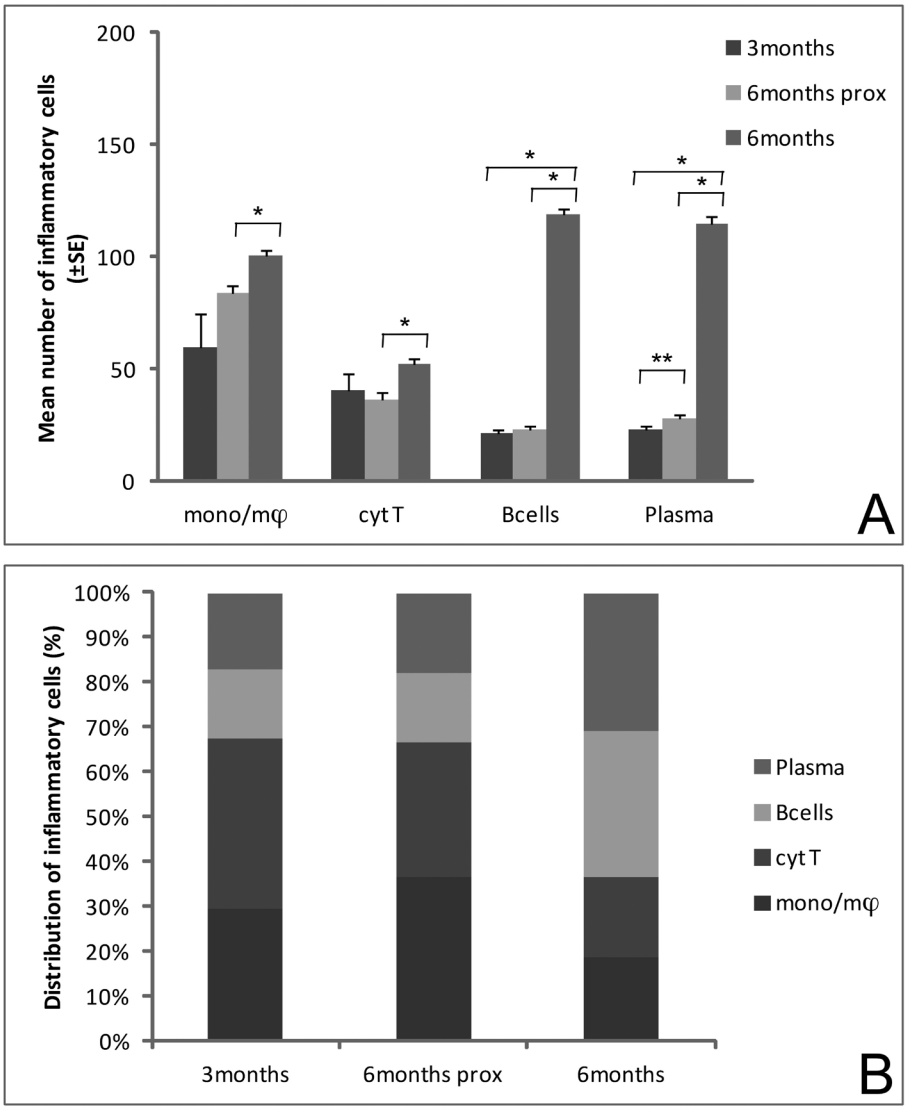

Figure 8.

*Increased numbers of inflammatory cells in SIM. (A) Mean numbers (mean±SE) of inflammatory cells per microscopic field (200x) were obtained by counting the immune cells in 5 randomly selected microscopic fields in the distal esophagus at 3 months, in the proximal non-SIM esophagus at 6 months, and at the distal SIM esophagus at 6 months. Inflammatory cells were more prevalent in the distal SIM than in the proximal non-SIM at 6 months (*p<0.0001), B cells and plasma cells were 4-fold more prevalent in SIM at 6 months than at 3 months, plasma cells were also more prevalent in non-SIM at 6months than at 3 months (**p<0.05). (B) A shift from predominantly Th1 effector cells at 3 months post-GEJ into predominantly Th2 effector cells at 6 months post-GEJ is observed.*

monocytes, macrophages, cytotoxic T cells, and a lower number of B cells and IgG producing plasma cells. The number of inflammatory cells was compared to GEJ rats at 6 months and is described in the next section (Figure 8).

Ki67 expression of the esophageal epithelial cells was shown at the basal layer of the SHAM esophagus. More cells expressed Ki67 in papillary hyperplasia, and the highest proliferation was found in focal SIM, where all epithelial cells expressed Ki67 (data not shown).

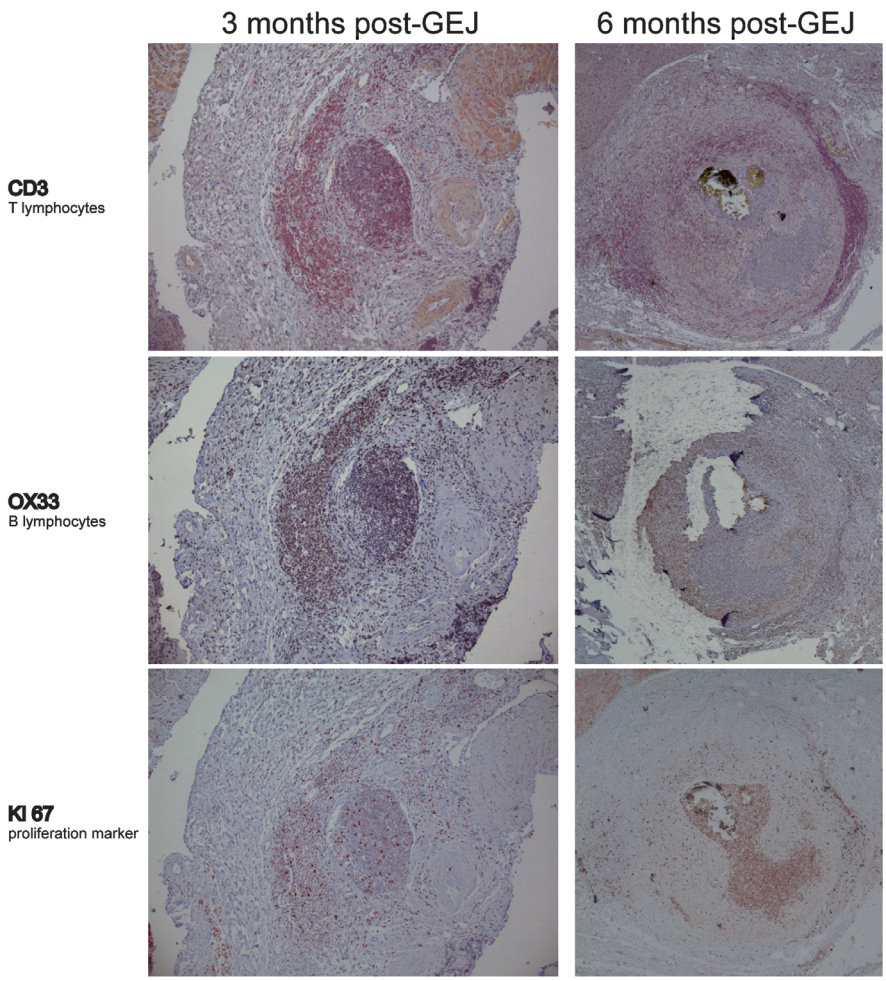

Figure 9.
The lymphoid aggregates at 3 months post-GEJ show randomly spread T cells (CD3), B cells (OX33), and proliferative (Ki67) cells. The large lymphoid follicle at 6 months post-GEJ contains strong central proliferation surrounded by a B cell zone (OX33) and a marginal T cell zone (CD3).

There was formation of lymphoid aggregates, which was not found in the esophagus of SHAM rats, but present at 3 months post-GEJ (Figure 2b). The lymphoid aggregates correlated in size with the significantly increasing mucosal thickness (r =0.67). They showed a distribution with a high number of T cells and a slightly lower number of B cells in the centre and surrounding zone. Ki67 expression was hardly active in the centre and slightly more present in the surrounding zone (Figure 9).

At 6 months post-GEJ

At 6 months, all GEJ rats (21/21) had complete SIM in the distal esophagus with a mean length of 3.96mm (±0.37) and this was confirmed by histology (H&E, PAS, CDX2, WE9). There was an increasing proliferation from the proximal to the distal esophagus, which was even clearer than the progress from proximal to distal at 3 months post-GEJ; Ki67 showed the highest and strongest expression in all epithelial cells of SIM (data not shown).

Papillary hyperplasia of the proximal esophagus was accompanied by macrophages and $CD8^+$ T cells, as was found in the distal segment of the GEJ rats at 3 months. The ulcerative areas in the medial and distal esophagus showed a higher number of these cells and were compared to the distal esophagus of the GEJ rats at 3 months. At 6 months post-GEJ, high numbers of B cells and IgG producing plasma cells were observed (Figure 7 and 8a). The inflammatory response had shifted from a predominance of monocytes, macrophages, and cytotoxic T cells at 3 months, to a predominance of B cells and plasma cells at 6 months post-GEJ (Figure 8b), indicating the activation of a Th2 immune reaction.

Large lymphoid follicles were formed in the esophageal mucosa, which correlated in size with the significantly increasing mucosal thickness at 3 and 6 months (r =0.68) (Figure 3). Of notice, in 4/21 rats, significantly larger lymphoid aggregates were detected underneath the SIM segment compared to the inflamed squamous epithelium (mean area 2.05±0.26 vs 0.04±0.02mm^2), none of them were rats with dysplasia or esophageal cancer. In the lymphoid aggregates in SIM segregated B and T cell areas were found on H&E (data not shown). These expressed macrophages and $CD8^+$ T cells, and strongly expressed CD3 T cells, B cells and IgG (Figure 9). Moreover, in contrast to smaller lymphoid aggregates at 3 months post-GEJ, Ki67 was highly active in the germinal centre, and T and B cells were organized in the surrounding marginal zone of the lymphoid aggregations (Figure 9).

Discussion

Present study in an established rat model showed that the development of SIM runs parallel with the immune shift from an early cell-mediated immune response during GERD into a late humoral reaction during BE. In the samples obtained at 3 months post-GEJ as well as in the proximal (SIM free) regions at 6 months post-GEJ large numbers of $CD8^+$ T cells and macrophages were found. This is indicative for a Th1-derived immune response indeed suggesting that a cell-mediated immune response (Th1) is strongly associated with GERD. A BE-like histology was particularly observed in the distal esophagus at 6 months post-GEJ. The inflammatory cells that characterized the SIM areas consisted predominantly of eosinophils, B cells, and IgG producing plasma cells, known as Th2-derived immune cells, and, occasionally, even large lymphoid aggregates were observed. This indicates that SIM is closely associated with both a temporal and spatial presentation of a chronic humoral immune response (Th2). Strikingly, SIM seems to develop in a patchy pattern in the submucosa progressing to the epithelial surface, rather than a continuous process of BE epithelium growing from the distal into the proximal esophagus (Table 1).

This study compliments the animal studies that found a greater destruction of the rat esophageal mucosa after exposure to duodenal juice than gastric juice [28, 31-33]. Our surgical model is characterized by chronic reflux causing the development of chronic mucosal inflammation in GERD, followed by SIM, and eventually EAC in some rats (1/21 rats in this study). The histological and immunohistochemical stages of our model were very similar to its equivalent in humans although a rat model can only mimic human disease. The gene expression profile [34, 35] as well as the Notch pathway expression [30] of esophageal SIM following surgically induced reflux in present rat model indicates that this indeed represents intestinal metaplasia of BE, and not proximal migration of jejunum.

In human studies, acid suppression therapy with PPIs is known to be effective in reducing inflammatory changes in the esophagus and therefore BE and EAC [36]. However, PPIs do not completely seem to protect from BE and EAC [37-40]. Acid suppression therapy mainly affects the acid component of the reflux and allows other components to persist. Esophageal exposure to bile acids is seen as a key factor in the etiology of GERD, BE and the development of EAC [41-44]. PPI therapy can apparently not fully protect the esophagus from the development of metaplasia or

adenocarcinoma by oxidative damage, irreversible dilated intercellular spaces of the esophageal epithelium or bile injury.

Physiological glands of the human esophagus are thought to play a role in the immune response during esophagitis and BE [23]. Rat esophagus and the proximal part of the stomach are non-glandular, thus the anatomy differs from that in human. However, the lymphoid aggregates, which develop during the inflammatory processes, resemble secondary lymphoid tissue. This supports the hypothesis that human isolated lymph follicles in BE are newly formed structures that react against severe inflammation. The mechanism of isolated lymph follicles or lymphoid tissue remains unknown.

A depressed cellular immunity is associated with decreased immune surveillance leading to cancer [25, 26, 45, 46]. As a depressed cellular immune response was observed in peripheral mononuclear cells in patients with BE [24], it may encourage the malignant transformation of BE. It has been argued that chronic inflammatory stimuli initially generate a Th1 pro-inflammatory cytokine response [47]. However, Th2 cytokine production is upregulated via COX2 generation and there is negative feedback leading to inhibition of the Th1 response. Both the Th1 and Th2 immune response are active in an environment with inflammatory stimuli. This balanced state between the cellular and humoral immune response exists in esophagitis, BE, and EAC. Pro-inflammatory cytokine induce neutrophil and T cell accumulation in the pathogenesis of acute reflux esophagitis [21, 48]. Where the cellular response is the major pathway for esophagitis development, both cellular and humoral immune responses are implicated in BE, with a predominant humoral immune profile characterizing BE [23, 49, 50]. This has lead to present hypothesis that the balance between the cellular and humoral immune response first leads to an activated cellular immune response in reflux esophagitis followed by the depressed cellular immune response and consequently a predominant humoral immune profile in BE.

In conclusion, present study shows that the shift from a cell-mediated immune response into a humoral immune response runs parallel to the development of intestinal metaplasia. This process can be distinguished in an initial and in a late phase, which coincides with the clinical and pathologic disorders GERD and BE, respectively. The up-regulation from a Th1- into a Th2-mediated immune response seems to trigger BE development. However, BE is a multi-factorial disease and the functional role of the immune response as well as the genetic immune status need further elucidation.

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CHAPTER 3

CELLULAR IMMUNE RESPONSE DETERMINES ONSET AND EXTENT OF REFLUX ESOPHAGITIS AND BARRETT'S ESOPHAGUS IN RAT

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Abstract

Background: Chronic gastro-duodenal reflux causes Barrett's esophagus (BE) that may progress to esophageal adenocarcinoma, which is currently the fastest growing malignancy in the Western population. The transition from reflux esophagitis into BE has been associated with a shift from the cellular (Th1) towards the humoral (Th2) immune response. Here, the function of the immune response for the initiation and persistence of BE was analyzed.

Materials and methods: Esophageal metaplasia and inflammation were determined by conventional histology and immunohistochemistry in a validated surgical rodent model in Th1 prone Lewis, Th2 prone Brown Norway and intermediate Th1/Th2 Wistar rats.

Results: While all three rat strains developed esophageal hyperplasia and severe esophagitis with predominantly Th1 immune cells, the time line and severity of the response showed marked differences. The mean Barrett's lesion was longer in Lewis than in Wistar and BN rats. Hyperplasia was seen in Lewis rats at 2 and 5 weeks after surgery with the highest number of monocytes and macrophages. At long term, all strains had developed BE, but most cytotoxic T cells and mast cells were seen early in Lewis rats at 9 and 12 weeks after surgery, and later in BN rats at 24 weeks after surgery.

Conclusion: These results imply that the development of BE metaplasia occurs under a Th1 predominant immune response, which then shifts towards Th2 predominance. This suggests that patients with a Th1 immune status may be more susceptible to the development of BE than Th2 prone patients.

Introduction

Ongoing gastro-esophageal reflux of acid and bile induces chronic inflammation of the esophagus [1-3]. It is as such an important risk factor for the development of both Barrett's esophagus (BE) and esophageal adenocarcinoma [4-6]. The incidence of esophageal adenocarcinoma has been rapidly increasing in the western population over the last three decades [7, 8]. Although reflux is a crucial factor in the pathogenesis of esophagitis and BE, pH, volume and composition of the refluxate by themselves do not explain the marked inter-individual differences in progress from esophagitis to BE and eventually esophageal adenocarcinoma [9-12].

Previously, we found that the transition from esophagitis to BE is associated with a shift from a cellular (Th1) towards a humoral (Th2) immune response [13, 14]. These findings suggest that a Th1 response is involved in BE development, whereas a Th2 response is more important to maintain the chronic status of BE.

Our hypothesis is that the host immune system plays a crucial role in the development of esophagitis and BE. If so, the immunological background of the host could influence the onset and outcome of esophagitis. If esophagitis is indeed primarily a Th1-based disease, we expect that a genetic background for a preferential Th1 response predisposes to more severe inflammation, hyperplasia and ulceration. On the other hand, Th2 prone individuals might be more sensitive to develop BE. It is well known that various rat strains have a genetic predisposition for either Th1 or Th2 immune response [15-19]. This is illustrated by the level of expression of specific markers, such as IL2 and IFNγ for cellular immunity, and IgE, IL4, IL5, IL9, IL10 and IL13 for humoral immunity. Figure 1 shows the ranking of the rat strains for a preferential Th1 and/ or Th2 immune response [15]. Based on this, we selected Lewis rats as Th1 representatives, Brown Norway rats as Th2 representatives, and Wistar rats as the intermediate T1/T2 strain and induced BE in these animals using a well established protocol [20-22]. To investigate our hypothesis, we then studied histology, immunohistochemistry, and their dynamics during the development of BE in these rats.

Figure 1.
Ranking of rat strains for a preferential Th1 and/ or Th2 immune response. Based on data from 15-19

Materials and methods

Animals

Six-week-old male Wistar, BN, and Lewis rats were obtained from Harlan, England and housed under standard pathogen-free conditions with a maximum of 3 animals per cage.

Experienced technicians performed all animal handling. The animals were fed a commercially available natural diet for rats (SDS, England) and had free access to tap water. After an acclimatization period of 1 week, the animals were randomly divided into three groups in a time course design of t = 0, 2, 5, 9, 12, and 24 weeks. These time intervals were based on previous studies, in which reflux esophagitis without BE was found at 12 weeks after surgical interference, and BE-like histology defined as specialized intestinal metaplasia (SIM) had developed at 24 weeks after surgery.

General health status and weight was monitored at least twice per week; weight loss of more than 20% of the preoperative body weight, severe regurgitation, aspiration not recovering within 24h, or apathetic behavior lead to exclusion of the animal from the study. The local experimental animal committee (Erasmus MC – University Medical Centre Rotterdam, The Netherlands) approved the experimental study protocol.

Surgical procedure

Based on a preliminary study (not shown), we designed a setup that was powered for a 30% mortality rate of this model to yield a total of 5 animals in each arm after a 6-months follow-up. Essentially the surgical procedure of Levrat [21] was followed with the modification of a gastrectomy instead of only ligation of the stomach. Briefly, the procedure consisted of gastrectomy with esophagojejunostomy. As controls, SHAM-operated rats were used to exclude a putative effect of the surgery itself. SHAM rats only had abdominal laparotomy, with palpitation of the esophagus and jejunum. No incisions were made in seven control rats. The rats were randomly sacrificed at 0, 2, 5, 9, 12, and 24 weeks post-GEJ. After sacrificing the rats, we took out the esophagus from below the larynx until the jejunum or stomach in non-operated rats, and a small piece of the jejunal anastomosis or gastro-esophageal junction was included to the esophageal sample. Swiss rolls were prepared with the luminal side at the inside and the distal end of the esophagus in the centre. Each Swiss roll was longitudinally divided in two, and either fixed in 10% neutral buffered formalin

for 24 hours, and embedded in paraffin, or frozen in Tissue tek (Sakura Finetek Europe, UK) and stored in the -80°C. The paraffin embedded samples were stored at room temperature until used for histology and immunohistochemistry.

Pathological analysis

Paraffin samples were cut at 4μm, deparaffinized in xylene, hydrated using a graded series of alcohol, and stained with routine hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Six levels of the Swiss rolls were stained for histopathological analysis. A specialized gastrointestinal pathologist assessed all slides for the presence of hyperplasia, ulceration, and columnar metaplasia. Ulceration as a consequence of reflux esophagitis was defined as large amounts of inflammatory cells that replace the epithelial layer. Specialized intestinal metaplasia (SIM) was defined as intestinal columnar epithelium with goblet cells above the anastomositic site marked by the black monofilament suture. Parts of squamous epithelium within SIM and a continuous basal membrane of SIM with the esophagus also differentiated the jejunum from SIM at the site of the anastomosis.

Immunohistochemical analysis

For immunohistochemistry, sections of 4μm were blocked for endogenous peroxidase activity with 1% H_2O_2 in 100% methanol for 30 minutes. Antigen retrieval was performed with 10mM 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 intestinal epithelium (anti-CDX2 clone AMT 28; 1:100 dilution; Novocastra Laboratories Ltd, Newcastle, UK) and proliferative cells (anti-Ki67; 1:500; BD Pharmingen, San Diego, USA). Binding of the primary antibody was visualized by the addition of Envision. Normal rat esophagus, rat colon, rat spleen and rat mesenteric lymph nodes were used as a control.

Frozen tissue sections were dried overnight at room temperature with silicagel. Sections were fixed with acetone and 0.05% H_2O_2 to quench endogenous peroxidase activity. Subsequently the sections were blocked with 10% normal rat serum for 20 minutes, followed by a 1 hour at room temperature incubation with primary antibodies against CD8, CD93, and CD68 (Serotec Ltd, Oxford, UK). Antibodies were used in a 1:100 dilution for CD8 and CD93 and a 1:2000 dilution for CD68. Subsequently the sections were washed with PBS, followed by incubation with a strepta-

vidin-labelled horseradish peroxidase (1:200; Dako, Glostrup, Denmark) for 45 min. After washing with PBS sections were visualized with DAB (3,3'-Diaminobenzidine) and counterstained with hematoxylin, sections were evaluated under a light microscope (Zeiss, Axioskop, Sliedrecht, The Netherlands). We used rat spleen tissue as a positive control, as negative controls an isotype control was used and the primary antibody was omitted.

Two independent observers (VM, JGK) evaluated the sections for the immunohistochemical staining, the mean error between observers was always <5%. To determine the grade of inflammation, the mean number of immune cells were determined from at least 3 fields for CD8 and CD93, or, in case of CD68, marked as negative, moderate (10-100 positive cells/field), and high (>100 positive cells/field). The slides were assessed using a Zeiss microscope (Axioskop 20, Carl Zeiss, Sliedrecht, The Netherlands) with a standard magnification (200x).

The incidence of esophageal lesions and the mean length of the SIM segment were determined on images, recorded with a Nikon camera (DS-5M-U1) and analyzed with Nikon Eclipse Net 2000 software (Nikon, Badhoevedorp, The Netherlands) that were calibrated with our Zeiss Axioskop 20 microscope.

Statistical analysis

The study was powered for the previously observed 30% mortality rate of this model to yield a total of 20 animals in each arm after 6-months follow-up. Mean differences between the Barrett's length of the rat groups and the cell expression were determined by Student's T-test. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted with the SPSS software package v11.1 (SPSS, Chicago, IL).

Results

Incidence of Esophagitis and BE in the GEJ Rat Model

In total, 110 rats were involved in this study, of which 7 were untreated control rats, 15 SHAM operated rats, 49 GEJ operated rats, 39 rats died after GEJ (16 Wistar, 11 Lewis and 11 Wistar), and 1 Lewis rat died pre-operative.

The esophageal histology findings in Lewis, Wistar and BN rats at 2, 5, 9, 12, and 24 weeks post-surgery are shown in Table 1. No macroscopic and microscopic lesions were observed in control and SHAM rats, whereas esophageal lesions were observed in all rats that underwent GEJ. Histologically, a normal rat esophagus has a thin epithelial layer with squamous cells, and few inflammatory cells in the submucosal layer (Figure 2.a). Marked squamous epithelial cell hyperplasia was observed in all GEJ rats. At 2 weeks after GEJ, hyperplasia was observed in the proximal and middle part of the esophagus of all rat strains. At 5, 9, 12, and 24 weeks after GEJ, hyperplasia was

Table 1a.
Incidence of esophageal lesions and histological changes after GEJ in Lewis rats

Period after operation (Lewis rats)	2 weeks (N=3)	5 weeks (N=6)	9 weeks (N=3)	12 weeks (N=3)	24 weeks (N=3)
Incidence of esophageal lesions (%)					
Squamous cell hyperplasia	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
Esophagitis	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
Barrett's esophagus	0 (0)	4 (66)	2 (66)	3 (100)	3 (100)

Table 1b.
Incidence of esophageal lesions and histological changes after GEJ in Wistar rats

Period after operation (Wistar rats)	2 weeks (N=4)	5 weeks (N=4)	9 weeks (N=3)	12 weeks (N=3)	24 weeks (N=4)
Incidence of esophageal lesions (%)					
Squamous cell hyperplasia	4 (100)	4 (100)	3 (100)	3 (100)	4 (100)
Esophagitis	4 (100)	0 (0)	1 (33)	3 (100)	4 (100)
Barrett's esophagus	0 (0)	0 (0)	1 (33)	1 (33)	4 (100)

Table 1c.
Incidence of esophageal lesions and histological changes after GEJ in BN rats

Period after operation (BN rats)	2 weeks (N=3)	5 weeks (N=4)	9 weeks (N=2)	12 weeks (N=2)	24 weeks (N=2)
Incidence of esophageal lesions (%)					
Squamous cell hyperplasia	3 (100)	4 (100)	2 (100)	2 (100)	2 (100)
Esophagitis	3 (100)	4 (100)	2 (100)	2 (100)	2 (100)
Barrett's esophagus	0 (0)	3 (75)	1 (50)	1 (50)	2 (100)

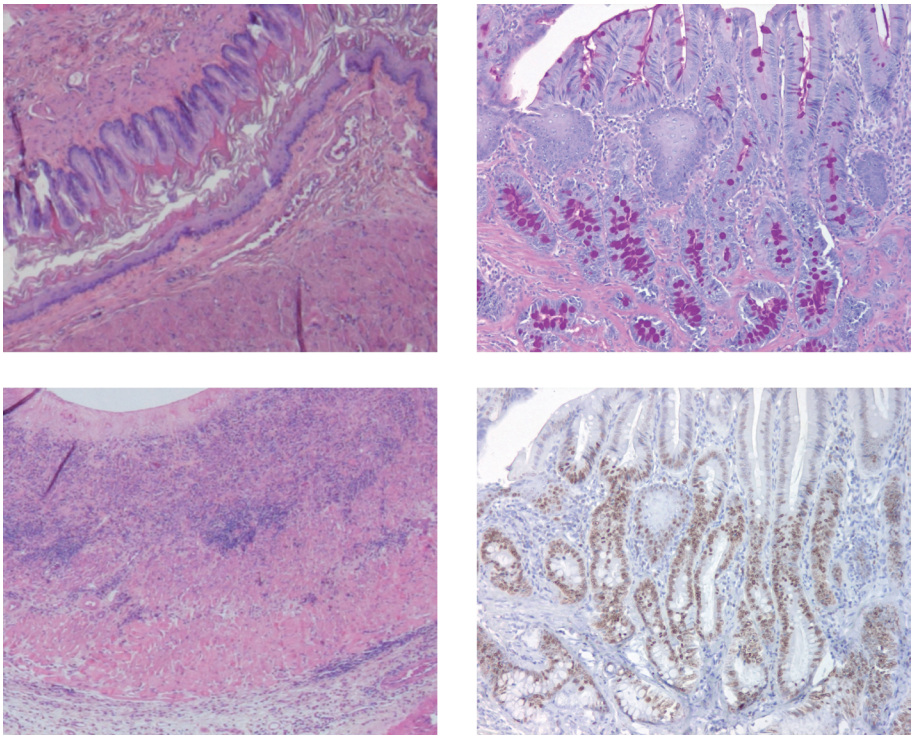


Figure 2.
Hematoxylin and eosin staining (H&E) of rat esophagus at 0 (SHAM), 9, and 24 weeks after GEJ. Figure 2a (Magnification 20x) shows twice the normal rat esophagus with squamous epithelium and few inflammatory cells in the submucosal layer. Figure 2b (Magnification 20x) shows the effect of reflux esophagitis at 9 weeks after GEJ with elongation of the lamina propria papillae, basal cell hyperplasia, and inflammatory cell infiltration with ulceration. Columnar epithelium with goblet cells was found in some areas of the esophageal lesions. Figures 2c-d are magnified at the distal esophagus (Magnification 10x). PAS was detected in the goblet cells but not in the squamous (hyperplastic) epithelium (Figure 2.c). Ki67 showed the proliferation of the esophageal lesions, whereas Ki67 was expressed in the nuclei of columnar epithelium in BE, whereas the expression in the crypts is the strongest (Figure 2.d).

always observed in the proximal esophagus, except for Wistar rats at 5 weeks after GEJ. Esophagitis was found in all rats after GEJ and characterized by elongation of the lamina propria papillae, basal cell hyperplasia, and inflammatory cell infiltration with ulceration (Figure 2.b). At 2 weeks after GEJ, esophagitis was observed in the distal part of the esophagus of all rat strains. At 5, 9, 12, and 24 weeks after GEJ, esophagitis was always observed in the middle and distal esophagus, except for Wistar rats at 5 weeks after GEJ. Columnar epithelium with goblet cells was found in some areas of the esophageal lesions. PAS positive material was only detected in the goblet cells but not in the squamous (hyperplastic) epithelium (Figure 2.c). Ki67 specific stain-

ing showed proliferation of the esophageal lesions, whereas Ki67 was expressed at the basal membrane of squamous epithelium (data not shown), and in the nuclei of columnar epithelium in BE (Figure 2.d). BE was observed onwards from 5 weeks after GEJ in Lewis and BN rats and its incidence and length increased over time (Table 2). The mean length of the Barrett's lesion was significantly different for Lewis versus BN rats at 9 weeks (p= 0.003), but not significantly different at 5 weeks (p= 0.875), at 12 weeks (p= 0.080), and at 24 weeks (p= 0.061).

There was a significant difference in the mean length of the Barrett's lesion for Lewis versus Wistar rats at 5 weeks (p= 0.025), at 9 weeks (p= 0.002), at 12 weeks (p= 0.002), and at 24 weeks (p= 0.007).

Expression and localization of inflammatory cells

To examine the associations between the immune response and development of BE, the dynamics of esophageal lesions and inflammatory cell expression were measured at 2, 5, 9, 12, and 24 weeks after GEJ in hyperplasia, ulceration and BE (Figure 3a-f). Cytotoxic T cells and mast cells were counted in all rats in all histological lesions and at all time-intervals. For macrophages a repetitive inter- and intra-observer variety occurred that made it not possible to perform cell-counting. Macrophages were marked as negative, moderate (10-100 positive cells/field), and high (>100 positive cells/field). High numbers of macrophages were seen in hyperplasia and BE of Wistar rats at 5 weeks, and in ulcerations at 9, 12, and 24 weeks after GEJ (figure 4.a). High numbers of macrophages were observed in hyperplasia of Lewis rats at 2, 5, 12, and 24 weeks, in ulcerations at 9, 12, and 24 weeks, while in BE only low to moderate numbers of macrophages were found (Figure 4.a). High numbers of macrophages were observed in hyperplasia of BN rats at 9 and 12 weeks, in ulcerations at 2, 5, 9, 12, and 24 weeks, while in BE only few macrophages were seen (Figure 4.a).

High numbers of cytotoxic T cells were observed in hyperplasia of BN rats at 9 weeks, in contrast to Lewis rats at 2, 5, 9, and 24 (p=0.06) weeks, however due to the low number of animals per group, statistical differences could not be shown (Figure 4.b). High numbers of cytotoxic T cells were observed in ulcerations of BN rats at 9 weeks, but moderate numbers at 2, 12, and 24 weeks. There was no statistical difference among BN rats at 24 weeks between hyperplasia and ulceration (p=0.06). Only at 12 weeks higher numbers of cytytoxic T cells were observed in Lewis rats (Figure 4.b). High numbers of cytotoxic T cells were also observed in BE of BN rats at 24 weeks, while lower numbers were observed at 9 and 12 weeks com-

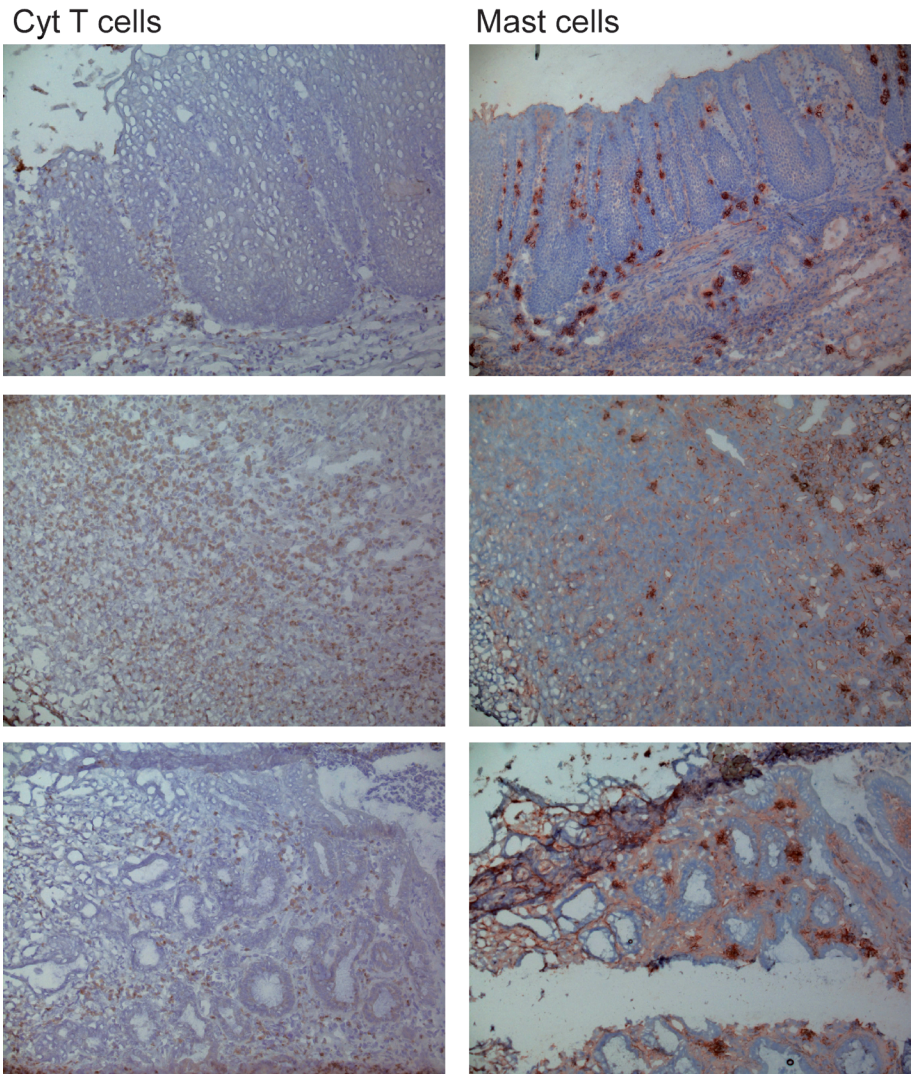
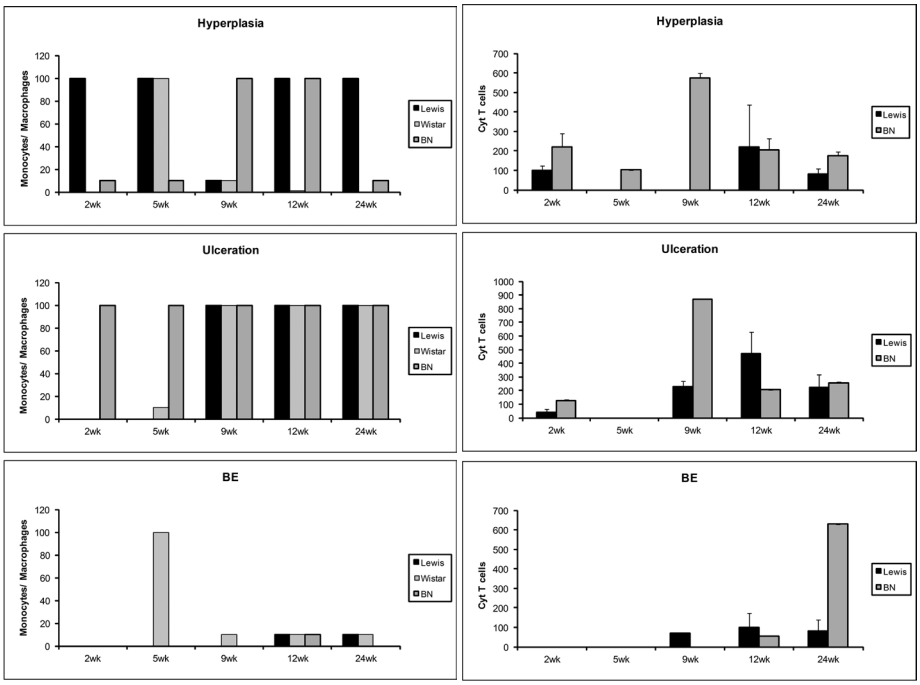


Figure 3.
Inflammatory cell expression was measured at 2, 5, 9, 12, and 24 weeks after GEJ in hyperplasia, ulceration and BE. Figure 3.a shows a brown-red staining of cytotoxic T cells in the submucosa of hyperplastic esophageal tissue. Figure 3.b shows a brown-red staining of cytotoxic T cells in the whole ulcerative tissue. Figure 3.c shows a brown-red staining of cytotoxic T cells in the submucosa of BE. Figure 3.d shows a brown-red staining of mast cells in the submucosa of hyperplastic esophageal tissue. Figure 3.e shows a brown-red staining of cytotoxic T cells in the whole ulcerative tissue. Figure 3.f shows a brown-red staining of cytotoxic T cells in the submucosa of BE.

pared to Lewis rats (Figure 4.b). There was no statistical difference among Lewis rats at 12 weeks between ulcerations and BE (p=0.07).

The number of mast cells were equally presented in both hyperplasia and ul-



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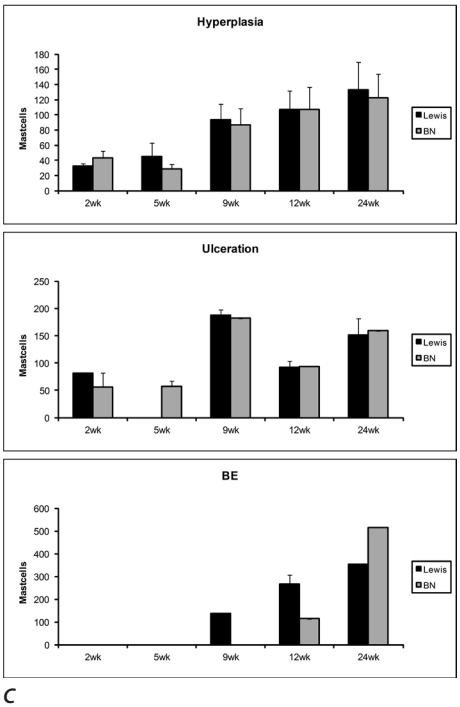

C

Figure 4

Macrophages were seen more often in hyperplasia and ulceration compared to BE (figure 4.a). Lewis rats showed the strongest expression in hyperplasia at 2, 5, 12, and 24 weeks, in ulceration at 9, 12, and 24 weeks, and few macrophages were seen in BE at 12 and 24 weeks. Wistar rats showed the strongest expression in hyperplasia at 5 weeks, in ulceration at 9, 12, and 24 weeks, and in BE at 5 weeks. BN rats showed the strongest expression in hyperplasia at 9 and 12 weeks, in ulceration at 9, 12, and 24 weeks, and few macrophages were seen in BE at 12 and 24 weeks. (Figure 4.a).

Cytotoxic T cells were observed strongly in hyperplasia and ulceration of BN rats at 9 weeks, and in BE of BN rats at 24 weeks. Lewis rats express more cytotoxic T cells in BE at 9 and 12 weeks (Figure 4.b).

*Mast cells were almost equally observed in hyperplasia and ulceration of BN and Lewis rats at the timeline. Lewis rats express more mast cells at 9 weeks than at 12 weeks in ulceration (*p<0.001), and in BE at 9 and 12 weeks, whereas BN rats express more mast cells in BE at 24 weeks (Figure 4.c).*

ceration of BN and Lewis rats at the timeline, with higher numbers being seen at 9, 12, and 24 weeks (Figure 4.c). There was a statistically significant difference among Lewis rats between 9 and 12 weeks in ulcerations (*$p<0.001$). There was no statistical difference among BN rats between 5 and 24 weeks in hyperplasia ($p=0.06$). The highest number of mast cells was observed in BE of BN rats at 24 weeks, but lower numbers were observed at 9 and 12 weeks compared to Lewis rats (Figure 4.c).

Discussion

We found that surgically induced chronic reflux in genetically different rats induced esophagitis leading to BE in all species. This process was associated with an active immune response. We found that all Th1 prone Lewis rats developed BE at 12 weeks after GEJ compared to 50% of Th2 prone BN rats and 33% of intermediate Wistar rats. Lewis rats also tended to develop a larger Barrett segment, implying that a Th1 predisposition is likely to be associated with the development of BE.

Depending on the predisposition of the Th1/Th2 immune response, an antigenic stimulus is likely to be presented to different effector cells, i.e., monocytes/ macrophages (CD68) and cytotoxic T cells (CD8) in animals with a preferential Th1 response and mast cells (CD93) in animals with a Th2 response. The Th1 effector cells were seen at the onset of the hyperplasia-esophagitis-BE cascade and Th1 prone Lewis rats expressed macrophages in an early stage compared to BN and Wistar rats. The high number of cytotoxic T cells in BE of BN rats indicate that Th2-prone animals are indeed able to generate a strong Th1 response. The number of mast cells increased in the hyperplasia-esophagitis-BE cascade and over time this was found in both Lewis and BN rats. Lewis rats had significantly more mast cells in esophageal ulcerations at 9 weeks compared to 12 weeks ($p<0.001$) and activated the mast cells in BE earlier than BN rats. Therefore, Lewis rats expressed an early Th1 immune response in hyperplasia and a strong and early Th2 immune response in BE.

This study was performed using an established surgical rat model for reflux esophagitis [20, 21]. In a pilot study, we used Wistar rats in order to test the effect of the surgical technique. This confirmed earlier findings that rats with esophagojejunostomy and ligated stomach without gastrectomy showed the same histology at 0, 3, and 6 months after surgery as rats undergoing esophagojejunostomy with gastrectomy [21]. The pH of the reflux was measured with pH paper strips (AlkaLife, Eindhoven, The Netherlands) at the surgical anastomosis and more proximal in the esophagus and confirmed that the pH showed a value of approximately 6.0 at all times. We did not find a variety in acidity in the refluxate comparing the two different surgery techniques (data not shown). However, more animals died after ligation of the stomach because of either necrosis or perforation of the stomach.

In present study, we ended up with a relatively low cohort of animals as a result of the low survival rate. The number of animals in all groups was however sufficient

to perform our statistical analysis and showed significant differences between the rats strains and in some cases between the various immune cells.

It is commonly accepted that the ability of a rodent to make Th1 or Th2 responses is influenced by animal housing conditions [23]. Indeed, various studies have demonstrated that prophylactic or therapeutic feeding of self-peptides to rodents could prevent experimental autoimmune diseases of various kinds [24, 25]. The regulatory T cells, induced by antigen feeding, that prevented these autoimmune diseases were generated in Peyer's Patches and had the characteristics of Th2 cells or Th3 cells [24, 25]. Functionally, these cells down-regulated the activity of tissue-damaging Th1 cells by secreting cytokines such as TGF-β [25]. While others have suggested that environmental factors such as hydrophobic bile acids and zinc deficiency play a crucial role in BE development [26], all animals in our study were kept under the same housing conditions and feeding, which allowed us to study only the differences resulting from the Th1 and Th2 prone immune response in the rat strains.

We are aware that a rat model can only mimic human disease. Rat esophagus and the proximal part of the stomach are non-glandular, thus the anatomy differs from that in humans. However, the lymphoid aggregates, which develop during the inflammatory processes, closely resemble those of human secondary lymphoid tissue [14]. This supports the hypothesis that human isolated lymph follicles play a role in the development of BE and might be a possible diagnostic criterion [27].

In a previous rat model study, a larger BE segment in BN rats compared to Sprague-Dawley rats was found. In contrast to pro-inflammatory cytokines, the Th2 cytokines such as IL-10 are increased in BE as compared to the levels in normal esophagus and esophagitis [28]. With regard to ranking of Th1/Th2 predisposition in rat strains (Figure 1 [15-19]), it is suggested that Sprague-Dawley rats are intermediate in the preference for a Th1 or Th2 immune response, thus comparable with Wistar rats. The present study also indicates that regarding BE the incidence was indeed more common and extent larger in Th2 prone BN rats than in intermediate Wistar rats. This is accompanied by infiltration of the BE mucosa with relatively high numbers of Th2 effector cells such as plasma cells and mast cells, although the absolute numbers of Th1 effector cells such as macrophages and cytotoxic T cells may also be high in BE. This is in line with previous human studies [13, 14]. Therefore, we expected the Th2 immune response to play a key role in the development of BE. However, in present study the Th2 -prone BN rats expressed more Th1 effector cells in BE, while the Th1-prone Lewis rats expressed more Th2 effector cells early

in BE. This suggests that the Th2 immune response plays a major role in BE, but still Th1-prone rats were found to be more susceptible to develop BE.

Polymorphism studies have suggested that hereditary or acquired individual differences, i.e. in immunological parameters, are associated with the development of BE [29]. Interestingly, when comparing BE between the three rat strains, the incidence was more common and extent of BE was larger in Lewis rats than in Wistar and BN rats. Since Lewis rats are known to be genetically Th1 predisposed, it appears that genetic differences in the immune response is involved in the development of BE [30]. The pathogenesis of the effect of the Th1 immune response on the development of BE remains unknown and needs further study.

In conclusion, this study shows that the genetic Th1 immune status of Lewis rats resulted in an earlier and more extended development of BE. This suggests that while BE patients have a Th2-type chronic inflammation in the esophagus, patients with a Th1 immune status might be more susceptible to the development of BE than Th2 prone patients. Identification and eventually inhibition of the immunological and genetic mechanisms might generate novel targets for BE and esophageal cancer prevention.

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CHAPTER 4

NCOI TNF-β GENE POLYMORPHISM AND TNF EXPRESSION ARE ASSOCIATED WITH AN INCREASED RISK OF DEVELOPING BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

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Abstract

Objective: Esophageal cancer development is a sequence that starts with reflux esophagitis (RE), followed by Barrett's esophagitis (BE), dysplasia, and finally esophageal adenocarcinoma (EAC). Tumor necrosis factor (TNF) is a potent antineoplastic agent, hence DNA-polymorphisms that reduce TNF levels potentially enhance development of BE and EAC. The aim of this study was to determine the impact of TNF gene variation on the RE-BE-EAC cascade.

Methods: DNA from 887 Caucasian participants (197 controls, 305 RE, 257 BE, 128 EAC) was tested for the gene polymorphism TNF-β *Nco*I, and TNF production was determined by TNF-α specific immunohistochemistry on esophageal biopsies from these BE (n=31) and EAC (n=4) patients.

Results: As compared to healthy controls the TNF-β *Nco*I A/A genotype was significantly more prevalent in BE (p=0.04) and EAC patients (p=0.02), but not in RE patients (p=0.1). While TNF-α protein levels were invariably high in esophageal biopsies from EAC patients, most esophageal BE samples showed low to moderate TNF levels.

Conclusions: Chronic inflammation, like in BE, markedly increase the risk of malignant transformation. In this study, the significantly higher frequency of the TNF-β *Nco*I A/A genotype and the local TNF expression indicate that the pro-inflammatory cytokine TNF plays a role in the development of BE and EAC.

Introduction

Esophageal adenocarcinoma (EAC) is the fastest rising malignancy of the Western world [1]. EAC frequently arises from Barrett's esophagus (BE), a chronic inflammatory condition characterized by a change in the normal esophageal epithelium into reflux esophagitis (RE) and intestinal metaplasia as a result of gastroesophageal and bile reflux [2,3], and genomic instability [4].

Both epidemiologic and functional studies have implicated chronic inflammation in the development of liver, esophagus, stomach, and colon cancer [5]. During this process, deregulated cytokine function between the immune and neoplastic cells can alter cell growth, differentiation, and apoptosis [6].

Tumor necrosis factor (TNF) is a key cytokine in both systemic inflammatory responses and anti-tumor activity [6,7]. Esophageal TNF expression is responsive to local concentrations of reflux components [8] and inflammatory cytokines [9], but is also controlled on a genetic level. A polymorphism in the promoter region of the TNF-β gene (Figure 1) [10] affects both the production of TNF-α and TNF-β [11,12], and deregulation has been associated with an increased risk of intestinal cancer development [13,14].

Present study assessed the association of the TNF-β (*NcoI*) polymorphism, and the local effect on TNF production in the RE-BE-EAC cascade.

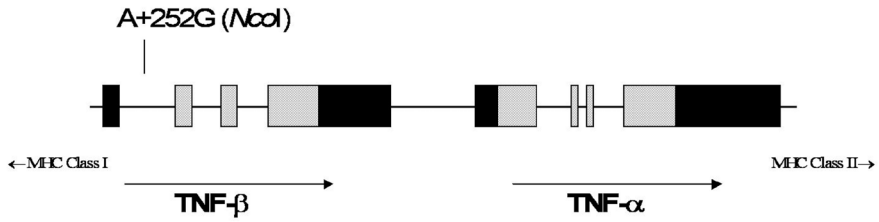

Figure 1

Genomic organization of the TNF genes in the MHC class III region at chromosome 6 (6p21). The location of MHC class I and II genes is indicated. Closed boxes refer to untranslated parts; open boxes to exons. The position of the bi-allelic NcoI restriction fragment length polymorphism in the first intron of the TNF-β gene is shown by the notation above the figure.

Material and methods

Study design

Between November 2002 and March 2006, all sequential patients with RE, BE, and EAC who visited the endoscopy unit of the Erasmus MC – University Medical Center Rotterdam or the IJsselland Hospital, Capelle aan den IJssel were invited to participate in this study. Participants underwent upper endoscopy for evaluation of reflux-related symptoms, surveillance of BE, or odynophagia or dysphagia. The presence of RE was confirmed at endoscopy and the length of any columnar-lined segment was determined by measuring the distance between the squamo-columnar junction and the proximal margin of the longitudinal gastric folds. Participants were only included if they had i) RE without the presence of BE, ii) BE defined as a columnar lined segment in the esophagus of ≥ 2 cm in length with specialized intestinal metaplasia at histology found in at least one of the biopsies taken, or iii) EAC defined at histology as an adenocarcinoma in BE or adenocarcinoma of the gastro-esophageal junction with more than 50% of the tumor mass in the esophagus. Biopsies were taken from patients with macroscopic signs of columnar metaplasia. Only samples from patients with histological BE were included in the BE group, but due to our criteria patients who only had histological evidence for BE at the z-line or at <2cm from the z-line were excluded from our study. In the patient population that we studied there were two patients with BE at 1cm and two with BE at 0cm. These four patients were excluded from our study.

Between October 2004 and April 2005, healthy volunteers (referred to as normal control subjects) were recruited from the general population via general practitioner practices. They were assessed by means of a questionnaire and were eligible as control if they had a negative history for reflux symptoms and of esophageal diseases. Patients who ever had reflux complaints, retrosternal pain, or regurgitation were excluded from the control group, as well those who used antacids, H2-receptor antagonists, or proton pump inhibitors.

This study was approved by the local institutional review boards of the participating centers. All participants were genetically unrelated Dutch Caucasians, and aged over 18. Prior to inclusion, all participants signed a written informed consent.

Genotyping of the TNF (NcoI) polymorphism

Genomic DNA was extracted from 5 ml of whole blood by standard procedures

(Wizard Genomic DNA Purification-kit; Promega, Madison, WI). Single nucleotide polymorphism (SNP) analysis of TNF-β (+252A/G) genotypes was performed on 20 ng DNA (Kbiosciences, Herts, UK) with a competitive allele-specific PCR system [15] by a technician unaware of the clinical findings for these patients.

Immunohistochemical analysis of TNF

From the 257 BE patients we selected those from which sufficient biopsy material from the BE segment was available (n=31) to further study TNF expression by immunohistochemistry. Exclusion criteria were BE patients using any type of acid suppression as medication. Paraffin samples were cut at 4mm, deparaffinized in xylene, hydrated using a graded series of alcohol, and stained with hematoxylin and eosin (H&E). An expert gastrointestinal pathologist blinded for the results of the genotyping, scored all H&E slides for squamous epithelium, RE, BE, and EAC. The sequential paraffin slide was used for TNF staining after inactivation of endogenous peroxidase activity with 1% H_2O_2 in 100% methanol for 30 minutes. Subsequently antigen retrieval was performed by boiling the slides in 10mM monocitric acid (pH 6.0) for 15 minutes, and allowing them to slowly cool down to room temperature. The slides were then blocked with non-immune serum for 20 minutes at room temperature. The sections were stained with a primary antibody against TNF-α (mouse-anti-human TNF; 1:100 dilution; ImmunoTools, Friesoythe, Germany). Binding of the primary antibody was visualized by the addition of a secundary biotinylated antibody (goat-anti-mouse IgG1; 1:200 dilution; DAKO, Glostrup, Denmark), and streptavidin-avidin-biotin-complex labeled with horseradish peroxidase (strep ABComplex; 1:200 dilution; DAKO). EAC biopsies (n=4 patients) were used as a positive control.

Two independent observers (VM, KvZ) evaluated the sections of the immunohistochemical stainings. The slides were assessed using a Zeiss microscope (Axioskop 20, Carl Zeiss, Sliedrecht, The Netherlands) with a standard magnification (200x), and the images were recorded with a Nikon camera (DS-5M-U1) and Nikon Eclipse Net 2000 software (Nikon, Badhoevedorp, The Netherlands).

Statistical analysis

The study was powered (80%) to allow detection of a 10% difference in allele distribution between the patient groups (significance level 5%).

Differences between allele distributions of the TNF *Nco*I polymorphism, as well

as differences between the patient groups in number, age, and gender were determined by Chi-square analysis. Age and sex corrected odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for the association between healthy controls and RE, BE, or EAC respectively by logistic regression analysis. Disease causality was calculated for RE, BE, and EAC, being a cascade, with ordinal regression. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted with the SPSS software package v11.1 (SPSS, Chicago, IL).

Results

Patient characteristics

In total, samples of 887 persons eligible for inclusion were collected. These consisted of 197 healthy controls, 305 patients with RE, 257 with BE, and 128 with EAC. Of the 257 BE patients, 19 were on PPI's. The characteristics of the participants are summarized in Table 1. In line with previous studies [16], male gender was more common in BE (p=0.04) and EAC (p=0.001) compared to the healthy subjects and the RE group. On average, patients with BE (p=0.002) and EAC (p=0.002) were slightly older than healthy controls and RE patients (Table 1). When comparing the EAC group with the BE group, the expected higher age (p=0.154) and prevalence of the male gender (p=0.005) of EAC patients were confirmed (Table 1).

Table 1.
Characteristics of the study population.

Patient data	Control	RE	BE	EAC
Number	197	305	257	128
Age	57±14	55±13	61±12**	63±10**
Male (%)	59	55	68*	82**
Length of BE segment (cm)	N.D.	0	4.1±2.4	N.D.

*p=0.04;**p<0.001, both in comparison with controls

The TNF NcoI A/A-genotype is more frequent in RE, BE, and EAC

Using the healthy controls as the reference, the association of a TNF-β polymorphism was tested for patients with RE, BE, or EAC. The allele frequencies for the *Nco*I (+252A/G) polymorphism (rs909253) among the 197 unrelated Dutch Caucasian controls was 39.1% (G) and 60.9% (A) respectively (Table 2). The allele frequency of the major allele (A) was 66.2% in RE, 69.5% in BE, and 71.1% in EAC (Table 2). The distribution of genotype frequencies for the polymorphism investigated was consistent with Hardy-Weinberg equilibrium in both the patient and control groups (p>0.05).

Comparing the raw data of the patient groups with the controls suggested that TNF-β (*NcoI*) A/G heterozygosity was observed more frequently in BE (OR=1.60; 95% CI:1.07-2.38) and EAC (OR=1.66; 95% CI:1.04-2.67), but not in RE (OR=1.23; 95% CI:0.83-1.80) (Table 3.a). However, after correction for age and gender between the four study groups the TNF-β (*NcoI*) A/A genotype was significantly more common

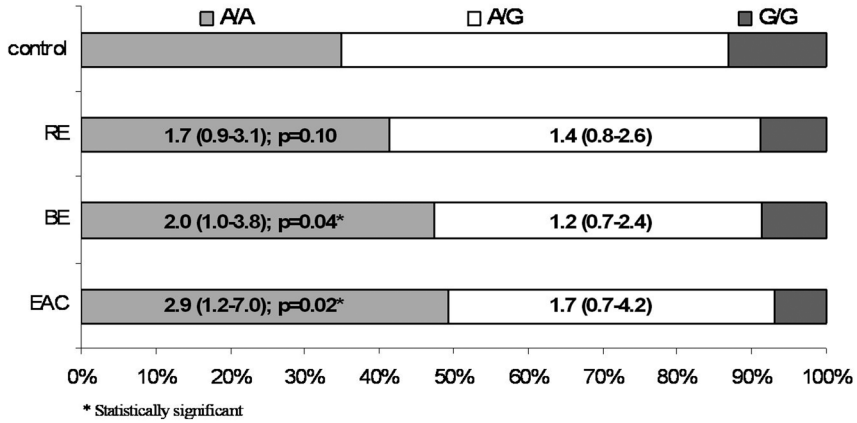

Figure 2

Distribution of the polymorphism in the TNF-β gene over the four study groups. The Odds Ratios show an increased risk for the development of BE and EAC, 2.0 and 2.9 respectively, in the homozygous A/A group compared to the healthy controls. An increase of the A allele can be observed.

Table 2.

Percentage distributions for the alleles of the TNF-β polymorphism tested

Alleles	**Control** n=197	**RE** n=305	**BE#** n=257	**EAC** n=128
A/A	69 (35%)	126 (41%)	122 (48%)	63 (49%)
A/G	102 (52%)	152 (50%)	113 (44%)	56 (44%)
G/G	26 (13%)	27 (9%)	22 (9%)	9 (7%)
Allele frequency				
A	0.609	0.662	0.695	0.711
G	0.391	0.338	0.305	0.289

31 random patients were tested for histological TNF expression

in BE (OR=1.98; 95% CI:1.03-3.81) and EAC (OR=2.87; 95% CI:1.17-7.05), but not in RE (OR=1.69; 95%CI:0.91-3.15) as compared to the control group (Table 3.b). The associated odds ratios significantly differed from controls for both BE (p=0.04) and EAC (p=0.02). The A allele shift towards BE and EAC indicates that the A/A genotype is predominantly associated with the presence of BE and EAC (Figure 2). Figure 2 shows the ordinal regression of disease and points out a decrease of RE and an increase of BE and EAC towards the A/A genotype. Thus, the balance in the RE-BE-EAC cascade is more on the RE side for the G/G genotype and shifts towards BE and EAC for the A/G group and even more for the A/A group.

Table 3.a

Comparison of the Odds Ratios calculated for the genotypes in the TNF-β polymorphism

Genotype	RE vs control OR (95%CI)	BE vs control OR (95%CI)	EAC vs control OR (95%CI)
A/A	0.70 (0.39-1.26)	0.76 (0.41-1.43)	0.63 (0.28-1.44)
A/G	1.23 (0.83-1.80)	1.60 (1.07-2.38)*	1.66 (1.04-2.67)**
G/G^	1 (control)	1 (control)	1 (control)

^ Minor allele; *p=0.04; **p=0.02

Table 3.b

Odds Ratios after correction for age and gender

Genotype	RE vs control OR (95%CI)	BE vs control OR (95%CI)	EAC vs control OR (95%CI)
A/A	1.69 (0.91-3.15)	1.98 (1.03-3.81)*	2.87 (1.17-7.05)**
A/G	1.41 (0.77-2.56)	1.24 (0.66-2.35)	1.71 (0.70-4.16)
G/G^	1 (control)	1 (control)	1 (control)

^ Minor allele; *p=0.04; **p=0.02

The TNF polymorphism is associated with gender

The RE-BE-EAC disease cascade is thought to progress more rapidly in males than in females [17]. This suggests a stronger correlation between causative agents and disease in males than in females. We tested for gender specific differences in disease stage distribution between the TNF-β (*NcoI*) genotypes.

Figure 3 divides the 3 genotypes for RE, BE and EAC in female (Figure 3.a) and male (Figure 3.b) patients. The Y-axis represents the chance of disease development in percentages and on the X-axis the age is shown. The genotypes are grouped per disease as A/A with black symbols, A/G with openwork, and G/G with gray symbols. Females with RE cross all BE genotypes around the age of 80, with the A/A genotype (black symbols) for RE and BE crossing at youngest age (Figure 3.a). This indicates that females with RE and the A/A genotype have a stronger causative relation with BE than the A/G and G/G genotype. BE and the EAC genotypes never cross before the age of 80, which means that there seems no causality between BE and EAC in females, but the causality between these diseases could appear at a higher age.

On average, the males in our study population seemed to develop BE at the age of 55 (Figure 3.b). However males with the A/A genotype do so at a younger age than males with an A/G or G/G genotype. Likewise the average age of EAC development is around 75 years of age, with the A/A genotype crossing some years earlier than the

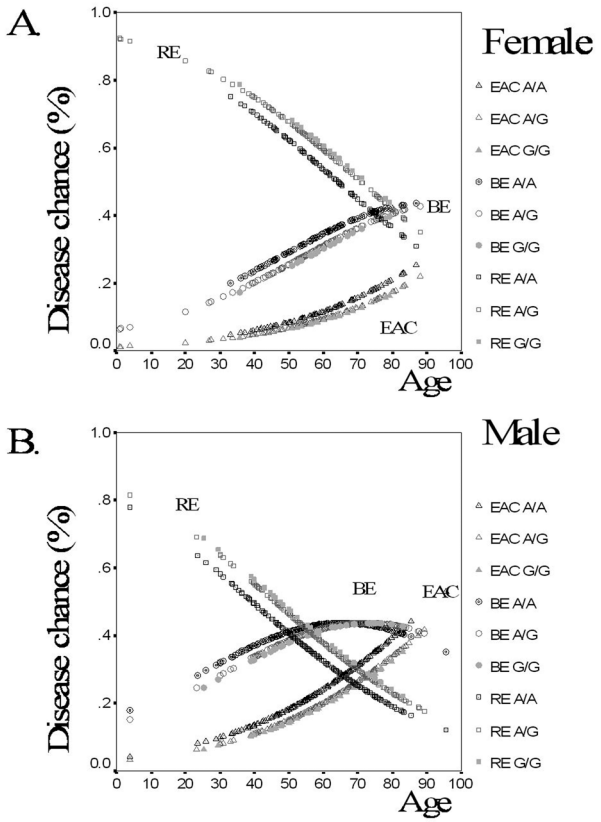

Figure 3

Causality for esophageal disease in females (A) and males (B). The RE-BE-EAC cascade represents a causal relation when the lines cross. The age is shown on the X-axis and the chance of developing esophageal disease is shown as percentage on the Y-axis.

A: The genotypes for RE and BE in females cross around the age of 80 years, and the A/A genotype (black) crosses at younger age when compared to the A/G and G/G genotype. The genotype-lines for BE and EAC in females never cross.

B: The genotypes for RE and BE in males cross between 50 and 55 years, and the A/A genotype (black) crosses at youngest age when compared to the A/G and G/G genotype. The genotype-lines for BE and EAC in males cross at approximately the age of 70, and again at youngest age for the A/A genotype.

A/G and G/G genotypes. These results indicate that male RE patients with a TNF-β (*NcoI*) A/A genotype have an increased risk for the development of BE into EAC.

RE, BE and EAC patients were then grouped together and compared for their TNF-β (*NcoI*) genotype with the healthy controls as a reference. TNF-β (*NcoI*) A/A homozygosity was significantly more frequent in the diseased group (OR=2.26; 95% CI:1.30-3.93), whereas the TNF-β (*NcoI*) A/G heterozygotic group was not (OR=1.15; 95% CI:0.89-2.59) (Table 4).

Table 4.

Odds ratios for the presence of the different TNF-β genotypes among patients with esophageal disease versus healthy controls

Genotype	Disease group vs controls OR (95%CI)	Significance p-value
A/A	2.26 (1.30-3.93)	0.004
A/G	1.15 (0.89-2.59)	0.130
G/G^	1 (control)	0.008

^ Minor allele

Differences in TNF levels between BE and EAC

We then sought to determine both the intensity and the histological site of TNF expression in BE mucosa by TNF-α specific immunohistochemical staining (Figure 4). EAC biopsies (n=4 patients) were used as a positive control and showed a strong TNF-α expression throughout the mucosa and submucosa (Figure 4.d). Typical examples of subsequently a negative, mild, and strong TNF-α expression (brown) in several BE sections are shown in Figure 4.a-c.

The staining for TNF-α was negative in 5/31 patients, including 2 patients from the A/A, and 3 patients from the A/G group. Mild TNF-α expression (19/31) was only seen in the homozygous groups, namely 9 with the A/A genotype and 10 with the

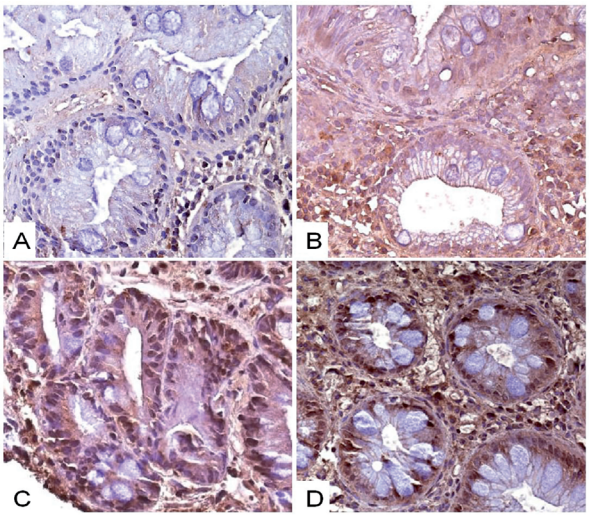

Figure 4

Typical examples of the TNF-α expression (brown) in BE sections. A negative staining (5.a), a mild staining in crypts and inflammatory cells (5.b), and a strong staining (dark brown) (5.c) are shown. TNF-α over-expression (dark-brown) in all crypt- and inflammatory cells is seen in EAC (5.d).

G/G genotype. Strong TNF-α expression was found in 7/31 patients of whom only 1 belonged to the homozygous A/A group, 3 to the A/G, and 3 to the G/G genotype.

Overall, there was a trend with the homozygous A/A group showing the lowest TNF-α expression while the highest expression was observed in the homozygous G/G group.

When analysing the tissue histology of the patients tested for TNF-α expression it was noted that the negative TNF score showed three patients without any pre-malignant tissue changes, one with hyperplasia and one with hyperplasia and ulceration; the mild TNF score showed twelve patients without tissue changes and seven with hyperplasia; the strong TNF score showed three patients without tissue changes, one with hyperplasia and five with hyperplasia and papillomatosis. The tissue inflammation shows a trend towards normal tissue without TNF expression, and stronger inflammation (hyperplasia, papillomatosis and ulceration) in strong TNF expression. This means that the inflammation correlates with the TNF expression.

Discussion

BE is a pre-malignant esophageal condition and the main risk factor for developing EAC. The progression from reflux esophagitis (RE) towards EAC follows a sequence of stages, including RE, BE, dysplasia, and eventually EAC [2, 18, 19]. The exact mechanism through which RE predisposes to EAC is currently unknown, but it has recently been shown that the chronic esophageal inflammatory characters of RE and BE play a crucial role in this process [3, 20].

Several studies showed that BE was associated with a pro-inflammatory genotype and a predominantly humoral immune response [21-23].

TNF, a pro-inflammatory cytokine [24], initiates signals for controlled cell proliferation and apoptosis to prevent cells for the pathogenesis of inflammatory, autoimmune, and malignant diseases [10]. TNF-α is expressed in the esophagus in response to inflammation, and it has been shown that TNF levels increase along the RE-BE-EAC cascade [24-26]. TNF gene polymorphisms that reduce the expression of TNF have been shown to contribute to the severity of infectious diseases and cancer [25]. We postulated that the BE and EAC development is largely dependent on the individual immune responses in the presence of RE. In this study we tested for an association between the functional TNF-β NcoI polymorphism, that decreases TNF production and its susceptibility for BE and EAC development in RE.

The allele frequencies among our 197 Dutch Caucasian healthy subjects for the TNF-β (*Nco*I) polymorphism (rs909253) was 39.1% (G) and 60.9% (A), respectively, which is similar to the European HapMap distribution of 35.8% (G) and 64.2% (A) [27]. The distribution of the three genotypes in the healthy cohort was G/G (13.2%), A/G (51.8%), and A/A (35%), which was also similar to the observed distribution in the European HapMap with 8.3% for G/G, 55% for A/G, and 36.7% for A/A. The slightly lower prevalence of both the G allele and G/G genotype in the European HapMap cohort compared to our healthy controls could be explained by the composition of the cohort, because the Hap Map project did not exclude reflux disease patients (Table 2). We therefore conclude that in spite of its relative small size our control population is a valid representation of the Dutch population.

The sequential RE, BE, and EAC patients who visited our endoscopy unit were included. This means that some patients were already in surveillance programs, but this did not lead to bias in age, gender or genotype. The age of our study groups is similar to the age in previous studies that describe the incidence and

prevalence of BE and EAC [17, 28, 29]. The mean age at BE presentation is 55y. As abovementioned, the genotype distribution has been compared with the European HapMap population and did not show (statistical) difference [27].

As compared to healthy controls the TNF-β *Nco*I A/A genotype was significantly more prevalent in BE (48%; p=0.04) and EAC patients (49%; p=0.02), but not in RE patients (41%; p=0.1). Thus, the homozygous A/A genotype was associated with an increased risk of 2.0 for BE, and 2.9 for EAC development, but there was no association with RE (Table 3.b). Above results indicate that the TNF-β A/A genotype predisposes to reflux related complications such as BE and EAC in the presence of gastroesophageal reflux, rather than that it predisposes to RE itself.

Males with an A/A genotype seem to develop BE and EAC at an earlier age then those with the A/G or G/G genotype (Figure 3.b). In women, this correlation between genotype and onset was only observed for BE, but not for EAC. This may be due to the fact that women tend to develop BE and EAC at later age [17], resulting in significantly less women with BE and EAC in our study cohort.

We did not attempt to make a statistical analysis of the histological TNF-α expression, because the number of available biopsy samples was too small. Unfortunately, we did not succeed to develop a reproducible esophageal TNF-β staining. While indeed an omission we believe that not testing for immunohistological TNF-β expression is only a minor problem because the functional TNF polymorphism affects both the TNF-α and the TNF-β level and therefore determining only TNF-α will provide us with a global idea of the total TNF production.

In our cohort, local TNF-α protein expression was invariably high in esophageal biopsies from EAC patients whereas it displayed marked variation in BE patients. In line with our study, a previous study showed that local TNF-α expression was upregulated in the progression of BE to EAC compared with normal squamous mucosa, but expression of TNF-α was not increased in RE patients [24]. EAC is seen as an immunological tumor [30], however the exact mechanism remains unknown. Cancer in BE arises through a sequence of genetics that endow the cells with six essential physiologic hallmarks of cancer; the ability to proliferate without exogenous stimulation, to resist growth-inhibitory signals, to avoid triggering apoptosis, to resist cell senescence, to develop new vascular supplies (angiogenesis), and to invade and metastasize [31]. Under normal conditions, the esophageal mucosa is in a state of 'controlled' inflammation regulated by a delicate balance of pro-inflammatory and anti-inflammatory cytokines. An environment with low levels of TNF-α is in imbalance and

cannot activate an optimal proliferation and apoptosis via the MAPK-pathway, NF-κB pathway, or TNFR superfamily. Hence, any aberration in the degradation of TNF-α can ultimately cause an increase in oncogene activation. In this study, we presented the lowest local TNF-α expression in BE tissue of the homozygous A/A group, indicating that people with a genetically determined down-modulation of TNF-α expression are more prone to develop BE and subsequently EAC.

In future studies, it might be relevant to investigate the TNF levels in inflamed esophageal squamous mucosa in BE patients to that of RE patients for evaluating a relation between disease causality and TNF genotypes. Unfortunately, we did not have a high number of RE biopsies nor squamous mucosa from BE patients of our polymorphism cohort, so that the relation between the TNF levels of these cohorts could not be investigated.

In conclusion, we demonstrate that the homozygous A/A TNF-β genotype is associated with a significantly increased risk for the development of BE and EAC as compared to the healthy population indicating that individual differences in TNF expression levels are important determinants in the development of EAC.

Acknowledgements

The authors would like to thank the investigators of the pro-inflammatory genotype study [23] for their permission to include the RE and BE patient materials in this study. We also like to acknowledge dr. H. van Dekken for the histology of the tissue samples, dr. Honkoop and dr. Wolters for the collection of materials from the EAC patients, and J. Francke, A. Heijens, and M. Ouwendijk for their assistance with the processing of the materials.

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CHAPTER 5

EXPRESSION, LOCALIZATION AND POLYMORPHISMS OF THE NUCLEAR RECEPTOR PXR IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

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Abstract

Background: The continuous exposure of esophageal epithelium to refluxate may induce ectopic expression of bile-responsive genes and contribute to the development of Barrett's esophagus (BE) and esophageal adenocarcinoma. In normal physiology of the gut and liver, the nuclear receptor Pregnane X Receptor (PXR) is an important factor in the detoxification of xenobiotics and bile acid homeostasis. This study aimed to investigate the expression and genetic variation of PXR in reflux esophagitis (RE), Barrett's esophagus (BE) and esophageal adenocarcinoma.

Methods: PXR mRNA levels and protein expression were determined in biopsies from patients with adenocarcinoma, BE, or RE, and healthy controls. Esophageal cell lines were stimulated with lithocholic acid and rifampicin. PXR polymorphisms 25385C/T, 7635A/G, and 8055C/T were genotyped in 249 BE patients, 233 RE patients, and 201 controls matched for age and gender.

Results: PXR mRNA levels were significantly higher in adenocarcinoma tissue and columnar Barrett's epithelium, compared to squamous epithelium of these BE patients ($p<0.001$), and RE patients ($p=0.003$). Immunohistochemical staining of PXR showed predominantly cytoplasmic expression in BE tissue, whereas nuclear expression was found in adenocarcinoma tissue. In cell lines, stimulation with lithocholic acid did not increase PXR mRNA levels, but did induce nuclear translocation of PXR protein. Genotyping of the PXR 7635A/G polymorphism revealed that the G allele was significantly more prevalent in BE than in RE or controls ($p=0.037$).

Conclusions: PXR expresses in BE and adenocarcinoma tissue, and showed nuclear localization in adenocarcinoma tissue. Upon stimulation with lithocholic acid, PXR translocates to the nuclei of OE19 adenocarcinoma cells. Together with the observed association of a PXR polymorphism and BE, this data implies that PXR may have a function in prediction and treatment of esophageal disease.

Background

Persistent regurgitation of gastroduodenal contents into the lower esophagus causes mucosal injury manifested as reflux esophagitis (RE).[1,2] As a complication of chronic RE, a Barrett's esophagus (BE) can develop.[3,4] BE is defined as an acquired condition in which the stratified squamous epithelium of the lower esophagus is replaced by specialized intestinal epithelium.[5] It is the sole commonly recognized risk factor for the development of esophageal adenocarcinoma (EAC).[6, 7] While the importance of acid and bile exposure in the development of BE is well established,[1,5,9,10] only a small percentage of BE patients will ultimately develop EAC. It remains largely unclear which factors control the rate of neoplastic progression in BE.[11] A growing body of evidence suggests that the intrinsic adaptive response to the toxic bile acids from the gastroduodenal contents is unable to prevent injury to the esophageal lining, thus suggesting a role for bile-induced signaling in the progression of BE.[12]

An important step in understanding the adaptive defence mechanism against toxic substances has been the identification and characterization of the nuclear pregnane X receptor (PXR).[13-16] PXR belongs to the nuclear receptor subfamily of ligand-activated transcription factors that play a key role in the regulation of biliary transport systems and enzymes that confer a protective role against toxic bile acids.[12] This group of nuclear receptors includes the constitutive androstrane receptor and the vitamin D receptor.[17,18] In humans, PXR is most abundantly found in the liver, the small intestine and the colon.[13,15,16,19] It is activated by a structurally diverse array of xenobiotics and endogenous compounds, including bile acids and steroid hormones.[13,17,18] Variability at the PXR genetic locus is therefore thought to be associated with pathophysiological changes in steroid, cholesterol or bile acid levels.[14] Polymorphisms in the PXR gene are associated with diseases such as inflammatory bowel disease and primary sclerosing cholangitis.[20,21] As these chronic inflammatory diseases are associated with aberrant bile acid metabolism, there may also be a link between PXR and BE.

The specific aim of this study was to explore the expression and distribution of PXR in BE and adenocarcinoma patients and analyse possible associations in the PXR gene with esophageal disease. We show that PXR expresses in tissue of BE and adenocarcinoma patients, and nuclear translocation in esophageal adenocarcinoma cells through bile acid stimulation. In addition, a link between PXR polymorphisms and esophageal disease was found.

Methods

Human specimens

For immunohistochemistry, biopsies were taken of adenocarcinoma tissue (n=19), columnar epithelium from BE patients (n=28) and squamous epithelium from RE patients (n=8). As healthy controls we included subjects that had no gastroesophageal reflux disease (GERD) symptoms or endoscopically detected aberrations of the esophagus (n=3). Table 1 gives patient characteristics of the population used for analysis of PXR mRNA levels. mRNA levels were determined in a total of 119 esophageal samples, counting biopsies from 11 adenocarcinoma patients, duplicate biopsies of both the squamous and the columnar epithelium from BE patients (n=21), squamous epithelium of RE patients (n=7), and squamous epithelium of healthy controls (n=5) without GERD symptoms or endoscopically detected aberrations of the esophagus. All BE patients had histologically confirmed intestinal metaplasia without high-grade dysplasia.

Characteristics of the group included in this study for genotyping are shown in Table 2. The total of 683 genetically unrelated Caucasians included 249 BE patients, 233 RE patients and 201 controls without any history of GERD symptoms, who all visited the endoscopy unit of the Erasmus MC-University Medical Center Rotterdam or the IJsselland Hospital in Capelle aan den IJssel between November 2002 and February 2005.[22] This study was approved by the institutional ethics review committees, and all patients gave informed consent before participating in the study.

Table 1
Patient characteristics for PXR mRNA analysis

	RE (n=7)	BE (n=21)	EAC (n=11)
Age, y (range)*	43 (21-60)	61 (34-78)	62 (42-73)
Male, (%)*	71	71	82
Type of epithelium	Sq	Sq, CE	tumor

Abbreviations: RE=reflux esophagitis; BE=Barrett's esophagus; EAC=esophageal adenocarcinoma; Sq=squamous epithelium; CE=columnar epithelium

*Groups did not differ significantly in gender. As expected, BE and EAC patients were somewhat older than RE patients

Table 2

Patient characteristics per group for genotyping

	HC (n=201)	RE (n=233)	BE (n=249)
Age, y (range)	57 (18-90)	54 (19-88)	61 (33-95)
Male, (%)	57	54	69
Length of BE segment, cm (SD)	na	0	4.23 (2.39)

Abbreviations: HC=healthy controls; RE=reflux esophagitis; BE=Barrett's esophagus; na=not applicable

Cell lines

The human adenocarcinoma cell line OE19 and human squamous epithelial cell line HET1A were obtained from the ATCC. OE19 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/l glutamine, 100 units/ml penicilline and streptomycin. HET1A cells were cultured in serum-free BRFF-EPM2 medium supplemented with 100 units/ml penicilline and streptomycin. Cells were maintained routinely at 37°C in 5% CO_2 humidified atmosphere. After a period of at least 24 hrs to allow cells to adhere they were stimulated with 10 µM of rifampicine, 50 µM lithocholic acid (LCA), or 50 or 100 µM taurolithochoic acid (TLCA) for 24 hrs.

Real-Time PCR mRNA quantification from human esophagus samples

Total RNA was extracted from tissue biopsies using TriReagent (Sigma, St Louis, MO) and purified using an RNeasy micro column kit (Qiagen, Hilden, CA). One-fortieth of a 1 µg cDNA synthesis reaction (iScript cDNA Synthesis Kit; Bio-Rad) was used in a 25 µl Real Time-PCR using SYBR GreenER (Invitrogen, Carlsbad, CA). The following primers were used for PXR gene amplification: 5'- ATGGCAGTGTCTGGAACTAC-3' and 5'- CAGTTGACACAGCTCGAAAG-3'. Duplicate samples were run three times in independent PCR-runs and the average level of PXR was normalized to GAPDH using the dCt method.[23]

Immunohistochemistry

Formalin fixed, paraffin embedded, five µm sections were mouned on glass slides. Microwave pretreatment in glycin-HCl/EDTA buffer (50 mM Glycin, 10 mM EDTA, pH 3.5) was performed for 10 min. After blocking for nonspecific antibody binding, sections were incubated overnight at 4°C with PXR antibody (diluted 1:200, Biolegend; San Diego, USA), followed by a biotin-labeled mouse anti-rabbit IgG (diluted

1:200; Dako, Glostrup, Danmark) and streptavidin-horseradish peroxidase (diluted 1:300, Dako) and visualized with diaminobenzidine. Aspecific background controls were done by omitting the primary antibody. Samples of the terminal ileum served as positive controls.

Confocal microscopy

Cells were cultured on coverslips washed with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde for 10 min. After washing, cells were permeabilized with 0.2% Triton X100 for 20 min and then blocked with 5% goat serum and 5% normal human plasma in PBS with 5% BSA. Cells were incubated with mouse IgG or anti-hPXR antibody (1:200; Biolegend, San Diego, USA) at 4°C overnight and then probed with 1:200 dilution of goat anti-rabbit Alexafluor 594 (Invitrogen; Oregon, USA). Hoechst 33342 was used to stain nuclei. Coverslips were mounted onto glass slides with gelvatol and visualized under a Zeiss LSM 410 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

Genotyping

Genomic DNA was extracted from 5 ml of whole blood by a wizard genomic DNA purification kit (Promega, Madison, USA). We analyzed polymorphisms -25385C/T, 7635A/G and 8055C/T as these should be informative for eight PXR polymorphisms and were observed by Zhang et al.[14] to have an effect on PXR function in humans. Assay validation setup was performed by K-Biosciences (Herts, UK) before performing a double blind analysis of PXR SNPs with a competitive allele-specific PCR system using primers designed in flanking region of the SNP located at -25385; TGGTCATTTTTTGGCAATCCCAGGTT[C/T]TCTTTTCTACCTGTTTGCTCAATCG at 7635; AGGAGCCATCCTCCCTCTTCCTCTC[A/G]CCCCCAACTTCTGGATTATGGGATG and at 8055; GCTTGCTGAGAAGCTGCCCCTCCAT[C/T]CTGTTACCATCCACAGGTGGCTTCC of the PXR gene NR1I2.

Statistical analyses

The study was powered (80%) to allow detection of a 10% difference in genotype distribution of the PXR polymorphisms between the groups by performing Chi-square analysis. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated by risk estimate analysis. All statistical analyses were conducted using SPSS v11.0 (SPSS, Chicago, IL) and 2-tailed significance was taken as $p<0.05$.

Results

PXR gene expression is elevated in BE and adenocarcinoma

PXR mRNA was determined in a group of 44 subjects with different esophageal pathologies by Real-Time PCR (Table 1). As shown in Figure 1A, levels of PXR mRNA were found consistently higher in columnar tissue compared to matching squamous tissue ($p<0.001$), in which levels of PXR transcripts were barely detectable. The relative levels of PXR mRNA in the BE columnar epithelium were higher than in squamous epithelium of RE ($p=0.003$, Figure 1B) and healthy controls ($p=0.002$, data not shown). Also PXR gene expression in tissue of adenocarcinoma patients was significantly higher than in squamous samples from BE patients and healthy controls. Comparing RE with controls, only one patient showed a strong increase in

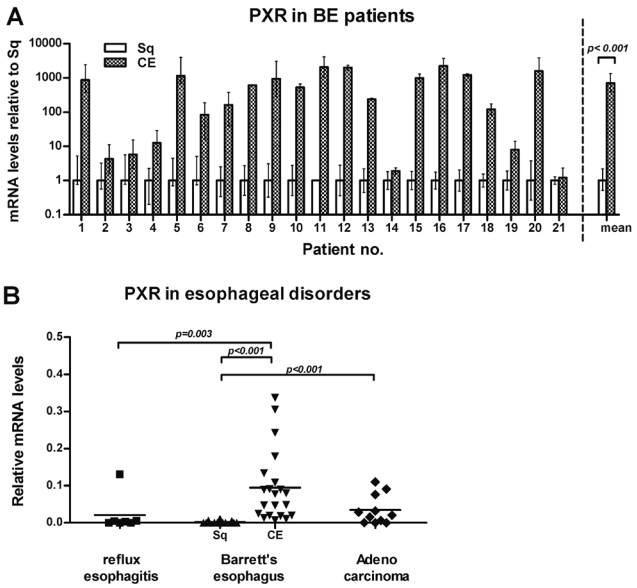

Figure 1

Relative mRNA levels of PXR in esophageal epithelium as determined by quantitative Real-Time PCR. (A) PXR levels in columnar epithelium (CE) are normalized to adjacent squamous epithelium (Sq) using 2(-ddCt) method[23] and are plotted for each of 21 Barrett's esophagus (BE) patients. Error bars express a range which is a result of incorporating the standard deviation into the calculation. The mean of this population renders a strong significant increase of PXR mRNA levels in CE compared to Sq of BE patients ($p<0.001$). (B) Relative PXR mRNA levels are calculated using 2(-dCt). Levels in Sq from patients with reflux esophagitis, and patients with BE are compared to CE from the esophagus of BE patients ($p=0.003$ and $p<0.001$ respectively). mRNA levels in tissue from adenocarcinoma patients did not differ statistically from CE of BE patients, but was significantly higher than all Sq tissues.

PXR mRNA and thus overall difference in mRNA levels between these two groups did not reach statistic significance (Figure 1B).

PXR protein distribution in BE and adenocarcinoma tissue

To test if the presence of PXR mRNA corresponded with the expression of PXR protein, esophageal biopsies of 39 patients were stained for PXR by immunohistochemistry. Figure 2 depicts representative stainings of PXR on esophageal biopsy

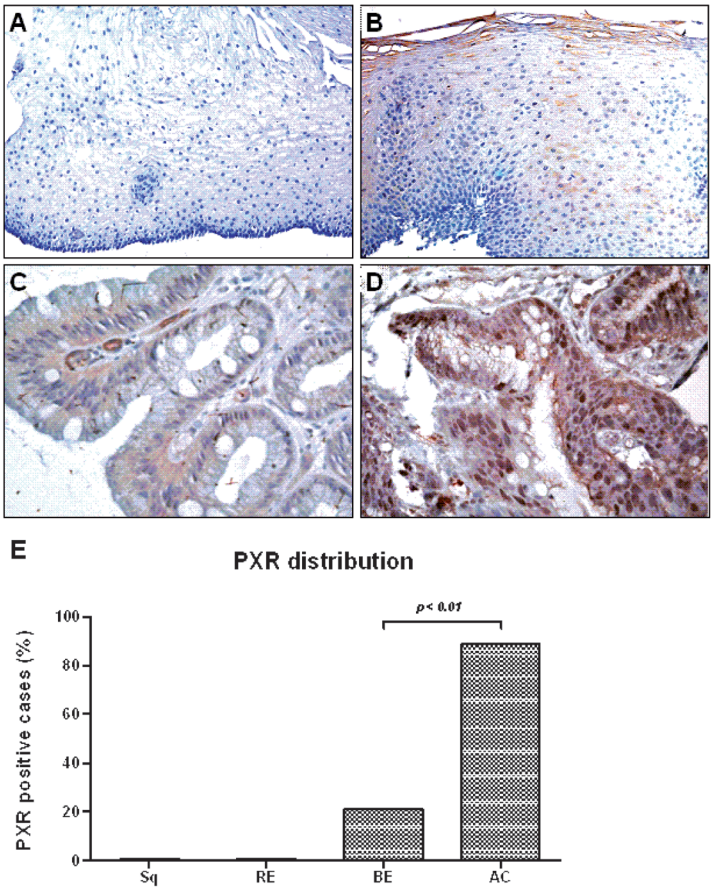

Figure 2

Result of PXR immunohistochemical staining on esophageal biopsy specimens. A) Esophagus of healthy controls is lined by a stratified squamous epithelium and is negative for PXR (200x). B) The esophageal mucosa of patients with reflux esophagitis is damaged and inflamed and demonstrates a weak signal for PXR (brown) in the cytoplasm of epithelium (200x). C) Barrett's epithelium is characterized by a single layer of columnar epithelium with the presence of typical goblet cells. Cytoplasmic as well as some nuclear PXR expression is observed (400x). D) Cells of adenoma tissue show high levels of nuclear PXR expression (400x). E) Quantification showed that the percentage of cases with PXR-positive nuclei was significantly higher in EAC than in BE ($p<0.01$).

specimens of healthy controls, and RE, BE, and adenocarcinoma patients. None of the normal squamous esophageal samples (n=3) stained positive for PXR (Figure 2A). Also, no specific PXR signal was detected in RE samples (Figure 2B). In patients with histologically confirmed BE (n=28), six cases of nuclear positivity were found (Figure 2C). In 17/19 adenocarcinoma patients, PXR expression was observed in the nuclei of cancer cells (Figure 2D). This was significantly higher compared to nuclear PXR expression in BE tissue ($p<0.01$, Figure 2E).

Exposure to bile acids does not affect PXR mRNA levels, but does induce nuclear translocation

PXR mRNA levels were analyzed in HET1A and OE19 cells upon stimulation with 50

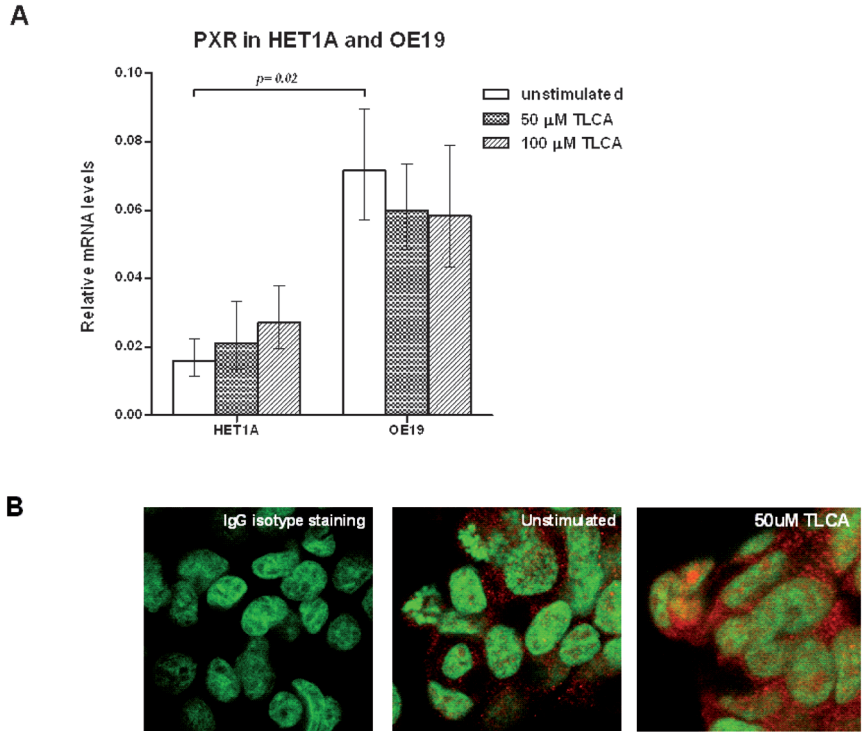

Figure 3

PXR mRNA levels and nuclear translocation of PXR protein in esophageal cell lines stimulated with bile acids. (A) PXR mRNA levels are significantly higher in OE19 than in HET1A (p=0.02). Bile stimulation with 50 µM or 100 µM of TLCA did not affect PXR mRNA levels compared to unstimulated conditions. (B) After immunofluorescent staining of PXR (red) and nuclei (green), localization in OE19 cells was visualized by a confocal laser microscope. In unstimulated cells, PXR was predominantly found in the cytoplasm. Upon 24 hours of stimulation with rifampicine (10 µM), LCA, or TLCA (50 µM), PXR translocated from the cytoplasm to the nuclei.

or 100 µM TLCA. PXR levels in the OE19 adenocarcinoma cell line were higher than in the squamous epithelial HET1A cells (p=0.02), but mRNA levels did not differ between unstimulated cells and cells stimulated with TLCA (Figure 3A). Figure 3B shows immunofluorescence of PXR in the nuclei of OE19 cells. More nuclear PXR staining was observed in cells stimulated with 10 µM rifampicine, 50 µM LCA, and 50 µM TLCA when compared to unstimulated cells. PXR staining was most intense for TLCA stimulated OE19 cells. In summary, exposure of adenocarcinoma cells to bile acids and xenobiotics induces nuclear translocation of PXR independent of PXR gene levels.

PXR polymorphism 7635AG is associated with BE

Polymorphisms at location 7635 and 8055C of the PXR gene have previously been found to be located in different linkage disequillibrium blocks and are thought to have an effect on PXR activity.[14] In our cohort the PXR gene polymorphisms were in Hardy-Weinberg Equilibrium. No significant association of SNP -25385C/T with BE or RE was found (p>0.5; data not shown). Allele frequencies of SNP 7635A/G and 8055C/T for patient and healthy control populations are listed in Table 3. Minor allele frequencies of these SNPs were in consensus with previous observations in European control cohorts.[14,24,25] Subjects carrying the SNP 7635G allele had an increased risk of BE (OR 1.36, 95% CI 1.03-1.79). In comparing genotype distributions, an increase was demonstrated in the minor allele frequency among BE patients as compared with RE patients and healthy controls for both 7635A/G and 8055C/T. For SNP 7635A/G this trend was statistically significant (p=0.037, Figure 4).

Table 3

Allele frequencies of PXR SNPs at locus 7635 and 8055

SNP locus	Allele	Allele, n (frequency) HC	RE	BE	HC vs RE, OR (95% CI)	HC vs BE, OR (95% CI)
7635	A G	267 (0.674) 129 (0.326)	294 (0.636) 168 (0.364)	298 (0.603) 196 (0.397)	1.18 (0.89-1.57)	***1.36 (1.03-1.79)***
8055	C T	321 (0.863) 51 (0.137)	381 (0.832) 77 (0.168)	397 (0.814) 91 (0.186)	1.27 (0.87-1.87)	1.44 (0.99-2.10)

Abbreviations: HC=healthy controls; RE=reflux esophagitis; BE=Barrett's esophagus

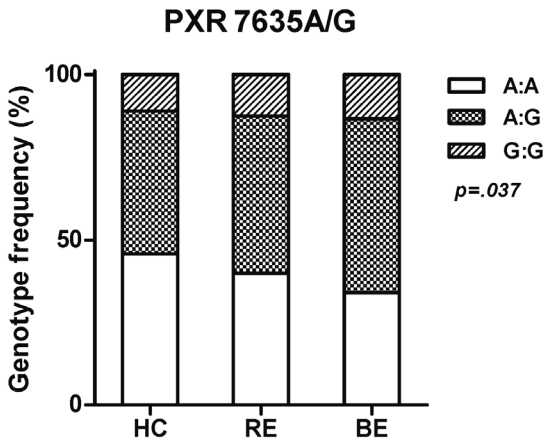

Figure 4

Genotype distributions of PXR polymorphisms across populations of healthy controls (HC), patients with reflux esophagitis (RE) and Barrett's esophagus (BE). The distribution of AA (open), AG (crosshatched) and GG (diaganol stripes) at locus 7635 of the PXR gene are depicted per patient group. As the pathologic condition of the esophagus progresses from healthy to RE to BE, prevalence of ancestral homozygous genotype decreases (p= 0.037).

Discussion

The precise pathophysiological mechanisms causing BE is still unclear, but the combination of gastric acid and bile acids from the gastroduodenal reflux is commonly acknowledged as the key factor in the development of BE.[26] At low pH, bile acids are thought to cause esophageal mucosal injury, which has been substantiated both *in vitro* and in animal model systems.[27,28,29]

The NR1I family of orphan nuclear receptors are known to prevent toxic accumulations of xenobiotics within cells by regulating a broad range of cellular transporters.[17, 30, 31] The nuclear receptor PXR is a member of this family and functions in the enterohepatic organs as detoxifier and regulator of bile acid homeostasis.[12-16] It can bind a variety of bile acids[32,33] and subsequently regulate the expression of a multitude proteins that transport bile acids across cell membranes.[34,35,36] These include the multidrug resistance (MDR)1 gene,[37,38] which encodes the efflux protein P-glycoprotein that removes xenobiotics from cells.[39] Other bile acid transporters that are induced by PXR include the multidrug resistance associated protein (MRP)2 and 3[40-42] and the organic anion transporting polypeptide (OATP)1 and 2.[42-44] From studies in mice it was concluded that the function of PXR is of particular importance when bile acid concentrations reach pathophysiologic levels.[33,45]

PXR expression is known in the liver and intestinal tract, but in cancer it has yet to be explored. In this study we aimed to explore the expression and significance of PXR in esophageal pathology. We did not detect PXR in normal squamous epithelium or in the squamous epithelium of RE patients. PXR did express at both mRNA and protein level in columnar epithelium, and was significantly lower in adjacent squamous esophageal epithelium of the same patient. In samples from adenocarcinoma patients nuclear expression of PXR was found. Translocation from cytoplasm to the nuclei of adenocarcinoma cells was observed *in vitro* after stimulation with rifampicine or litholic acid.

Previous studies have suggested that PXR expression in cancer cells can interfere with the metabolism and responsiveness to chemotherapeutics, such as irinotecon and tamoxifen.[46,47] They suggest this drug resistance involves the metabolizing enzyme CYP3A4, one of the key target genes of PXR.[15] These effects on the metabolism of anticancer agents are especially important considering that PXR ligands include endogenous steroids and bile acids, as well as numer-

ous environmental chemicals and dietary constituents. It has yet to be investigated whether higher levels of PXR in the esophagus also affects responsiveness to chemotherapy.

Given the relatively low rare allele frequency for SNP 8055C/T our population size may have been insufficient to detect a statistically significant association. Validation of our findings will require a well-characterized population from a multicenter study. Recent studies associate PXR polymorphisms with other pathogenic conditions of the gastrointestinal tract, such as inflammatory bowel disease[20] and primary sclerosing cholangitis.[21] Since associations with the two PXR SNPs in this study are in line with previous findings in IBD,[20] this draws attention on a possible link of the functional effect of these SNPs with chronic inflammation. It is well known that inflammation, through the activation of NF-κB pathway leads to a decrease of CAR, PXR and RXR-alpha expression and the expression of their target genes. In addition, it has recently been shown that the mutual repression between PXR and NF-κB signaling pathways provides a molecular mechanism linking xenobiotic metabolism and inflammation.[48]

Although it cannot be ruled out that the observed link between BE and PXR levels is not the cause but only the consequence of the metaplasia from squamous to intestinal-type mucosa, the link with PXR-activity associated SNPs suggest a active role of PXR in BE pathophysiology. Further research should focus on the biologic function of PXR in BE and EAC, especially because PXR protein expression was observed predominantly in the cytoplasm of Barrett's epithelial cells and not in the nucleus. For a complex disease such as BE, development and validation of representative animal models are of great importance in order to investigate if PXR plays a protective role in the development of BE or has a detrimental effect on neoplastic progression.

Conclusions

In summary, PXR which is normally not present in the squamous esophageal epithelium, expressed highly in the columnar esophageal epithelium of BE patients and tumor tissue of EAC patients. Upon stimulation with lithocholic acid, PXR translocates to the nuclei of OE19 adenocarcinoma cells. Together with the observed association of a PXR-activity associated SNPs and BE, this data implies that PXR may have a function in predicting progression and treatment of esophageal disease.

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CHAPTER 6

FUNCTIONAL SINGLE NUCLEOTIDE POLYMORPHISM OF EPIDERMAL GROWTH FACTOR IS ASSOCIATED WITH THE DEVELOPMENT OF BARRETT'S OESOPHAGUS AND OESOPHAGEAL ADENOCARCINOMA

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Abstract

Introduction: Reflux oesophagitis (RO) and Barrett's oesophagus (BO) can cause oesophageal adenocarcinoma (OAC). The oesophageal mucosa in the RO-BO-OAC cascade is chronically exposed to gastro-oesophageal reflux. Epidermal growth factor (EGF) plays an important role in the protection and repair of mucosal damage, and non-physiologic levels are associated with gastrointestinal tumors.

Aim: To determine the functional effect of EGF gene polymorphisms on RO, BO, and OAC development.

Methods: A cohort of 871 unrelated Dutch Caucasians consisted of 198 healthy controls, 298 RO patients, 246 BO patients, and 129 OAC patients. The frequency of the EGF production associated 5'UTR A+*61*G polymorphism was determined in these four groups. EGF immunohistochemistry was performed on BO biopsies.

Results: EGF expression was significantly lower in the G/G genotype compared to the A/G (p=0.008) and A/A (p=0.002) group. The G/G genotype was significantly more prevalent in RO (OR=2.6; 95% CI:1.3-5.2), BO (OR=3.0; 95% CI:1.5-6.2), and OAC (OR=4.1; 95% CI:1.7-9.7) than in controls.

Conclusion: The G allele is associated with reduced EGF expression and increased risk for RO, BO and OAC development. This indicates that reduced mucosal protection resulting from genetically decreased EGF expression enhances oesophageal tumor development.

Introduction

Oesophageal adenocarcinoma (OAC) is rising faster than any other epithelial malignancy in the Western world since 1970 [1]. OAC frequently arises from reflux oesophagitis (RO) and Barrett's oesophagus (BO), a chronic inflammatory condition characterised by a change in the normal oesophageal epithelium into intestinal metaplasia as a result of persistent severe reflux [2-4], and genomic instability [5].

Several twin-studies have provided evidence for a genetic component in RO [6, 7]. The presence of an inherited genetic component impacting on the individual predisposition to develop BO has been accumulated over the last three decades [8], and the only predisposing factor in families with BO and OAC seems to be RO [9, 10].

The stratified squamous epithelium of the healthy oesophagus possesses a variety of intrinsic defenses that enable it to resist acid-peptic reflux, divided in pre-epithelial defense, epithelial defense, and post-epithelial defense [11, 12]. Growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are associated with epithelial proliferation and restitution that are the two key mechanisms involved in oesophageal epithelial defense against acid, maintaining epithelial integrity and enabling rapid repair after injury [13]. Multiple signaling pathways are activated by binding of TGF-α and EGF with their receptor EGFR [14, 15], resulting in proliferation and differentiation of epithelial tissues [16, 17].

Basal EGF levels are in part determined at genetic level, and variation at a single nucleotide polymorphism (SNP) within the EGF gene has been associated with altered EGF production [18]. This SNP in the 5' UTR of the EGF gene has been associated with the risk of various tumors, such as oesophageal adenocarcinoma, gallbladder cancer, ovarian cancer, and gastric cancer [19-22]. For all three malignancies, it was reported that hetero- and homozygous G allele carriers had an increased risk for cancer compared with non-carriers. However, these associations were in contrary to others that showed an increased risk for A allele carriers with renal cell carcinoma, gastric cancer, and oesophageal squamous cell carcinoma [18, 21, 23].

Several studies have reported on the expression of TGF-α, EGF, and EGFR in the human oesophageal mucosa, both at the level of RNA and at protein level [24-27]. EGF expression in BO was significantly higher compared to both squamous epithelium and gastric mucosa, while EGF expression was depleted in RO [24]. BO showed local EGF expression and an over-expression of both TGF-α and EGFR suggesting a deregulation of important proliferation control mechanisms in these epithelial cells.

EGFR over-expression in oesophageal adenocarcinoma correlated with advanced pathologic tumor classification and lymph node metastasis [28].

BO is an ideal model to study cancer genetics and EGF is suggested to be involved in oesophageal cancer development [29]. We postulated that the functional EGF 5' UTR G allele is associated with individual susceptibility for RO, BO, or OAC, and affects local EGF expression in the oesophagus.

Material and methods

Study design

Between November 2002 and March 2006, all subsequent patients with RO, BO, and OAC who visited the endoscopy unit of the Erasmus MC – University Medical Centre Rotterdam or the IJsselland Hospital, Capelle aan den IJssel were invited to participate in this study. The overall response rate was 85%. Participants underwent upper endoscopy for evaluation of reflux-related symptoms, surveillance of BO, or odynophagia or dysphagia. The presence of RO was confirmed at endoscopy and the length of any columnar-lined segment was determined by measuring the distance between the squamo-columnar junction and the proximal margin of the longitudinal gastric folds. Participants were only included if they had i) RO without the presence of BO, ii) BO defined as a columnar lined segment in the oesophagus of ≥ 2 cm in length with specialised intestinal metaplasia at histology found in at least one of the biopsies, or iii) OAC defined at histology as an adenocarcinoma in BO or at the gastro-oesophageal junction with more than 50% of the tumor mass in the oesophagus. The response rate of the patients was 86.3%. Not all biopsies fitted the above-mentioned histological criteria, therefore approximately 50% of RO patients could be included, 90% of BO patients and 95% of OAC patients.

Between October 2004 and April 2005, healthy volunteers (referred to as normal controls) were recruited from the general population of general practitioners practices. Subsequent adult patients visiting the participating general practices were examined by means of a validated questionnaire. Subjects were eligible for inclusion if they never had reflux complaints, retrosternal pain, or regurgitation, nor used antacids, H2-receptor antagonists, or proton pump inhibitors. We performed age, sex and race frequency matching during the selection of controls. The response rate was 48.9%.

All individuals of the four study groups donated 10ml of whole blood.

The study was approved by the local institutional review boards of both participating hospitals. All participants were genetically unrelated Dutch Caucasian people, and aged over 18. Prior to inclusion, all participants signed a written informed consent.

Genotyping of the EGF 5' UTR polymorphism

Genomic DNA was extracted from 5 ml of whole blood by standard procedures

(Wizard Genomic DNA Purification-kit; Promega, Madison, WI). Single nucleotide polymorphism (SNP) analysis of EGF (*+61A/G*) genotypes was performed by a technician unaware of the clinical findings for these patients on 20 ng DNA (Kbiosciences, Herts, UK) with a competitive allele-specific PCR system [30] using primers designed in flanking region of SNP CAAGGGTTGT[A/G]GCTGGAACTTTCCATCAGT located at position +61 in the 5'UTR (rs4444903).

Immunohistochemical analysis of EGF

The local institutional review board only allowed us to use remnants of routine biopsy samples from the Erasmus MC – University Medical Centre Rotterdam. From the 246 BO patients that participated in this study only 37 BO patients had sufficient high quality biopsy material of the BO segment left to allow an immunohistochemical study of oesophageal EGF and EGFR expression. Paraffin samples were cut at 4mm, deparaffinised in xylene, hydrated using a graded series of alcohol, and stained with hematoxylin and eosin (H&E). The two sequential paraffin slides directly following the slide used for histological evaluation were used for EGF and EGFR analysis. The local institutional review board did not allow extensive testing for the presence of EGF and EGFR levels in OAC biopsy samples, because a preliminary screening (see results) of four samples revealed that over-expression both EGF and EGFR was to be expected in all OAC samples. For EGF staining endogenous peroxidase activity was inactivated with 0.3% H_2O_2 in 100% methanol for 30 minutes. Subsequently antigen retrieval was performed by boiling the slides in 10mM monocitric acid (pH 6.0) for 15 minutes, and allowing them to slowly cool down to room temperature. Antigen retrieval for EGFR was performed by 5 minutes incubation with Prot K at room temperature, followed by one washing with ice cold sterile PBS. The slides were then blocked with 10 % normal human plasma for 20 minutes at room temperature. The sections were stained using a primary antibody against EGF (anti-EGF clone EGF-10; 1:200 dilution; Sigma, St. Louis, MO) or EGFR (anti-EGFR clone F4; 1:250 dilution; Sigma). Binding of the primary antibody was visualised by the addition of anti-mouse Envision labeled with horseradish peroxidase (DAKO, Glostrup, Denmark).

Two independent observers (VM, KvZ) evaluated the sections of the EGF and EGFR stainings, while blinded for the score of the other observer, and unaware of the patient characteristics and genotype. To determine EGF and EGFR expression, the number of stained cells was scored per microscopic field as 0 (negative), 1

(mild, 1-100 positive cells/field), and 2 (strong, >100 positive cells/field). The slides were evaluated using a Zeiss microscope (Axioskop 20, Carl Zeiss, Sliedrecht, The Netherlands) with a standard magnification (200x), and the images were recorded with a Nikon camera (DS-5M-U1) and Nikon Eclipse Net 2000 software (Nikon, Badhoevedorp, The Netherlands). The mean variation in scores between observers was found to be <5%, and the average of the two observations was used to analyze the relation with the EGF genotypes.

Statistical analysis

The study was powered (80%) to allow detection of a 10% difference in allele distribution between the patient groups (significance level 5%). Differences between allele distributions of the EGF 5'UTR polymorphism, as well as differences between the patient groups in number, age, and sex were determined by Chi-square analysis. Age and sex corrected odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for the association between healthy controls and RO, BO, or OAC respectively by logistic regression analysis. Disease causality was calculated with ordinal regression for the RO-BO-OAC cascade in relation to sex. The mean immunohistochemical EGF expression was compared between the three genotypes with ANOVA. A difference between the median immunohistochemical EGF expression per genotype was calculated with the Wilcoxon non-ranked sum test. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted with the SPSS software package v11.1 (SPSS, Chicago, IL).

Results

Patient characteristics

In total, 871 participants were included; 198 controls, 298 RO, 246 BO, and 129 OAC patients. Table 1 shows that male gender was more common in BO and OAC than among controls and RO patients. On average, patients with BO and OAC were older than controls and RO patients. When comparing the OAC with the BO group, slightly higher age and higher prevalence of males were found in the OAC patients.

Table 1.
Characteristics of the study population.

Patient data	Control	RO	BO[#]	OAC
Number	198	298	246	129
Age (+ SD)	56±15	55±14	61±12	63±10
Male (%)	57.6	54.7	67.9	82.2
Length of BO segment (cm)	N.D.	0	3.9±2.1	N.D.

[#] 37/246 biopsy specimens were stained for EGF and EGFR

G/G-genotype is more prevalent among RO, BO, and OAC patients

Using controls as a reference, the association of an EGF polymorphism was tested for RO, BO, or OAC patients. Allele frequencies for the EGF 5'UTR polymorphism among the 198 controls were 32.8% (G) and 67.2% (A) respectively. The allele frequency of the major allele (A) was 60.9% in RO, 59.3% in BO, and 58.1% in OAC.

The distribution of genotype frequencies for the polymorphism investigated was consistent with Hardy-Weinberg equilibrium in both the patient and control groups (p>0.05). Comparing the patient groups with the controls showed that EGF G/G homozygosity was observed more frequently in RO (OR=2.62; 95% CI:1.33-5.15), BO (OR=2.85; 95% CI:1.42-5.75) and OAC (OR=3.12; 95% CI:1.42-6.85) compared to controls. The adjustment for age and sex showed that EGF 5'UTR G/G homozygosity was significantly more common in RO (OR=2.61; 95%CI:1.32-5.15), BO (OR=3.04; 95% CI:1.49-6.17) and OAC (OR=4.12; 95% CI:1.76-9.65) compared to the controls (Figure 1). There was no significant difference in the frequency distribution of EGF G/G genotype in BO versus RO (OR=1.00; p=0.74), in OAC versus RO (OR=1.46; p=0.35), and OAC versus BO (OR=1.19; p=0.65). Figure 1 shows that the frequency of A/A genotype decreases gradually for RO, BO and finally for

OAC, while there is a clear increase for the G/G genotype frequency. This G-allele shift taken together with the odds ratios for RO (p=0.006), BO (p=0.002) and OAC (p=0.001) indicates that the G/G genotype is predominantly associated with the presence of RO, BO and OAC.

Risk stratification was calculated for RO, BO and OAC patients together named reflux-associated disease with the controls as a reference. The EGF A/G genotype frequency did not differ between diseased patients and controls (OR=1.05; 95% CI:0.74-1.49). However, EGF G/G homozygosity was observed more frequently in the diseased group (OR=2.76; 95% CI:1.45-5.24; p=0.002).

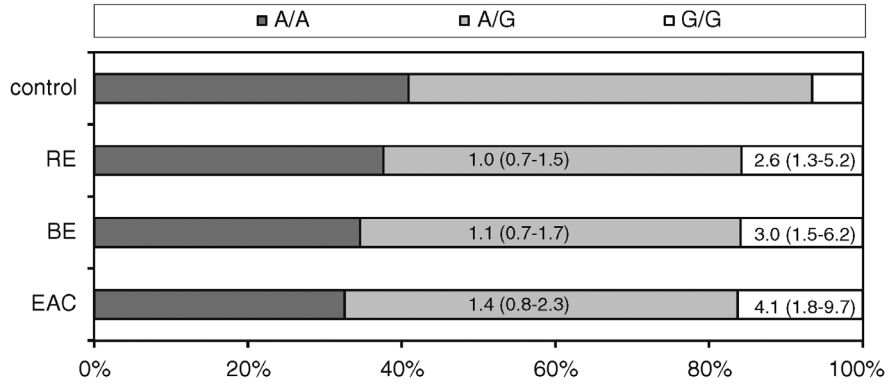

Figure 1

Distribution of the polymorphism in the EGF (position +61) gene demonstrated for the four study groups. The comparison of the Odds Ratios (OR) shows an increased risk for the development of RO, BO and OAC, with p-values of 0.006 (RO), 0.002 (BO) and 0.001 (OAC), respectively, in the homozygous G/G group compared to the healthy controls. A clear shift from the A-allele (dark grey) in the control group towards the G-allele (white) in the RO, BO, and OAC groups can be observed in the bars.

The EGF polymorphism in BO is associated with sex

The ordinal regression model was used to test for sex specific differences in disease stage distribution between the EGF 5'UTR genotypes.

Figure 2 shows the distribution of the three genotypes for RO, BO and OAC in male (Figure 2A) and female (Figure 2B) patients. The Y-axis represents the chance of disease development in percentages and on the X-axis the age is shown. The three genotypes are grouped per disease as lines of coloured symbols. Females with RO cross all BO genotypes around the age of 80, with the G/G genotype for RO

and BO crossing at younger age (RO: grey symbols, BO: red, OAC: blue) (Figure 2B). This suggests that females with RO and the G/G genotype have a stronger causative relation with BO than the A/A and A/G genotype. BO and the OAC genotypes never cross before the age of 80, which means that there seems no current causality between BO and OAC in females, but the causality between these diseases could appear at a higher age.

Males with RO cross BO at the age of approximately 55, and do so at a younger age than males with an A/G or A/A genotype (G/G-RO: grey, -BO: red, -OAC: blue). The development of BO toward OAC appears at approximately 80 years of age, with the G/G genotype crossing some years earlier than the A/A and A/G genotypes (Figure 2B). These results indicate that male RO patients with an EGF 5'UTR G/G genotype have an increased risk for BO and OAC development, as a RO-BO-OAC disease cascade.

Figure 3 shows the distribution of the three genotypes per disease as A/A with red squares, A/G with green squares, and G/G with blue squares. The Y-axis represents the predicted probability for disease in percentages and on the X-axis the age is shown. All lines are double and to be separated in males [top] and females [bottom]. The G/G genotype is associated with a higher probability for the develop-

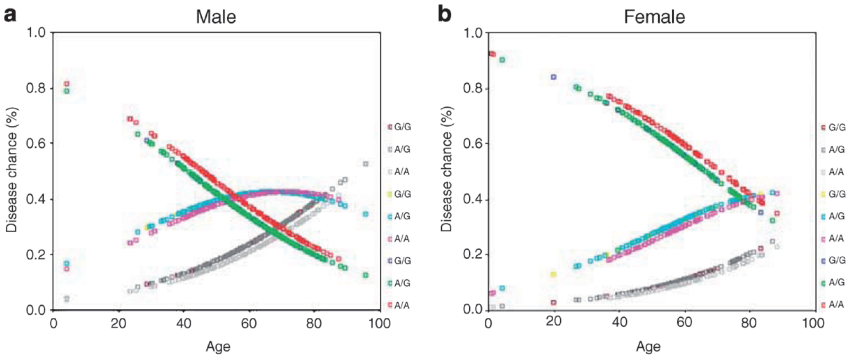

Figure 2

Ordinal regression model for the causality of oesophageal disease in males (A) and females (B). The RO-BO-OAC cascade represents a causal relation when the lines cross. The age is shown on the X-axis and the chance of developing oesophageal disease is shown as percentage on the Y-axis.

A: Male patients develop RO from BO around the age of 60, and OAC from BO around the age of 80. The G/G genotype crosses the RO-BO line and BO-OAC line at youngest age.

B: The genotypes for RO and BO in females cross around the age of 80 years, and the G/G genotype (RO-grey circles with BO-red circles) crosses at younger age when compared to the A/A and A/G genotype. The genotype-lines for BO and OAC in females never cross.

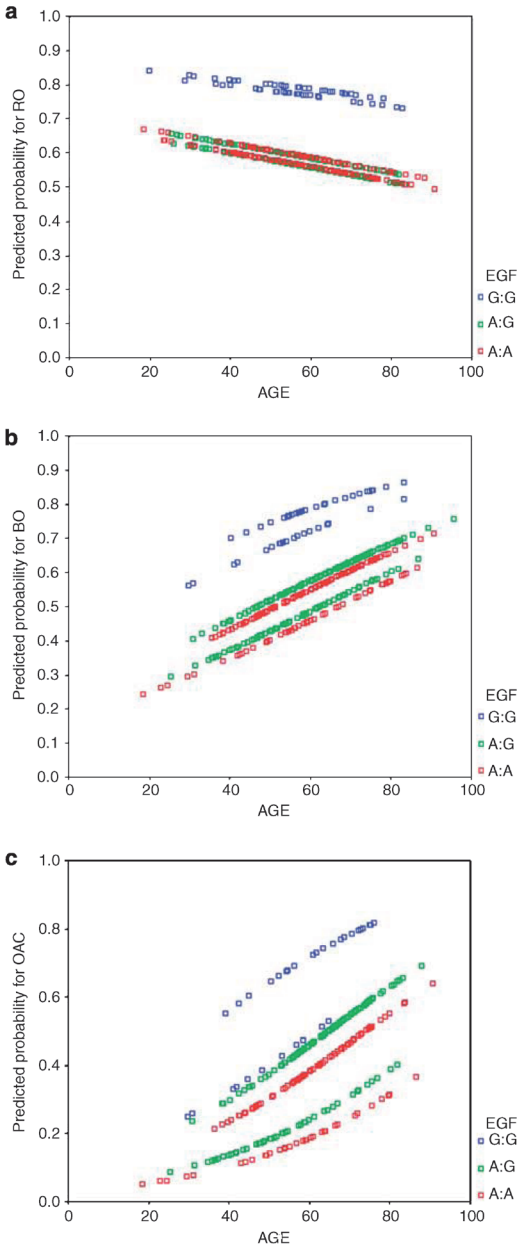

Figure 3
Logistic regression of the EGF (+61A/G) gene for the RO, BO, and OAC development of male and female patients. The age is shown on the X-axis and the predicted probability of oesophageal disease is shown as percentage on the Y-axis. The G/G genotype (blue lines) is associated with an increased risk for RO, BO, and OAC. For all instances the upper line represents the male individuals, the lower line the female patients. Note that that the lines representing female patients follow the same pattern as the male curves but seem shifted in time by approximately 20 years.

ment of RO (Figure 3A), BO (Figure 3B), and OAC (Figure 3C). Comparing all female graphs with the male graphs for BO and OAC points out that females form similar curves as males, but 20 years of age later (Figure 3A-C).

EGF expression in BO in relation to genotype

EGF and EGFR expression might influence development of RO, BO, or OAC. Therefore, the variation of EGF and EGFR expression in BO biopsies per genotype was determined (Figure 4). EGF staining was observed in the cytoplasm of epithelial cells and inflammatory cells. BO biopsies varied in EGF expression (Figure 4A-C) and all OAC biopsies tested showed a strong EGF expression in the tumor (data not shown).

The EGF staining was negative in 0% of A/A, 5% of A/G, and 43% of G/G patients. The positive biopsies displayed mild staining in 38% of A/A, 68% of A/G, and 57% of G/G patients. Strong EGF expression was found in 62% of A/A, 27% of A/G,

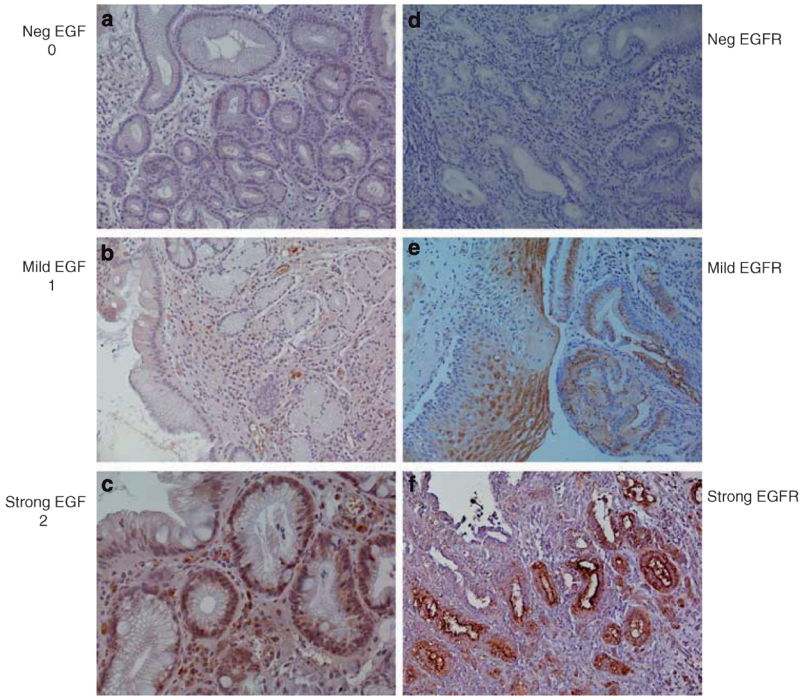

Figure 4

Typical examples of the EGF and EGFR expression. EGF was scored as 0 (negative), 1 (mild, 1-100 positive cells/microscopic field), and 2 (strong, >100 positive cells/microscopic field) on BO tissue of patients from our BO polymorphism cohort. A: Negative staining for EGF on BO tissue B: Mild EGF staining (brown) of metaplastic epithelial cells and submucosal inflammatory cells is alternated by blue epithelial and inflammatory cells C: EGF staining is strongly present in all metaplastic epithelial cells and in most inflammatory cells D: Negative staining for EGFR on BO tissue E: EGFR staining (brown) of metaplastic epithelial cells is present in RO tissue (left) and BO tissue (right) F: The EGFR staining is strongly present in all OAC metaplastic epithelial cells and also in BO tissue.

and 0% of G/G patients. The mean EGF expression (ANOVA p=0.067) did not differ between the 3 genotype-groups (A/A, A/G and G/G). However, comparisons of EGF expression among A/A versus A/G (p=0.13), A/G versus G/G (p=0.032), and A/A versus G/G (p=0.0093) genotype carriers showed that the carriage of the G allele was associated with a significantly lower local EGF expression (Figure 5A).

EGFR expression is known to be present in (pre-) malignant tissues. Strong EGFR expression was observed in near to all biopsies of our BO cohort (28/33) (Figure 4D-F). The EGF expression was calculated for 37 biopsies, but 4 stains of the EGFR slides were unreliable and taken out of the calculations. The median EGFR expression per EGF genotype (A/A versus G/G p=0.83) showed no significant difference (ANOVA p=0.094) (Figure 5B).

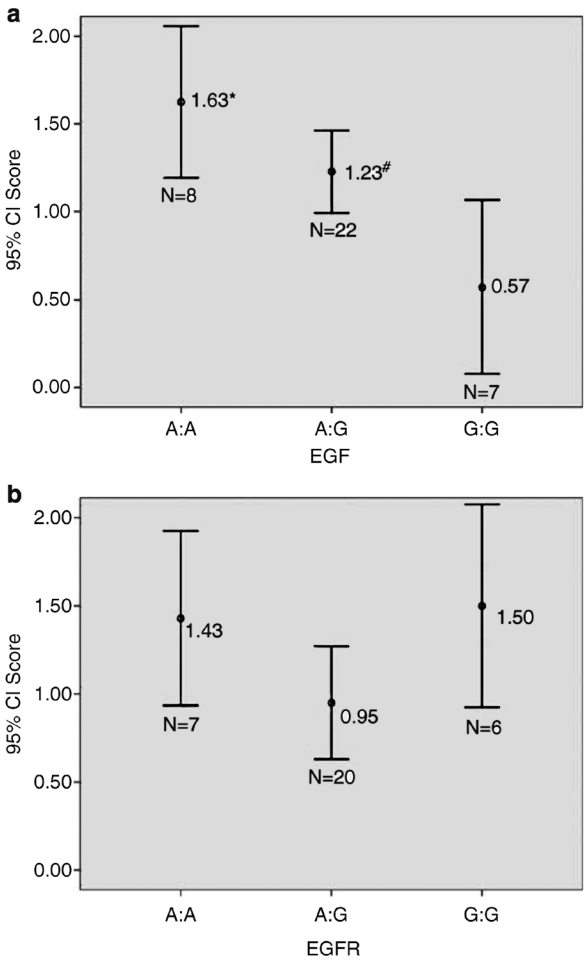

Figure 5
The median immunohistochemical EGF and EGFR scores in a subset of the BO cohort are presented in the boxplot. A: The G/G genotype shows a significant lower local EGF expression in BO epithelium compared to the A/A and A/G carriers. The A/A genotype significantly expresses more EGF than the A/G carriers. B: The EGFR staining is not significantly different between the three genotype-groups.

Discussion

Persistent severe gastro-oesophageal reflux of gastric acid and bile is associated with the development of RO and BO, which are the main risk factors for developing OAC [2, 3]. Since EGF/EGFR has been implicated in the progression of BO into OAC [31], the EGF genotype may serve as a biomarker to assess the risk of malignant transformation in surveillance programs for patients with BO.

The EGF (*+61A/G*) polymorphism is associated with individual risk of oesophageal, gastric, gall bladder, ovarian, and renal cell cancer in a variety of studies [18-21, 32]. Our study shows an association between the functional EGF 5' UTR G allele and RO-BO-OAC development.

The EGF 5' UTR G allele frequency showed a significantly increased odds ratio of 2.6 for RO, 3.0 for BO, and 4.1 for OAC, and the 5'UTR G allele was found to be closely associated with local lower EGF expression levels in BO. The reduced EGF production in the G/G genotype could therefore mediate the development of BO and OAC. To our knowledge, we are the first to show the association of the functional EGF G/G genotype with both an enhanced chance of RO, BO, and OAC development, and a decreased EGF expression in BO tissue.

A hospital-based case-control study on the association between genetic polymorphisms firstly needs large subgroups to show an association. For that purpose we sampled groups of patients that by comparison with other papers are substantial, but confirmation and validation with further studies is needed.

The allele frequencies in the control cohort we used to test this polymorphism was close to equal with the frequencies reported for the European HapMap population [33]. Eventually all man at higher age develop RO, BO and OAC, as shown in our ordinal regression model. As a consequence, the control group has fewer male cases and a slightly younger age, while older males had RO complaints. The RO-BO-OAC disease cascade is thought to progress more often and faster in males than in females [34]. This suggests a stronger correlation between causative agents and disease in males than in females, as we showed in present study with the ordinal regression model.

The number of BO biopsy samples that was available in sufficient quantities for use in our immunohistochemical EGF expression study was small (n=37), we did however observe a significantly lower EGF expression in the G/G genotype compared with A/G or A/A carriers (p=0.0093). The precise mechanisms underlying the EGF

gene interaction in BO- and OAC-risk are probably complex. Previous studies firstly showed that the studied EGF polymorphism was functional and associated with EGF serum levels. The G allele was linked to elevated EGF production in lymphocytes of malignant melanoma patients [35], and serum of patients with gastro-oesophageal reflux disease [19]. Secondly, low EGF serum levels may increase the risk of renal cell carcinoma [18]. In this study we found that there was a significantly lower EGF expression in BO cases carrying G/G homozygote than the A/A or A/G carriers.

Since EGF is abundant in saliva and oesophageal mucosa, serum EGF levels may not entirely reflect the endoluminal milieu of the oesophagus. Indeed a study on local EGF expression in RO, BO, and OAC indicates that EGF was depleted in RO, the EGF expression of BO epithelium was mostly superficial, and EGF over-expression was observed in all histological OAC samples [24]. These observations are very similar to our findings and support our hypothesis that a genetically predetermined decreased EGF expression results in an increased risk for RO, BO, and OAC development.

EGFR expression was observed intracellular in RO, BO, and OAC, as shown in previous studies [25]. The finding of EGFR over-expression in all BO biopsies is in line with the previously reported EGFR over-expression in a wide variety of cancers of the gastrointestinal tract [36-38]. High EGFR levels might well represent a negative feedback loop resulting from low EGF levels. This would suggest that elevated EGFR levels could be an indirect result of gastrointestinal neoplasia and cancer development [39], rather than a direct trigger for neoplastic development [40]. That high EGFR levels not likely cause tumor development is further supported by the finding that EGFR over-expression in oesophageal adenocarcinoma is associated with advanced pathologic tumor classification and lymph node metastasis [28], but not with an increased risk for tumor development or survival. In line with this, a previous study showed that not EGFR but the TGF-α expression differentiated between the survival of oesophageal cancer patients [41]. Finally, an EGF/EGFR peptide array study demonstrated that EGF activation is significantly more prominent in squamous epithelium than in BO, concluding that EGF is an important growth factor for normal oesophageal tissue regulation through binding with EGFR. Although the EGFR expression is stronger in BO compared to squamous epithelium, the activation of EGFR in BO is lower [40]. This suggests that EGFR is a major receptor, but the growth factors EGF and TGF-α determine the outcome of disease. The decreased EGF activity in the oesophagus could therefore trigger the promotion of a tumor environment, thereby either directly or indirectly affecting the risk for OAC development [42, 43].

In conclusion, we demonstrate that the homozygous G/G genotype of the EGF 5'UTR polymorphism is associated with a significant decrease in local EGF levels and an increased risk for the development of RO, BO and OAC. The genetically determined decreased EGF levels could result in less EGFR activation, leading to neoplastic changes, like RO, BO, and OAC, but this hypothesis needs validation in other independent cohorts.

Acknowledgements

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CHAPTER 7

MYO9B IS ASSOCIATED WITH AN INCREASED RISK OF BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

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Abstract

Background: Reflux esophagitis (RE) and Barrett's esophagus (BE) are predisposing factors for development of esophageal adenocarcinoma (EAC), the solid tumor with the fastest rising incidence in the Western world. This RE-BE-EAC cascade involves multiple host factors and consequently multiple genes. Polymorphisms in the 3' region of myosin IXB (Myo9B) are associated with chronic inflammatory gastrointestinal disorders like celiac disease and ulcerative colitis, assuming that variation in Myo9B influences the intestinal permeability.

Aim: To determine esophageal expression and the genetic variation of the Myo9B gene in the RE-BE-EAC cascade

Methods: DNA from 886 Caucasian participants (198 non-reflux controls, 305 RE, 254 BE, 129 EAC) was collected for the determination of the Myo9B gene polymorphism (rs2305764). Esophageal Myo9B expression was determined on biopsies from normal, RE, BE, and EAC epithelium.

Results: Genotype G/G was more common in BE (p=0.032), and EAC (p=0.046), but not in RE (p=0.126) compared to the control group. Cytoplasmic Myo9B expression was determined in RE, BE, and EAC, but most prominent in epithelial cells of BE and EAC.

Conclusions: Genetic variation of Myo9B may play a role in the etiology of BE and EAC by increasing the permeability of the epithelial barrier.

Introduction

The incidence of esophageal adenocarcinoma (EAC) has increased rapidly in North America and Western Europe and is most pronounced in Caucasian men over 50 years of age [1, 2]. Established risk factors for the development of EAC include reflux esophagitis (RE) and Barrett's esophagus (BE) [3, 4]. BE is characterized by the replacement of normal squamous epithelium with metaplastic columnar epithelium under the influence of inflammation as a consequence of persistent severe gastroesophageal reflux [5], and genomic instability [6]. In addition to genetic variation in inflammatory factors also other factors that indirectly affect the inflammatory response may lead to increased susceptibility for BE and EAC.

The genome is the complete human's hereditary information and it is encoded in DNA or RNA. Two sequenced DNA fragments from different individuals can contain a difference in a single nucleotide (A, T, C or G). In this case, Myo9B has a variation in 2 alleles: A and G. Almost all single nucleotide polymorphisms (SNPs) have only two alleles that occur more frequently in non-coding regions than in coding regions. In general, natural selection is acting and fixating the allele of the SNP that constitutes the most favourite genetic adaptation. Serial analysis of gene expression in Barrett's related adenocarcinomas showed a leading role for chromosome 19, that carries several gene groups relevant to carcinogenesis [7]. Genetic variation in the 3'region of the myosin IXB (Myo9B) gene on chromosome 19 was associated with inflammatory intestinal disorders, like celiac disease and inflammatory bowel disease [8-11]. The selected tag SNPs were able to capture most of the common genetic variation present in this region, by virtue of the strong correlation between the known variants in this region [12, 13]. However, conflicting data exist on the involvement of polymorphisms in this region and inflammatory disorders. In 2005, Monsuur et al reported a significant association between celiac disease and a common polymorphism (rs2305764) in intron 28 of Myo9B [14]. However, subsequent smaller British, Italian, and Swedish studies have failed to replicate these findings [15-18].

Myo9B is a Rho family GTPase activating protein involved in epithelial cell cytoskeletal organisation [19]. Human Myo9B is expressed in intestinal epithelium which is altered upon differentiation and genetic variation could cause impaired intestinal permeability [20]. A defect in the intestinal barrier function by genetic variation could be a factor for the genesis of intestinal inflammation [21, 22]. Increased

mucosal permeability is recognized as one of the earliest histological changes in gastroesophageal reflux disease patients [23].

Therefore, we postulated that genetic variation in Myo9B is associated with individual susceptibility for other gastro-intestinal inflammatory diseases, including RE, BE, and EAC. To investigate this hypothesis, we tested esophageal Myo9B expression and the association with SNP (rs2305764) in a case–control study.

Material and methods

Study design

Between November 2002 and March 2006, all subsequent patients with RE, BE, and EAC who visited the endoscopy unit of the Erasmus MC – University Medical Center Rotterdam or the IJsselland Hospital, Capelle aan den IJssel were invited to participate in this study. Participants underwent upper endoscopy for evaluation of reflux-related symptoms, surveillance of BE, or odynophagia or dysphagia. The presence of RE was confirmed at endoscopy and the length of any columnar-lined segment was determined by measuring the distance between the squamo-columnar junction and the proximal margin of the longitudinal gastric folds. Participants were only included if they had i) RE without the presence of BE, ii) BE defined as a columnar lined segment in the esophagus of ≥ 2 cm in length with specialized intestinal metaplasia at histology found in at least one of the biopsies taken, or iii) EAC defined at histology as an adenocarcinoma in BE or adenocarcinoma of the gastro-esophageal junction with more than 50% of the tumor mass in the esophagus. Biopsies were taken from patients with macroscopic signs of columnar metaplasia. Only samples from patients with histological BE were included in the BE group, but due to our criteria patients who only had histological evidence for BE at the z-line or at <2cm from the z-line were excluded from our study. In the patient population that we studied there were three patients with BE at 1cm and two with BE at 0cm. These five patients were excluded from present study. The diagnosis of BE and EAC is usually made by 2 two experienced gastro-intestinal pathologists.

Between October 2004 and April 2005, healthy volunteers (referred to as normal control subjects) were recruited from the general population via general practitioner practices. They were assessed by means of a questionnaire and were eligible as control if they had a negative history for reflux symptoms and esophageal disease. Patients who had a past history of antacids, H2-receptor antagonists, or proton pump inhibitor use, or suffered from reflux complaints, retrosternal pain, or regurgitation were excluded from the control group.

This study was approved by the local institutional review boards of the two participating hospitals. All participants were genetically unrelated Dutch Caucasians, and aged over 18. This study was approved by the local ethical review boards and all participants signed a written informed consent prior to participation in this study.

Genotyping of the Myo9B (rs2305764) polymorphism

Genomic DNA was extracted from 5 ml of whole blood by standard procedures (Wizard Genomic DNA Purification-kit; Promega, Madison, WI) and put in sterile 96 Well polystyrene suspension culture microplates (Greiner-Bio, Alphen a/d Rijn, Netherlands). Analysis of the Myo9B polymorphism (rs2305764) was performed by a competitive allele-specific PCR system (Kbiosciences, Herts, UK) on 20 ng DNA by a technician who was unaware of the histopathological findings for the patients and controls [24].

Immunohistochemical analysis of Myo9B

A subpopulation of the 886 participants (i.e. patients with available paraffin embedded samples) were further studied for local esophageal Myo9B expression in biopsy specimens. BE patients using any type of acid suppression as medication were excluded. We collected samples of non-reflux controls (n=20), RE (n=24), BE (n=63), and EAC (n=40) patients.

Paraffin samples were cut at 4μm, deparaffinized in xylene, hydrated using a graded series of alcohol, and stained with hematoxylin and eosin (H&E). An expert gastrointestinal pathologist blinded for the results of the genotyping, scored all H&E slides for squamous epithelium, RE, BE, and EAC. The sequential paraffin slide was used for Myo9B staining after inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in 100% methanol for 20 minutes. Subsequently antigen retrieval was performed by boiling the slides in 10mM monocitric acid (pH 6.0) for 15 minutes, and allowing them to slowly cool down to room temperature. The slides were then blocked with donkey serum and normal human plasma for 20 minutes at room temperature. The sections were stained using a primary antibody against myosin IXB (goat-anti-human Myo9B; 1:100 dilution; Santa Cruz, Heidelberg, Germany). Binding of the primary antibody was visualized by the addition of a secundary biotinylated antibody (donkey-anti-goat IgG1; 1:200 dilution; DAKO, Glostrup, Denmark), and streptavidin-avidin-biotin-complex labeled with horseradish peroxidase (strep ABComplex; 1:200 dilution; DAKO). EAC staining always showed a strong cytoplasmic expression. As negative controls for IHC an isotype control was used, and the primary antibody was omitted. Two independent observers (VM, KvZ) evaluated the immunohistochemical stainings. The slides were assessed using a Zeiss microscope (Axioskop 20, Carl Zeiss, Sliedrecht, The Netherlands) with a standard magnification (200x), and the images were recorded with a Nikon camera (DS-5M-U1) and Nikon Eclipse Net 2000 software (Nikon, Badhoevedorp, The Netherlands).

Statistical analysis

The study was powered (80%) to allow detection of a 10% difference in allele distribution between the patient groups (significance level 5%). Differences between allele distributions of the Myo9B polymorphism, as well as differences between the patient groups in number, age, and gender were determined by chi-square analysis. Age and sex corrected odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for the association between healthy controls and RE, BE, or EAC respectively by logistic regression analysis. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted with the SPSS software package v11.1 (SPSS, Chicago, IL).

Results

Patient characteristics

In total, 886 subjects were recruited, including 198 healthy controls, 305 patients with RE, 254 with BE, and 129 with EAC were included in this study. Of the 254 BE patients, 17 were on any type of acid suppression as medication. The patient characteristics are summarized in Table 1. Male gender was more common in BE ($p<0.05$) and EAC ($p<0.005$) compared to the healthy subjects and the RE group. On average, patients with BE ($p<0.005$) and EAC ($p<0.005$) were slightly older than healthy controls and RE patients (Table 1). When comparing the EAC group with the BE group, the expected higher age ($p=0.154$) and prevalence of male gender ($p=0.005$) of EAC patients were confirmed (Table 1).

Table 1.
Characteristics of the study population.

Patient data	Control	RE	BE	EAC
Number	198	305	254	129
Age	56±16	55±15	61±14**	63±10**
Male (%)	59	55	69*	82**
Length of BE segment (cm)	N.D.	0	4.2±1.9	N.D.

*p=0.05 **p<0.005

G/G-genotype is more frequent in BE and EAC, but not in RE

Using the 198 non-reflux controls as a reference, the association of a Myo9B polymorphism was tested for patients with RE (n=305), BE (n=254), and EAC (n=129). The allele frequencies for the Myo9B polymorphism (rs2305764) among the 198 unrelated, Dutch Caucasian controls was 41.9% (A) and 58.1% (G) respectively (Table 2). This is comparable to the European HapMap distribution of 42.2% (A) and 57.8% (G). The allele frequency of the major allele (G) was 60.8% in RE, 63.4% in BE, and 62.4% in EAC (Table 2). The distribution of the three genotypes in this cohort was G/G (41%), A/G (34%), and A/A (25%). The European HapMap shows a comparable distribution for G/G (35%), A/G (47%), and A/A (19%). The distribution of genotype frequencies for the polymorphism investigated was consistent with Hardy-Weinberg expectations in both the patient and control groups ($p > 0.05$).

Myo9B A/G heterozygosity was observed more frequently in EAC (OR=4.52;

Table 2.
Percentage distributions for the alleles of the Myo9B polymorphism tested

Alleles	Control N=198	RE n=305	BE n=254	EAC n=129
G/G	81 (41%)	110 (36%)	102 (40%)	50 (39%)
A/G	68 (34%)	151 (50%)	118 (47%)	61 (47%)
A/A	49 (25%)	44 (14%)	34 (13%)	18 (14%)
Allele frequency				
G	0.581	0.608	0.634	0.624
A	0.419	0.392	0.366	0.376

95% CI:1.99-10.25), but not in RE (OR=1.58; 95% CI:0.74-3.37), and BE (OR=2.28; 95% CI:0.87-5.95) compared to the control group. Myo9B G/G homozygosity in RE (OR=1.87; 95% CI:0.84-4.15) was not different compared to our control group, but it was observed more frequently in BE (OR=2.96; CI:1.10-7.99) and EAC (OR=2.23; CI:1.01-4.92) (Table 3), indicating that the G/G genotype is significantly associated with the presence of BE and/or EAC.

Table 3.
Comparison of the Odds Ratios calculated for the genotypes in the Myo9B polymorphism

Genotype	RE vs control OR (95%CI)	BE vs control OR (95%CI)	EAC vs control OR (95%CI)
G/G	1.87 (0.84-4.15)	2.96 (1.10-7.99)	2.23 (1.01-4.92)
A/G	1.58 (0.74-3.37)	2.28 (0.87-5.95)	4.52 (1.99-10.25)
A/A*	1 (control)	1 (control)	1 (control)

* Minor allele

Myo9B immunohistochemistry

Immunohistochemical staining with Myo9B has to our knowledge never been performed. Myo9B specific staining revealed that the localization of Myo9B in esophageal biopsy specimens is exclusively present in the esophageal epithelium. (Figure 1). In RE tissue Myo9B expression was observed in low intensity and primarily in the cytoplasm of the basal layer and not in the squamous epithelium itself (Figure 1.A and 1.B). In contrast, in BE and EAC there was strong cytoplasmic expression of Myo9B (Figure 1.D and 1.F). Figure 1.C and 1.E represent the negative controls.

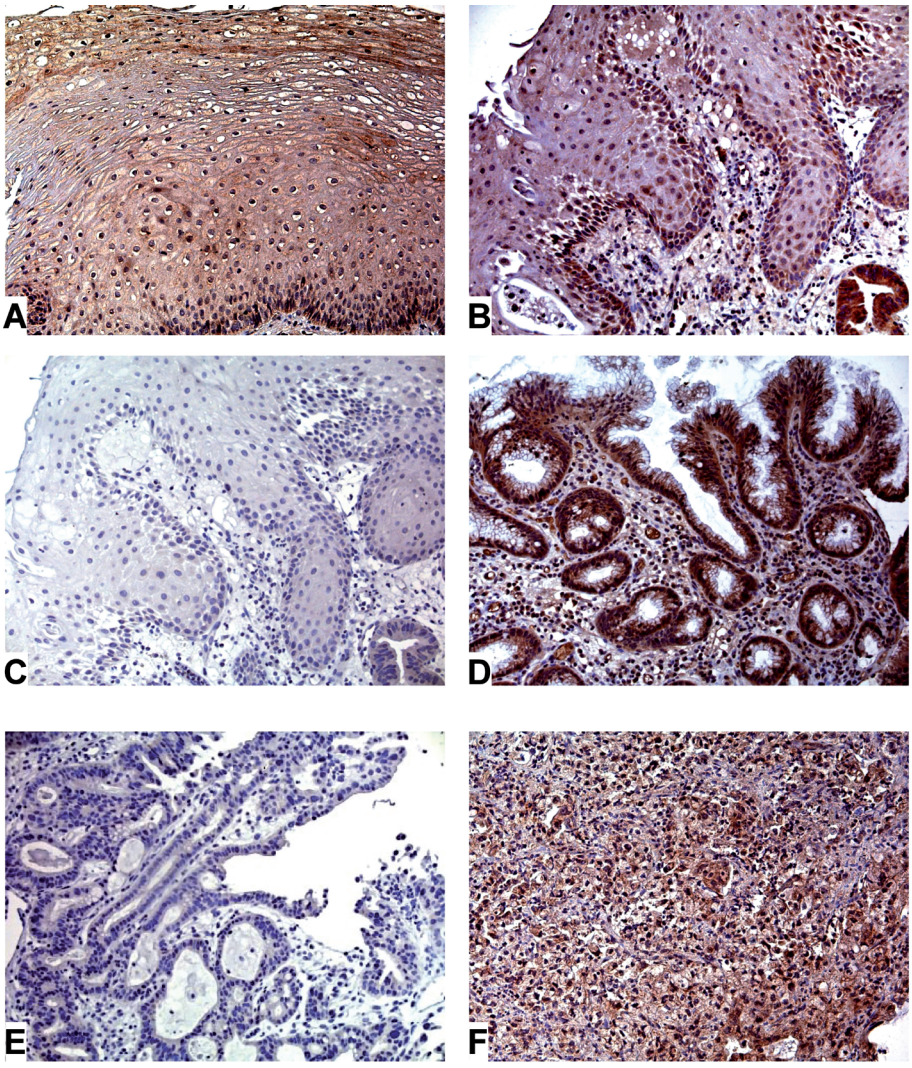

Figure 1

Typical examples of the Myo9B expression. Myo9B expression is present in the cytoplasm of epithelial cells. Myo9B is expressed in the basal layer of RE (n=24) (1.A/B), no expression is seen in the negative control (1.C). Myo9B is over-expressed in epithelial cells of BE (n=63) (1.D), absent in the negative control (1.E), and over-expressed in epithelial cells of EAC (n=40) (1.F)

Discussion

In current study, we found that the homozygous G/G group of the Myo9B polymorphism was associated with an increased risk for BE (OR: 2.96; CI: 1.10-7.99), and EAC

(OR: 2.23; CI: 1.01-4.92) development. Also the heterozygous the A/G genotype was associated with an increased risk for EAC development (OR: 4.52; CI: 1.99-10.25). There was no clear association between the tested SNP and RE (Table 3).

To our knowledge no other studies, than a study from our group that was published in Abstract[25] have focused on Myo9B in relation to RE, BE and EAC and this is the first study to show an association between Myo9B and esophageal neoplasia. Although association of homozygous A/A and heterozygous A/G genotypes of Myo9B have been reported to more than one intestinal inflammatory disease [14, 26], its role remains controversial because of failed replication efforts [15-18, 27]. There are a number of potential reasons for discrepancy between these studies, most well recognized in complex genetic trait studies [28], e.g. heterogeneity between the British, Italian, Swedish, and Dutch populations. In the Dutch study, a statistically significant and replicable association was found for a common variant (rs2305764) in intron 28 of Myo9B and celiac disease ($p=2.1x10^{-6}$) [14]. The frequency of the A allele in these Dutch controls of the celiac study was 37.9%, very similar to South Spanish controls (37,1%), but different from North Spain (39%), Italy (40%), UK (42.3%), Norway (42.6-43.1%), and our healthy controls. The allele frequencies among our 198 Dutch Caucasian control subjects for the Myo9B polymorphism (rs2305764) was 58.1% (G) and 41.9% (A), respectively, which is comparable to the European HapMap distribution of 57.8% (G) and 42.2% (A). The distribution of the three genotypes in this cohort was G/G (41%), A/G (34%), and A/A (25%), and again the European HapMap shows a comparable distribution of 35% for G/G, 47% for A/G, and 19% for A/A. The slight differences in the percentages of the European HapMap cohort compared to our healthy controls can be explained by the composition of the cohort, as the Hap Map project did not exclude persons with gastroesophageal reflux disease (Table 2). This confirms the quality of the reflux-free, healthy control group included in present study on the Dutch population.

The sequential RE, BE, and EAC patients who visited our endoscopy unit were included. This means that some patients were already in surveillance programs, but this did not lead to bias in age, gender or genotype. The age of our study groups is similar to the age in previous studies that describe the incidence and prevalence of BE and EAC [29-31]. The mean age at BE presentation is 55 years. As above-mentioned, the genotype distribution has been compared with the European HapMap population and did not show (statistical) difference [32].

Genetic variation in the 3'region of the myosin IXB (Myo9B) gene seems impor-

tant to the gastrointestinal inflammatory response [33], as SNPs in this gene predispose to celiac disease and inflammatory bowel disease [21]. However, it remains unclear at present how genetic variation affects cellular mechanisms involving Myo9B.

Only a subgroup of patients with long-standing reflux esophagitis will develop Barrett's esophagus. The exact mechanism remains unknown, but immunological, environmental and genetic factors likely play a role. Our Myo9B polymorphism indicates that the homogenous G/G genotype predisposes to BE and EAC, and might be (one of the) key factor(s) in patients with long-standing reflux esophagitis at risk for BE and EAC, while the Myo9B polymorphism is not a risk factor for reflux esophagitis itself. The exact mechanism needs further elucidation in future studies.

Myo9B is predominantly expressed in highly motile cells such as leukocytes; undifferentiated cells express Myo9B in the cell periphery, while its localization is more cytoplasmic in differentiated cells of human myelocyte cell lines [20]. We here show for the first time that there is also local Myo9B expression in the esophagus. Our results showed a strong Myo9B cytoplasmic expression in RE at the basement membrane, and strong expression in the columnar epithelial cells of BE and EAC (Figure 1). Perhaps the strong local expression of Myo9B in BE and EAC is induced by the local inflammation as apparently reflux per se is not sufficient to generate an increased epithelial permeability, because high expression is not manifested in RE patients. Interestingly it was reported that all BE patients have a significant and dramatic transepithelial leak [34] and this may be the result of the aberrant Myo9B expression that we observed in these patients. Larger studies need to be performed to define a role for local Myo9B in the esophageal stages of RE, BE, and EAC.

In conclusion, we demonstrate that the homozygous G/G Myo9B (rs2305764) genotype is significantly associated with a increased risk for the development of BE and EAC. This suggests that the local epithelial barrier function is essential for the predominant development of BE and EAC in patients with RE.

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CHAPTER 8

CONVERSION OF METAPLASTIC BARRETT'S EPITHELIUM INTO POST-MITOTIC GOBLET CELLS BY γ-SECRETASE INHIBITION

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Translational Impact Box

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 re-growth following treatment.

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 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 [1-3]. In BE, the multi-layered 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 [4]. 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 [5-7]. An unsuspected adenocarcinoma is identified in approximately 30-40% of esophagi that are resected for high-grade dysplasia, [8-13]. 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 [14]. Although the management of high-grade dysplasia is controversial, most institutes consider esophagectomy if the diagnosis is confirmed by pathology [12, 13, 15].

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 rapid conversion of all proliferative cells into differentiated goblet cells [16]. The activation of Notch signaling is critically dependent on an intramembrane protease complex termed γ-secretase [17-19]. This protease complex is also implicated in the pathogenic processing of the amyloid precursor protein in Alzheimer's disease [20]. 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 [16, 21-24], while (pre-)clinical studies have revealed a single major side effect of γ-secretase inhibitors: the induction of goblet cells in the intestine [25].

Multiple Notch pathway components are expressed in intestinal crypts and, together, constitute a functional signaling pathway [16, 21, 26, 27]. 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 [28-32].

Results

Notch signaling in human biopsy specimens

To study several parameters of Notch signaling, we used immunohistochemistry on serial sections of normal human colon (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 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 [33, 34]. The prototype human *HES* gene, *HES1*, is controlled by Notch signaling in the intestine [16, 21]. 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 [16, 21, 35], as in other tissues [36], 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 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 [37]. 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 [16, 21, 24], readily reduced *HES1* mRNA levels (Fig. 3B).

Notch signaling in BE rat model

We then sought to investigate the effects of γ-secretase inhibitor treatment in a

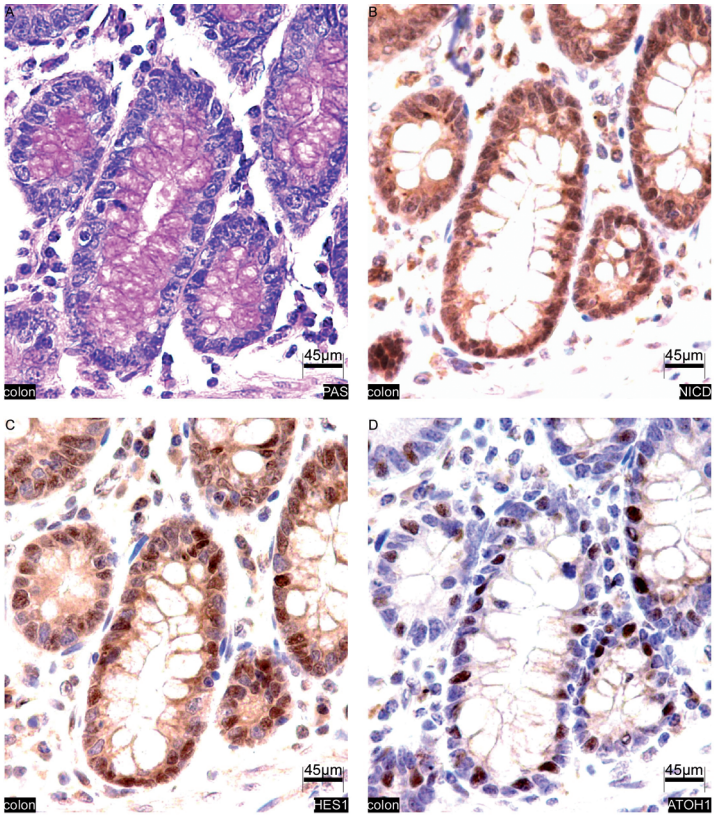

Fig. 1.
Notch pathway components in serial sections of the human colon.
A: *PAS staining for goblet cells (pink) in crypt structures of the colon.*
B: *NICD staining (brown) occurs in virtually all epithelial nuclei, indicative of active Notch signaling. Note the negative (blue) nuclei of stromal cells.*
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 colon. Note that ATOH1 is repressed by active Notch signaling.*
Scale 1:0.000045

well-validated rat model for BE [28-32] 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

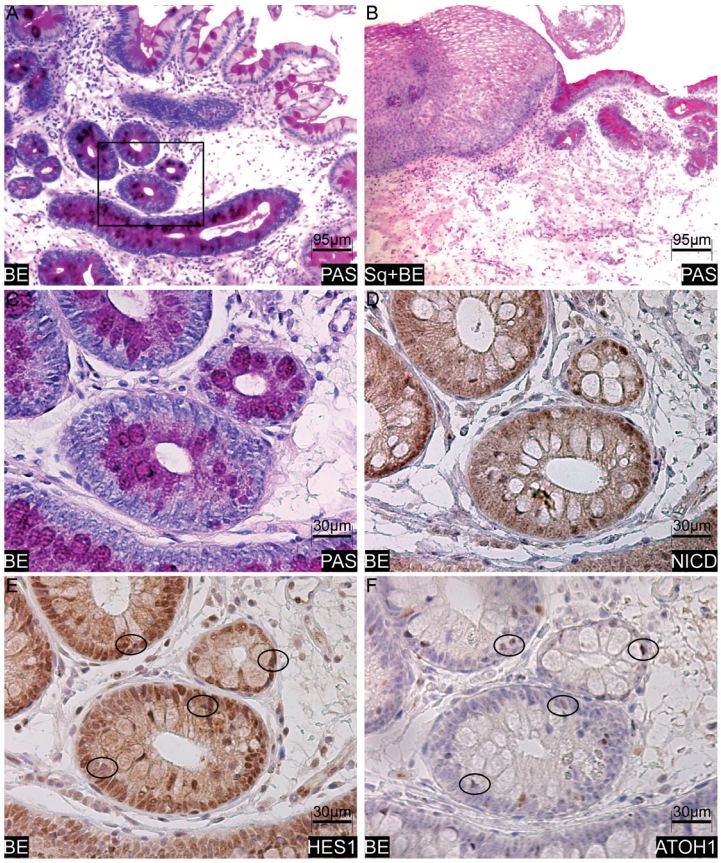

Fig. 2.

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. Note that ATOH1 is repressed by active Notch signaling.*

Scale: A,B 1:0.000095; C,D,E,F 1:0.00003

immunohistochemistry (Fig. 4E). Nuclear ATOH1 staining, although clearly present, was only observed in a few scattered cells (Fig. 4F).

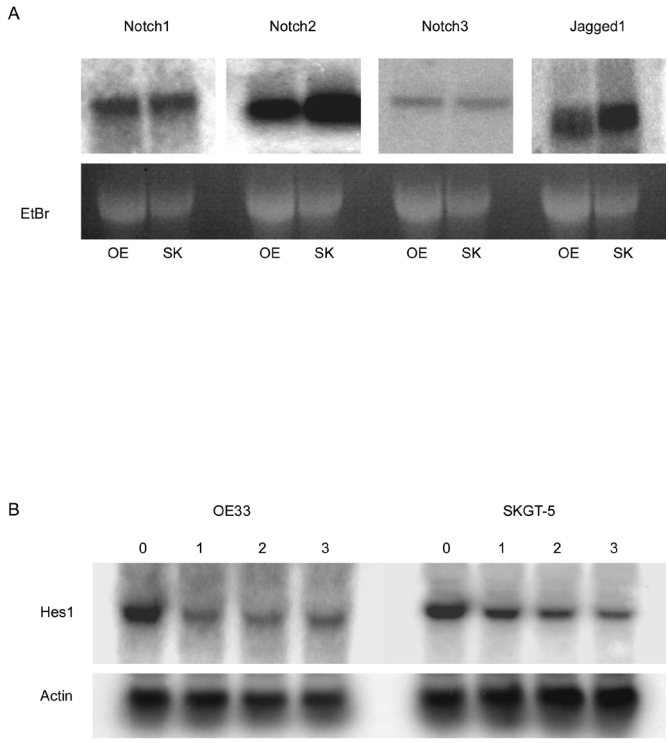

Fig. 3.

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 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 [24] caused efficient goblet cell conversion in the small intestine of rats after five daily intra-peritoneal injections at 30 mmol/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),

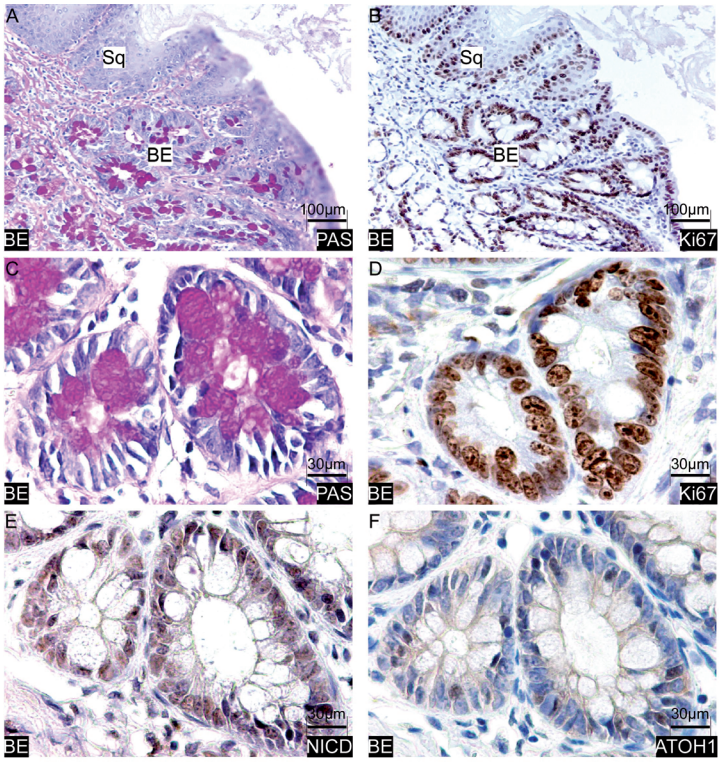

Fig. 4.

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 present 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 intra-nuclear staining in the rat BE, indicative of active Notch signaling.*

F: *TOH1 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.

Scale: A,B 1:0.0001; C,D,E,F 1:0.00003

and healthy control rats that had squamous epithelium lining the normal esophagus (data not shown).

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 [16, 21], 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 on 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 derepressed 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 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.

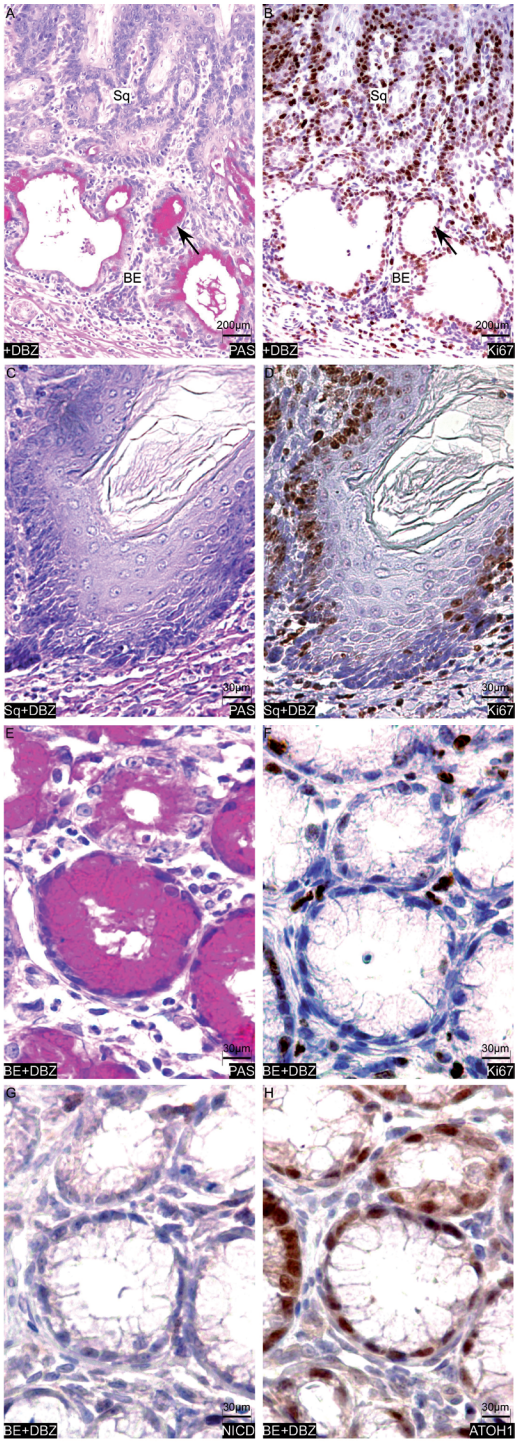

Fig. 5.

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 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 an intra-nuclear 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 derepression of ATOH1 gene expression, which controls the goblet cell fate.*

Sq, Squamous epithelium; BE, Barrett's Esophagus epithelium

Scale: A,B 1:0.0002; C,D,E,F,G,H 1:0.00003

Discussion

The golden standard for diagnosing BE is the histology of columnar epithelium with goblet cells [38]. 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 [4]. Surgical resection of the esophagus takes place when patients are at the high-grade dysplasia or EAC stage of disease [13]. 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 [4].

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[16].

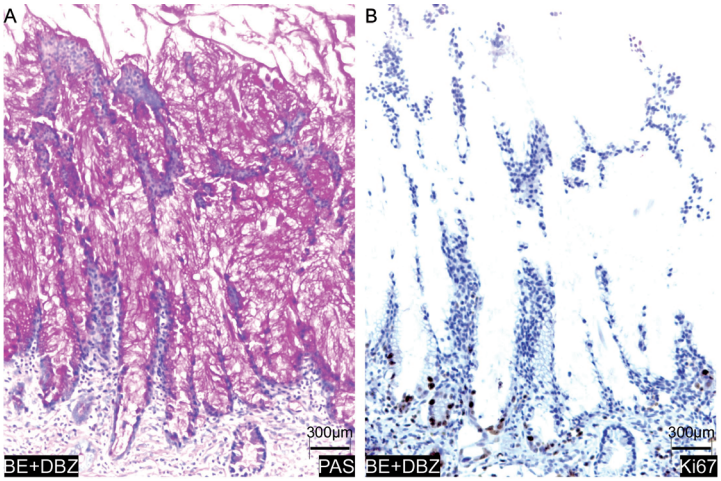

Fig. 6.
DBZ treatment can induce virtually complete exfoliation of BE epithelium.
A,B: *Serial sections of a BE epithelial region showing the extensive effects of DBZ treatment. PAS staining (pink)* ***(A)*** *and Ki67 staining (brown)* ***(B)*** *reveal that post-mitotic goblet cells have dissolved 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.*
Scale 1:0.0003

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 [16, 21, 24], readily reduced mRNA levels of the Notch target gene *HES1*, which is indicative of Notch pathway inhibition. When applied in 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 [28, 30-32].

This study indicates that Notch inhibition by DBZ in BE mirrors the effects on the normal absorptive epithelium of the intestine [16] 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 [25]. Phase II studies have already taken place to test the safety, tolerability and response to γ-secretase inhibitors [39-41]. Since the lesions in BE reside in a tissue environment that essentially appears to be refractory to the principle 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 premalignant condition.

Methods

Histology All histology was performed as described elsewhere [16, 21].

Antibodies For immunohistochemistry, serial sections of 4 mm 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-Math1, 1:3000; [42], 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, Salisburg, UK) and SKGT-5 [37], cultures were incubated for the indicated number of days in 200 nM of DBZ in cell culture flasks (Greiner bio-one). 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-*Hind*III 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*, *Hind*III-*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 carried out all of the animal handling. After an acclimatization period of 1 week, the animals were operated on. BE was induced on twelve rats by gastrectomy with esophagojejunostomy, as previously reported [21, 28-31]. 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 mmol/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

Hans Clevers and Johan van Es are inventors on patent applications that claim the use of Notch inhibitors as a treatment for intestinal diseases.

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CHAPTER 9

Summary and Discussion

The incidence of esophageal adenocarcinoma (EAC) has been rising dramatically in Western countries over the last four decades [1,2]. The impact of EAC on an individual is devastating because current treatment options are limited and the odds of survival remain low. Barrett's esophagus (BE) represents the only identified precursor lesion and most important risk factor for EAC. Patients with BE have a 30- to 125-fold greater risk of developing EAC than the general population [3].

The incidence of BE itself has also increased markedly since the 1970's. This increase was once felt to be due to the increased use of diagnostic upper endoscopy, however, data from our own center provided compelling evidence that in The Netherlands, the incidence of BE increased from 14.3/100,000 person years in 1997 to 23.1/100,000 person years in 2002 in the general population independent of the number of upper endoscopies [4].

Although genetic factors might predispose individuals to EAC, the rapid rate of increase in incidence must be due to non-genetic factors, both behavioral and environmental. BE is predominantly a disease of middle-aged white males. The prevalence of BE increases until a plateau is reached in the seventh decade. The most well-documented risks for the presence of BE include increasing age, male gender, Caucasian ethnicity, cigarette use, increased body mass index, severe and long standing gastroesophageal reflux disease (GERD), and absence of H. pylori infection.

Cigarette use rose in Western countries during the first decades of the 19th century but declined steadily since the 1970s, so this seems unlikely to explain the increased incidence of BE, although the prolonged latency between exposure to cigarettes and development of BE could still be an explanation. If this were the case, one could expect a subsequent decline in BE incidence over the next two decades.

Obesity promotes symptomatic and asymptomatic GERD through mechanical effects [5]. In addition, adipose tissue is metabolically active [6], and secreted adipokines have been associated with the development of a number of cancers [7,8]. The rapid rise in incidence in EAC may thus in part be due to the increasing prevalence of obesity.

H. pylori infection causes chronic gastritis which eventually can lead to development of atrophic gastritis with decreased production of gastric acid [9]. This explains the observation in a range of studies of an inverse association between H. pylori infection and both BE and EAC. This is further in line with the fact that the rise in BE and EAC in many countries correlated with the decline in H. pylori preva-

lence, both as a result of a birth cohort effect with changes in infection transmission, as well as due to H. pylori eradication therapy.

All together, BE is uncommon in the general population (1.3-5.6%) and the risk of EAC is low for an individual BE patient. However, the diagnosis of BE can have considerable impact on an individual patient. It is therefore imperative that treating physicians provide accurate information and appropriate counseling concerning risks of cancer and expectations from surveillance or treatment strategies. Screening strategies based on reflux symptoms alone will miss almost 50% of BE patients [10]. The current symptom- and risk factor-based screening concepts are general and probably lead to over-screening. More specific markers are needed and it would be helpful to further understand the process of development of GERD, BE and eventually EAC to identify the patients at risk.

The aim of the work described in this dissertation was to identify the histological and immunohistochemical characteristics in the development of GERD and BE, to evaluate biomarkers and genetic factors involved in the GERD-BE-EAC cascade, and eventually to introduce a possible option for therapeutic intervention.

In **Chapter 2**, the development of BE is represented in a rodent surgical model with esophagojejunal reflux. We found that reflux esophagitis preceded the development of BE in Wistar rats. All rats develop BE 6 months after surgical esophagojejunostomy and gastrectomy (GEJ). The development of BE started in a patchy design at the submucosal layer and was considered an immune-mediated disease. We revealed an early stage of esophageal tissue damage (0-3 months post-GEJ) characterized by esophagitis and increased numbers of macrophages and cytotoxic T cells without significant metaplasia, and a late stage of esophageal tissue damage (3-6 months post-GEJ) characterized by intestinal metaplasia with goblet cells compatible with BE, with a marked influx of eosinophils, B cells, and plasma cells. There is now overwhelming evidence supporting the association of GERD and BE. However, the role of individual constituents of the gastric and duodenal reflux in the development of BE and its associated complications still remain uncertain. Patients on PPIs and post-gastrectomy are still at risk for BE. This suggests a possible role for duodenal contents including bile acids [11, 12]. Our GEJ rat model showed the development of BE after duodenal reflux. The conversion from squamous into columnar epithelium was a mimic of human BE histology. Parallel with the development of intestinal metaplasia, a transition of the immune response pattern was noted. In esophagitis, the submucosal layer

showed a cell-mediated Th1-like inflammation profile, while in BE, the submucosal layer contained a predominant humoral Th2-like inflammation profile. This is in line with previous observation in humans. In chapter 2, we show that this switch of the immune response is related to the development of BE in an inflamed esophageal mucosa.

The esophageal epithelium is embryologically, morphologically, and functionally related to the skin epithelium, which is recognized as a major immunological organ. Along the same lines, we can consider the esophagus an immunological organ. The reflux of gastric and duodenal content may activate T cells in the submucosal layer that in turn release cytokines and chemokines. The cytokine profile of the mucosal immune response might explain the outcome of gastroesophageal reflux, the severity of mucosal injury, and even the relapse of esophagitis [13]. Thus, esophageal damage may start in the submucosal layer and predominantly depend on cytokine secretion. Indeed, in our rat model, the inflammatory infiltration occurred early and appeared to be restricted to the submucosal layer. In contrast, mucosal alterations, such as basal cell proliferation and papillary cell hyperplasia occurred after two weeks (**Chapter 2 and 3**). After 5 weeks, the first intestinal metaplasia appeared in the submucosal layer.

We then tested for a functional role of the immune response in the development of esophagitis and BE in our GEJ rat model in several rat strains with different immunological background (**Chapter 3**). We found that surgically induced chronic reflux in genetically different rats induced esophagitis leading to BE in all species. This process was associated with an active immune response. We found that all Th1-prone Lewis rats developed BE at 12 weeks after GEJ compared to 50% of Th2-prone BN rats and 33% of intermediate Wistar rats. Lewis rats also tended to develop a larger Barrett segment, implying that a Th1 predisposition is likely to be associated with the development of BE. Depending on the predisposition of the Th1/Th2 immune response, an antigenic stimulus is likely to affect different effector cells, i.e., monocytes, macrophages and cytotoxic T cells in animals with a preferential Th1 response and mast cells in animals with a Th2 response. The Th1 prone Lewis rats showed Th1 effector cells at the early onset of the hyperplasia-esophagitis-BE cascade as well as Th2 effector cells in an early stage compared to BN and Wistar rats. Therefore, Lewis rats expressed an early Th1 immune response in hyperplasia and a strong and early Th2 immune response in BE. This suggests that a Th1-predominant immune status may predispose to the development of BE.

The pro-inflammatory tumor necrosis factor (TNF) is a key cytokine in both systemic inflammatory responses and anti-tumor activity [14, 15]. Esophageal TNF expression is responsive to local concentrations of reflux components [16] and inflammatory cytokines [17], but is also controlled on a genetic level. A polymorphism in the promoter region of the TNF-β gene [18] affects both the production of TNF-α and TNF-β [19, 20], and deregulation has been associated with an increased risk of intestinal cancer development [21, 22]. In **Chapter 4**, we show a positive association between the functional TNF-β NcoI polymorphism, that decreases TNF production, and susceptibility to BE and EAC development in patients with chronic gastro-esophageal reflux. While TNF-α protein levels were invariably high in esophageal biopsies from EAC patients, most esophageal BE samples showed low to moderate TNF levels. In this study, the significantly higher frequency of the TNF-β **Nco**I A/A genotype and the local TNF expression in patients with BE and EAC indicate that the pro-inflammatory cytokine TNF plays a role in the development of BE and EAC.

In BE, oxidative stress has been strongly linked to disease progression [23, 24]. This oxidative stress can be induced in epithelial cells by exposure of these cells to both bile acid [25] and acid components of refluxate [26]. In normal physiology of the gut and liver, the nuclear Pregnane X Receptor (PXR) is an important factor in the detoxification of xenobiotics and bile acid homeostasis. In **Chapter 5**, we show that PXR was highly expressed in adenocarcinoma tissue and columnar Barrett's epithelium, compared to squamous epithelium of these BE patients ($p<0.001$), and esophagitis patients ($p=0.003$). PXR appeared to be activated upon bile acid stimulation. A genetic association was observed between the PXR polymorphism and BE. Together, these data imply that PXR may have a function in progression of esophageal disease.

The stratified squamous epithelium of the healthy esophagus possesses a variety of intrinsic defenses that enable it to resist acid-peptic reflux, divided in pre-epithelial defense, epithelial defense, and post-epithelial defense [27, 28]. Growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) are associated with epithelial proliferation and restitution that are the two key mechanisms involved in esophageal epithelial defense against acid, maintaining epithelial integrity and enabling rapid repair after injury [29]. Multiple signaling pathways are activated by binding of TGF-α and EGF with their receptor EGFR [30, 31], resulting in proliferation and differentiation of epithelial tissues [32, 33]. In **Chapter 6**, we show that a genetic variant of EGF is associated with reduced EGF expression and increased risk for esophagitis, BE and EAC development. This indicates that

reduced mucosal protection resulting from genetically decreased EGF expression enhances esophageal tumor development.

Myo9B is predominantly expressed in the immune system since macrophages purified from Myo9B knockout mice exhibit defects in chemotactic motility [34, 35]. Genetic variation in the 3'region of the myosin IXB (Myo9B) gene on chromosome 19 has been associated with inflammatory intestinal disorders, like celiac disease and inflammatory bowel disease [36-39]. These diseases are often characterized by increased paracellular permeability in the intestinal epithelium [40, 41]. Since Myo9B is also expressed in the intestinal epithelial cell [42], it could play key roles in Rho mediated regulation of the mucosal barrier, disruption of which could also contribute to celiac disease and inflammatory bowel disease. Increased mucosal permeability is recognized as one of the earliest histological changes in GERD patients [42]. In **Chapter 7**, we show that, genetic variety of Myo9B was associated with an increased risk for BE and EAC development. Cytoplasmic Myo9B expression was determined in esophagitis, BE, and EAC, but most prominent in epithelial cells of BE and EAC. Genetic variation of Myo9B might play a role in the etiology of BE and EAC by increasing the permeability of the epithelial barrier.

Since the metaplastic columnar epithelium of BE is similar to colonic epithelium, the Notch pathway, which is a signaling cascade that is central to both normal and neoplastic colonic development, might be involved. In **Chapter 8**, we show using esophageal 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, a known inhibitor of the Notch pathway, was used to treat rats with surgically induced BE. 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. These data imply that local application of Notch inhibitors may present a simple therapeutic strategy for BE conversion.

Conclusions

The research presented herein identified immunological targets in rodent models

with GERD-like disease, in the development of esophagitis and BE. This thesis suggests that patients with a Th1 immune status may predispose to the development of BE. These findings are supported by the positive association between the genetic variation of the pro-inflammatory cytokine TNF-α and the development of BE and EAC, but not esophagitis.

The progression of squamous epithelium in the healthy esophagus into metaplastic columnar epithelium in BE is a multifaceted process. One of the culprits which elicits a defensive cellular response is DNA damage. The mechanisms of damage become synergistic when bile acids are combined with acid. DNA damage becomes more pronounced and the cells are forced to adapt, incur mutations, and progress to cancer. This supports our findings that genetic variation in EGF and Myo9B, that both influence mucosal permeability and defense, are associated with the development of BE and EAC. PXR is activated upon bile acid stimulation, and may promote submucosal inflammation in GERD preceding BE development.

We then found that the Notch pathway is active in BE and EAC. 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 Notch inhibitors may present a simple therapeutic strategy for BE.

Future directions:

The esophageal mucosal damage can be caused by other environmental factors including esophageal microbial flora. Therefore, we looked at bacteria at several stages of the esophagitis-metaplasia-EAC cascade and found that Corynebacteria, S. Aureus, Veillonella and Streptococcus may be related to BE (not published). Esophageal and mucosal bacteria need to be further evaluated.

We tested for a functional role of the immune response in the development of esophagitis and BE in our GEJ model in several mouse strains with different immunological background. None of the mice developed BE at 16 weeks after surgery, but Th2 prone GEJ mice showed a trend towards a more extensive esophagitis segment in the esophagus compared to other mouse strains. To determine the role of the immune response in esophagitis and BE, studies with knockout mice would be helpful.

Although BE is a pre-malignant condition, a therapeutic strategy like Notch inhibition would prevent EAC. Further studies are required to optimize a method of Notch delivery and, importantly, to determine the nature of any epithelial regrowth following treatment.

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Samenvatting en Discussie

De incidentie van het adenocarcinoom van de slokdarm (EAC) is de laatste 4 decennia sterk gestegen in de westerse landen [1, 2]. De diagnose is ingrijpend voor een patiënt, want de huidige mogelijkheden voor behandeling zijn zeer beperkt en de overlevingsfactor is laag. Barrett slokdarm (BE) is de enige bekende voorbode van EAC en ook de belangrijkste risicofactor. BE patiënten hebben een verhoogd risico van 30- tot 125-maal om EAC te ontwikkelen in vergelijking met de normale populatie [3].

De incidentie van BE is duidelijk toegenomen sinds de 70-er jaren. Deze groei werd eerst gewijd aan de toename van het aantal maagonderzoeken, maar data van onze eigen onderzoeksgroep toonden voldoende bewijs dat de incidentie van BE in de gemiddelde populatie in Nederland werkelijk toegenomen is van 14.3/100000 persoonsjaren in 1997 naar 23.1/100000 persoonsjaren in 2002, onafhankelijk van het aantal uitgevoerde maagonderzoeken [4].

Genetische factoren kunnen de aanleiding zijn tot de ontwikkeling van EAC, maar de snelle groei van de incidentie komt eerder door gedrags- en milieufactoren dan door een genetische invloed. BE is hoofdzakelijk een ziekte van blanke mannen op middelbare leeftijd. De prevalentie van BE neemt toe totdat een plateaufase bereikt is rond de leeftijd van 70 jaar. De grootste risicofactoren voor BE zijn ouder worden, mannelijk geslacht, caucasisch ras, roken, overgewicht, hevig en langdurig zuurbranden bij gastro-oesofageale reflux ziekte, en de afwezigheid van een H. pylori infectie.

Roken nam in westerse landen gedurende de eerste decennia van de 19e eeuw toe, maar neemt weer gelijkmatig af sinds 1970, dus het lijkt onwaarschijnlijk dat roken van invloed is op de toegenomen incidentie van BE, alhoewel de vertraagde latentietijd tussen sigarettengebruik en de ontwikkeling van BE nog steeds een verklaring zou kunnen zijn. Als dit laatste het geval is, dan wordt de komende 2 decennia een afname van de BE incidentie verwacht.

Overgewicht veroorzaakt symptomatisch en asymptomatisch zuurbranden door een mechanische storing [5]. Bovendien is vetweefsel metabool actief [6], en de uitscheiding van adipokines is geassocieerd met de ontwikkeling van verschillende soorten kanker [7, 8]. De snelle toename van EAC kan komen door de toename van overgewicht en vetzucht in de bevolking.

Een chronische maagontsteking door de bacterie H. pylori kan tot verlies van de slijmbekleding van de maag lijden en daardoor verminderde productie van maag-

zuur [9]. Dit verklaart waarom vele studies een omgekeerd evenredige associatie tussen H. pylori infectie en zowel BE als ook EAC gevonden hebben. Andere studies tonen aan dat de groei van BE en EAC in veel landen correleert met de afgenomen prevalentie van H. pylori, onder andere als een gevolg van verminderde overdracht tijdens de geboorte maar ook door H. pylori eradicatie therapie.

Samenvattend concluderen we dat BE relatief weinig voorkomt in de gemiddelde populatie (1.3-5.6%) en het risico op de ontwikkeling van EAC voor de individuele patiënt laag is. Desalniettemin heeft de diagnose 'Barrett slokdarm' een groot effect op de individuele patiënt. Het is dan belangrijk dat de arts nauwkeurige en duidelijke informatie geeft over mogelijke behandelingen en het risico op kanker. Verder moet het nut van poliklinische controle uitgelegd worden. Wanneer wordt gescreend op basis van klachten van gastro-oesofageale reflux (zuurbranden) dan worden 50% van BE patiënten over het hoofd gezien [10]. De huidige screening is gebaseerd op symptomen en risicofactoren, maar deze aanpak is te algemeen en voert ertoe dat te veel mensen gescreend worden. Er zijn specifieke markers nodig, zodat het proces van gastro-oesofageale reflux ziekte dat leidt tot BE en uiteindelijk EAC ontdekt wordt, zodat de risicopatiënten geïdentificeerd kunnen worden.

Het doel van dit promotieonderzoek was om de histologische en immunologische kenmerken in de ontwikkeling van gastro-oesofageale reflux ziekte en BE te bepalen, om biomarkers en genetische factoren te vinden die betrokken zijn bij de cascade van reflux oesofagitis-BE-EAC, en uiteindelijk om een mogelijke optie voor de therapeutische interventie van BE te introduceren.

In **Hoofdstuk 2** is de ontwikkeling van BE in een chirurgisch rattenmodel met gallige reflux in de slokdarm aangetoond. Wij hebben gevonden dat reflux oesofagitis voorafgaat aan de ontwikkeling van BE in Wistar ratten. Alle ratten ontwikkelen een Barrett slokdarm op 6 maanden na de operatie met de techniek van oesofagojejunostomie en gastrectomie (GEJ). In een Barrett slokdarm is gezond plaveiselcel epitheel vervangen door cilindrisch epitheel. Het nieuwe cilindrisch epitheel is darmachtig weefsel en daarom heet het proces intestinale metaplasie. We toonden in ons rattenmodel aan dat de ontwikkeling van BE begint als een onregelmatige verdeling van cilindrisch epitheel in de onderhuid en dit wordt gezien als een immuun gestuurde ziekte. We ontdekten een vroeg stadium van weefselschade in de slokdarm (0-3 maanden na GEJ) gekarakteriseerd door oesofagitis en een grote hoeveelheid macrofagen en cytotoxische T cellen zonder significante metaplasie, en een laat stadium van weefselschade in de slokdarm (3-6 maanden

na GEJ) gekarakteriseerd door intestinale metaplasie met slijmbekercellen (goblet cellen) zoals bij mensen, met een duidelijke instroom van eosinofielen, B cellen en plasma cellen. We hebben inmiddels voldoende bewijs dat er een associatie bestaat tussen reflux ziekte en BE. Echter, de rol van individuele bestanddelen uit de maagzuur en gallige reflux in de ontwikkeling van BE en de geassocieerde complicaties blijft onbekend. Mensen die maagzuurremmers nemen of patiënten na gastrectomie (operatieve verwijdering van de maag) hebben nog steeds een risico op BE. Dit suggereert dat er een rol is weggelegd voor componenten uit de dunne darm inclusief galzuren [11, 12]. Ons GEJ rattenmodel toonde de ontwikkeling van BE na gallige reflux uit de dunne darm. De conversie van plaveiselcel- in cilindrisch epitheel lijkt histologisch precies op humane BE. Parallel met de ontwikkeling van intestinale metaplasie vindt een transitie plaats van de immuun respons. Bij oesofagitis zagen we in het onderhuids weefsel een cel-gemedieerde Th1-achtige ontsteking, terwijl bij BE voornamelijk een humorale Th2-achtige ontsteking in het onderhuids weefsel plaatsvond. Dit komt overeen met voorafgaande BE studies in mensen. In Hoofdstuk 2 laten we zien dat omslag van de immuun respons is gerelateerd aan de ontwikkeling van BE door chronische reflux.

Het slokdarmepitheel lijkt embryologisch, morfologisch en functioneel op epitheel van de huid, dat wordt gezien als een groot immunologisch orgaan. Daarom kunnen we de slokdarm ook beschouwen als een immunologisch orgaan. Reflux van maagzuur en dunne darm inhoud kunnen T cellen activeren in het onderhuids weefsel waardoor cytokines en chemokines vrijkomen. Het cytokine profiel van de onderhuidse immuun respons zou het resultaat van gastro-oesofageale reflux kunnen verklaren, zoals de ernst van de onderhuidse schade en het ontstaan van oesofagitis [13]. De oesofageale schade dus kan beginnen in de onderhuid en voornamelijk afhangen van de cytokine uitscheiding. In ons rattenmodel zagen we inderdaad dat er een vroege infiltratie van de ontsteking plaatsvond die beperkt bleef tot de onderhuid. Echter, 2 weken na de operatie (GEJ) traden onderhuidse veranderingen op, zoals proliferatie van de basale cellen en papillaire hyperplasie (**Hoofdstuk 2 and 3**). Na 5 weken ontstond de eerste intestinale metaplasie in de onderhuid.

Vervolgens werd de functionele rol van de immuunrespons onderzocht tijdens de ontwikkeling van oesofagitis en BE met behulp van ons GEJ rattenmodel bij verschillende rattenstammen met uiteenlopende immunologische achtergronden (**Hoofdstuk 3**). We zagen dat chirurgisch geïnduceerde chronische reflux bij genetisch verschillende ratten leidde tot oesofagitis en BE in alle stammen. Dit proces

was geassocieerd met een actieve immuun respons in het slokdarmweefsel. We vonden dat alle Lewis ratten met een bekende Th1-voorkeur BE ontwikkelden op 12 weken na GEJ in vergelijking met 50% van de BN ratten met Th2 voorkeur en 33% van de Wistar ratten met gemengde Th1/Th2 voorkeur voor de immuun respons. Lewis ratten hadden de neiging om een langer BE segment te ontwikkelen, wat betekent dat een Th1 voorkeur waarschijnlijk geassocieerd is met de ontwikkeling van BE. Afhankelijk van de voorkeur voor de Th1 of Th2 immuun respons, treedt een antigeen stimulans op die van invloed is op het vrijkomen van de verschillende effector cellen, bijvoorbeeld monocyten, macrofagen en cytotoxische T cellen bij de Th1 respons en mestcellen bij de Th2 respons. De Th1 Lewis ratten hadden een vroege expressie van Th1 effector cellen in de hyperplasie-oesofagitis-BE cascade, terwijl juist de Th2 effector cellen werden gezien in een vroeger stadium van BE ontwikkeling dan bij BN en Wistar ratten. Hieruit kunnen we concluderen dat Lewis ratten een vroege Th1 immuun respons tonen bij hyperplasie en een vroege en uitgebreide Th2 immuun respons tonen in BE weefsel. Onze bevindingen lijken erop te wijzen dat een Th1 voorkeur voor de immuun respons tijdens chronische reflux de aanzet kan zijn voor de ontwikkeling van BE.

De pro-inflammatoire tumor necrosis factor (TNF) is betrokken als een belangrijke cytokine bij zowel de systemische ontsteking als ook bij anti-tumor activiteit [14, 15]. TNF expressie in de slokdarm is een reactie op locale concentraties van reflux componenten [16] en inflammatoire cytokines [17], maar wordt ook gereguleerd op een genetisch niveau. Een polymorfisme in het promoterdeel van het TNF-β gen [18] heeft invloed op zowel de productie van TNF-α als TNF-β [19, 20], en deregulatie is geassocieerd met een verhoogd risico op de ontwikkeling van intestinale kanker [21, 22]. In **Hoofdstuk 4** toonden we een positieve associatie aan tussen het functionele TNF-β NcoI polymorfisme, dat de TNF productie negatief beïnvloedt, en de aanleg voor de ontwikkeling van BE en EAC bij patiënten met chronische gastro-oesofageale reflux. Hoewel het TNF-α eiwitgehalte zeer hoog was in slokdarm biopten van patiënten met EAC, brachten de meeste BE biopten een laag tot gemiddeld TNF eiwitgehalte tot expressie. In deze studie tonen we aan dat de significant hogere frequentie van het TNF-β ***Nco*I** A/A genotype en de locale TNF expressie bij patiënten met BE en EAC indiceren dat TNF als pro-inflammatoire cytokine een rol speelt in de ontwikkeling van BE en EAC.

Bij BE wordt oxidatieve stress gekoppeld aan progressie van de ziekte [23, 24]. De oxidatieve stress kan geïnduceerd worden in epitheelcellen door blootstelling van

de cellen aan zowel galzuren [25] als zure componenten in reflux [26]. De normale fysiologie van de darm en lever bevat de nucleaire Pregnane X Receptor (PXR) die een belangrijke rol speelt in het ontgiften van xenobiotica en de galzuurhomeostase. In **Hoofdstuk 5** toonden we aan dat er een hoge PXR expressie is in EAC en Barrett epitheel in vergelijking met het plaveiselcel epitheel van deze BE patiënten ($p<0.001$), en oesofagitis patiënten ($p=0.003$). PXR werd geactiveerd door stimulatie met glazuren. Een genetische associatie werd gezien tussen het PXR polymorfisme en BE. Samenvattend kunnen we concluderen dat PXR betrokken is bij de progressie van ziekten aan de slokdarm.

Het plaveiselcel epitheel van de gezonde slokdarm bevat een verscheidenheid aan intrinsieke afweermechanismen, dat leidt tot een natuurlijke weerstand tegen zure-gallige reflux, verdeeld in een pre-epitheliale afweer, een epitheliale afweer en een post-epitheliale afweer [27, 28]. Groeifactoren zoals epidermal growth factor (EGF) en transforming growth factor-α (TGF-α) worden geassocieerd met epitheliale proliferatie en compensatie, dat 2 mechanismen zijn die een hoofdrol spelen in de oesofageale epitheliale afweer tegen zuur, waardoor de epitheliale integriteit behouden blijft en snel herstel van schade plaatsvindt [29]. Multipele signaalroutes worden geactiveerd bij de binding van TGF-α en EGF met hun receptor EGFR [30, 31], dat resulteert in proliferatie en differentiatie van epitheel [32, 33]. In **Hoofdstuk 6** tonen we aan dat een genetische variant van EGF geassocieerd is met lage EGF expressie en een verhoogd risico op de ontwikkeling van oesofagitis, BE en EAC. Onze resultaten suggereren dat verminderde mucosale bescherming, door genetisch verlaagde EGF expressie, de ontwikkeling van slokdarmkanker stimuleert.

Myo9B komt voornamelijk tot expressie in het immuunsysteem aangezien macrofagen van Myo9B knockout muizen defecten vertonen in de chemotactische motiliteit [34, 35]. Genetische variatie in het 3'einde van het myosin IXB (Myo9B) gen op chromosoom 19 wordt geassocieerd met intestinale ontstekingsziekten, zoals coeliakie en inflammatory bowel disease (IBD) [36-39]. Deze ziekten worden vaak gekarakteriseerd door verhoogde paracellulaire permeabiliteit van het intestinale epitheel [40, 41]. Aangezien Myo9B ook tot expressie komt in intestinale epitheelcellen [42], zou het een rol kunnen spelen in de Rho gemedieerde regulatie van de mucosa barrière, en verstoring van de barrière zou kunnen leiden tot coeliakie en IBD. Verhoogde mucosa permeabiliteit wordt al vroeg gezien in de histologie van patiënten met gastro-oesofageale reflux ziekte [42]. In **Hoofdstuk 7** tonen we aan, dat een genetische variant van Myo9B geassocieerd is met een verhoogd risico op de ontwikkeling van BE en EAC. Myo9B

expressie werd aangetoond in het cytoplasma van oesofagitis, BE en EAC, en sterke expressie werd voornamelijk gezien in de BE en EAC epitheelcellen. Genetische variatie van Myo9B zou betrokken kunnen zijn bij het ontstaan van BE en EAC vanwege verhoogde permeabiliteit van de epitheel barrière.

Aangezien het metaplastische cilindrische epitheel van BE lijkt op darmepitheel, zou de Notch route, die een centrale signaleringscascade is voor zowel de normale als neoplastische ontwikkeling van darmweefsel, betrokken kunnen zijn bij BE ontwikkeling. In **Hoofdstuk 8**, tonen we in slokdarm biopten aan dat de Notch route niet actief is in normaal plaveiselcel epitheel van de slokdarm, maar dat Notch zeer actief is in de gebieden van slokdarm waar cilindrisch Barrett epitheel is ontstaan. Door middel van remming van de Notch route met dibenzazepine, een bekende remmer van Notch, hebben we bepaald of BE verwoest of geconverteerd kon worden in ons chirurgische GEJ rattenmodel met BE. Zoals eerder is aangetoond in normaal darmepitheel, converteerde Notch remming de proliferatieve Barrett cellen in compleet gedifferentieerde goblet cellen, terwijl het normale plaveiselcel epitheel ongeschonden bleef. In enkele gevallen was het Barrett epitheel volledig uitgekleed, waarbij een kale submucosa overbleef. De locale applicatie van Notch remmers zou een simpele therapeutische strategie kunnen zijn voor conversie van BE.

Conclusies

Het onderzoek dat hier gepresenteerd is identificeert de immunologische processen in een rattenmodel met reflux-achtige ziekte tijdens de ontwikkeling van reflux oesofagitis en BE. Dit proefschrift stelt voor dat patiënten met de Th1 immuunstatus neigen tot de ontwikkeling van BE. Deze bevindingen worden ondersteund door de positieve associatie tussen de genetische variant van de pro-inflammatoire cytokine TNF en de ontwikkeling van BE en EAC, maar juist niet van reflux oesofagitis.

De progressie van plaveiselcel epitheel in de gezonde slokdarm naar metaplastisch cilindrisch Barrett epitheel is een veelzijdig proces. De DNA schade lokt een defensieve cellulaire respons uit. De schade wordt synergistisch wanneer galzuren gecombineerd worden met maagzuur. Wanneer de DNA schade toeneemt worden de cellen gedwongen zich aan te passen, waardoor mutaties ontstaan, die uiteindelijk leiden tot kanker. Dit ondersteunt onze resultaten, omdat we aantonen dat genetische variatie in DNA van EGF en Myo9B, die beide betrokken zijn bij de mu-

cosa permeabiliteit en -afweer, geassocieerd is met de ontwikkeling van BE en EAC. PXR wordt geactiveerd door galzuur stimulatie en zou de ontsteking in de submucosa van gastro-oesofageale reflux ziekte die vooraf gaat aan de ontwikkeling van BE kunnen bevorderen.

Vervolgens hebben we uitgevonden dat de Notch route geactiveerd is in BE en EAC. Notch remmers converteren het proliferatieve Barrett epitheel naar compleet gedifferentieerde goblet cellen, terwijl het plaveiselcel epitheel ongeschonden blijft. Deze data impliceren dat locale applicatie van Notch remmers een simpele therapeutische strategie vormt voor conversie van Barrett epitheel.

Verder onderzoek:

De schade van de slokdarmweefsel kan door verschillende omgevingsfactoren veroorzaakt worden waaronder ook de microbiotische flora van de slokdarm. We hebben de bacteriële flora onderzocht op verschillende stadia van de reflux oesofagitis-BE-EAC cascade en vonden dat Corynebacteria, S. Aureus, Veillonella en Streptococcus gerelateerd zijn aan BE (niet gepubliceerd). Bacteriën in de slokdarm en op de mucosa moeten in vervolgstudies onderzocht worden.

We hebben de functionele rol van de immuun respons tijdens de ontwikkeling van reflux oesofagitis en BE in ons GEJ model bij meerdere muizenstammen met een verschillende immunologische achtergrond getest. Geen enkele van de muizen ontwikkelde binnen 16 weken na de GEJ operatie een Barrett slokdarm, maar muizen met een voorkeur voor de Th2 immuun respons toonden een trend richting een uitgebreide oesofagitis in vergelijking met de andere muizenstammen. Om de rol van de immuun respons bij reflux oesofagitis en BE te bepalen, zouden studies met knockout muizen ons verder kunnen helpen.

Alhoewel BE een premaligne stadium is, zou een therapeutische strategie met Notch remming de ontwikkeling van EAC kunnen voorkomen. Verder onderzoek is nodig om de veilige toediening van Notch te optimaliseren en, zeer belangrijk, om de aard van het epitheel te bepalen dat opkomt nadat Notch remming als therapie is toegepast.

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