Predisposing Role of Immunologic Determinants in the Etiology of Barrett's Esophagus

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Predisposing Role of Immunologic determinants in the Etiology of Barrett's Esophagus

Predisponerende rol van immunologische determinanten in de etiologie van een Barrett-oesofagus

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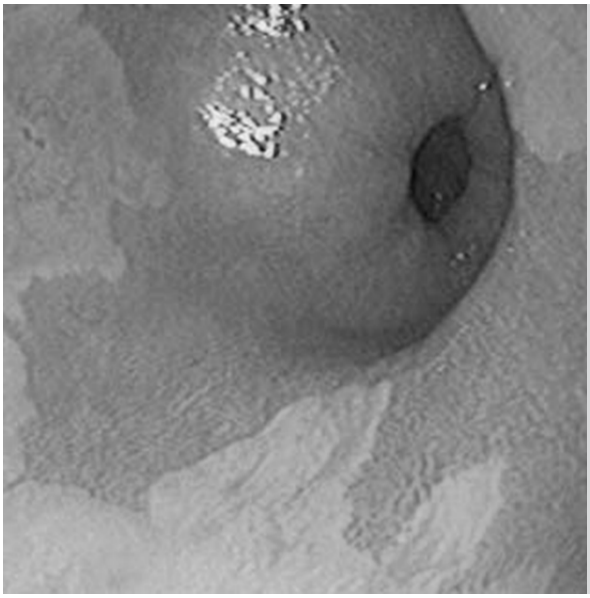

Chapter 1

General introduction

Leon M.G. Moons

GASTROESOPHAGEAL REFLUX DISEASE

Gastroesophageal reflux disease (GERD) is defined as the presence of symptoms or lesions that can be attributed to reflux of gastric contents into the esophagus. In the Western world, GERD represent one of the most common gastrointestinal problems [1,2]. Cardinal manifestations of GERD are the presence of reflux symptoms such as heartburn, and the presence of gastroesophageal reflux. Heartburn is very common in Western populations with over 40% of the general population having monthly symptoms, and approximately 20% having symptoms on at least a weekly basis [1].

GERD is a spectrum encompassing a broad range of conditions. Of all patients referred for upper GI endoscopy for heartburn as their major symptom, 20-40 % have a reflux esophagitis, 6-12% have a Barrett's esophagus (BE), and the remaining have endoscopy-negative GERD [3-10]. The diagnosis of severe reflux esophagitis is important as treatment can prevent the development of reflux related complications such as bleeding, stricture, and ulcers. Another important complication of chronic exposure to gastroesophageal reflux is the development of esophageal adenocarcinoma (EAC). Gastroesophageal reflux is considered to be the key element in the development of EAC since severe, longstanding, and frequent episodes of gastroesophageal reflux is associated with a seven- to eightfold increased risk of EAC. [2,11-13].

THE ESOPHAGITIS-BE-EAC SEQUENCE

EAC is an important cause of cancer-related deaths each year, and its incidence is rapidly rising [14,15]. Once EAC is detected, most patients are in an advanced disease stadium with distant metastasis already present. EAC is therefore associated with a poor prognosis with a mortality rate exceeding 90%. EAC is believed to develop secondary to gastroesophageal reflux and is thought to follow a sequence of disease stages beginning with inflammation (esophagitis), followed by the development of BE, low grade and high grade dysplasia, and eventually EAC (Figure 1). Progression to the different disease stages is paralleled by biological changes such as increased angiogenesis and proliferation, and decreased apoptosis, immune surveillance, cell cycle control and cell-cell-adhesion. Progression is also accompanied by acquired genetic alterations such as p53- and p16 gene mutations, aneuploidy, and loss of APC-gene heterozygosity (Figure 1).

The most important known risk factor for the development of EAC is the presence of BE, which is associated with a more than 30 fold excess risk of EAC [16,17]. BE is present in 6-12% of patients with reflux-related complaints[18], and 0.2-2% in the general population[19]. In this thesis we set out to identify new risk factors for BE in the GERD population, and to reveal new mechanisms involved in its development, this chapter will focus on the progression of reflux esophagitis towards BE.

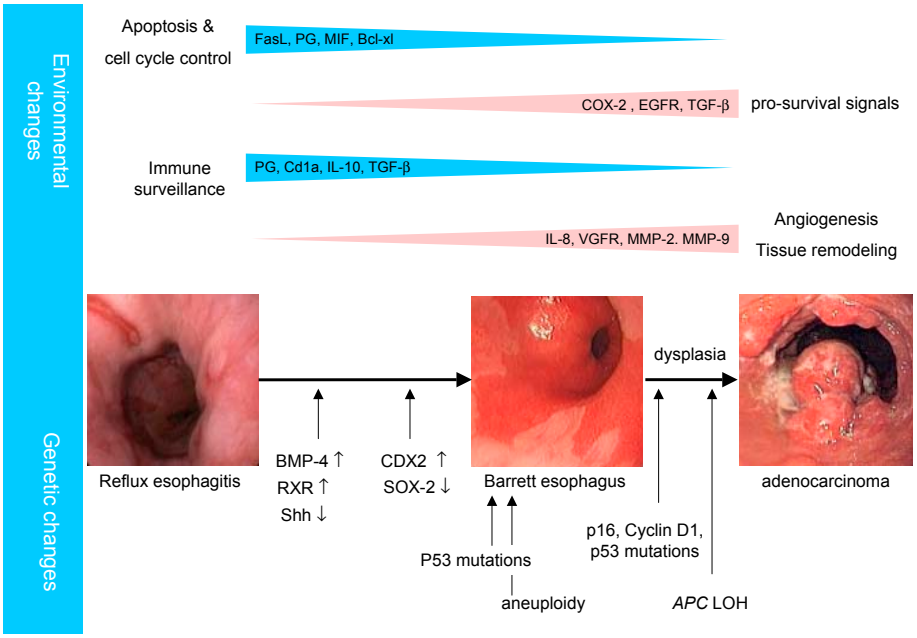

Figure 1: The inflammation-Barrett's esophagus-esophageal adenocarcinoma sequence. Esophageal adenocarcinoma (EAC) is thought to develop after consecutive disease stages beginning with inflammation, followed by Barrett's esophagus (BE), low grade- and high grade dysplasia, and eventually EAC. Progression to the different disease stages is accompanied by epithelial changes such as genetic changes and altered expression of epithelial differentiation regulators (CDX2, SOX-2, BMP-4, RXR, and Sonic hedgehog (Shh)), as well as changes in the surrounding environment resulting in increased epithelial survival, increased angiogenesis, decreased apoptosis and cell cycle regulation, decreased immune surveillance. PG; prostaglandins, EGFR; epithelial growth factor receptor, MMP; metalloproteinase, MIF; macrophages inhibiting facto, VEGF; vascular endothelial growth factor.

BARRETT'S ESOPHAGUS

Definition of Barrett's esophagus

BE is defined by the replacement of squamous epithelium at the lower part of the tubular esophagus by specialized intestinal epithelium (SIE), characterized by acid mucin containing goblet cells observed at histology (Figure 2). BE is therefore a histological diagnosis, which requires biopsies from the columnar lined segment to differentiate between gastric type metaplastic epithelium and BE.

Intestinal differentiation

BE epithelium consists of all four intestinal cell types including absorptive enterocytes, goblet cells, enteroendocrin cells, and occasionally Paneth cells. As specialized intestinal epithelium in BE has many similarities with intestinal epithelium in the intestine, it may well be that intestinal differentiation in the esophagus is regulated in the same way.

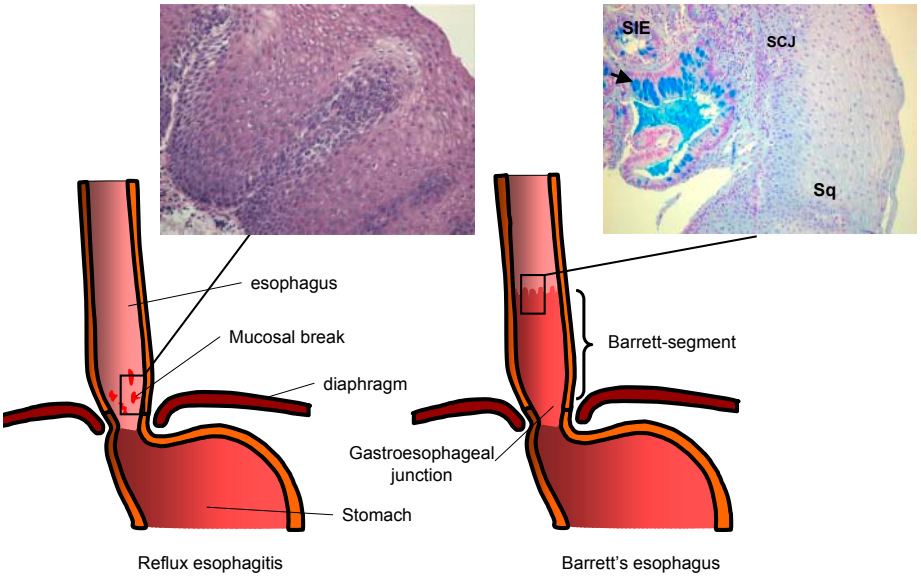

Figure 2: Anatomical localization and histological characteristics of reflux esophagitis and Barrett's esophagus. Mucosal erosions or ulcerations can develop in response to gastroesophageal reflux (reflux esophagitis). Histologically, esophagitis is characterized by papill elongation, basal hyperplasia, and appearance of intraepithelial eosinophils. BE is also thought to develop secondary to gastroesophageal reflux and is defined as the replacement of native squamous epithelium (Sq) by specialized intestinal epithelium (SIE). The histological picture shows the neo-squamocolumnar junction (SCJ) with squamous epithelium just beside SIE in an Alcian Blue stain, showing goblet cells in blue (arrow).

Self-renewal of the intestine is dependent on maintenance and migration of differentiated cells along the crypt-villus axis. If the stem/progenitor cell compartment is depleted the production of new epithelial cells is halted, resulting in villus atrophy and eventually desquamation of the epithelial lining. In the intestine four different epithelial cells are present; absorptive enterocytes, or a cell of the secretory cell lineage such as Paneth cells, goblet cells, and enteroendocrin cells. These cells do not derive directly from the pluripotent stem cells, but are produced from different populations of committed progenitor cells. Determination of cell fate is tightly regulated by signaling molecules. Different signals and expression of nuclear transcription factors determine the commitment of a specific progenitor population to a specific cell lineage.

A key protein in intestinal differentiation is the homeobox protein CDX2. Expression of CDX2 is detected at the time of morphogenesis in the visceral endoderm of mouse intestine [20] and continues to be present throughout adulthood, but then is normally restricted to the intestine [21]. It is detectable in the crypts of the intestine as well as in the villi [22,23]. Exogenous expression of CDX2 in IEC6 cells, an undifferentiated rat intestinal cell line which does not express CDX2, causes differentiation of IEC6 cells into goblet cells and absorptive enterocytes [24]. Similar observations have been made in an animal model, in which ectopic expression of CDX2 induced the development of metaplastic changes of the gastric antrum, and in Helicobacter pylori-related intestinal metaplasia

of the human stomach [25,26]. These metaplastic changes of the mouse gastric antrum were also characterized by the development of goblet cells and absorptive enterocytes, and the expression of intestine specific proteins such as MUC2, alkaline phosphatase, villin, guanylyl cyclase C and Trefoil factor 3 [27]. In contrast, heterozygous CDX2 knockout mice developed polyp-like lesions in their colon during the first 3 months of life, which lacked CDX2 expression [28]. These lesions were composed of heterotopic, well differentiated stratified squamous epithelium, stomach and small intestinal mucosa [29]. It was concluded that CDX2 directs epithelial differentiation toward a caudal phenotype.

As CDX2 seems to be a key regulator of intestinal differentiation, CDX2 may also be associated with the development of BE. Identifying the pathways leading to CDX2 expression in esophageal epithelial cells may provide insight in the mechanisms leading to the development of specialized intestinal epithelium. This pathway may also be a suitable target for chemoprevention for BE in GERD patients at risk of developing BE.

Risk factors involved in the development of BE

Development of BE in the population with GERD is associated with several risk factors such as the presence of a hiatal hernia, increasing age, male sex, Caucasian race, severe, long duration, and frequent episodes of GERD symptoms, and genetic predisposition. Some of them will be discussed in more detail.

Gastroesophageal reflux

Gastroesophageal reflux is thought to be the key element causing mucosal injury, leading to the development of reflux esophagitis, BE and EAC. BE is thought to be the continuum of severe gastroesophageal reflux, as BE is associated with higher amounts of acid and biliairy reflux, and is more frequently associated with abnormal esophageal motility than patients with reflux esophagitis [30,31]. Not surprising, the risk of BE increases in patients with conditions associated with esophageal motility disorders such as achalasia [32] and scleroderma [33] with a BE prevalence of 11% and 13% respectively. Abnormalities of esophageal motility in Barrett's esophagus are however identical to patients with severe reflux esophagitis [31,34], suggesting that additional factors than increased gastroesophageal reflux and abnormal esophageal clearance are needed for the development of BE.

Such a factor may be refluxate composition. Patients with dysplastic Barrett's epithelium or adenocarcinoma have more gastro-duodenal reflux into their esophagus than patients with uncomplicated BE and reflux esophagitis [35,36]. Reflux of acid alone has been shown to be able to induce mucosal injury, but the deleterious effect of reflux is aggravated by the addition of bile acids [37]. Especially when the refluxate's pH is between 3-5, bile acids are extremely injurious to the esophageal mucosa [38,39]. The risk of BE in the presence of mixed acid and bile reflux is a three-fold higher as compared to acidic reflux alone [37,40].

Reflux components may have a direct effect on cellular processes by stimulating several pathways resulting in the induction of protein expression such as chemokines (IL-8, TNF-α, and MIP-3α), cell cycle regulators (cyclin D1), growth factors (VEGF, EGFR), stress molecules (HSPs) [41] and differentiation markers (CDX2). All these biological pathways modulate cellular behavior itself, but also the surrounding microenvironment and thereby the interplay between epithelial- and stromal cells such as fibroblasts and immune cells. Especially bile acids have recently been shown to modulate mucosal homeostasis and epithelial differentiation via binding to nuclear receptors in epithelial- and stromal cells [42,43].

There is also another indirect mechanism in which gastroesophageal reflux is able to influence mucosal homeostasis. One of the earliest events leading to mucosal injury is the effect of reflux on intercellular junctions resulting in dilation of intercellular spaces and increase in membrane permeability [44,45]. It is therefore conceivable that impairment of the mucosal integrity by gastroesophageal reflux will result in exposure of the underlying lamina propria and its inflammatory cells to luminal components such as food antigens, bile acids, gastric chemicals, and bacterial products. This interaction will probably evoke an immune response with secretion of all kinds of biological active components.

Hiatal Hernia

Hiatal hernia refers to the herniation of parts of the abdominal contents through the esophageal hiatal of the diaphragm. This is believed to develop secondary to widening of the diaphragmatic hiatus, pulling up of the stomach by esophageal shortening, and pushing up of the stomach by increased intra-abdominal pressure. The frequency of hiatal hernia, like that of esophagitis, increases with age. The overall effect is that of increased esophageal acid exposure. The presence of a hiatal hernia is associated with symptoms of gastroesophageal reflux, increased prevalence and severity of reflux esophagitis, as well as BE and EAC [46]. In a study of 229 patients with BE and 229 patients with non-erosive GERD, the presence of BE was strongly associated with a hiatal hernia, more reflux episodes, and excess smoking and alcohol [47]. Cameron et al. looked at the prevalence and size of hiatal hernia in BE [48]. A hiatal hernia was found in 96% of patients with classical BE, 72% of patients with short segment (<2 cm) BE, 71% of patients with esophagitis, and 29% of controls with no esophagitis. Among patients with hiatal hernias, those with BE had wider hiatal orifices, and longer hiatal hernias compared to patients with and without reflux esophagitis [48].

Genetic predisposition

In addition to various of the above mentioned anatomic and environmental risk factors, there also seems to be a genetic component [49-52]. This is e.g. indicated by familial clustering of BE. In these families, the condition inherits in an autosomal dominant fashion

with incomplete penetrance [50]. As most BE is sporadic, for an individual to develop BE and hence EAC, environmental exposures need to interact with genetically determined characteristics that define one's personal susceptibility. This indicates that in the majority of cases, these inherited genetic factors are likely to be polymorphisms in multiple genes rather than single gene mutations.

Single nucleotide polymorphisms are variations in the gene present in at least 1% of the population. These variation can be present in the promotor region regulating transcription levels, in an exon changing the protein's tertiary structure and function, or in a non-coding region influencing mRNA stability or mRNA splicing variants. In genes encoding for inflammatory proteins such as cytokines, membrane receptors, enzymes, or intracellular signaling molecules, several polymorphisms have been described. Some of these polymorphisms are shown to have functional effect such as increased production of IL-12p70 associated with the IL-12B C-residue at position +1188 in the 3'UTR region, and are associated with several inflammatory conditions (Figure 3).

SURVEILLANCE AND SCREENING

Currently, patients with BE are invited to participate in surveillance programs with repeated endoscopy every three years in order to detect early progression to EAC [53]. Early recognition of high grade dysplasia or intramucosal tumors (T1 and T2 tumors) increases survival from EAC. As only 1 of the 200 BE patients will progress to EAC [18], this approach is topic of debate, and new methods are developed to identify those BE patients at increased risk of progression to EAC. Future improved surveillance programs will however still be hampered by another problem. More than 90% of the patients presenting with EAC were not recognized for having BE at an upper endoscopy before detection of their EAC [54-56]. This may be explained by the fact that a large group of

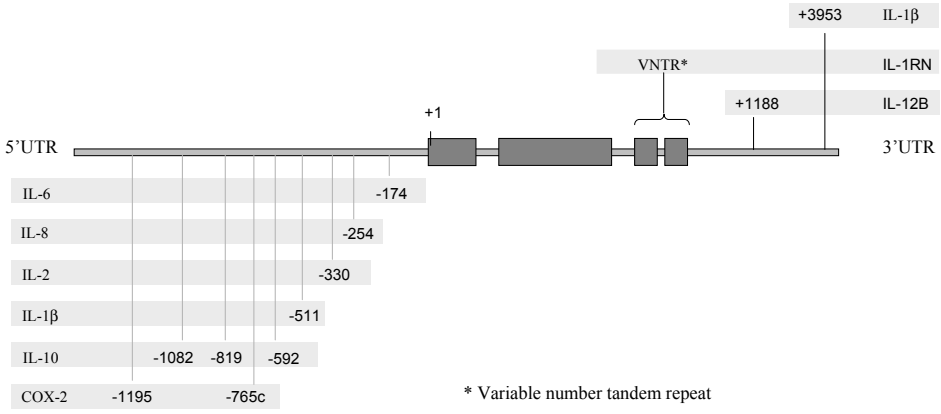

Figure 3 A schematic overview of genetic polymorphisms studied in this thesis.

patients with gastroesophageal reflux disease do not seek medical consultation for their reflux-related symptoms, or are not screened for the presence of BE. It seems likely that surveillance programs complemented by screening for BE in the GERD population could decrease EAC mortality and morbidity. Current guidelines do not recommend screening endoscopy, as this is not cost effective [57]. The two major risk factors for BE, reflux esophagitis and obesity, are very common in patients with GERD, and using these criteria for screening would soon overtax health resources. Only in patients with "alarm" signals such as chronic persistence of pyrosis and regurgitation of more than five years, screening endoscopy is advized. A recent study by Chak et al. showed that only two-third of the patients with EAC and only one-third of patients with adenocarcinoma of the gastroesophageal junction recall chronic reflux symptoms. They showed that using the cut-off point of 5 years of chronic reflux symptoms as a criterion will falsely exclude patients for screening. This is also supported by other studies which showed that severity of reflux symptoms can not predict findings at endoscopy [5,58]. As anamnestic criteria are insufficient for identifying those patients who would benefit from screening endoscopy, new non-invasive methods are needed to support screening. It is likely that the identification of markers associated with a high risk of developing BE, will follow from a better understanding of the etiology of BE.

This thesis was initiated to gain more insight in the immune mechanisms involved in the development of BE. In the following sections, different aspects of the progression towards BE are discussed.

INFLAMMATION AND BE

Inflammation and progression to BE

Chronic inflammation is thought to play an important role in progression to the different stages of disease. Reflux-induced inflammation of the esophagus precedes the development of BE, and severity of inflammation correlates with the development of BE and EAC [59,60]. Moreover, use of anti-inflammatory drugs such as non-steroid anti-inflammatory drugs (NSAIDs) and aspirin have been shown to diminish mucosal inflammation and result in a lower incidence of EAC and BE in both humans and animals [61-66]. This would be

Table 1

Inflammatory condition	Associated neoplasm	Inflammation induced by
Gastritis	Stomach cancer	*Helicobacter pylori*
Chronic pancreatitis	Pancreatic carcinoma	Alcohol, choledocholithiasis
Inflammatory bowel disease (IBD)	Colorectal cancer	Causes of IBD
Reflux oesophagitis	OAC	gastroduodenal content
Chronic cholecystitis	Gall bladder cancer	Bacteria, cholecystolithiasis
Hepatitis	Hepatocellular carcinoma	Hepatitis B and/or C virus
Sinusitis	Sinonasal adenocarcinoma	Wood dust

in line with observations made in other parts of the digestive tract, as population-based studies have shown that susceptibility to cancer increases when tissues are chronically inflamed (Table I) [67,68].

These links have been confirmed in a number of animal models, especially gastric (H. pylori) [69], liver (cholangitis), and colon (colitis) cancers [70]. It is therefore conceivable that chronic inflammation of the esophagus is involved in esophageal carcinogenesis, and that the presence of severe inflammation may predispose to progression towards BE.

Role of the mucosal immune response in the etiology of mucosal damage

A mixed inflammatory cell infiltrate is a common feature of acid and bile damage to the native esophageal mucosa, especially around the stem-cell rich areas of the basal mucosal compartment and papillae [71-73]. Recruitment of this inflammatory infiltrate by chemotaxis is crucial for the development of mucosal injury. Firstly, chemokines are expressed at increased levels in reflux esophagitis and BE as compared to controls, and expression levels of chemokines correlate with severity of disease and extent of the mucosal erosions [74,75]. This relation is also observed in patients with non-erosive reflux disease (NERD), where mucosal IL-8 levels were higher in patients with little evidence of damage to the esophageal mucosa at endoscopy and basal hyperplasia and papilla elongation at histology, than in NERD patients without these mucosal changes [75]. These increased IL-8 levels correlate well with numbers of intraepithelial neutrophils [75]. Secondly, reflux induced mucosal erosions can be prevented by treatment with ketotifen (an anti-inflammatory agent), superoxide dismutase (a radical scavenger), or with the intraperitoneal injection of anti-neutrophil serum [76-79]. These studies show that TNF-α and IL-8 are involved in the attraction of a mixed leukocyte infiltrate, which in turn is responsible for the development and extent of mucosal erosions by generating reactive oxygen species (ROS) (Figure 4) [76-79]. Whether inflammatory cells are the most important source of ROS, is however still under debate as another study showed that only 30-45% of ROS could be attributed to neutrophils [80].

Differences in mucosal inflammation between reflux esophagitis and BE

There are other mechanisms than ROS induced mucosal damage involved in the progression towards BE. BE is associated with increased acid exposure time, decreased lower esophageal sphincter pressure, and decreased motility as compared to moderate reflux esophagitis [34,83]. This difference is however absent between patients with severe reflux esophagitis and BE [34]. It is therefore conceivable that other mechanisms than severe reflux alone are involved in progression towards BE.

It has been noticed that an adaptive immunity is recruited to the site of inflammation in BE. This is illustrated by the following observations: i) T lymphocytes become more numerous in tissues where metaplastic foci develop [71], ii) After endoscopic ablation

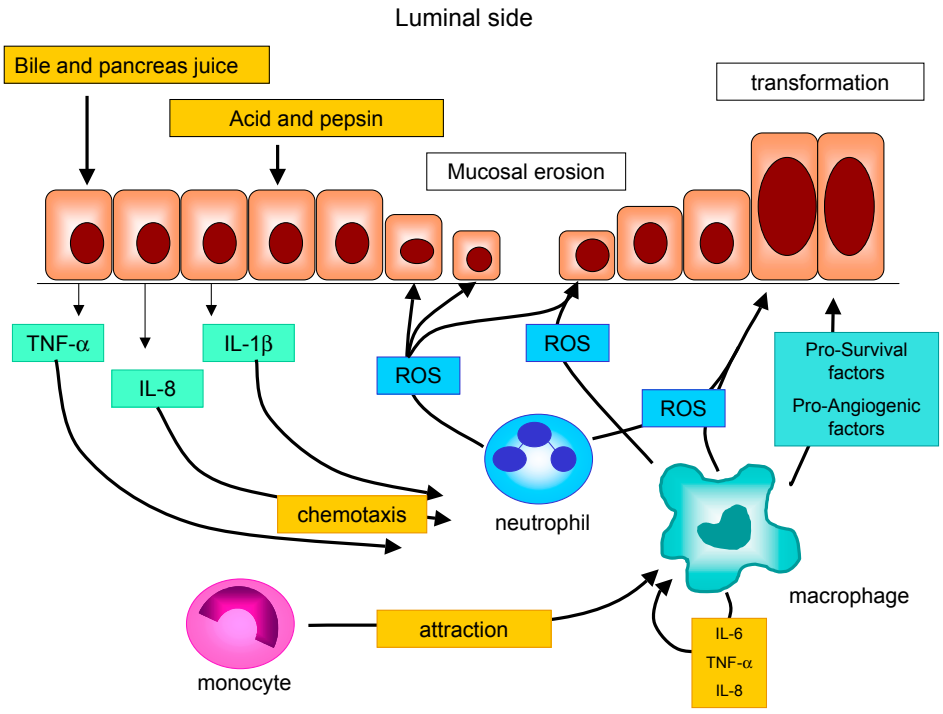

Figure 4: A proposed inflammation induced pathway leading to mucosal erosions and transformation of epithelial cells. Exposure of the esophagus to reflux of gastroduodenal content elicits an inflammatory response by inducing pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-8. This results in activation of the innate immune response by attraction and activation of neutrophils, monocytes, dendritic cells, natural killer cells, and macrophages. Neutrophils and macrophages are the source of reactive oxygen species (ROS) which can either result in increased epithelial damage, but is also capable of both directly damaging DNA and affecting the DNA repair machinery, thereby enhancing genetic instability of affected cells. Macrophages are potent sources of growth- and pro-angiogenic factors which additional provide pro-survival signals to transformed epithelial cells [81,82].

therapy, persistent BE is associated with a T-cell infiltrate, which is absent in the neo-squamous islands [84]. These findings suggest that lymphocytes in BE are not only secondary to gastroesophageal injury or loss of epithelial integrity, but their presence may also be stimulated by reflux-independent factors. The exact role of inflammation in the development of BE is still unclear, but several immunological characteristics of BE have been described. In a study comparing cytokine levels in biopsies taken from the proximal region of the BE segment (near the neosquamocolumnar junction) to biopsies taken from the distal region, an inflammatory gradient was observed with pro-inflammatory cytokines such as IL-1β, TNF-α and IL-8 being expressed at increased levels in the proximal region, whereas the distal region was associated with expression of regulatory cytokines such as IL-10 [85]. This is in line with findings at the esophageal-cardiac junction in patients with GERD, where interaction between squamous and columnar cells was associated with the strongest inflammatory response. BE is also associated with increased

expression of regulatory cytokines such as IL-4 and IL-10, while reflux esophagitis on the other hand is associated with increased transcription of pro-inflammatory cytokines such as IL-1β, IFN-γ and IL-8 [86]. The inflammatory signaling molecule NFκB has been shown to be increasingly activated in the inflammation-BE-EAC sequence [87], indicating that sustained inflammatory stimulation is a key feature in esophageal carcinogenesis. These findings suggest that BE is an adaptive tissue response to increased tissue damage secondary to gastroesophageal reflux, and that an increased pro-inflammatory response may therefore precede the change in mucosal lining.

HYPOTHESIS

Type I and type II immune responses

Antigens are presented to $CD4^+$ T cell populations via MHC II molecules on antigen presenting cells, such as dendritic cells, macrophages and B cells. These CD4+ T cells can differentiate into two types of effector cells, called Th1 and Th2 cells (Figure 5). Pathogens

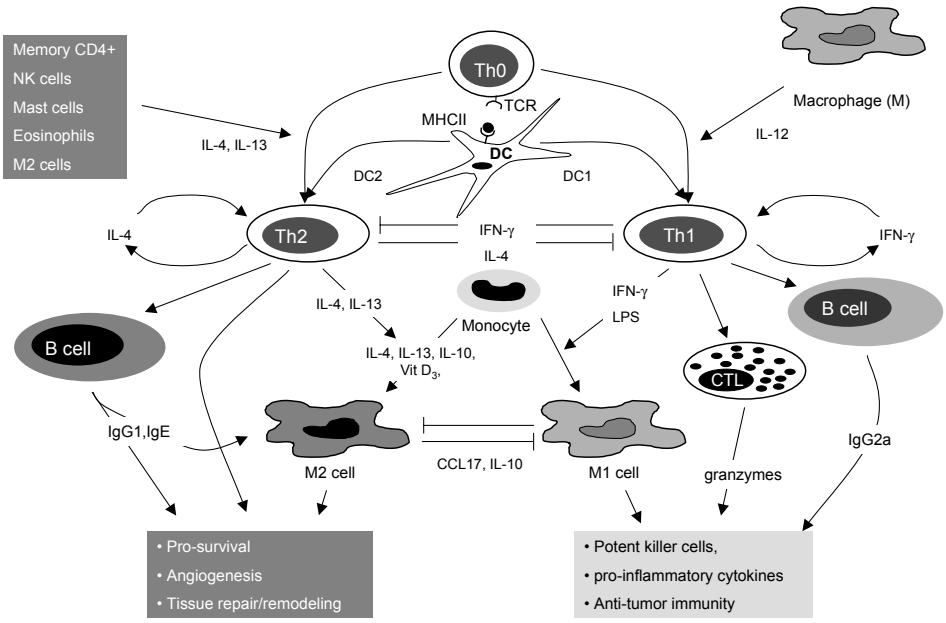

Figure 5 A simplified schematic overview of the polarization of adaptive immune responses. Activated antigen presenting cells such as dendritic cells (DCs) direct naïve T cells (Th0) to differentiate into either Th1 or Th2 cells. This differentiation into either Th1 or Th2 cells is dependent on cytokines, and on specific characteristics obtained by DCs during antigen processing, activation, and antigen presentation. Th1 cells direct cellular immune responses by classical activation of macrophages (M1), stimulation of a cytotoxic T cell (CTL) response, and direct B cells to make opsonizing antibodies. Th2 cells initiate the humoral immune response by alternative activation of macrophages (M2), and by inducing B cells to produce IgE and IgG1. For clarity, this schematic overview does not provide information on the role of regulatory cells such as regulatory T- and B cells, which are able to modulate these differentiation and activation pathways.

that accumulate in large numbers inside macrophages and dendritic cells vesicles tend to stimulate the differentiation of Th1 cells, whereas extracellular antigens tend to stimulate the differentiation of Th2 cells [88]. Th1 cells produce a distinct set of cytokines such as IFN-γ, IL-2 and lymphotoxin, and direct cellular immune responses by activating the microbicidal properties of macrophages and induce B cells to make IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. Th2 cells initiate the humoral immune response by activating naïve antigen specific B cells to produce IgM antibodies. These Th2 cells can subsequently stimulate the production of different isotypes, including IgA and IgE, as well as neutralizing and/or weakly opsonizing subtypes of IgG. Typical Th2 cytokines are IL-4, IL-13, IL-9, and IL-5. Th2 cells also influence macrophage differentiation, as Th2 cytokines are shown to activate the alternative activation route of macrophages, also referred to as M2 cells (Figure 5)[89]. M2 cells are a distinct population of macrophages which are, in contrast to their classically activated counterparts (M1 cells), involved in tissue repair by promoting angiogenesis, tissue remodeling, proliferation, and inhibiting cellular immune responses [89,90]. M1 cells on the other hand are potent killers and are important in bactericidal and anti-tumor immune responses. Th1/M1/cell mediated immune responses are referred to a type I and Th2/M2/humoral immune responses are referred to as type II. Both type I and II immune responses are extreme polarized phenotypes of human immunity, and therefore most inflammatory responses in the human body are a mix of both type I and II. The mucosal environment is greatly influenced by the phenotype of the immune response, and differences in immune composition and polarization influence disease outcome [91].

Th2 immune responses and cancer

Type II immune responses are believed to promote tumor development [67,92]. Immune compromized mice are more susceptible for the development of spontaneous carcinogenesis, which are either aggressive sarcomas or intestinal epithelial carcinomas. IFN-γ is the most important cytokine involved, as IFN-γ deficient mice are also more susceptible for carcinogenesis [93,94]. Experiments with mice infected with either Th1- or Th2 immune response inducing pathogens, have shown that type I responses were associated with a strong suppression of neoplastic growth in comparison to type II immune responses. This effect could be aggravated in IL-10 deficient mice, where the inhibitory effect correlated with increased serum IFN-γ levels [95]. This indicates that suppression of cellular immune responses results in decreased tumor surveillance. When a chronic mutagenic stimulus such as ROS, coexists with decreased tumor surveillance, the risk of tumor development increases. Enhanced anti-tumor immune activity was noticed in B cell deficient mice [98,99], and it is only recently that two mechanisms of B cell promoted carcinogenesis have been identified (Figure 6). B cells were shown to be responsible for suppression of natural killer cell and T cell mediated anti-tumor immunity, by influencing IFN-γ production at time of activation. In a HPV16 skin cancer model, products secreted

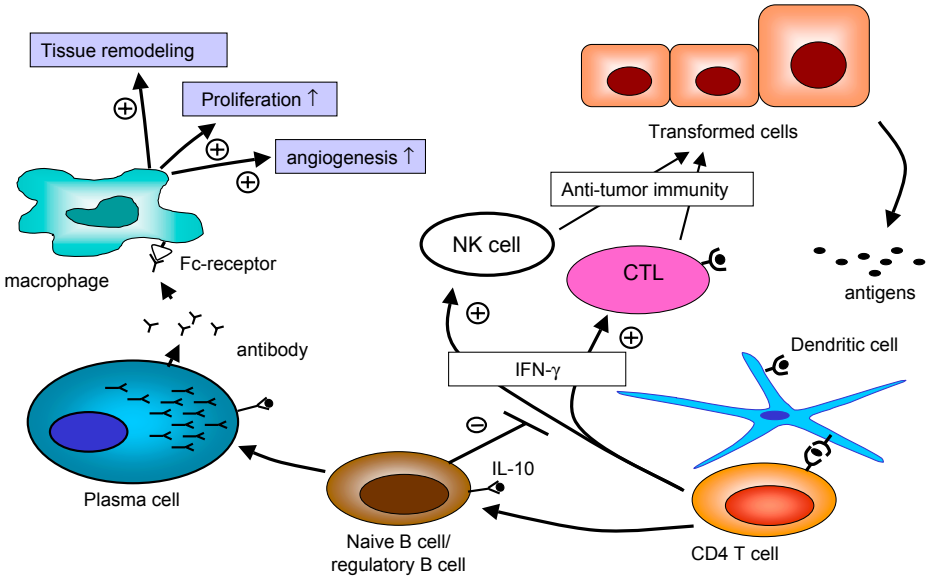

Figure 6: Humoral immunity and tumor promotion. Antigens expressed by transformed cells or other source, are taken up by dendritic cells. These antigen presenting cells activate responding T cell populations by presenting the antigens to CD4+ T cells, CD8+ T cells, and B cells, thereby generating an adaptive immune response. B cells can contribute to tumor development by i) inhibition of a cellular anti-tumor immune response mediated by activated cytotoxic T cells (CTLs) and natural killer (NK) cells, by influencing the IFN-γ and IL-10 balance at time of activation [96], and ii) by secretion of soluble factors (most likely antibodies), which are able to induce recruitment of innate immune cells, secretion of pro-angiogenic compounds, growth factors, and tissue remodeling [97].

by activated B cells were shown to be responsible for tumor development by recruitment of innate immune cells, inducing angiogenesis and proliferation, and remodeling of the extracellular matrix with upregulation of metalloproteinases (MMP) 2 and 9 (Figure 6). Interruption of this pathway resulted in a significant delay in carcinogenesis [97].

CONCLUSION

EAC is a lethal cancer, whose incidence is rising. BE is the most important known risk factor for the development of EAC, which develops secondary to gastroesophageal reflux. It is still largely unknown why only a subset of patients exposed to gastroesophageal reflux develop BE, and why only a small proportion of BE patients progresses to EAC. Inflammation seems to be a key feature of progression to BE and EAC, although the precise etiology is largely unknown. It can be hypothesized that, like in other (pre-)malignant disorders developing secondary to chronic inflammation, inflammation in BE promotes tumor development by obtaining specific characteristics, which distinguish it from the inflammatory response observed in reflux esophagitis. Development of a type II immune

response has been associated with such a tumor promoting microenvironment. The pathways leading to the development of such a type II microenvironment are unknown. It is however useful to identify the key factors leading to a type II microenvironment, as these factors may prove to be powerful targets for chemoprevention of BE and EAC in the GERD population, or may be used for screening purposes.

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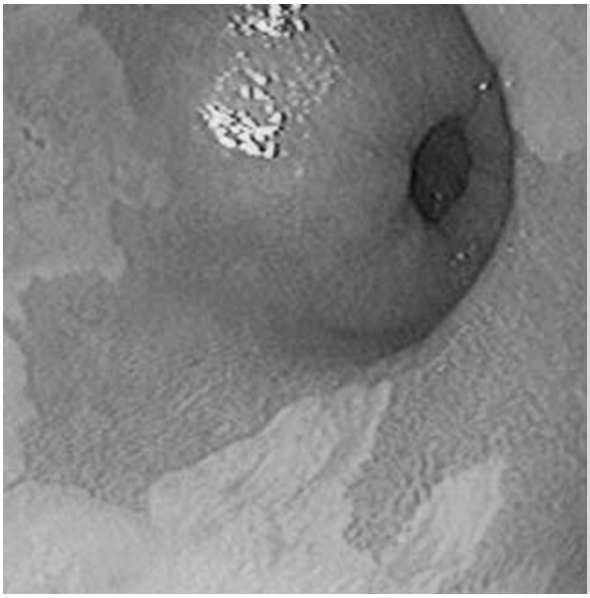


Chapter

II

Aims and outline of the thesis

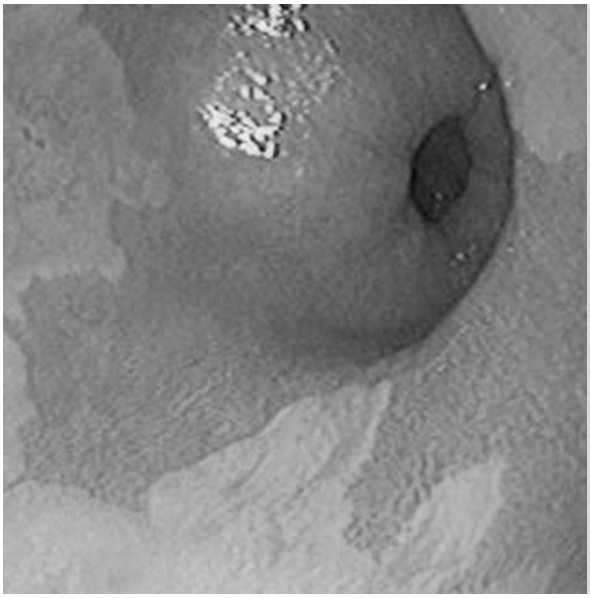
AIMS AND OUTLINE OF THE THESIS

The studies described in this thesis were undertaken to gain more insight in the role of chronic inflammation in the esophagus and progression towards BE. As mentioned above, chronic inflammation in the esophagus plays a role in disease progression, but its precise role is still largely unclear.

As Th2 inflammatory responses are likely to promote tumorigenesis, we investigated whether inflammation in BE has characteristics of a type 2 immune response (Chapter III). Number and type of inflammatory cells were determined in both BE and reflux esophagitis.

Progression to BE is seldom observed in humans, making it difficult to observe the mechanisms involved in its development. We therefore studied whether individual susceptibility for progression to BE or EAC could be determined by cytokine gene polymorphisms in chapter IV, V, and VI. Genotypes of the key cytokines IL-12, IL-10, COX-2, IL-2, IL-6, IL-8, IL-1β, IL-1RN were determined in patients with BE and compared to patients with endoscopically proven reflux esophagitis.

To investigate whether the development of Barrett's epithelium is regulated by key transcription factors of normal intestinal differentiation, we have studied the expression of CDX2 in BE and reflux esophagitis (Chapter VII). As bile acids have been implicated in mucosal immunity, we tested whether a bile acid receptor was active in BE, and whether stimulation of this receptor resulted in immune regulation (Chapter VIII). Finally, this thesis is concluded by a general discussion that incorporates our findings into existing knowledge from literature. Possible mechanisms for inflammation associated esophageal carcinogenesis, and change in mucosal lining will be discussed (Chapter IX).



Chapter

III

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Barrett's esophagus is characterized by a predominant humoral inflammatory response

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ABSTRACT

Introduction: Barrett's esophagus (BE) is thought to be an intermediate step in the progression from reflux esophagitis (RE) to esophageal adenocarcinoma. Premalignant conditions associated with chronic inflammation are often associated with the development of a more pronounced humoral immune response during progression of disease. The aim of this study was to determine whether BE is also associated with a more pronounced humoral immune response when compared to RE.

Materials and methods: Immunohistochemical studies were performed to quantify the mean numbers of Th2 effector cells (plasma cells and mast cells), Th1 effector cells (macrophages and CD8+ T cells), to detect the antibody classes produced by plasma cells (IgA, IgG, IgM or IgE), and to determine the presence of isolated lymph follicles (segregated B- and T cell areas, follicular dendritic cells (CD23), and expression of CXCL13) in 124 esophageal biopsies of 20 patients with BE and 20 patients with RE.

Results: BE and RE contained equal numbers of Th1 effector cells, but BE had a 4-fold higher number of plasma cells ($p<0.001$) and a 2.4-fold higher number of mast cells ($p<0.001$). Most plasma cells expressed IgG, but IgE+ plasma cells were also detected in BE. In line with this, isolated lymph follicles were observed in 4/20 (20%) patients with BE, but not in RE.

Conclusion: We therefore conclude that the inflammatory response is skewed towards a more pronounced humoral immune response when RE progresses to BE. It may be that this shift, which is similar to what is found in other chronic inflammatory conditions contributes to an increased cancer risk in BE.

INTRODUCTION

Barrett's esophagus (BE) is considered to be a complication of chronic gastro-esophageal reflux disease (GORD).[1,2] Prolonged exposure of the lower part of the esophagus to gastro-duodenal contents induces an inflammatory response, which in 6-12% of patients with GORD-related complaints, results in the replacement of squamous epithelium by specialized intestinal epithelium (SIE).[3]

Like other chronic-active inflammatory conditions of the digestive tract such as infection with Helicobacter pylori[4] and inflammatory bowel disease,[5] chronic esophagitis is associated with an increased risk of cancer.[6] Premalignant conditions that develop in the presence of a chronic inflammation are often associated with changes in the inflammatory response such as down-regulation of cell-mediated immunity and the presence of a more pronounced humoral immune response.[7,8,9,10] The resulting environment is thought to facilitate the development of tumors as predominant humoral environments promote angiogenesis,[11,12] A predominant cellular immune response rather tends to inhibit angiogenesis.[13] In addition, a depressed cellular immunity is associated with decreased immunosurveillance.[10,14,15,16]

BE is thought to be an intermediate step in the progression from reflux esophagitis to the development of esophageal adenocarcinoma. In the presence of BE, the annual risk of developing adenocarcinoma is approximately 0.5%.[17] It is however unknown whether the chronic inflammatory response changes when RE progresses to BE. A shift towards a more pronounced humoral immune response might contribute to the increased propensity for neoplastic progression of BE. The aim of this study was to investigate whether the composition of the inflammatory infiltrate in SIE is indeed characterized by a more pronounced humoral immune response.

MATERIALS AND METHODS

Patients and Materials

Patients with BE, as defined by a columnar lined esophagus of ≥ 2 cm and the presence of specialized intestinal metaplasia containing goblet cells (SIE) in at least on of the biopsies, and patients with erosive RE, were enrolled in this study after having given informed consent. The study was approved by the Central Committee on Research Involving Human Subjects of the Erasmus MC- University Medical Center Rotterdam in The Netherlands in 2002. Endoscopic grading of reflux esophagitis in squamous epithelium of patients with RE and BE was performed according to the Los Angeles (LA) classification.[18] Since there is no endoscopic classification for signs of inflammation in the Barrett-segment itself, the endoscopic assessment of inflammation within the BE segment was based on the presence of inflammation-related changes such as erosions and ulcers. In BE, biopsies

were taken of the columnar lined segment from each quadrant at every 2 cm over its complete length. In RE, biopsies were taken from the squamous epithelium next to erosions in the esophageal mucosa. Biopsies of each patient were pooled, formaline-fixed, and paraffin-embedded. The presence of SIE, squamous epithelium, and the grade of light microscopic inflammation present in the biopsies, was evaluated by an experienced pathologist in sectioned slides stained with hematoxylin & eosin (H&E). The grade of inflammation was evaluated at the light microscopic level using the Ismail-Beigi classification for inflamed squamous epithelium of patients with RE,[19] and by using the updated Sydney system for the inflammation in glandular epithelium of patients with BE [20,21]. Biopsies containing SIE, or histologically confirmed inflamed squamous epithelium, were used for further analyses of the inflammatory infiltrate.

Immunohistochemistry

Biopsy samples were serially sectioned at 4 µm, mounted on adhesive slides, dried overnight at 37°C, and deparaffinized with xylene. Antigen retrieval was performed in 10 mM monocitric acid (pH 6.0) at 100°C for 15 min (anti-CD8, anti-CD68, anti-CD20, anti-CD23, anti-α tryptase, anti-CXCL13, anti-CD4 and anti-CD138) or by incubating with pronase at 37°C for 10 min followed by cooling at 4°C (anti-IgA, anti-IgG, anti-IgM, anti-IgE). After cooling, the samples were blocked with non-immune serum for 30 min at room temperature. The sections were stained using primary antibodies against CD8+ T cells (anti-CD8; 1:100 dilution), macrophages (anti-CD68; 1:400), plasma cells (anti-CD138; 1:250), mast cells (anti-α-tryptase ; 1:12,500), B cells (anti-CD20; 1:400), follicular dendritic cells (anti-CD23; ready to use), anti-CXCL13 (1:100), T helper cells (anti-CD4; 1:100), anti-IgA (1:25), anti-IgG (1:250), anti-IgM (1:1,000), and anti-IgE (1:50). To evaluate the presence of IgE expressing plasma cells, a CD138 and IgE double staining was performed as previously described [22]. As formalin-fixed and paraffin-embedded samples could not be used for this purpose, additional snap frozen biopsies were obtained from the BE-segment of 5 patients with BE. The presence of SIE was confirmed in a H&E stain. Binding of the primary antibody was visualized by the addition of either a biotinylated rabbit anti-mouse secondary antibody, or a biotinylated swine anti-rabbit secondary antibody, or a biotinylated donkey anti-rabbit secondary antibody followed by incubation with

Table 1 Patient characteristics

	Barrett's oesophagus	Reflux oesophagitis	P-value
Number of patients	20	20	
Number of biopsies	71	53	
Mean age (±SD)	71(± 4)	53(± 3)	0.03
Gender (% males)	65%	45%	0.2
Hiatal hernia	18/20	17/20	0.6
Length of BO (range)	6.0 (2-12)	-	
Reflux symptoms (%)	0%	100%	
Acid suppressant medication	19/20	10/20	< 0.01

a streptavidine-alkaline phosphatase complex. Apart from CXCL13 (R&D systems Inc., Minneapolis, USA) all other antibodies were obtained from DAKO, Glostrup, Denmark. A red color was developed using new-fuchsine substrate, and a blue color was developed using fast blue substrate.

The mean number of inflammatory cells in each patient was calculated by counting the various immune cells in 10 randomly selected microscopic fields of tissues (magnification 400x) by two observers. The mean number of inflammatory cells was obtained for squamous epithelium of patients with RE, lamina propria of patients with RE, and from SIE of patients with BE. All individual patient results were used for calculating the mean number of inflammatory cells per patient group.

Statistical analyses

All continuous variables were statistically analyzed by the Mann-Whitney-U test and expressed as mean ± standard error of the mean (SE). Nominal variables were analyzed by the chi-quadrate test. A two-sided *p*-value <0.05 was considered as statistically significant. All statistical analyses were conducted by using SPSS 11.0 (Chigaco, Illinois, USA).

Table 2. Classification of inflammation in BE and RE biopsies

	Endoscopic classification (n=20)		Histological classification (n=20)	
Barrett's esophagus	LA-classification		Updated Sydney classification#	
	NI*	20	0	-
	A	-	1	4
	B	-	2	9
	C/D	-	3	7
Reflux esophagitis	LA-classification		Ismael-Beigi classification ξ	
	NI	-	0	-
	A	4	1	17
	B	12	2	-
	C/D	4	3	3

* NI means no signs of inflammation observed at endoscopy.The inflammation observed at endoscopy was graded according the Los Angelas classification. [13] The grade of inflammation was evaluated at light microscopy using the Ismail-Beigi classification for inflamed squamous epithelium of patients with RE ξ,[19] and by using the updated Sydney system #[20,21] for the inflammation in glandular epithelium of patients with BE.

RESULTS

Patient characteristics

A total of 40 patients were enrolled in this study. Twenty of them had RE as defined by the combined finding of macroscopic signs of erosions at endoscopy, and microscopic findings of inflammation on histology. The other twenty patients had BE as defined by SIE in biopsies of the columnar lined segment. Hundred and twenty-four biopsies were obtained in these patients, consisting of 71 biopsies with SIE from the 20 BE patients, and 53 biopsies with inflamed squamous epithelium from the 20 RE patients. Patient characteristics are given in Table 1

Ten biopsies of 9 patients with RE also contained lamina propria beneath the inflamed squamous epithelium of the esophagus. Although signs of inflammation were not observed within the Barrett's epithelium at endoscopy (Table 2), subsequent evaluation using light microscopy showed signs of inflammation ranging from mild to severe in the biopsies with SIE (Table 2).

In RE, the finding of inflammation visible at endoscopy was confirmed at light microscopic evaluation of the biopsies taken next to the erosions (Table 2). The use of a proton pump inhibitor (PPI) or a histamine $(H)_2$ antagonist by half of the patients with RE did

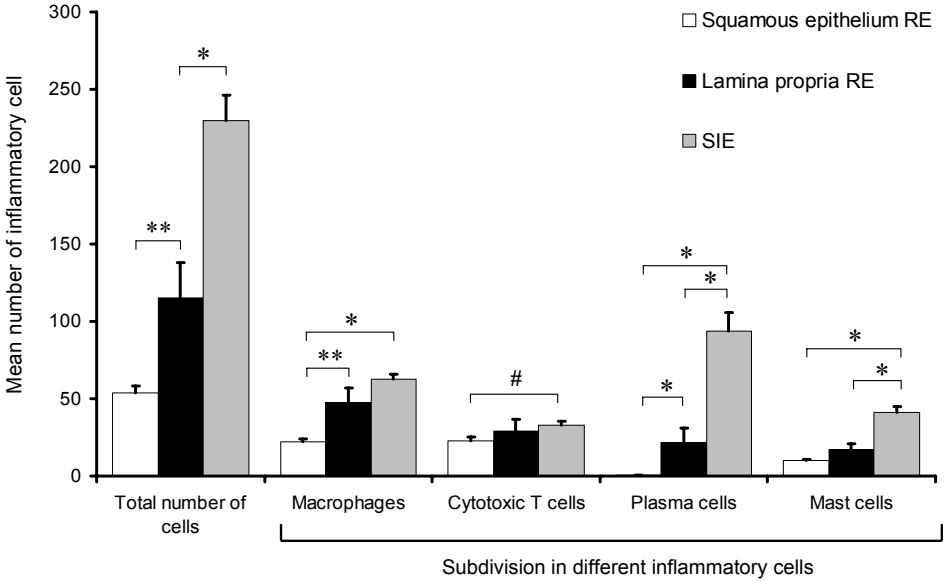

Figure 1: Increased numbers of plasma cells in BE. Mean numbers (mean±SE) of inflammatory cells per microscopic field (400x) were obtained by counting the immune cells in 10 randomly selected microscopic fields in squamous epithelium and lamina propria of patients with RE, and SIE of patients with BE. Inflammatory cells were more prevalent in both the lamina propria of RE and SIE than in squamous epithelium (** $p<0.05$, # $p<0.01$, and * $p<0.001$). Plasma cells were only observed in the lamina propria of RE and SIE. SIE and lamina propria of RE contained equal mean numbers of macrophages and CD8+ T cells, but SIE had a 4 fold higher number of plasma cells (* $p<0.001$)and a 2.4-fold higher number of mast cells (* $p<0.001$).

not affect the grade of esophagitis and severity of inflammation seen at the endoscopical and histological level (p=0.5 and p=0.7, respectively). Based on these findings we have assumed that the biopsies obtained in this study were representative for the whole spectrum of inflammation in BE and RE.

BE is associated with more predominant humoral immune response

Th1 cells are the key regulators of cell-mediated immunity,[23] whereas Th2 cells are associated with humoral immune responses.[24] A relative increase of Th2 cells in BE as compared to RE, was assessed by quantifying the numbers Th1 and Th2 effector cells in immunohistochemical stained sections of esophageal biopsies of patients with BE and RE. Macrophages and CD8+ T cells were used as markers for Th1 effector cells, as these cells play an important role in cell-mediated immunity. Immune cells related to the humoral response such as plasma cells and mast cells, were used as markers for Th2 effector cells.Clear differences were observed in the mean numbers and proportion of the inflammatory cell population between BE and RE. The inflammatory infiltrate in squamous epithelium of patients with RE mainly consisted of CD8+ T cells, mast cells and macrophages located between the squamous epithelial cells, while plasma cells were not observed. In BE, the total number of inflammatory cells per microscopic field was higher as compared to squamous epithelium of patients with RE (p<0.001) (Figure 1). This

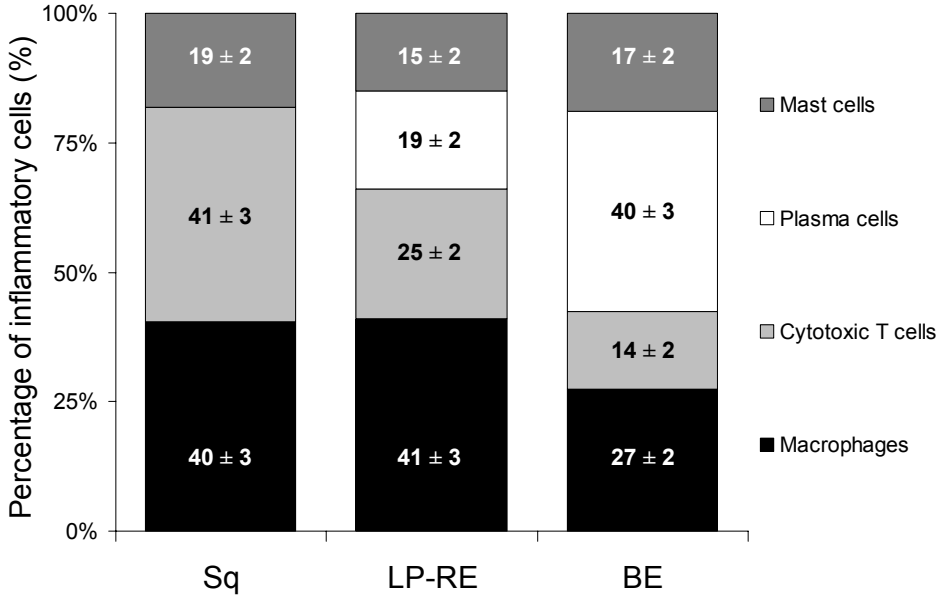

Figure 2: Mean proportion of inflammatory cells in RE and BE. The proportion of plasma cells was higher in SIE than in squamous epithelium and lamina propria of patients with RE (p<0.001).The proportion of mast cells and macrophages were similar between all three tissues. The proportion of CD8+ T cells was only higher in squamous epithelium than in SIE (p<0.01), but not between lamina propria of RE and squamous epithelium of RE, and lamina propria of RE and SIE.

was due to higher mean numbers of all the separate inflammatory cells analyzed (Figure 1). However, the inflammatory cells did not increase at the same rate. The inflammatory cell population in BE had a relative higher proportion of plasma cells ($p<0.001$), and a decreased proportion of CD8+ T cells ($p<0.001$), and macrophages ($p<0.01$), while there was no difference in the proportion of mast cells ($p=0.5$) (Figure 2). This indicates that BE is associated with a higher number of Th2 effector cells and RE with a higher number of Th1 effector cells.

Observed difference in inflammatory response is independent on the type of epithelium

Most of the inflammatory cells in SIE were located in the lamina propria between the glandular epithelium, except for CD8+ cells, as these cells were also located between glandular epithelial cells. Plasma cells were also only observed in the lamina propria of SIE and not in squamous epithelium. To determine whether the observed differences were not due to the inability of inflammatory cells to infiltrate squamous epithelium as easy as lamina propria, the inflammatory infiltrate in the lamina propria of patients with RE was also analyzed. The lamina propria of RE contained a 2 fold higher number of inflammatory cells as compared to squamous epithelium of the same patients (Figure 1). Plasma

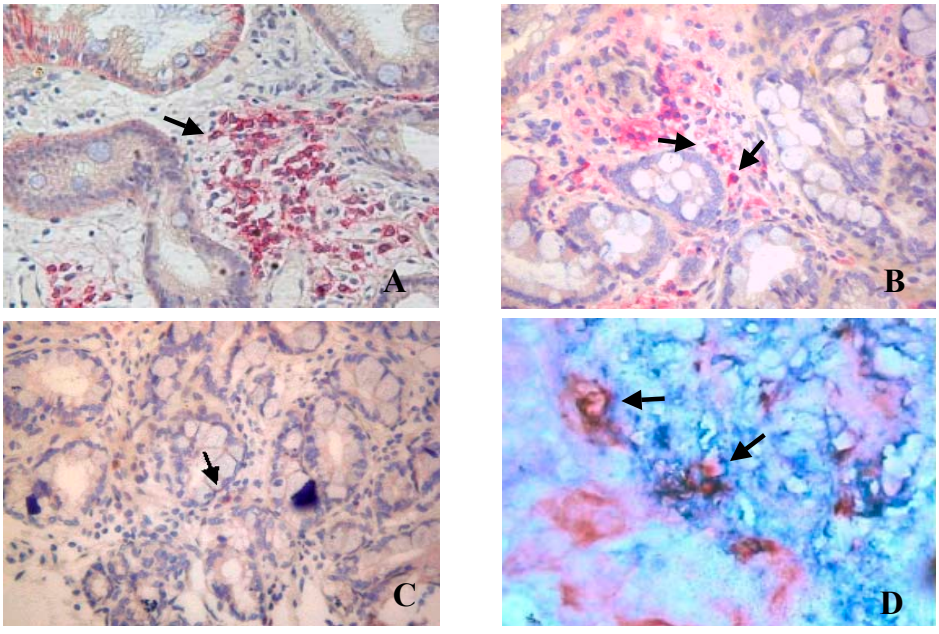

Figure 3: Isotypes of immunoglobulins (Ig) on Ig+ cells in the lamina propria of patients with SIE. A) Large numbers of CD138+ cells (red) were observed in the lamina propria of patients with BE (indicated by arrow). B) A large proportion of these cells expressed IgG (red). C) A smaller proportion of the cells (red) was positive for IgA (indicated by arrows). D) A double staining for CD138 (blue) and IgE (red) showed IgE expressing plasma cells (purple) (arrow) in BE

cells were also observed in the lamina propria of patients with RE. This suggests that inflammatory cells do infiltrate the lamina propria in greater numbers than squamous epithelium. However, in agreement with the earlier findings, the lamina propria of RE contained less plasma cells and mast cells ($p<0.001$), but equal numbers of macrophages and CD8+ T cells when compared to BE (Figure 1). The relative proportion of plasma cells was therefore also higher in BE than in lamina propria of RE ($p<0.001$) (Figure 2). The proportion of macrophages was lower ($p=0.024$), and the proportion of mast cells and CD8+ T cells was similar (Figure 2). This indicates that the inflammation in BE is characterized by a more pronounced humoral immune response, and that this is independent on differences in type of epithelium.

Plasma cells produce IgG and IgE.

To support the hypothesis that a more pronounced Th2/humoral type immune response is present in BE, the production of different immunoglobulins (IgM, IgA, IgE, and IgG) by plasma cells was determined. In both RE and BE, the majority of Ig producing (Ig^+) cells (Figure 3A) expressed IgG (>90%) (Figure 3B), with a small fraction expressing IgA, IgM, and IgE (Figure 3C). IgE+ plasma cells were present in the lamina propria of SIE, as demonstrated using a double stain of CD138 (plasma cells) and IgE performed on 5 snap-frozen biopsies of patients with BE. The majority of IgE+ cells were IgE+ mast cells, although a fraction of the IgE+ cells were indeed IgE producing plasma cells (Figure 3D). This is in line with the presence of a humoral immune response with Th2 characteristics in BE.

Formation of isolated lymph follicles in Barrett's esophagus

Consistent with the observation of a pronounced humoral immune response in BE, lymphoid aggregates were detected in the lamina propria of 8/20 (40%) patients with BE and in 2/9 (22%) patients with RE from who lamina propria was obtained (Figure 4A). These lymphoid aggregates consisted of large numbers of B cells and CD4+ T cells, and small numbers of CD8+ T cells and monocytes/ macrophages. The isolated lymph follicle (ILF) specific organization was studied by the detection of segregated B- and T cell localization, follicular dendritic cells (FDC). Lymphoid aggregates with a segregated localization of B cells in the center and CD4+ T cells at the periphery were observed in 6/8 patients with BE (Figure 4BC), and in 0/2 patients with RE. CD23+ cells were only located in 4/6 of the lymphoid aggregates with clear B- and T cell segregation in BE (Figure 4D), but not in 4/8 patients with BE and 2/2 patients with RE.

The presence of the B lymphocyte chemoattractant CXCL13 was evaluated, in order to determine whether molecules associated with early formation of ILFs are released into the esophageal mucosa. CXCL13 was found to be predominantly located in the periphery of all lymphoid aggregates where it was associated with extracellular fibrils (Figure 4E). Only a few CXCL13+ cells could be observed in the center of lymphoid aggregates. Apart

from their localization in well organized lymphoid structures, a few CXCL13+ cells were also located in less organized lymphoid aggregates consisting of only a few B- and T cells (Figure 4F).

DISCUSSION

Although BE often has no endoscopic signs of inflammation, it is becoming apparent that active inflammation is ongoing in the metaplastic tissue.[21] We observed a chronic active inflammation in SIE (Figure 1), which was characterized by an increase in Th2 effector cells (plasma cells and mast cells), and equal numbers of Th1 effector cells (macrophages and CD8+ T cells) compared to RE (Figure 1). BE is therefore characterized by a higher proportion of plasma cells than RE. As class-switching of IgG to IgE is predicted to require significant amounts of Th2 cytokines,[25] the presence of IgE+ plasma cells in BE (Figure 2C) provides additional evidence for the presence of a humoral immune response with Th2 characteristics.[26] Another important clue for the development of a humoral immune response is provided by the detection of isolated lymph follicles (ILF) in 20% of patients with BE, whereas these structures were absent in patients with RE (Figure 3).

Ours is the first study, which extends a previous observation that SIE was associated with increased mRNA levels of Th2 type cytokines IL-4 and IL-10 at a cellular level.[21] Taken together, these findings support the hypothesis that the inflammatory response

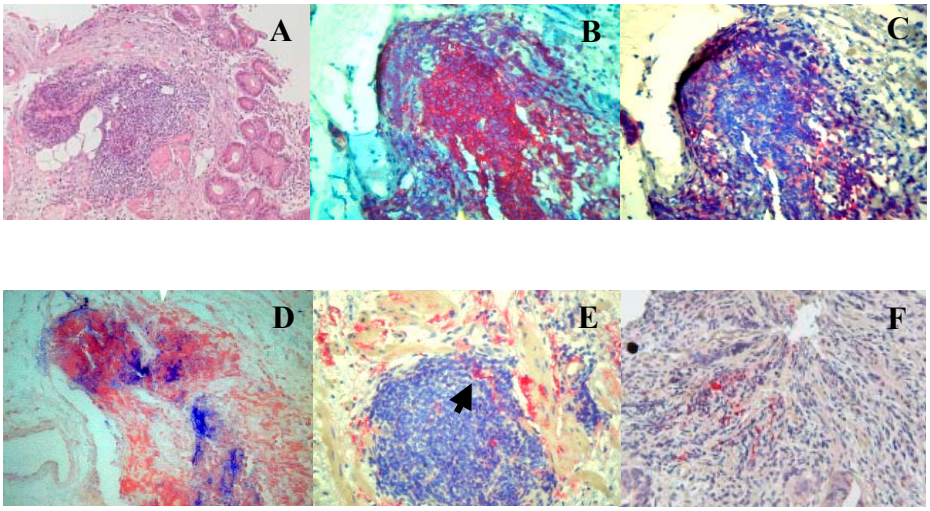

Figure 4: Isolated lymph follicles (ILF) in the mucosa of patients with BE. A) ILFs were observed in lamina propria of patients with BE (H&E stain). These ILFs were characterized by B) segregated B cell areas (red) C) segregated T cell (red) areas and D) the presence of CD23+ follicular dendritic cells (blue). E) Extranodal lymphoid follicle formation associated CXCL13 (red) was mainly present in the periphery of the ILF or lymphoid aggregate associated with extracellular fibrils. F) CXCL13 was also detected in cells located at small lymphoid aggregates without ILF specific organization.

shifts towards a more humoral immune response when RE progresses to BE. A shift towards a more humoral immune response in premalignant lesions is often associated with a depression of the cell-mediated immune response.[7,8,9,10] Not surprisingly, this is also reported for BE,[27] and esophageal adenocarcinoma.[28] Dysplastic esophageal epithelial cells in BE produce the cell-mediated immune-suppressive cytokine IL-10, whereas non-dysplastic epithelial cells do not.[29,30] In addition, increased COX-2 expression, especially when associated with esophageal carcinogenesis, is known to inhibit Th1 cytokine expression in favor of upregulation of Th2 cytokines.[31,32]

The presence of a humoral immune response in BE is likely to be inflammation-driven, rather than the result of the replacement of squamous epithelium by SIE for the following reasons. First, BE differs on the higher proportion of plasma cells from lamina propria as well as squamous epithelium of patients with RE, regardless of the difference in total cell numbers. This indicates that the differences observed between BE and RE are not due to differences in the ability of inflammatory cells to migrate to the different layers of the mucosa. Second, most of the plasma cells present in BE expressed IgG, This immunoglobulin is known to be mainly induced in the presence of inflammation in intestinal mucosa.[33] In non-inflamed intestinal mucosa most of the plasma cells express IgA. In SIE however, IgA+ cells only made up a small proportion of the plasma cells, indicating that the relative proportion of plasma cells in SIE differs fundamentally from non-inflamed intestinal mucosa. Whether an antigenic stimulus is responsible for the observed differences in SIE is unknown. PCR-based analyses of the humoral immune response showed however that the response was at least oligoclonal (results not shown).

Lymphoid tissue, located in the mesenteric lymph nodes and in Peyer's patches, is involved in the induction of immune responses as they provide the structure where antigens are presented to T helper cells and naïve B cells. In response to chronic inflammation, isolated lymph follicles (ILFs) may also develop in the human small intestine.[34,35] ILFs are lymphoid aggregates consisting of segregated B- and T-cell areas and germinal centers, similar to Peyer's patches. Characteristic for germinal centers are follicular dendritic cells, which are stromal cells that play an important role in the selection of memory B cells during the germinal center reaction.[36] Because of these structures, ILFs have been suggested to be inductive sites for mucosal immune responses.[37] ILFs were observed in 4 patients with BE but not in RE (Figure 4B-D). IgG expressing plasma cells were also observed in the vicinity of these ILFs. These structures may therefore well be inductive sites for the observed humoral immune response. As not all lymphoid aggregates were ILFs, the presence of key factors involved in the formation of ILFs was evaluated. One of these key factors is CXCL13, a chemokine which is required for normal formation of mucosa associated lymphoid tissue,[38,39] and which has been shown to be induced in several inflammatory conditions.[40,41] CXCL13 was shown to be produced by cells in the ILFs in BE (Figure 4E), but was also detected closely associated with extra cellular fibrils at the periphery of all lymphoid aggregates. In addition, CXCL13 was also found

to be produced by cells in very small groups of lymphoid cells (Figure 4F). This suggests that expression of CXCL13 is an early step in the formation of these structures, but that the inflammatory response in RE does not provide the additional factors required for ILF formation.

In conclusion, we have demonstrated that in comparison to RE, BE is associated with increased proportions of plasma cells and formation of isolated lymph follicles. In addition, IgE expressing plasma cells were observed in the lamina propria of patients with BE, which is suggestive for an immune response with Th2 characteristics. These findings indicate the presence of a more pronounced humoral immune response in BE than in RE. Such a shift in the inflammatory response has been associated with an increased risk for the development of cancer in other chronic inflammatory conditions, and might contribute to the malignant propensity of BE. Identification and eventually inhibition of the mechanisms responsible for this shift may generate novel targets for esophageal cancer prevention.

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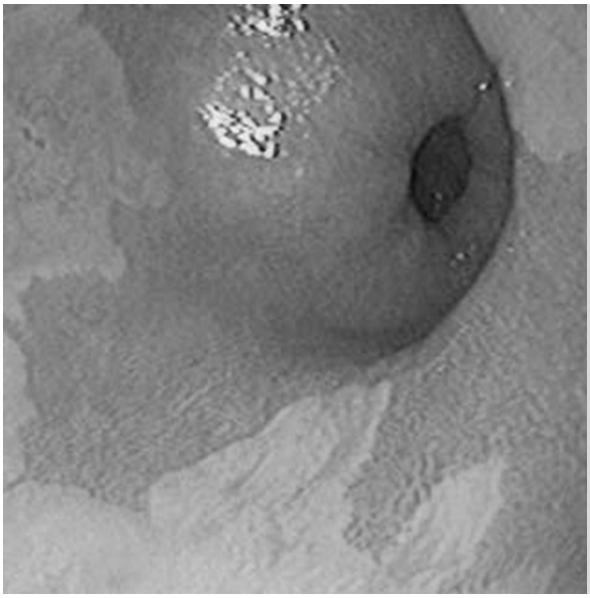

Chapter

IV

Accepted for publication in Carcinogenesis

A pro-inflammatory genotype is associated with Barrett's esophagus

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ABSTRACT

Introduction: Severity of mucosal inflammation is shown to be associated with Barrett's esophagus (BE) development in animals. It has therefore been postulated that a strong pro-inflammatory host response predisposes to BE.

Aim: To determine the impact of cytokine gene polymorphisms on the development of BE.

Methods: The multiplex SNaP-shot™ method was used to determine IL-12 (A+1188C), IL-10 (C-592A; C-819T, A-1082G), IL-8 (A-251T), IL-6 (G-174C), and IL-2 (G-330T) gene polymorphisms in 255 patients with BE, and 247 patients with reflux esophagitis.

Results: The presence of the IL-12B C-allele, which is associated with increased IL-12p70 expression, was more frequently observed in BE than in reflux esophagitis patients (OR 1.8 95%CI 1.2-2.7; p=0.007). The risk of BE was increased in patients in whom the IL-12 genotype coincided with a hiatal hernia (OR 2.9 95%CI 1.32-6.58; p=0.008). The IL-10_{-1082} GG genotype, which is associated with higher IL-10 levels, was also associated with a decreased risk of BE when it was associated with the IL-12-high genotype, indicating IL-10 dependent downregulation of IL-12p70 expression. A combination of the IL-12 AA genotype and the IL-10 AA or AG genotypes was associated with reflux esophagitis (OR 1.4 95%CI 1.05-1.85; p=0.011).

Conclusion: A genetic profile predisposing to a strong pro-inflammatory host response mediated by IL-12p70, and at least partially dependent on IL-10, is associated with BE. This risk further increases when this genotype coincides with a hiatal hernia, suggesting that exposure of the esophageal mucosa to gastroesophageal reflux in the presence of a pro-inflammatory genetic background is a driving force in the development of BE.

INTRODUCTION

Chronic inflammation predisposes to the development of gastrointestinal malignancies [1,2]. This is also true in the esophagus, where the progression from reflux esophagitis towards esophageal adenocarcinoma follows a sequence of different stages including esophagitis, Barrett's esophagus (BE), dysplasia, and eventually esophageal adenocarcinoma. The presence of BE is associated with a 0.5% annual risk of developing esophageal adenocarcinoma [3], and is therefore the most important risk factor for this disorder.

There is increasing evidence that a more severe esophageal inflammation predisposes to the development of BE. In animal models, the inflammation was more intense in animals developing BE and esophageal adenocarcinoma as compared to animals that did not [4,5]. Progression towards BE could be prevented in these animals by the use of COX-2 inhibitors, which reduced the level of mucosal inflammation. BE is associated with a more predominant humoral immune response (Th2) with increased expression of IL-4 and IL-10, whereas reflux esophagitis was associated with a cellular immune response (Th1) expressing high IL-1β, IL-8, and IFN-γ [6,7]. In a BE-segment, the proximal region was associated with more increased levels of pro-inflammatory cytokines as compared to the distal region [8]. The distal region on the other hand was associated with increased expression of regulatory cytokines [8]. This suggests that the change in mucosal lining of the esophagus may be paralleled by a shift in mucosal immunity, and may be preceded by a strong pro-inflammatory mucosal immune response. Levels of cytokine expression and interaction between key cytokines and inflammatory cells direct mucosal immunity, and thereby the outcome of disease [9]. It is therefore conceivable that genetic regulation of cytokine expression levels, resulting in increased or decreased levels of these cytokines in the esophageal mucosal immune response, may alter individual susceptibility to BE.

In the genes encoding for IL-12, IL-10, IL-8, IL-6, and IL-2 single nucleotide polymorphisms (SNPs) have been reported, which are associated with altered expression levels of these inflammatory agents. The C-residue at position +1188 in the IL-12B gene encoding for the IL-12 p40 subunit is associated with high expression of the pro-inflammatory cytokine IL-12p70 by monocytes [10,11]. Expression of the regulatory cytokine IL-10, is partially dependent on polymorphisms in the IL-10 promotor, with the IL-10 $C_{-592}C_{-819}G_{-1082}$ (CCG) allele being associated with the highest mucosal IL-10 levels as compared to the IL-10 CCA and ATA haplotypes [12-14]. The A-residue at position –251 in the gene encoding for IL-8 is associated with a 2-5-fold increased transcription of IL-8 [15-17], the G-residue at position -174 in the promoter of IL-6 with lower IL-6 expression by inflammatory cells [18], and the IL-2 G-residue at position -330 with lower IL-2 expression [19]. Esophageal mucosal IL-8 and IL-6 levels have been demonstrated to be induced by gastroesophageal reflux, and are increasingly expressed in BE [7,20-22].

Whether a severe pro-inflammatory immune response also predisposes to the development of BE in humans has not been established yet. This is partially due to the fact

that progression from reflux esophagitis towards BE is only infrequently observed in the clinical situation [23], and the majority of patients already have developed BE at the initial endoscopic examination. We postulated that genetic factors controlling the mucosal inflammatory response could affect the individual susceptibility for the development of BE. This study therefore assessed the association between polymorphisms encoding for higher or lower levels of IL-12, IL-10, IL-8, IL-6, and IL-2 and BE. As gastroesophageal reflux is the key mechanism of mucosal inflammation, we included patients with reflux esophagitis as controls and compared them with patients with BE.

MATERIALS AND METHODS

Study design

All consecutive Caucasian patients, who were eligible to be included and visited the endoscopy unit of the Erasmus MC- University Medical Centre Rotterdam and the IJsselland Hospital, Capelle aan de IJssel, the Netherlands between November 2002 and February 2005, were invited to participate in this study. The study was approved by the institutional ethical review boards of both hospitals. Participants were included when they either had BE or reflux esophagitis without the presence of BE. In total 1186 gastroscopies were performed in patients referred to the endoscopy for the evaluation of reflux-related symptoms, odynophagia or dysphagia, suspected extraesophageal manifestations of gastroesophageal reflux disease, or surveillance of BE. Eleven hundred and fifteen patients gave written informed consent (response rate 94%, 96% for reflux esophagitis patients and 89% for BE patients). Most patients were invited to participate in this study after upper endoscopy had been performed and reflux esophagitis or BE was diagnosed. Eighty-five percent of the included BE patients already participated in a surveillance program, and were invited to participate in this study prior to endoscopy by a postal letter (n=213). Fifteen percent of the BE patients were newly recognized during the inclusion period. All participants were genetically unrelated Dutch Caucasian patients. Information was collected on the endoscopic presence of a hiatal hernia, reflux esophagitis and BE, as well as on age, gender, and use of acid suppressant medication. Patients were only included if they either had a BE defined as having a columnar lined segment in the distal esophagus of ≥2 cm in length with specialized intestinal epithelium (SIE) found in at least one of the biopsies, or endoscopic evidence of reflux esophagitis in the absence of a BE. The presence of SIE was routinely determined in haematoxylin & eosin stained slides by an experienced gastrointestinal pathologist. The severity of esophagitis in the squamous epithelium was endoscopically graded according to the Los Angeles (LA)-classification, with grade A being the least severe and grade D being the most severe grade of esophagitis [24,25]. A hiatal hernia was considered to be present if gastric folds were observed in columnar lined epithelium above the hiatal impression.

Genotyping of cytokine polymorphisms

Blood was obtained by venapunction and used for DNA extraction using standard procedures. The IL-12 A+1188C (rs3212227), IL-10 C-592A (rs1800872), C-819T (rs1800871), and G-1082 (rs1800896), IL-8 A-251T (rs4073), IL-6 G-174C (rs1800795), and IL-2 T-330G (rs2069762) polymorphisms were determined by SNaP-shot multiplex PCR as described previously [26]. Briefly, PCR was performed in one 10-plex PCR reaction. PCR was carried out in a T-gradient 96-well Thermal cycler (Biometra, Goettingen, Germany) in a 10 µL volume, containing 1µl 10X PCR buffer (Invitrogen, Breda, the Netherlands), 0.2 mmol/L deoxynucleotide triphosphates (Invitrogen), 1.25 mmol/L $MgCl_2$ (Invitrogen), 0.06 unit Platinum Taq-Polymerase (Invitrogen), and 40 ng template DNA. PCR conditions were 94°C for 3 minutes (denaturation); 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds; followed by a single final extension step of 5 minutes at 72°C. PCR products were subsequently incubated (37°C for 45 minutes) with 4 µL Exo-SAP-IT (Amersham, Roosendaal, the Netherlands) to digest contaminating deoxynucleotide triphosphates and PCR primers. Enzymes were deactivated at 75°C for 15 minutes. Multiplex genotyping was performed by single base extension (SBE) using SnaPShot (Applied Biosystems, Nieuwekerk aan de IJssel, the Netherlands) as described previously [26]. SBE products were mixed with deionized formamide containing Genescan 120 LIZ size standard and denatured at 95°C for 5 minutes and analyzed on an ABI Prism 3100 genetic analyzer using Genescan Analysis software (version 3.7). PCR and SBE primers are listed in Table I

Statistical analysis

The study was powered (80%) to detect a 10% difference in allele distribution between the two patient groups (significance level 5%). We nominated BE patients and EAC patients as cases and patients with reflux esophagitis as controls. Differences between the allele distributions of cytokine polymorphisms in the studied populations were determined by Chi-quadrate analysis. Age and sex corrected odds ratios (ORs) with corresponding 95% confidence intervals (CIs) for the risk of developing BE were calculated by logistic regression analysis. The positive associations were then tested in multivariate analysis including all tested polymorphisms, age, and gender. As only the IL-12B A1188C polymorphism was shown to be positive in the multivariate analysis, a Bonferroni correction for multiple comparisons was calculated for this association. The association between a hiatal hernia and the length of the BE-segment was calculated by Mann-Whitney testing. A two-sided p-value ≤ 0.05 was considered as statistically significant. All statistical analyses were conducted by using SPSS 11.0 (SPSS, Chicago, Illinois, USA).

Table I Primers used for genotyping of the studied polymorphisms

Polymorphism		Primers
IL-10 –592	Forward	5′-AGCTGAAGAGGTGGAAACAT- 3′
	Reverse	5′- TATCCTCAAAGTTCCCAAGC– 3′
	SBE*	5′- AACTGACTAAACTTCGTGCCACGTCGT ATTTTACTTTCCAGAGACTGGCTTCCTACAG- 3′
IL-10 –819	Forward	5′-ACAGTAGGGTGAGGAAACCA- 3′
	Reverse	5′- TGACCCCTACCGTCTCTATT– 3′
	SBE*	5′- ACTGACTAAACTTCGTACCACGTCGTGC TATTTTATAGTGAGCAAACTGAGGCACAGAGAT- 3
IL-10 –1082	Forward	5′-CACACAAATCCAAGACAACAC- 3′
	Reverse	5′- AAAGATGGGGTGGAAGAAGT– 3′
	SBE*	5′- CTTACTTTCCTCTTACCTATCCCTACTTCCCC– 3′
IL-12B +1188	Forward	5′- GATAATTTCTATCTGATTTGCTTT- 3′
	Reverse	5′- GGCAACTTGAGAGCTGGAA– 3′
	SBE*	5′– AGGATCACAATGATATCTTTGCTGTATTTGTATAGTT– 3′
IL-8 –251	Forward	5′-TGTTCTAACACCTGCCACTC-3′
	Reverse	5′-CATTTAAAATACTGAAGCTCCACA–3′
	SBE*	5′-TGGTACTATGATAAAGTTATCTAGAAATAAAAAAGCATACA-3′
IL-6 -174	Forward	5′- CTAGCCTCAATGACGACCTA - 3′
	Reverse	5′- GGGCTGATTGGAAACCTTAT – 3′
	SBE*	5′- AACTGAGTACACTAGGTGGATTGTGCAATGTGACGTCCTTTAGCAT-′3
IL-2 –330	Forward	5′-CTTGTCCACCACAATATGCT-3′
	Reverse	5′-TGCAATTAACGCCTTCTGTA-3′
	SBE*	5′-AACTGACTAGACTAGGTGCCACGTCGTGAACCACAATATGCTATTCA CATGTTCAGTGTAGTTTTA-3′

* single base extension (SBE)

RESULTS

Patient characteristics

In total, 1,115 patients were subjected to upper GI endoscopy for analysis of reflux-related symptoms (n=902) or surveillance of BE (n=213). Of these, 247 (22%) patients had reflux esophagitis without BE (mean age 55±14 years, 54% male) (Figure 1). Patients with a columnar lined segment ≥2 cm and with SIE in one of the biopsies were included in the BE-group. In total, 255 patients met these criteria (mean age 63±12 years, 69% male) (Figure 1). Forty-six percent of the patients with reflux esophagitis used acid inhibiting mediation and 72% of those with BE. Endoscopic findings are shown in Table II. Male gender was more frequently observed in BE (OR 1.9 95%CI 1.3-2.7; $p<0.001$) as compared

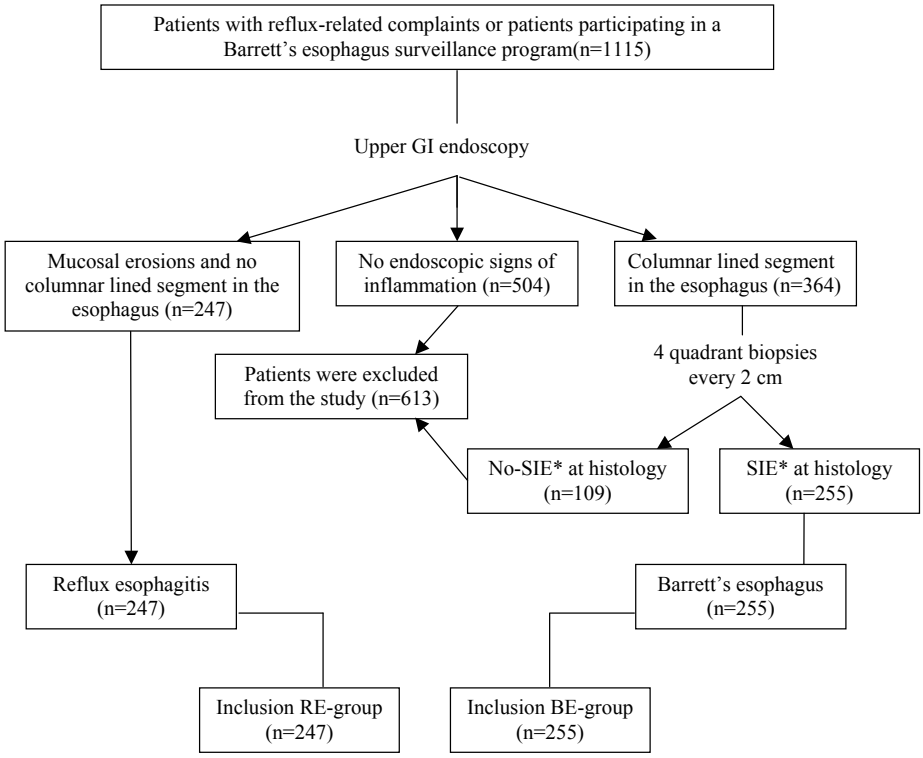

Figure 1: flowchart of inclusion of patients. * SIE; Specialized intestinal epithelium.

to reflux esophagitis. Patients with BE were also slightly older than patients with reflux esophagitis (p<0.001).

Table II Endoscopic findings in the included patients.

	Reflux esophagitis (n=247)	Barrett's esophagus (BE) (n=255)	*p*-value
Age (yrs)	55±14	63±12	0.000
Gender (male%)	133 (54%)	176 (69%)	0.000
Length of the BE-segment (cm±SD)	-	4.1±2.4	0.000
Grading of reflux esophagitis according to the LA-classification			
No-inflammation	0%	130 (51%)	
A	57 (23%)	13 (5%)	
B	151 (61%)	53 (21%)	
C	32 (13%)	41 (16%)	
D	7 (3%)	18 (7%)	
Hiatal hernia	161 (65%)	217 (85%)	0.000

Table III Allele distribution of the IL-10, IL-12B, IL-6, IL-2 and IL-8 polymorphisms

Genotype	Phenotype		χ^2
	Reflux esophagitis (n=247)	Barrett's esophagus (n-255)	*p*-value
IL-12B A1188C			
AA	180 (73%)	163 (64%)	0.031
AC	63 (25%)	86 (34%)	0.044
CC	4 (1.6%)	6 (2.4%)	0.557
IL-10 C-592A			
CC	162 (66%)	145 (57%)	0.055
CA	73 (29%)	98 (38%)	0.045
AA	12 (5%)	12 (5%)	0.936
IL-10 C-819T			
CC	162 (66%)	145 (57%)	0.055
CT	73 (29%)	98 (38%)	0.045
TT	12 (5%)	12 (5%)	0.936
IL-10 A-1082G			
AA	63 (25%)	74 (29%)	0.337
AG	110 (45%)	127 (50%)	0.237
GG	74 (30%)	54 (21%)	0.024
IL-8 T-251A			
TT	76 (31%)	80 (31%)	0.884
TA	122 (49%)	131 (52%)	0.657
AA	49 (20%)	44 (17%)	0.456
IL-6 G-174C			
GG	91 (37%)	92 (37%)	0.993
GC	120 (48%)	123 (48%)	0.992
CC	37 (15%)	38 (15%)	0.922
IL-2 G-330T			
TT	135 (55%)	132 (52%)	0.636
TG	90 (37%)	103 (41%)	0.414
GG	21 (8%)	18 (7%)	0.546

Association analyses of single nucleotide polymorphisms with either BE or reflux esophagitis.

All individual SNPs were tested for an association with either reflux esophagitis or BE. The distribution of the different genotypes in the reflux esophagitis and BE population is shown in Table III. The genotype frequencies of IL-8, IL-6, and IL-2 polymorphisms were not differently distributed between BE or reflux esophagitis (Table III), and these gene polymorphism were therefore not preferentially associated with either disease state. The IL-12B C-allele was more frequently observed in patients with BE (91/255; 36%) than in

Table IV The risk of having BE in a multivariate analyses

Variable	OR	CI 95%	*p*-value
Age (old age)	1.04	1.02- 1.06	0.000
Gender (male)	1.93	1.32- 2.71	0.000
Hiatal hernia (yes)	2.59	1.67- 4.35	0.000
IL-2 –330 G	0.95	0.70- 1.28	0.713
IL-6 –174 C	1.07	0.76- 1.33	0.956
IL-8 –251A	0.86	0.66- 1.14	0.291
IL-10 –592 C	1.10	0.74- 1.63	0.647
IL-10 –819 C	1.10	0.74- 1.63	0.647
IL-10 –1082 G	0.77	0.55- 1.08	0.127
IL-12B +1188 C	1.82	1.17- 2.69	0.007

patients with reflux esophagitis (67/247; 27%, p=0.031) (Table III), and was associated with the presence of BE (OR 1.8 95%CI 1.04-2.22; p=0.007).

The IL-10 polymorphisms at position -592 and -819 were completely linked to each other; i.e. the C-residue at position IL-10_{-592} was always found in combination with a C-residue at position IL-10_{-819}, and the A_{-592}-residue with a T_{-819}-residue. The IL-10 -592 and -819 polymorphisms were not significantly associated with the presence of BE (Table III) (p=0.078). Only the IL-10_{-1082} GG genotype was more frequently observed in patients with reflux esophagitis (74/247;30%) than in patients with BE(54/255; 21%; p=0.024) as compared to the other IL-10_{-1082} genotypes (OR 1.6 95%CI 1.06-2.39; p=0.024).

The association between the IL-12B C-allele and BE remained significant in a multivariate analysis (OR 1.8 95%CI 1.17- 2.69; p=0.007), but the IL-10_{-1082} GG genotype and reflux esophagitis did not (Table IV). The association between the IL-12B C-allele and BE was also significant after correction for multiple comparisons by the conservative Bonferroni correction. The IL-12B C-allele was not associated with gender, age, grade of inflammation, length of BE-segment, or hiatal hernia (data not shown).

The presence of a hiatal hernia is associated with BE

It is known that the presence of a hiatal hernia is associated with a more severe reflux induced mucosal inflammation [27-29]. In the tested population of 255 BE patients, a hiatal hernia was associated with a longer BE-segment (4.3±2.5 cm; n=217) as compared to patients without this endoscopic finding (3.1±1.1 cm; n=38) (p=0.016). A hiatal hernia was present in 39/70 (56%) of patients with grade A reflux esophagitis, in 142/204 (70%) patients with grade B, and in 90/98 (92%) patients with grade C or D reflux esophagitis (p<0.001). The presence of a hiatal hernia was associated with a BE (OR 2.6 95%CI 1.67-4.35; p<0.001).

Table V The risk of BE in patients with an IL-12B polymorphism and hiatal hernia.

Genotype and hiatal hernia	Phenotype		OR (95%CI)	*p*-value
	Reflux esophagitis (n=247)	Barrett's esophagus (n=255)		
IL-12B$_{C\ or\ CC}$ & hiatal hernia	37 (17%)	71 (31%)	2.9 (1.32-6.58)	0.008
IL-12B$_{C\ or\ CC}$ without hiatal hernia	20 (9%)	13 (5%)	0.75 (0.48-1.16)	0.150
IL-12B$_{AA}$ & hiatal hernia	104 (47%)	125 (54%)	1.13 (0.95-1.36)	0.175
IL-12B$_{AA}$ without hiatal hernia	58 (27%)	23 (10%)	0.50 (0.35-0.72)	0.000

The combined presence of the IL-12B C-allele and a hiatal hernia is associated with an increased risk of BE.

As both the presence of a hiatal hernia and the IL-12B C-allele displayed a positive association with BE, we evaluated whether the IL-12B C-allele in combination with a hiatal hernia was associated with BE. The combined presence of a hiatal hernia and the IL-12B C-allele was more prevalent in patients with BE (71/255; 31%) than in patients with reflux esophagitis (37/247; 17%), and this combination was even stronger associated with BE (OR 2.9 95%CI 1.32-6.58; p=0.008) (Table V) as compared to patients with the IL-12B C-allele without a hiatal hernia. In contrast, absence of the IL-12B C-allele combined with the absence of a hiatal hernia was associated with a significantly decreased risk of BE (OR 0.50 95%CI 0.35-0.72; p<0.001) (Table V).

A pro-inflammatory gene profile is associated with an increased risk of BE

It was tested whether the IL-10$_{-1082}$ GG genotype would modify the association between IL-12 and BE. (Table VI). The prevalence of BE or reflux esophagitis was equally distributed between patients with IL-12B$_{CA\ or\ CC}$/IL-10$_{GG}$ and IL-12B$_{AA}$/IL-10$_{non\text{-}GG}$ (Table VI). The combination IL-12$_{CA\ or\ CC}$/IL-10$_{non\text{-}GG}$ was more frequently observed in BE (70/255; 28%) than in patients with reflux esophagitis (45/247; 18%), and was associated with BE (OR 1.7 95%CI 1.1-2.7; p=0.014) (Table VI). The combination IL-12$_{AA}$/IL-10$_{GG}$ was more often observed in

Table VI Gene profiles consisting of the combination of IL-12B and IL-10 polymorphisms and the risk of developing BE.

Genotype and hiatal hernia	Phenotype		OR (95%CI)	*p*-value
	Reflux esophagitis (n=247)	Barrett's esophagus (n=255)		
IL-12B $_{C\ or\ CC}$ & IL-10$_{GG}$	22 (50%)	22 (50%)	0.98 (0.72-1.34)	0.912
IL-12B.$_{C\ or\ CC}$ & IL-10$_{non\text{-}GG}$	45 (39%)	70 (61%)	1.77 (1.12- 2.81)	0.014
IL-12B $_{AA}$ & IL-10$_{GG}$	52 (62%)	32 (38%)	0.71 (0.54- 0.95)	0.011
IL-12B $_{AA}$ & IL-10$_{non\text{-}GG}$	128 (49%)	111 (51%)	1.0 (0.83- 1.18)	0.920

patients with reflux esophagitis (52/247; 21%) than in patients with BE (32/255; 12.5%), and was negatively associated with BE (OR 0.6 95%CI 0.34- 0.99; p=0.011).

DISCUSSION

This is to our knowledge the first study reporting a link between the IL-12 C-residue at position +1188, which is associated with increased expression of IL-12p70, and an increased risk of BE in a population with gastroesophageal reflux disease (OR 1.8 95%CI 1.17- 2.69; p=0.007). Our findings implicates a role for high IL-12p70 levels in the development of BE. IL-12p70 has a pro-inflammatory function by favoring and maintaining Th1 differentiation, and inducing Th1 cytokine synthesis (mainly IFN-γ) [46]. IL-12p70 is therefore believed to be a major link between innate immunity and the recruitment and polarization of adaptive immunity towards a cellular immune response (Th1). The finding of an increased prevalence of the IL-12p70-high genotype (IL-12B C-allele) in BE, indicates that a strong host cellular immune response may be involved in the pathogenesis of BE in humans, as was suggested from observations in animal models[4,5]. The association between the IL-12B C-allele and BE, was modified by the presence of a hiatal hernia, which increased the risk of BE (OR 2.9 95%CI 1.32-6.58; p=0.008) (Table V). This supports the hypothesis that an increasing severity of esophageal inflammation induced by increased exposure to the gastroesophageal refluxate and a pro-inflammatory host response predisposes to development of BE.

Although IL-2, IL-6, and IL-8 gene polymorphisms are also associated with increased transcriptional levels of their respective cytokines, we did not observe a link between these polymorphisms and BE. This may be partially explained by a limitation of this study. We selected a control group consisting of patients with endoscopically confirmed reflux esophagitis, since this confirms the presence of gastroesophageal reflux and its ability to induce mucosal erosions. As we have not investigated severity, duration, content, and pattern of gastroesophageal reflux, this introduces the possibility that significantly fewer control patients had similar reflux characteristics as the patients with BE. As it has been shown that both IL-8 and IL-6 expression are induced by reflux components[20,22,33], this raises the possibility that their may be a selection bias based on differences in the composition and extent of gastroesophageal reflux.

We did observe a link between the IL-10_{-1082} GG genotype and reflux esophagitis (Table III), but that this association was not significant in a multivariate regression analysis (Table IV). This is however in contrast to what has been recently reported by Gough et al. [34], who showed that homozygosity for IL-10 -1082 allele 2, which is associated with higher IL-10 levels, was linked to male BE patients (OR 1.84 95%CI 1.04- 3.28; p=0.035). Although not stated by the authors, this allele is likely to be the G-residue at position -1082, as it is associated with higher IL-10 levels [12,13]. Failure to replicate a genetic association

in a complex disease is a common observation [35,36]. The contrasting findings could relate to differences in ethnic composition of the studied populations, as our population consisted exclusively of Dutch Caucasians. In addition, the frequency of the IL-10_{-1082} GG-genotype (22%) in our population was within the reported range for Caucasian populations (20%-28%) [37-39]. It is noteworthy that the reported frequency of the IL-10 -1082 allele 2 in the study of Gough et al. [34] was lower, i.e., 15%. Finally, it is still possible that non-registered confounders such as smoking or weight differences are involved.

In this study, IL-10 genotypes interacted with the IL-12B polymorphism, and may have modified the risk of BE associated with the IL-12B C-allele. Homozygosity of the IL-10_{-1082} G-allele was more prevalent in patients with reflux esophagitis (OR 1.6 95%CI 1.06-2.39; p=0.024) (Table III), and when combined with the IL-12B C-allele decreased the risk of BE (OR 1.0 95%CI 0.5-1.9; p=0.1) (Table VI). Patients with a IL-12B AA genotype (low IL-12p70 expression) even had a significantly lower risk of BE when this genotype was present in combination with the IL-10_{-1082} GG genotype (OR 0.6 95%CI 0.34-0.99; p=0.011)(Table VI). As the IL-10_{-1082} GG genotype is associated with higher IL-10 expression [14], and IL-10 inhibits IL-12p70 expression by activated dendritic cells, the IL-10_{-1082} GG genotype may decrease the IL-12 p70 production, and in this way the risk of BE. This is also evidenced by a recent observation that IL-10 genotypes interacted with IL-12 genotypes on the level of IL-12p70 expression [32]. The IL-10_{-1082} GG genotype was shown to decrease IL-12p70 expression by LPS stimulated dendritic cells in spite of the presence of a IL-12p70-high genotype [32]. These findings further supports the notion that increased expression of IL-12p70 might be important in the pathogenesis of BE.

This interaction between IL-12B and IL-10 polymorphisms in the production of IL-12p70 may also provide an explanation why IL-10 polymorphisms itself were only weakly associated with BE in this study. Firstly, although BE is associated with increased levels of IL-10[7,8], this was not tested in relation to expression of IL-12. A strong pro-inflammatory response in the esophagus could be mediated by IL-12p70, whereas IL-10 levels may tune the inflammatory response by lowering IL-12p70 expression. Secondly, BE is the end result of a process leading to its development. Inflammatory characteristics in biopsies from the columnar epithelium of a BE-segment may not correlate well with the mucosal characteristics in the squamous epithelium just before the change in mucosal lining, as it is believed that the columnar epithelium has a protective effect on reflux related chemical corrosion. IL-10 was shown to be highly expressed in the distal region of the BE-segment, suggesting that progression of BE may be associated with increased IL-10 expression [8]. This is further supported by the observation that dysplastic epithelium is able to express IL-10 [8], and that IL-10 polymorphisms are associated with esophageal adenocarcinoma [34]. This suggests that IL-10 is mainly involved in neoplastic progression in BE, and to a lesser extent in the development of BE itself.

A hiatal hernia is associated with a higher total esophageal acid exposure, increased acid clearance time, and severity of esophagitis [27,28]. Its presence is therefore likely

to be associated with increased reflux-related mucosal damage and the induction of a pro-inflammatory tissue response. Patients in whom a high IL-12 p70 genotype (IL-12B C-allele) coincided with a hiatal hernia, had a higher risk of BE (OR 2.9 95%CI 1.32-6.58; *p*=0.008)(Table V). We could not detect an association between the grade of endoscopic esophageal inflammation and IL-12B C-allele in patients with reflux esophagitis. Nor did we observe a relationship between the IL-12B C-allele and the length of the BE-segment (data not shown). A fraction of the population was already diagnosed with BE, and used acid suppressant medication for their reflux-related symptoms. Moreover, 46% of the patients with reflux esophagitis used acid suppressant medication as well at the time of upper GI endoscopy. This may have influenced mucosal appearance at the time of endoscopy, and could therefore have resulted in an underestimation of the endoscopic grade of inflammation . Secondly, endoscopic grading of esophagitis by the LA-classification uses macroscopic criteria as marker of the extent of inflammation, but does not give information about the extent of inflammation at the mucosal level. Characteristics of the local immune response may therefore be more important for tissue remodeling than the endoscopic grade of inflammation.

In conclusion, this is the first study providing evidence that the IL-12 C-allele (high IL-12p70 expression) predisposing towards a strong pro-inflammatory host response is involved in the development of BE in humans. This risk increased when the IL-12B C-allele coincided with a hiatal hernia (OR 2.9). This indicates that genetic regulation of cytokine expression levels, and their interaction with local conditions, such as a hiatal hernia which is associated with increased gastroesophageal reflux, contributes to a delicate process in which timing and interaction is of crucial importance for changing mucosal homeostasis. Future studies are needed to elucidate and confirm the role of IL-12p70 and the IL-12B polymorphisms in the pathogenesis of BE. Our findings add knowledge to the growing interest into the role of the inflammatory reaction in the pathogenesis of BE.

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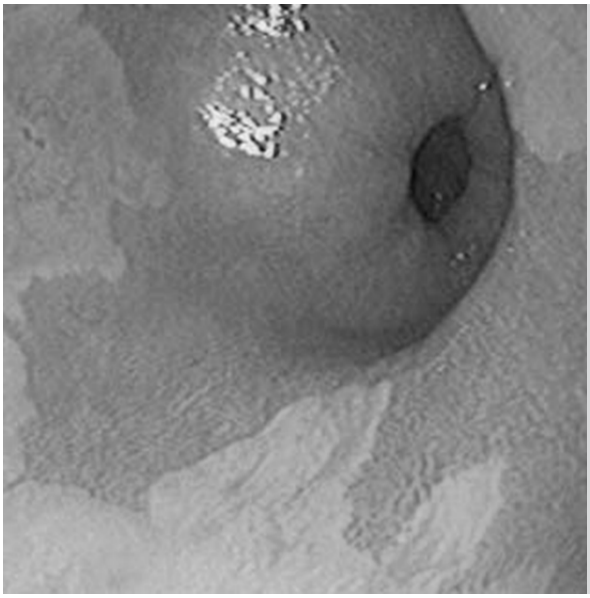

Chapter V

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IL-1B and IL-1RN gene polymorphisms are not associated with Barrett's esophagus

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ABSTRACT

Introduction: Chronic inflammation secondary to gastroesophageal reflux is known to play an important role in the etiology of Barrett's esophagus (BE). IL-1β expression is induced by gastroesophageal reflux, and pro-inflammatory IL-1β signaling is involved in altered epithelial and muscular cell behavior. Polymorphisms in the IL-1B and IL-1RN locus, known to influence IL-1β expression levels, have already been shown to be involved in the inflammation-metaplasia-adenocarcinoma sequence of the stomach, with higher levels of IL-1β being associated with intestinal metaplasia and adenocarcinoma.

Aim: This study set out to determine whether IL-1B and IL-1RN polymorphisms are associated with BE.

Materials and Methods: The IL1B (T-511C; C+3954T) and IL-1RN (VNTR) gene polymorphisms were determined in 255 Dutch patients with BE, and 247 patients with endoscopically confirmed reflux esophagitis by SNaP-shot multiplex PCR (IL-1B –511 & IL-1B+3953), and size separation of the PCR-fragments (IL-1RN).

Results: There was no difference between the distribution of IL-1B and IL-1RN polymorphisms and either reflux esophagitis or BE. The polymorphisms were also not associated with the grade of inflammation and the presence of a hiatal hernia.

Conclusion: Genetic regulation of IL-1β production does not play a role in the etiology of Barrett's esophagus in a Dutch population.

INTRODUCTION

Gastroesophageal reflux is a known risk factor for the development of esophageal adenocarcinoma (EAC) [1], a cancer with a dismal prognosis and rapidly rising incidence. [2,3]. The most important known risk factor for the development of EAC is the presence of a Barrett's esophagus (BE). This acquired condition is believed to develop secondary to chronic esophageal exposure to reflux of gastroduodenal content [4]. As the presence of complaints related to gastroesophageal reflux is associated with an 8-fold increased risk of BE [4], it seems likely that reflux esophagitis is involved in the development of BE.

An early event of esophageal inflammation is the increased expression of cytokines such as IL-8, IL-1β and TNF-α [5-8]. It is part of an innate pro-inflammatory response to irritating stimuli or mucosal damage, resulting in chemotaxis of leukocytes, local inflammatory effects, and tissue remodeling [9-11]. It is thought that a chronic active inflammation of the esophagus triggers tissue remodeling, which results in differentiation towards specialized intestinal epithelium. IL-1β expression is induced upon exposure to gastroesophageal reflux [6,8,12], and has been shown to influence epithelial and muscular cell behavior [9,12,13].

A key feature of the replacement of squamous epithelium by specialized intestinal epithelium is expression of homeobox proteins CDX1 and CDX2. Both are key regulators of normal intestinal differentiation , and their expression has been associated with the presence and development of specialized intestinal epithelium in the esophagus [14-16]. IL-1β signaling via NFκB has been shown to induce expression of homeobox proteins CDX1 and CDX2 in the esophagus [9,15,17,18].This was also shown in the stomach, were increased IL-1β levels in Helicobacter pylori infected patients was associated with an increased incidence of specialized intestinal epithelium of the stomach and gastric cancer. [19,20]

Once released from cells, mature IL-1β encounters two antagonistic molecules(I) the soluble form of the type I receptor, which tightly binds IL-1β, and (II) the IL-1 receptor antagonist, which competes with IL-1β for cell surface receptor occupancy. This balance between agonists and antagonists determines the net effect of IL-1β signaling [21]. In the gene encoding for IL-1β (IL-1B), there are at least two biallelic base exchange polymorphisms, one located at position −511 in the IL-1 promoter region [22] and one at position +3953 in the fifth exon [23]. The IL-1B T-allele at position −511 is associated with increased mucosal IL-1β levels in patients infected with Helicobacter pylori [19], as is also the IL-1B T-allele at position at +3953 [24]. In the gene encoding for the IL-1 receptor antagonist (IL-1RN) a variable number penta-allelic 86-bp tandem repeat (VNTR) polymorphism is present of which the short allele (2 repeats), also referred to as allele 2, is associated with decreased levels of IL-1 receptor antagonist [25].

As IL-1β signaling may be important in the pathogenesis of BE, we have tested whether IL-1β −511 & +3953, and IL-1RN (VNTR) genetic polymorphisms are associated with BE.

Patients with reflux esophagitis were taken as controls, as these patients are exposed to gastroesophageal reflux, but did not develop the mucosal changes as observed in BE and EAC.

MATERIALS AND METHODS

Study design

All consecutive Caucasian patients, who were eligible to be included and visited the endoscopy unit of the Erasmus MC- University Medical Centre Rotterdam and the IJsselland Hospital, Capelle aan de IJssel, the Netherlands between November 2002 and February 2005, were invited to participate in this study. The study was approved by the institutional ethical review boards of both hospitals. All patients included gave written consent. Participants were included when they either had BE or reflux esophagitis without the presence of BE. In total 1186 gastroscopies were performed in patients referred to the endoscopy for the evaluation of reflux-related symptoms, odynophagia or dysphagia, suspected extraesophageal manifestations of gastroesophageal reflux disease, or surveillance of BE. Eleven hundred and fifteen patients gave written informed consent (response rate 94%, 96% for reflux esophagitis patients and 89% for BE patients). Most patients were invited to participate in this study after upper endoscopy had been performed and reflux esophagitis or BE was diagnosed. Eighty-five percent of the included BE patients already participated in a surveillance program, and were invited to participate in this study prior to endoscopy by a postal letter (n=213). Fifteen percent of the BE patients were newly recognized during the inclusion period. All participants were genetically unrelated Dutch Caucasian patients. Information was collected on the endoscopic presence of a hiatal hernia, reflux esophagitis and BE, as well as on age, gender, and use of acid suppressant medication. Patients were only included if they either had a BE defined as having a columnar lined segment in the distal esophagus of ≥2 cm in length with specialized intestinal metaplasia (SIE) found in at least one of the biopsies, or endoscopic evidence of reflux esophagitis in the absence of a BE. The presence of SIE was routinely determined in haematoxylin & eosin stained slides by an experienced gastrointestinal pathologist. The severity of esophagitis in the squamous epithelium was endoscopically graded according to the Los Angeles (LA)-classification, with grade A being the least severe and grade D being the most severe grade of esophagitis [26,27]. A hiatal hernia was considered to be present if gastric folds were observed in columnar lined epithelium above the hiatal impression.

Genotyping of cytokine polymorphisms

Blood was obtained by venapunction and used for DNA extraction using standard procedures. The IL-1B C-511T and IL-1B C+3953T polymorphisms were determined by

SNaP-shot multiplex PCR as described previously [28]. PCR and SBE primers are listed in Table I. The IL-1RN (VNTR) polymorphism exists of different numbers of a 86- basepair tandem repeat. Allele 1 consists of 4 repeats, allele 2 of 2 repeats, allele 3 of 5 repeats, allele 4 of 3 repeats and allele 5 of 6 repeats. Differentiation between the different alleles was performed by size separation of the PCR-fragment created with the primers located at both sides of the of the area of interest, as was previously described [25].

Statistical analysis

The study was powered (80%) to detect a 10% difference in allele distribution between the two patient groups (significance level 5%). We nominated BE patients as cases and patients with reflux esophagitis as controls. Differences between the allele distributions of cytokine polymorphisms in the studied populations were determined by Chi-quadrate analysis. A two-sided *p*-value ≤ 0.05 was considered as statistically significant. All statistical analyses were performed with SPSS 11.0 software (SPSS, Chicago, Illinois, USA).

RESULTS

Patient characteristics

In total, 1,115 patients were subjected to upper GI endoscopy for analysis of reflux-related symptoms (n=902) or surveillance of BE (n=213). Of these, 247 (22%) patients had reflux esophagitis without BE (mean age 55±14 years, 54% male). Patients with a columnar lined segment ≥2 cm and with SIE in one of the biopsies were included in the BE-group. In total, 255 patients met these criteria (mean age 63±12 years, 69% male). Forty-six percent of the patients with reflux esophagitis used acid-inhibiting medication and 72% of those with BE. Endoscopic findings are shown in Table II. As expected from

Table 1 Primers used for the genotyping of IL-1B and IL-1RN

Polymorphism		primers
IL-1B –511	Forward	5′- GTCAGGAGCCTGAACCCTG - 3′
	Reverse	5′- AGCCCTCCCTGTCTGTATTG – 3′
	SBE*	5′-ACTGACTAAACTTGGTGCCACGTCTACCTTGGGTGCTGTT CTCTGCCTC- 3′
IL-1B +3953	Forward	5′-GCCTGCCCTTCTGATTTTAT- 3′
	Reverse	5′-CGTGCAGTTCAGTGATCGTA- 3′
	SBE*	5′-AACTAGCTAAACTAGGTACCACGTCATCACATAAGCCTCGTT ATCCCATGTGTC- 3
IL-1RN (VNTR)	Forward	5′- CCCCTCAGCAACACTCC - 3′
	Reverse	5′- GGTCAGAAGGGCAGAGA - 3′

* single base extension (SBE)

Table II Patient characteristics

	Reflux esophagitis	Barrett's esophagus	*p*-value
Number of patients included	247	255	0.000
Age, mean (SD)	55±14	63±12	0.000
Male (%)	54%	69%	0.000
Length of the BE-segment (cm±SD)	-	4.1± 2.4	
Grading of reflux esophagitis according to the LA-classification			
No-inflammation	0%	130 (51%)	
A	57 (23%)	13 (5%)	
B	151 (61%)	53 (21%)	
C	32 (13%)	41 (16%)	
D	7 (3%)	18 (7%)	
Hiatal hernia	161 (65%)	217 (85%)	0.000

other BE cohorts, male gender was more frequently observed in BE (OR 1.9 95%CI 1.3-2.7; $p<0.001$) as compared to reflux esophagitis. Patients with BE were also slightly older than patients with reflux esophagitis ($p<0.001$).

IL-1B –511, +3953, and IL-1RN (VNTR) polymorphisms are not associated with BE.

None of the individual polymorphisms was associated with either reflux esophagitis or BE (Table III). The combinations of IL-1B –511 & +3953, and IL-1RN polymorphisms were also not significantly associated with the grade of endoscopic inflammation ($p=0.557$, $p=0.309$, and $p=0.938$ respectively), or with the presence of a hiatal hernia ($p=0.347$, $p=0.297$, $p=0.307$ respectively). As it has been shown that net IL-1β levels are genetically determined by the polymorphism combination of IL-1B –511 and IL-1RN polymorphisms, we tested whether the combination associated with high IL-1β levels (IL-1B –511 TT and IL-1RN allele 2/allele 2) was associated with an increased risk of BE. Although the combination IL-1B –511 TT and IL-1RN 2/2 was more prevalent in patients with reflux esophagitis (10/247; 4%) than in BE patients (6/255; 2%), this was not significant ($p=0.737$). This combination was also not associated with increased inflammation ($p=0.553$) or the presence of a hiatal hernia ($p=0.775$)

DISCUSSION

The presence of chronic inflammation in the esophagus is thought to be involved in the development of BE [6,11,29,30]. This prompted us to investigate whether a genetic profile associated with increased expression of IL-1β was associated with BE. In this study, the IL-1B and IL-1RN polymorphisms were not associated with the presence of BE. We

therefore conclude that genetic regulation of IL-1β does not have a significant effect on the pathogenesis of BE.

Genetic polymorphisms association studies often provide conflicting data on associations between gene polymorphisms and a disease [31,32]. Previously, Gough et al. observed an increased prevalence in IL-1RN allele 2 distribution in BE patients when compared to patients with reflux esophagitis , but also no difference between IL-1B polymorphisms [33]. The difference between the Gough et al. study and our study may be partially due to differences in risk factor distribution, ethnic background, socio-economic differences, and other confounders not yet recognized. The IL-1RN allelic distribution for BE and EAC was similar between the Dutch and the English population, however differed significantly among reflux esophagitis patients in both countries (11.4% in the Dutch vs. 2% in the English population, $p<0.0001$). In line with an earlier report [34], our data suggest that the IL-1RN polymorphism is not associated with an increased risk on the development of BE. These findings and reports on IL-1RN (VNTR) frequencies [35-37], suggest that the association found by Gough et al. may indeed result from a skewed allelic distribution in their population. Other studies have to be performed to answer the question whether IL-1RN polymorphism are indeed involved in the pathogenesis of BE, although it seems very likely that it does not.

Although an increase in IL-1β expression is an early event of esophageal inflammation [6,8,38], IL-1β probably does not play a direct role in the development of BE. The combination of the IL-1B -511 TT genotype and homozygosity of IL-1RN allele 2, which is associ-

Table III genotype distribution

Genotype	Reflux esophagitis (n=247)	Barrett's esophagus (n=255)	*p*-value
IL-1B -511			
CC	116 (47%)	127 (50%)	0.687
CT	106 (43%)	106 (41%)	0.542
TT	25 (10%)	22 (9%)	0.230
IL-1B -3954			
CC	141 (57%)	150 (59%)	0.674
CT	87 (35%)	96 (38%)	0.817
TT	19 (8%)	9 (3%)	0.051
IL-1RN (VNTR)			
1/1	133 (54%)	142 (56%)	0.310
1/2	74 (30%)	78 (31%)	0.736
1/3	6 (2%)	9 (3%)	0.938
1/4	1 (<1%)	- (0%)	0.116
2/2	30 (12%)	24 (9%)	0.248
2/3	2 (<1%)	1 (<1%)	0.212
2/4	1(<1%)	1 (<1%)	0.351

ated with the highest levels of IL-1β, was not associated with BE in this Dutch cohort. This conflicting data in respect to carcinogenesis in the stomach, may partially be explained by the powerful inhibiting capacities of IL-1β on acid secretion [39,40]. Gastroesophageal reflux is prerequisite for the development of BE, and a genetic composition associated with lower acid secretion, may counterbalance the effect of IL-1β on BE development. Another explanation may be provided by the fact that the IL-1β dependent induction of the CDX1 or 2 transcription is dependent on the level of promotor methylation. In normal esophagus, the promotor is silenced by methylation of the 5' region [18]. When this methylation is completely or partially reduced, induction of transcription by NFκB can result in CDX 1 and 2 expression [9,17,18]. It may be that for differentiation towards specialized intestinal epithelium other factors are needed, such as bile acids[17], dietary habits [41], or other inflammatory agents than IL-1β, to reduce the level of promotor methylation. This demethylation may be more important for IL-1β dependent CDX2 expression than genetic regulation of the IL-1β levels in the presence of a strong IL-1β inducer such as gastroesophageal reflux.

In conclusion, we could not observe a difference between IL-1B and IL-1RN polymorphisms and the development of BE in a population exposed to gastroesophageal reflux. This suggests that increased IL-1β levels do not play a role in the development of BE, and that other inflammatory factors than IL-1β have to be identified for their role in esophageal carcinogenesis.

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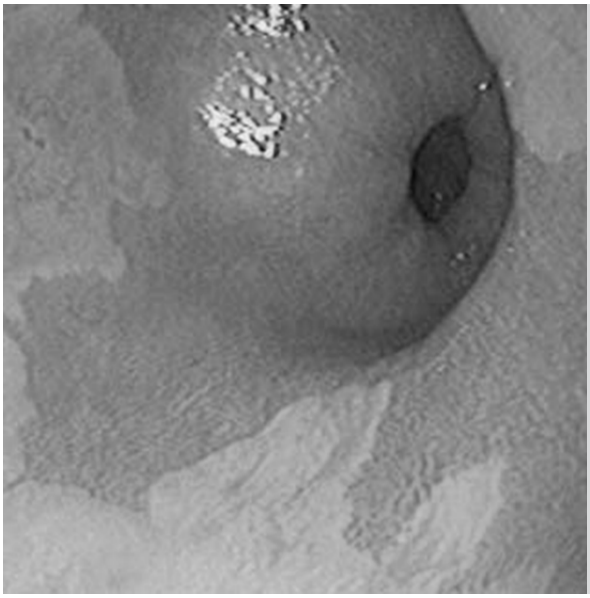

Chapter

VI

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COX-2 CA-haplotype is a risk factor for the development of esophageal adenocarcinoma

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ABSTRACT

Background: Neoplastic progression of Barrett's esophagus (BE) towards esophageal adenocarcinoma (EAC) is associated with increased expression of cyclooxygenase 2 (COX-2). Increased COX-2 expression and enzyme activity is linked to the COX-2 CA haplotype, which consists of two gene polymorphisms in the COX-2 promoter.

Aim: to study the impact of COX-2 haplotypes on the risk of developing EAC in patients with different forms of gastroesophageal reflux disease including BE.

Methods: DNA was obtained from a total of 635 Dutch Caucasian patients comprising of 140 patients with EAC, 255 with BE, and 240 with reflux esophagitis. COX-2 haplotypes were based on the gene polymorphisms at -765C/G and -1195A/G, as determined by PCR-RFLP.

Results: The tested population contained 170 (14%) CA- (-765C and -1195A) haplotypes, 829 (65%) GA-, and 271 (21%) GG-haplotypes, and no GC-haplotypes. The haplotype distribution in patients with reflux esophagitis and BE was similar (CA 12%, GA, 68%, GG 21%), but differed significantly from that in patients with EAC (CA 21%, GA 58%, GG 20%). Particularly, the CA-haplotype was more common ($p<0.001$) in EAC patients. CA-carriership was associated with EAC (OR 2.8 95% CI 1.3-6.2; $p=0.008$), with homozygosity for the CA-allele being statistically most significantly associated (OR 6.1; 95% CI 1.6-24.2; $p=0.01$).

Conclusion: The COX-2 CA-haplotype is more frequently observed in patients with EAC than in patients with BE and reflux esophagitis. These data suggest a direct link between COX-2 activity and neoplastic progression in patients with BE and reflux esophagitis.

INTRODUCTION

Esophageal carcinogenesis is believed to be a multi-step process which involves the consecutive stages of progressions of reflux esophagitis towards Barrett's esophagus (BE), development of low grade and high grade dysplasia in BE, and eventually the development of esophageal adenocarcinoma (EAC). Persistence of severe gastroesophageal reflux related symptoms is associated with an increased risk of EAC development [1, 2]., suggesting that the chronic esophageal inflammation developing secondary to gastroesophageal reflux is elemental for esophageal carcinogenesis [3]. This is supported by the finding that the risk of EAC development in animal models is affected by the severity of esophageal inflammation [4, 5]. As the development of EAC is directly linked to chronic inflammation it is likely that local levels of inflammatory mediators reflects the risk of esophageal carcinogenesis [6].

During the progression of reflux esophagitis into BE and EAC there is a significant increase in the local levels of the cyclooxygenase 2 (COX-2) enzyme [7] and its product prostaglandin E_2 (PGE_2) [8, 9]. Modulation of the immune system through the administration of COX-2 inhibiting agents such as aspirins or non-steroidal anti-inflammatory drugs (NSAIDs) resulted in a reduction of EAC formation in animals[4, 5], and was associated with a lower incidence of EAC in retrospective human studies [10-14]. This suggests that COX-2 activity is important in the early stages of EAC development. Also during tumor progression there seems to be a function for COX-2 since patients with tumors expressing high levels of COX-2 have a worse prognosis compared to those with low COX-2 expressing tumors [7] [15], and high levels of PGE_2 are associated with more aggressive tumor behavior [16].

It is still poorly understood why only a small proportion of all people exposed to gastroesophageal reflux develop either BE or EAC. It is conceivable that genetic factors are involved in this variety of disease outcomes, as gene polymorphisms may modulate the inflammatory host response and thereby the individual susceptibility for a specific disease phenotype. Esophageal COX-2 expression is responsive to local concentrations of reflux components [17, 18] and inflammatory cytokines [19, 20], but is also controlled on a genetic level. The promoter of the COX-2 encoding gene (PTGS2), contains several single nucleotide polymorphisms (SNPs) of which only the SNPs -765_{GtoC} and -1195_{AtoG} were shown to have a direct effect on COX-2 expression and activity [21-23]. The COX-2 –765 C-allele is associated with a 10-fold increased production of the COX-2 enzyme products prostaglandin (PG) E_2 and PGD_2 [23, 24], whereas the COX-2 –1195 A-allele is associated with increased COX-2 expression [22].

We postulated that genetically predetermined increased COX-2 activity levels would predispose people exposed to gastroesophageal reflux to EAC development. To test this hypothesis the impact of COX-2 haplotypes on the variety of disease phenotypes was assessed in patients sharing the same pathogenetic mechanisms. Patients with reflux

esophagitis were taken as control group and were compared to patients with BE or EAC, as these patients all have been exposed to gastroesophageal reflux, but did not develop the mucosal changes as observed in the progression towards BE and EAC.

MATERIALS AND METHODS

Study design

Between November 2002 and February 2005, patients with reflux esophagitis, BE, or EAC who visited the endoscopy unit of the Erasmus MC- University Medical Center Rotterdam, the IJsselland Hospital in Capelle aan de IJssel, and the Academic Medical Center in Amsterdam were invited to participate in this study. The study was approved by the local institutional review boards. Prior to inclusion, all patients gave their written informed consent. All subjects were genetically unrelated Dutch Caucasian patients. Participants underwent an upper endoscopy for evaluation of reflux-related complaints, odynophagia, or problems with passage of food through the esophagus. Participants were included when they either had BE, reflux esophagitis without the presence of BE, or EAC. In total 1251 gastroscopies were performed in patients referred to the endoscopy unit by family physicians, internists, cardiologists or otorhinolaryngologists for the analysis of reflux-related symptoms, suspected extraesophageal manifestations of gastroesophageal reflux disease, or surveillance of BE. Eighty-five percent of the included BE patients already participated in a surveillance program, and were invited to participate in this study. Fifteen percent of the BE patients were newly recognized during the inclusion period. The length of the columnar lined segment was determined endoscopically by measuring the distance between the squamocolumnar junction and the proximal margin of the longitudinal gastric folds. Patients with metaplastic changes shorter than 2 cm were excluded form the study. Of 364 patients with a columnar lined segment of at least 2 cm, 109 patients did not have specialized intestinal metaplasia in any of their biopsies. These patients were also excluded from the study. A total of 724 patients underwent a diagnostic gastroscopy for analysis of symptoms. In 240 patients reflux esophagitis was observed at endoscopic examination in absence of a columnar lined segment. The presence of reflux esophagitis was endoscopicaly graded according to the Los Angeles (LA)-classification [25]. A total of 140 patients were diagnosed with esophageal adenocarcinoma based on histologic examination of biopsies taken from a tumor located for more than 50% in the esophagus. The tumor was either protruding from a BE-segment of from the gastro-esophageal junction. In the majority of EAC patients, the primary diagnosis was made in a local hospital and these patients were referred to the Erasmus MC Rotterdam, or the AMC Amsterdam, for staging and treatment.

Genotyping of PTGS2 -765 and -1195 polymorphisms

Genomic DNA was extracted from 5 ml of whole blood by standard salt-out procedures. The PGST2 haplotypes were determined by amplifying the proximal promoter region by polymerase chain reaction (PCR) using the following primers: -765 forward 5'-AGG CAG GAA ACT TTA TAT TGG-3', -765 reverse 5'-ATG TTT TAG TGA CGA CGC TTA-3', -1195 forward 5'-ccctgagcactacccatgat-3', and -1195 reverse 5'-gcccttcataggagatactgg-3'. 50 ng DNA was added to 20 pmol/L of each primer, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 1 unit Taq polymerase (Roche) in a total volume of 25 µl. An 391 bp fragment was generated by the following PCR-procedure: an initial step at 94°C for 2 min was followed by 35 amplification steps (94°C 45 sec, 60°C 45 sec and 72°C for 1 min), followed by a final extension step at 72°C for 10 min. The polymorphisms were determined by restriction fragment length polymorphisms using the restriction endonuclease *Aci*I (New England Biolabs, Frankfurt, Germany) for the PTGS2 -765 polymorphism and *Pvu*II (New England Biolabs) for the PTGS2 -1195 polymorphism.

Statistical analyses

The study was powered (80%) to detect a 10% difference in allele distribution between the two patients groups (significance level 5%). Differences between the allele distribution of the PTGS2 -765 and -1195 polymorphisms, as well as differences between the patients groups in age, sex and gender were determined by Chi-square analysis. Age and sex corrected odds ratio (OR) and 95% confidence intervals (95% CI) was calculated for association with EAC by logistic regression analysis. A two-sided p-value ≤ 0.05 was considered to be statistically significant. All statistical analyses were conducted by using SPSS 11.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Patient characteristics

In total, 240 patients with reflux esophagitis, 255 with BE, and 140 with EAC were included in the study. The patient characteristics are shown in Table I. In line with previous studies [26, 27], the male gender was more frequently found in the BE (OR 1.9 95% CI 1.3-2.7; $p<0.001$) and EAC (OR 6.7 95% CI 3.7- 11.9; $p<0.001$) than in the reflux esophagitis group. On average patients with EAC ($p<0.001$) and BE ($p<0.001$) were slightly older than reflux esophagitis patients (Table I). Also when comparing our EAC group with the BE group the expected higher prevalence of the male gender ($p<0.001$) and age of EAC patient ($p=0.04$) was observed (Table I).

Table I Patient characteristics

	reflux esophagitis n=240	Barrett's esophagus n=255	Esophageal adenocarcinoma n=140
Age, mean ± SD (year))	56 ± 14	62 ± 13	65 ± 11
Male gender (%)	127 (53%)	173 (68%)	124 (88%)
BE length, mean ± SD (cm)	0	4.1 ± 2.4	nd*
Reflux esophagitis present (%)#	240 (100%)	126 (50%)	nd
No inflammation	0%	129 (50%)	
Grade A	55 (23%)	13 (5%)	
Grade B	143 (59%)	55 (22%)	
Grade C	35 (15%)	40 (18%)	
Grade D	7 (3%)	18 (5%)	
Use of proton pump inhibitor therapy	108 (45%)	212 (83%)	nd
Hiatal hernia	156 (65%)	209 (82%)	nd

*nd: no data available, # grading scored according to LA classification

Allele distribution

It has recently been demonstrated that the PTGS2 -765 and -1195 polymorphisms have a functional effect on COX-2 expression and enzyme activity [22, 23]. Using reflux esophagitis patients as the reference we tested for an association of these two polymorphisms with BE and EAC. The allele frequencies among the 635 Dutch Caucasian subjects for -765C (rs 20417) Were 75% GG, 23% GC and 2% CC. This is in line with findings in other studies in Caucasian people (67-70% GG, 29-31% GC and 1.6-3% CC) [28-31]. To our knowledge, this information is not available for the polymorphism at position 1195 (rs689466). When both polymorphisms were investigated separately, there was a statisti-

Table II Allele distribution of the PTGS2 gene polymorphisms in patients with reflux esophagitis, BE, and EAC.

genotype	phenotype		
	Reflux esophagitis (n=240)	Barrett's esophagus (n=255)	Esophageal adenocarcinoma (n=140)
PTGS2 G-765C			
GG	183 (76%)	201 (79%)	92 (66%)
GC	57 (24%)	50 (20%)	41 (29%)
CC	- (0%)	4 (1.5%)	7 (5%)
allele frequency			
G	0.881	0.886	0.804
C	0.119	0.114	0.196
PTGS2 G-1195A			
AA	154 (64%)	155 (61%)	83 (59%)
AG	76 (32%)	85 (33%)	54 (39%)
GG	10 (4%)	15 (6%)	3 (2%)
allele frequency			
A	0.800	0.775	0.786
G	0.200	0.225	0.214

cally significant difference of the PTGS2 -765 allele distribution between the different patient groups (Table II). When reflux esophagitis patients were compared with BE and EAC patients, the PTGS2 -765 CC genotype was more frequent in patients with BE (reflux esophagitis 0% vs. BE 1.6%; p=0.088), and in patients with EAC (reflux esophagitis 0% vs. EAC 5%; p<0.001). As there was only a small non-significant difference between reflux esophagitis and BE, but a significant difference between reflux esophagitis or BE and EAC patients (p=0.008), the CC-genotype was predominantly associated with the presence of EAC. The allele distribution of the PTGS2 -1195 polymorphism was not different between reflux esophagitis, BE, and EAC.

CA-haplotype is associated with a higher frequency of EAC

Based on the two polymorphisms tested, a haplotype analysis was performed. In the studied population, three different haplotypes could be distinguished. The tested population contained 170 (14%) CA-haplotypes (C_{-765} and A_{-1195}) , 829 (65%) GA-, 271 (21%) GG-haplotypes, and no CG-haplotypes (0%). As a consequence of the latter, the –765 C-allele was always associated with the –1195 A-allele in this population. The CA-haplotype was more frequently observed in patients with EAC than in patients with BE and reflux esophagitis (Figure 1). It was therefore investigated whether the CA-haplotype was a risk-allele for the development of EAC. BE patients carrying only a single CA-allele had a 2.0 –fold increased risk for developing EAC (95% CI 1.287-3.47; p=0.004), while BE patients homozygous for the CA-allele had a 3.8-fold increased risk (95%CI 1.1-14.6; p=0.048;Table III).

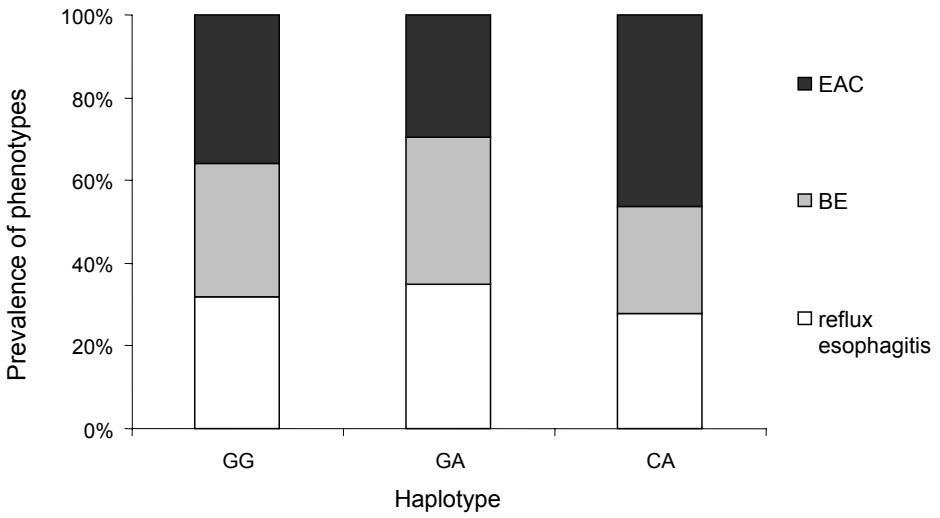

Figure 1. Disease distribution per haplotype. The CG haplotype (C-765 and G-1195) was absent from the tested population. The distribution of reflux esophagitis (white), BE (gray) and EAC (black) per haploytpes (GA, GG and CA) is given. The CA-haplotype displays a strong association with the presence of EAC as compared to the GG- and GA-haplotype (p<0.001).

Table III The relative risk of different COX-2 haplotypes for the presence of EAC.

PTGS2 Genotype	haplotype	n	EAC vs. reflux esophagitis		EAC vs. BE		EAC vs. BE+reflux esophagitis	
			OR	95% CI+ *p*-value	OR	95% CI+ *p*-value	OR	95% CI+ *p*-value
-765_{GG}- *x* 1195_{GG}	GG/GG	28	1.9	(0.4- 10.2; *p*=0.46)	0.7	(0.2-2.5; *p*=0.5)	0.9	(0.3- 3.4; *p*=0.9)
-765_{GG} *x* -1195_{AG}	GA/GG	174	1.2	(0.6-2.3; *p*=0.54)	1.3	(0.7-2.2; *p*=0.4)	1.3	(0.8-2.1; *p*=0.4)
-765_{GG} *x* -1195_{AA}	GA/GA	274	-	-	-	-	-	-
-765_{GC} *x* -1195_{AA}	GA/CA	107	1.5	(0.7- 3.1; *p*=0.25)	2.0	(1.1- 3.8; *p*=0.03)	1.8	(1.1- 3.2; *p*= 0.045)
-765_{GC} *x* -1195_{AG}	GG/CA	41	2.9	(1.1-7.7; *p*=0.036)	2.8	(1.2-6.5; *p*=0.02)	2.8	(1.3-6.2; *p*=0.008)
-765_{CC} *x* -1195_{AA}	CA/CA	11	-	-	3.8	(1.1-14.6; *p*=0.048)	6.1	(1.6-24.2; *p*= 0.01)

In patients with reflux esophagitis, the presence of a CA-haplotype was only a risk factor for EAC development if it was associated with the presence of a GG-haplotype (OR 2.9 95%CI 1.1-7.7; *p*=0.036) but not when associated with the GA-haplotype (OR 1.5 95%CI 0.7- 3.1; *p*=0.25). Heterozygosity of the CA-allele was therefore not associated with an increased risk of EAC development in patients with reflux esophagitis (OR 1.7 95%CI 0.9-3.0; *p*=0.083). Homozygosity for the CA-haplotype was not observed in the reflux esophagitis population. As reflux esophagitis is considdered the obligate precondition for BE we also compared the allele frequencies of these two groups combined with the frequency in the EAC group. When BE patients and reflux esophagitis patients were taken together and compared with EAC, patients heterozygous for the CA-haplotype had a higher risk of developing EAC than patients without the CA-haplotype (OR 1.9, 95% CI 1.2-3.0, *p*=0.007). BE patients homozygous for the CA-haplotype had an even higher risk (OR 6.1, 95% CI 1.6-24.2, *p*=0.01) (Table III). These results indicate that the PTGS2-CA haplotype is a risk allele for EAC, but primarily in patients with BE. There was also a higher risk of EAC if the CA-haplotype was combined with a GG-haplotype instead of a GA-haplotype (Table III).

DISCUSSION

Gastroesophageal reflux is a major risk factor for the development of either BE and EAC [1-3]. It is however still poorly understood why only a small proportion of people exposed to gastroesophageal reflux, indeed develop BE or EAC. COX-2 is an important modulator of immune reactivity in the inflamed esophagus, and COX-2 enzyme activity is associated with EAC development [4, 5, 10-14]. Polymorphisms in the gene encoding for COX-2 (PTGS2) are known to modulate COX-2 expression and enzyme activity, and we hypothesized that this genetic variation may modulate the individual susceptibility for BE and EAC. We have therefore performed a genetic association study to investigate the impact of COX-2 polymorphisms on the progression of people exposed to gastroesophageal reflux to either BE or EAC. Patients with endoscopically observed reflux esophagitis were therefore compared to patients with endoscopically and histologically proven BE

and EAC. It is shown in this study that the COX-2 $C_{-765}A_{-1195}$ haplotype, which is associated with increased COX-2 enzyme expression and activity, is more prevalent in patients with EAC (OR 3.8 95%CI 1.1-14.6; p=0.048). This suggests that increased enzyme activity associated with increased levels of PGE_2 may be important in the progression of BE to EAC.

Although several polymorphisms are present in the PTGS2 gene, thus far only two of these polymorphisms ($-765_{C/G}$ and $-1195_{A/G}$) have been shown to exert an effect on COX-2 production [21-23, 32]. The C-residue at position –765 is associated with increased COX-2 enzyme activity, resulting in higher levels of the COX-2 enzyme products PGE_2 and PGD_2 [23]. In the present study, the polymorphism at position –765 was associated with a significant increase in the risk of EAC (Table II). This suggests that increased levels of COX-2 enzyme products, , results in the generation of a tumor promoting environment in the esophagus, thereby either directly or indirectly (e.g. through changes in PGE_2 levels) affecting the risk of EAC development. PGE_2, the major reaction product of the COX-2 enzyme, is associated with increased proliferation, decreased apoptosis, and increased angiogenesis. Increased PGE_2 production is therefore associated with an increased risk of cancer development [19]. In addition increased local PGE_2 levels are known to induce the modulation of the ongoing inflammation towards a T helper 2 (Th2) type immune response [19, 33]. In line with this, we and others recently showed that progression from reflux esophagitis to BE is associated with a shift towards a more Th2 type immune response [34, 35]. This Th2 shift results in the generation of a pre-malignant environment similar to the situation in other pre-malignant chronic inflammatory conditions such as Helicobacter pylori induced gastric carcinoma [36], hepatitis B and C induced hepatocellular carcinoma [37], and HPV associated cervix carcinoma [38].

The polymorphism at position –1195 was shown to be associated with increased COX-2 transcription [22]. In our study there was no significant association between EAC development and the polymorphism at position –1195. Perhaps the in vivo relevance of the c-MYB binding site that is created by the -1195_{GtoA} change is less important in the presence of strong inducers of COX-2 expression such as reflux components [17, 18], and inflammatory cytokines [19, 20] than the in vitro model used to test the effect of this polymorphism on COX-2 promotor activity [22]. Alternatively this might be explained by a lack of the CG haplotype in our population, rendering it impossible to test the -1195 polymorphism independent of the potentially more dominant effects of the -765 polymorphism. The finding of an association of the COX-2 $C_{-765}A_{-1195}$-haplotype with EAC suggests that genetic regulation of COX-2 expression and enzyme activity might play a role in the progression towards EAC.

The significance of genetic regulation of COX-2 expression and enzyme activity in carcinogenesis has also been shown in other inflammation-related cancers such as colorectal cancer [39], lung cancer [40], bladder cancer[41], and esophageal squamous cell carcinoma [22]. Expression of COX-2 has also been implicated in the development and tumor behavior of EAC. Increased COX-2 expression has been shown in 41-94% of BE

patients as compared to no or only mild COX-2 expression in inflamed and non-inflamed squamous epithelium [17, 42-45]. This percentage increases to 83% in dysplasia, and to 99-100 % in EAC [7, 42, 43]. In a recent prospective study, increased COX-2 expression was associated with more advanced local tumor growth, more often nodal involvement, increased local recurrence, and a poor survival [15]. This confirms earlier findings by Buskens et al. [7], who showed a prognostic significance of increased COX-2 expression for survival, distant metastases, and local recurrence in 145 patients with EAC. Whether COX-2 is mainly involved in the early formation of BE, or in progression to dysplasia and EAC, is still under debate. In some studies, the level of COX-2 expression did not differ between dysplastic and non-dysplastic Barrett's epithelium, and between BE and EAC [44, 45]. Other studies however, found increased COX-2 expression with progression to high grade dysplasia and EAC [7, 43, 46]. In our study, differences in haplotype distribution were only observed between EAC and reflux esophagitis or BE, and not between reflux esophagitis and BE. This would suggest that COX-2 polymorphisms affect the progression of BE towards EAC, but not the progression of reflux esophagitis towards BE. Furthermore, the CA-haplotype displayed a stronger association with EAC when analyzed in BE than in reflux esophagitis patients. This is in line with the assumption that BE is a more advanced stage in the reflux-BE-EAC cascade, and that other factors relevant for EAC development are already active in BE patients, resulting in an augmented neoplastic tendency of the CA haplotype associated increased PGE_2 production.

Our data support the presumed beneficial effect of NSAIDs induced COX-2 inhibition for the prevention of EAC development in patients with reflux esophagitis and BE [4, 5, 10-14], and suggest that testing for the COX-2 polymorphisms may contribute to risk stratification. However as in our study only Dutch Caucasian patients were included, further studies are needed to elucidate whether testing for COX-2 CA-haplotypes is useful in the identification of patients at an increased risk for EAC development. The distribution of the –765 C-allele in the Dutch population is in line with reported frequencies in other Caucasian populations [28-31]. This decreases the chance that the difference observed in the study is due to a selection bias in the Dutch population, and suggests that COX-2 polymorphisms may alter the disease outcome in the presence of prolonged exposure to gastroesophageal reflux.

This is a genetic association study comparing patients with reflux esophagitis and BE with EAC. Therefore, there is a risk that the association between the COX-2 CA-haplotype and the presence of EAC is based on a type 2 statistical error. Future studies should confirm this association with more patients in each group. Another limitation of this study is the selection of a control group. We have selected a control group consisting of patients with reflux esophagitis but without mucosal changes as can be found in patients with BE and EAC. We have chosen these control group, as the mucosal inflammation clearly confirms the presence of gastroesophageal reflux and its ability to induce mucosal erosions. We thereby assume that this group has more or less the same reflux characteristics

as compared to patients with BE and EAC. As we have not investigated the severity, duration, and pattern of gastroesophageal reflux, the content of the gastroesophageal refluxate, dietary habits, this may introduce the possibility that only a small proportion of the control patients have similar reflux characteristics as the patients with BE. This is of course also true for the comparison between BE and EAC. This may lead to an over- or underestimation of the impact of different genotypes and may result in the incorrect conclusion that COX-2 haplotypes are not associated with the progression of reflux esophagitis to BE. It also cautions drawing too robust conclusions on a direct causal relation between the COX-2 haplotype and the development of EAC. Future studies should include pH-metry, refluxate composition examination, and a dietary questionnaire, to guarantee a control group exposed to the same degree and type of reflux esophagitis, and with the same dietary habits. This can in our opinion only be achieved in a prospective study were most of the determinants can be controlled. Finally, to investigate whether COX-2 polymorphisms are indeed involved in the progression of patients with BE to EAC, a prospective study is indicated with long-term follow-up of a cohort of patients with reflux esophagitis and BE in order to investigate whether patients with the CA-haplotype have indeed an increased risk of developing EAC.

In conclusion, we show that the homozygous COX-2 CA-haplotype is associated with a significantly increased risk of developing EAC. Others previously showed that the CA-haplotype is associated with increased expression and enzyme activity of COX-2 [21-23]. This suggests that the local chronic Th2 type inflammatory environment that is induced by increased COX-2 activity plays a major role in esophageal carcinogenesis, supporting the concept that modulation of esophageal inflammation by aspirin, NSAIDs, or selective prostaglandin receptor antagonists will reduce the risk of EAC development in patients with BE.

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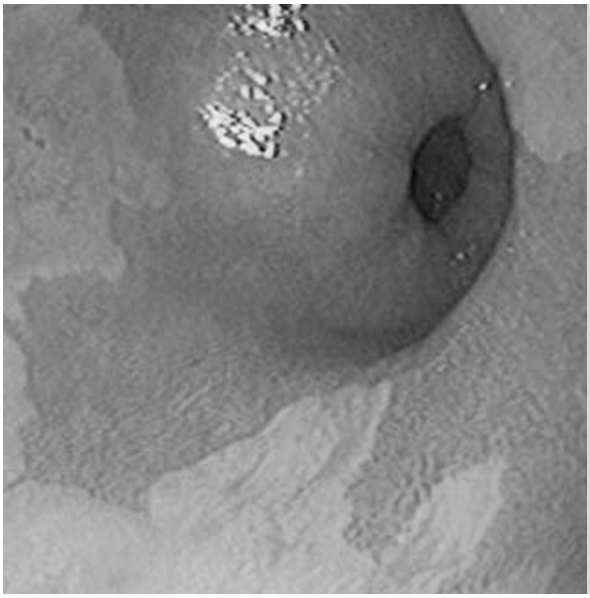

Chapter

VII

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The homeodomain protein CDX2 is an early marker of Barrett's oesophagus

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ABSTRACT

Background: Barrett's esophagus (BE) is defined by the replacement of squamous epithelium by specialized intestinal epithelium (SIE). Transcription factors associated with normal intestinal differentiation may be involved in the development of BE. One of the key regulators of intestinal differentiation is thought to be CDX2. To evaluate if CDX2 is involved in the development of BE, expression of CDX2 was determined in BE, in squamous epithelium and adenocarcinoma of the esophagus.

Methods: CDX2 expression was assessed in 245 samples from the esophagus. These consisted of 167 biopsies of the columnar lined segment and 38 squamous epithelial biopsies of 39 patients with histologically confirmed BE, of whom 10 also had an adenocarcinoma, In addition, 40 biopsies of 20 patients with reflux esophagitis without BE were evaluated. The presence of CDX2 protein was detected by immunohistochemistry in 138 formalin-fixed paraffin-embedded biopsies of 16 patients with BE, 4 patients with an esophageal adenocarcinoma and 20 patients with reflux esophagitis. Semi-quantitative detection of *Cdx*2 and Muc2 mRNA was performed by RT-PCR on RNA isolated from 88 snap frozen biopsies of BE and squamous epithelium of 19 patients with BE, and when present from esophageal adenocarcinoma.

Results: In 53/79 biopsies taken from the columnar lined segment SIE was present, in which CDX2 protein was observed in all epithelial cells. However, CDX2 protein was not observed in biopsies containing only gastric metaplastic epithelium (26/79), or in squamous epithelium (0/40) of patients with reflux esophagitis ($p<0.001$). *Cdx*2 mRNA was detected in all biopsies with goblet cell specific Muc2 transcription, which is indicative for the presence of SIE. Low levels of Cdx2 mRNA were also observed in 6/19 squamous epithelium samples taken 5 cm above the squamo-columnar junction of patients with BE.

Conclusion: CDX2 protein and *Cdx*2 mRNA are strongly associated with the presence of SIE in the esophagus. As *Cdx*2 mRNA was also present in one-third of BE patients with endoscopically normal appearing squamous epithelium, expression of *Cdx*2 may precede morphological changes observed in BE. Therefore, pathways involved in the induction of *Cdx*2 transcription in squamous epithelial cells are likely to play a role in the development of Barrett's esophagus.

INTRODUCTION

Barrett's esophagus (BE) is a complication of chronic gastro-esophageal reflux disease (GERD). Although 20-30% of the Western population has regular GERD-related complaints [1], it is poorly understood why only 3-5 % of these patients develop long segment BE [2]. BE has a prevalence of 0.03% in the Western population [3] and its development in patients with GERD is associated with obesity at a young age [4], increased duodeno-gastro-esophageal reflux [5, 6], complaints at an earlier age [5], a familiar predisposition [7], and a combination of obesity, presence of a hiatal hernia and male gender [6]. Patients with BE have an increased risk of developing adenocarcinoma in the esophagus with an annual incidence of 0.5% per year [8]. Once these patients have developed adenocarcinoma, the prognosis is poor with a 5-year survival rate of 5-20% [9].

BE is characterized by the metaplastic replacement of squamous epithelial cells of the lower part of the esophagus by specialized intestinal epithelium (SIE), which is associated with the presence of goblet cells and the expression of intestinal markers such as MUC2 [10], alkaline phosphatase [11], villin [12], and sucrase-isomaltase [13]. The genetic events responsible for this process are largely unknown.

Transcription factors, which play an important role in normal intestinal differentiation, may also play a role in the development of SIE in the esophagus. CDX2 is such a transcription factor, and belongs to the caudal-related homeobox gene family[14]. CDX2 expression in the gastrointestinal tract is intestine-specific, with a tightly regulated anterior boundary in the duodenum [15]. CDX2 is involved in early differentiation and maintenance of intestinal epithelial cells, characterized by the formation of multilayered structures with microvilli [14]. CDX2 also induces intestine-specific transcription of the genes encoding MUC2, alkaline phosphatase and sucrase-isomaltase [14, 16-18]. Therefore, CDX2 is considered to be an important factor in the development and differentiation of intestinal epithelium [19, 20].

As BE is characterized by the development of SIE in the esophagus, CDX2 may also play a role in the development of BE. To investigate if CDX2 expression is associated with BE and if its expression may precede the morphological changes observed in BE, we determined its expression in columnar epithelium of patients with BE, in squamous epithelium of patients with reflux esophagitis only, and in esophageal adenocarcinoma. Here, we demonstrate that CDX2 is expressed in SIE, but not in squamous epithelium of patients with reflux esophagitis and in gastric metaplastic epithelium. Furthermore, Cdx2 mRNA was also detected in squamous epithelium of one-third of patients with BE, suggesting that CDX2 is indeed involved in the development of BE and that its expression may precede morphological changes observed in BE.

MATERIALS AND METHODS

Patients and Materials

CDX2 expression was analyzed in 245 esophageal samples. These consisted of 167 biopsies of the columnar lined segment and 38 squamous epithelial biopsies of 39 patients with histologically confirmed BE, of whom 10 also had an esophageal adenocarcinoma, and 40 biopsies of 20 patients with reflux esophagitis without BE.

In 138 biopsies, consisting of 79 biopsies of the columnar lined segment, 19 esophageal adenocarcinoma, and 40 squamous epithelium biopsies of the esophagus, CDX2 protein was detected by immunohistochemistry (Table 1). The four quadrant biopsies taken at 2 cm intervals from the columnar lined segment were pooled, formalin-fixed, and paraffin-embedded. Biopsies of the colon were used as a positive control.

Biopsies from a second group of patients, not related to the first group, were used for mRNA analysis, as this analysis could not be performed on the formalin-fixed paraffin embedded samples. One-hundred and seven esophageal biopsies were collected at endoscopy from 19 patients with BE (Table 1), of whom 6 patients also had esophageal adenocarcinoma. Biopsy specimens were obtained from the columnar mucosa of the esophagus (n=38), the adenocarcinoma if present (n=12), and the squamous epithelium 5 cm above the neosquamous-columnar junction (n=38). For each of these locations, the biopsies of each location (2 of the BE segment, 2 of the squamous epithelium, and when present 2 of the esophageal adenocarcinoma) of individual patients were pooled, snap frozen and used for RNA extraction (see below). An additional biopsy was taken next to the previous biopsies from the BE-segment, and was used for the histological evaluation of the presence of SIE (n=19). All columnar segments lining the esophagus at endoscopy of both groups of patients had a length of 3 cm or more. Biopsies of the colon were used as positive control for *Cdx*2 mRNA. The study was approved by the Central Committee on Research Involving Human Subjects in The Netherlands in 2002.

Table 1. Patient characteristics

Patient characteristics		Nº of patients	Nº of biopsies	Age Mean (SD)	Male %
First group					
IHC[a]	Adenocarcinoma	4	19	78.5 (2.7)	75%
	Barrett's oesophagus	16	79	70.8 (14)	66%
	Reflux oesophagitis	20	40	61.8 (11.6)	71%
Second group					
RT-PCR[a]	Adenocarcinoma	6	12	68.9(11.5)	57%
	Barrett's oesophagus	19	76	65.1(15.1)	55%

[a] Abbreviations used: IHC, immunohistochemistry; RT-PCR, reverse transcriptase PCR.

Histological analyses

Sections from the biopsies and part of the biopsies taken for RNA analysis, were stained with haematoxyline and eosin (H&E) and evaluated for the presence of SIE and/or adenocarcinoma. Alcian blue at pH 2.5 staining was performed to facilitate the detection of mucin producing goblet cells [21]. The inflammatory response in biopsies of patients with reflux esophagitis and BE, which were used for immunohistochemistry, was graded by the Ismail-Beigi classification [22] for squamous epithelium and by the updated Sydney system [23, 24] for columnar epithelium.

Immunohistochemistry

Biopsy samples were serially sectioned at 4 µm, mounted on adhesive slides, dried overnight at 37 °C, and deparaffinized with xylene. Antigen retrieval was performed in 10 mM monocitric acid at pH 6.0 at 100 °C for 15 min. After cooling, the samples were blocked with non-immune serum for 30 minutes. The sections were stained using the primary antibody against CDX2 (1:100 diluted; Biogenex, San Ramon, USA). Followed by the addition of a biotinylated rabbit secondary antibody (DAKO, Glostrup, Denmark) and streptavidine-alkaline phosphatase complex (DAKO, Glostrup, Denmark). A red color was developed using new-fuchsine substrate.

Semiquantitative RT-PCR

Total RNA was isolated using TRIzol-reagent (Invitrogen, Groningen, The Netherlands), and remaining chromosomal DNA was subsequently removed with the DNA-free RNA kit (Zymo, Orange, USA). Semiquantitative reverse transcription (RT)-PCR was performed using the intron-spanning primers *Cdx*2 5′-CCCAGCGGCCAGCGGCGAAACCTGT / 5′-TATTTGTCTTTTGTCCTGGTTTTCA and *Muc*2 5′-CAGGATGGCGCCTTCTGCTA / 5′-ATGCTGCTCCAAGCTGAGGT. Levels of *Cdx*2 and *Muc*2 mRNA were standardized to levels of β-actin using the primers β-actin5'-CAAGGCCAACCGCGAGAAG / 5'-CAGGGTACATGGTGGTGCC. cDNA was synthesized with the use of avian myeloma virus reverse transcriptase (Promega, Madison, USA). Primers were annealed by cooling down from 70°C to room temperature, followed by cDNA synthesis by incubation for 30 min at 42°C. PCR-reactions (total volume of 25 µl) contained 1 µl of the cDNA solution, 1× PCR-core buffer (Promega), 2 mM $MgCl_2$, 0.4 µM forward and reverse primer, 200 µM of each nucleotide (Promega) and 0.02 U/µl Taq polymerase (Promega).

PCR conditions were 35 cycles at 94°C (30 s); 55°C (30 s) and 72°C (1 min). PCR-products were size-separated on a 2% agarose gel and stained with ethidium bromide. Band size and intensity were determined with the Kodak 1D software version 3.5 (Kodak, Rochester, USA). Bands were standardized against a housekeeping gene, β-actin, as described previously [25].

Statistical analyses

All continuous variables were expressed as mean ± SEM. Statistical analyses were done by using the Fisher's exact test for immunohistochemistry and the Mann Whitney U test for the semi-quantitative RT-PCR data. A two-sided *p*-value <0.05 was considered statistically significant.

RESULTS

Histology

SIE was observed in 53/79 (67%) of the biopsies from the columnar lined segment and was absent in 26 (33%) biopsies. The biopsies without SIE existed of gastric type (cardiac or fundic-type) epithelium. SIE was also absent in all 40 reflux esophagitis biopsies, which contained only squamous epithelium in all biopsies.

The inflammatory response in BE was graded according to the updated Sydney system, in which the inflammation is divided in 4 categories based on a acute component (numbers of neutrophils and eosinophils) and a chronic component (mononuclear cell count) in the epithelium [24]. This system was originally developed for glandular epithelium of the stomach, but has also been shown to be useful in the inflammatory classification of BE [24]. The acute component of inflammation in BE samples ranged from mild to severe in the majority of biopsies, with 4 BE patients showing a grade 1, 7 BE patients a grade 2, and 5 BE patients a grade 3 inflammation, according to the updated Sydney classification (Table 2).

The chronicity of the inflammation ranged from mild to severe, with five BE patients showing a grade 1, seven patients showing a grade 2, and four BE patients showing a grade 3. The inflammation in the 40 squamous epithelium biopsies of the 20 patients with reflux esophagitis was graded as grade 1 in seven patients, grade 2 in seven patients and grade 3 in six patients, according to the Ismail-Beigi classification (Table 2). For this reason, we assumed that the biopsies were representative for the whole spectrum of inflammation in both BE and reflux esophagitis.

Table 2. Histological classification of inflammation

Reflux oesophagitis		Barrett's oesophagus		
Histological classification		Histological classification		
Ismail-Beigi [a]	n=20	Updated Sydney [b]	n=16	
			Acute	Chronic
1	7/20 (35%)	1	4/16 (25%)	5/16 (31%)
2	7/20 (35%)	2	7/16 (44%)	7/16 (44%)
3	6/20 (30%)	3	5/16 (31%)	4/16 (25%)
		4	0	0

[a] Scored according to reference [22]

[b] Scored according to reference [24]

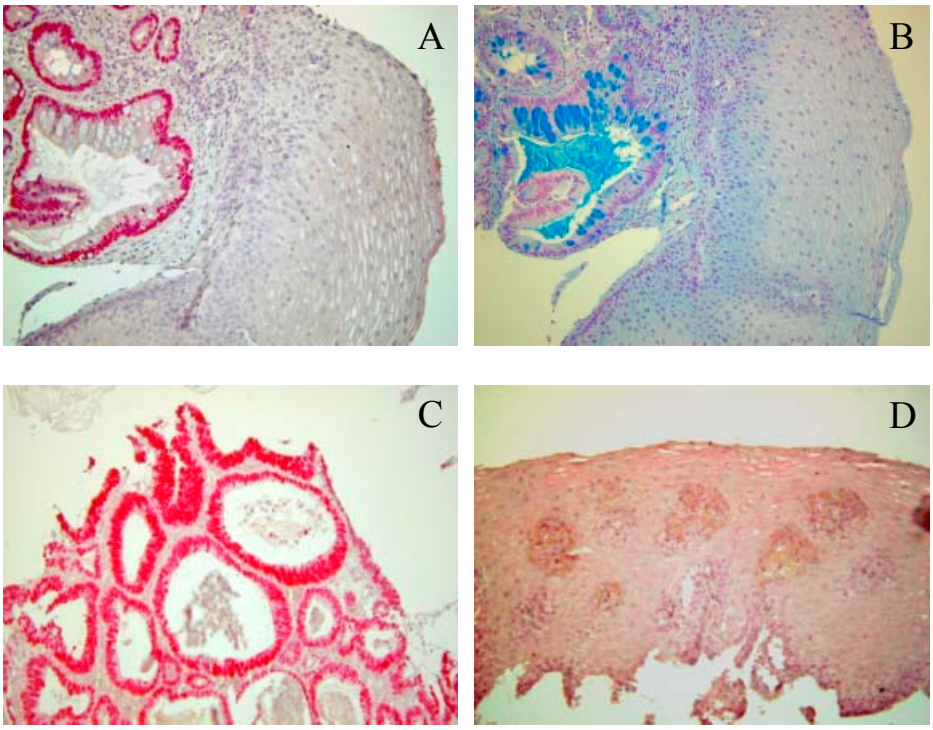


Figure 1: CDX2 protein in Barrett's esophagus, esophageal adenocarcinoma, and reflux esophagitis. (A) A positive nuclear stain (red) for CDX2 was observed in 16/16 samples of Barrett's esophagus. A representative slide of patient 10 is shown. (B) he presence of CDX2 was associated with goblet cells, which are characteristic of Barrett's esophagus, as was shown in an Alcian Blue at pH 2.5 stain of a serially sectioned slide of the same patient. (C) CDX2 was also present in 4/4 adenocarcinomas, which can be seen in a slide of patient 3. (D) CDX2 was absent in the squamous epithelium of all patients with reflux esophagitis (0/20).

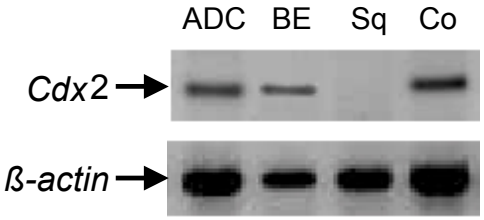
CDX2 expression

All esophageal biopsies (53 SIE, 26 gastric type, 19 esophageal adenocarcinoma, and 40 inflamed squamous epithelium) were analyzed for presence of CDX2 protein by immunohistochemistry. All 53 biopsies with SIE had a positive nuclear stain for CDX2 of the epithelium (Figure 1A). This staining was associated ($p< 0.001$) with the presence of goblet cells, as detected in serially sectioned slides with Alcian Blue at pH 2.5 staining (Figure 1B). Nuclear CDX2 expression was also observed in all 19 esophageal adenocarcinoma samples (Figure 1C). Cytoplasmic staining of CDX2 was not observed in any of the samples. Nuclear or cytoplasmic staining of CDX2 protein was absent in the 26 gastric type epithelium biopsies and in 40 squamous epithelium biopsies (Figure 1D).

Molecular analysis

Presence of Cdx2 mRNA was evaluated in the second group of 19 patients with BE by RT-PCR, and this was normalized for β-actin transcript levels (Figure 2a). For all 19 pa-

A



B

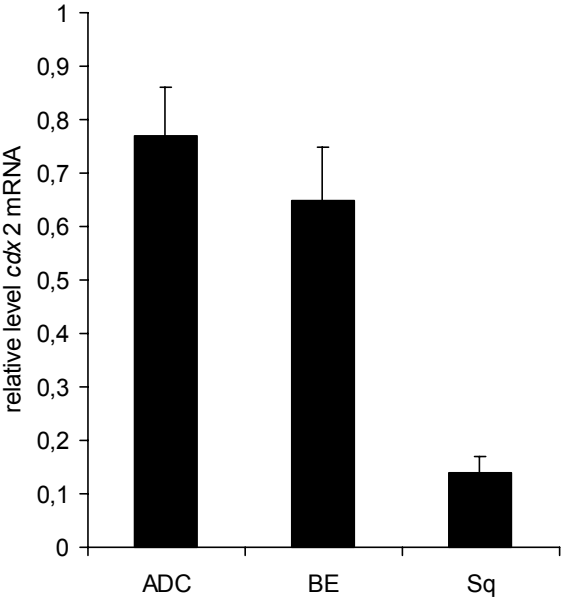


Figure 2: Cdx2 mRNA in Barrett's esophagus, squamous epithelium and esophageal adenocarcinoma. (A) *Cdx*2 mRNA was detected by RT-PCR (95 bp fragment) in 4/6 esophageal adenocarcinoma (ADC) samples, in 13/19 Barrett's esophagus (BE) samples, in 6/19 squamous epithelium (Sq) samples and in colon control tissue (Co). (B) Relative mRNA levels of *Cdx*2 in squamous epithelium, BE and esophageal adenocarcinoma. Levels of *Cdx*2 mRNA were normalized against β-actin. The mean *Cdx*2 mRNA levels in squamous epithelium (n=6) were significantly lower than the levels observed in BE (n=13) (:$p<0.01$). The relative mRNA levels of *Cdx*2 did not differ significantly between BE (n=13) and esophageal adenocarcinoma (n=4) (NS: $p<0.1$).

tients, the presence of SIE was confirmed in the biopsies taken adjacent to those used in the transcriptional analysis. *Cdx*2 mRNA was detected in 13/19 BE segments and in 4/6 esophageal adenocarcinomas (Figure 2b). The levels of *Cdx*2 mRNA did not significantly differ between BE and esophageal adenocarcinoma (p=0.9) (Figure 2b).

In order to determine if expression of *Cdx*2 precedes the morphological changes seen in BE, levels of *Cdx*2 mRNA were also determined in squamous epithelium biopsies obtained 5 cm above the neosquamous-columnar junction of patients with BE (Figure 3). Low levels of *Cdx*2 mRNA were present in 6/19 (32%) samples of squamous epithelium (Figure 3). The relative levels of *Cdx*2 mRNA in the squamous epithelium were significantly lower ($p \leq 0.01$) than those observed in BE tissue (Figure 2b). The presence of goblet cells, characteristic for BE, was evaluated by the detection of goblet cell specific *Muc*2 mRNA. *Cdx*2 mRNA was present in all *Muc*2 positive samples. Furthermore, samples without the presence of *Cdx*2 mRNA did not have *Muc*2 transcription. In only three BE samples with *Cdx*2 transcription, *Muc*2 mRNA was absent (Figure 3). *Muc*2 transcripts were also not detected in any of the squamous epithelium samples.

DISCUSSION

In this study, we demonstrated that CDX2 protein was present in BE containing SIE, as was recently reported by others in BE [26, 27]. CDX2 expression was also detected in esophageal adenocarcinoma. In contrast, no CDX2 protein was observed in biopsies containing gastric type epithelium of the distal esophagus of patients with BE, nor in squamous epithelium of patients with reflux esophagitis without BE. Low levels of *Cdx*2 mRNA were however detected in approximately one third of the squamous epithelium samples of patients with BE. The presence of *Cdx*2 mRNA also correlated with goblet cell specific *Muc*2 mRNA in BE samples (Figure 3).

The homeobox protein CDX2 is involved in the differentiation and maintenance of intestinal epithelium [14]. Expression of CDX2 is detected at the time of morphogenesis in the visceral endoderm of mouse intestine [28] and continues to be present throughout adulthood, but then is normally restricted to the intestine [29]. It is detectable in the crypts of the intestine as well as in the villi [15] and is suggested to be a key regulator of intestinal differentiation [19]. Exogenous expression of CDX2 in IEC6 cells, an undifferentiated rat intestinal cell line which does not express CDX2, causes differentiation of IEC6 cells into goblet cells and absorptive enterocytes [14]. Similar observations have been made in an animal model, in which ectopic expression of CDX2 induced the development of metaplastic changes of the gastric antrum, and in Helicobacter pylori-related intestinal metaplasia of the human stomach [30, 31]. These metaplastic changes of the mouse gastric antrum were also characterized by the development of goblet cells and absorptive enterocytes, and the expression of intestine specific proteins such as MUC2,

alkaline phosphatase, villin, guanylyl cyclase C and Trefoil factor 3 [21]. In contrast,

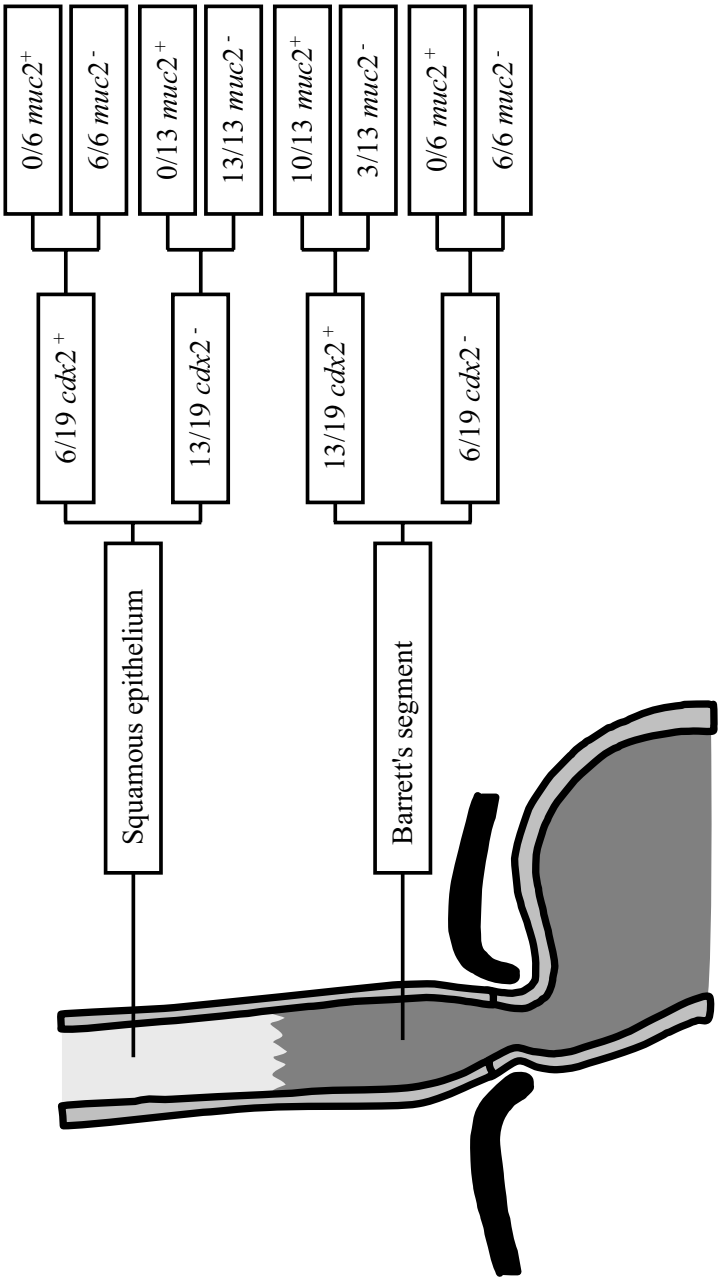


Figure 3: Schematic overview of the distal esophagus, summarizing the transcription of Cdx2 mRNA and goblet cell-specific Muc2 mRNA in biopsies taken from the Barrett's segment and the squamous epithelium of patients with Barrett's esophagus.

heterozygous CDX2 knockout mice developed polyp-like lesions in their colon during the first 3 months of life, which lacked CDX2 expression [32]. These lesions were composed of heterotopic, well differentiated stratified squamous epithelium, stomach and small intestinal mucosa [33]. It was concluded that CDX2 directs epithelial differentiation toward a caudal phenotype. For these reasons, CDX2 expression is believed to be an early marker of intestinal differentiation and may therefore play a role in the development of SIE in the lower part of the esophagus, as observed in BE.

Although all additional biopsies taken from the BE-segment for histological evaluation in the group of patients in whom *Cdx*2 mRNA was determined showed SIE, *Cdx*2 mRNA was not detected in 6/19 (32%) BE segments. In the biopsies taken from these segments, *Muc*2 transcription was absent, which suggests that goblet cells were not present in these samples. As goblet cells are a hallmark of BE, these biopsies may have contained another type of columnar epithelium, probably gastric type epithelium as was detected in 26/79 (33%) biopsies of the BE segment in this study. This is in agreement with findings in another study, which reported that goblet cells were only found in 51% of patients with 3-4 cm columnar-like epithelium of the esophagus on a first endoscopy [34]. This increased to 88.9% after 3 endoscopies [34]. This suggests that the absence of SIE in the biopsies taken from the columnar lined segment might be due to sampling error. Since in this study, only 1 of the 6 patients negative for *Cdx*2 mRNA had an esophageal adenocarcinoma and no dysplasia was observed in the adjacent biopsies taken for routine screening in the other 5 patients, it is unlikely that a neoplasia, which is associated with a decreased number of goblet cells, was present in these biopsies.

In order to assess whether CDX2 is an early marker for the metaplastic replacement in the esophagus, *Cdx*2 mRNA levels were also determined in reflux-exposed squamous epithelium of patients with BE. Low levels of *Cdx*2 mRNA were indeed observed in 6/19 (32%) of the squamous epithelium samples tested (Figure 3). In addition, transcription of *Muc*2 was not detected in any of these samples, which excludes the possibility that SIE was covered by a stratified epithelial layer. This indicates that healthy appearing squamous epithelium 5 cm above the squamo-columnarjunction of the esophagus in a subset of patients with columnar metaplasia of the distal esophagus may already have undergone molecular changes, which may make them prone to the development of SIE, although this needs to be determined in a longitudinal follow-up study of patients with reflux esophagitis without BE. Patient-to-patient variation in the extent of reflux, the severity of inflammation, and the effect of the medication used, may well explain why not all squamous epithelium samples of patients with BE contained detectable amounts of *Cdx*2 mRNA.

The development of BE is associated with pathologic reflux of acid [35] and/or bile [36]. Taken together with recent reports that CDX2 expression can be induced in keratinocytes by prolonged exposure to acid [37], *Cdx*2 transcription may be an early step in the metaplastic replacement of esophageal squamous epithelium by SIE. We hypothesize that

inflammation in the esophagus caused by duodeno-gastro-esophageal reflux induces CDX2 expression in a subset of patients. Pathways involved in de novo CDX2 expression in esophageal squamous epithelium may be important for the development of Barrett's esophagus. Elucidating these pathways may result in a greater understanding why only a subset of GERD patients develop Barrett's esophagus.

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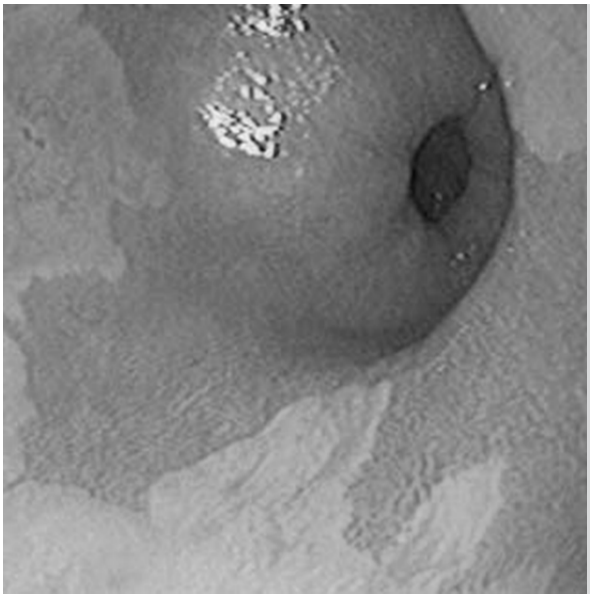

Chapter

VIII

Submited to Am J Gastroenterol

Bile acid stimulated expression of the Farnesoid X Receptor enhances the immune response in Barrett Esophagus

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ABSTRACT

Introduction: Barrett's esophagus (BE) is a premalignant condition of the esophagus. It is a consequence of mucosal injury from chronic gastroesophageal reflux, in which bile acids are an important toxic component. The Farnesoid X Receptor (FXR) is a nuclear receptor involved in regulation of bile acid synthesis, transport, and absorption. FXR activation is also involved in the induction of the innate immune response. This suggests that FXR is involved in the pathogenesis and inflammation seen in BE.

Methods: mRNA levels of FXR and the FXR regulated genes; ileal bile acid binding protein (IBABP), small heterodimer partner (SHP), and chemokines IL-8 and macrophage inflammatory protein 3α (MIP3α) were determined by RT-PCR. Protein expression was determined by immunohistochemistry.

Results: FXR was not expressed in squamous epithelium of healthy subjects (n=7), but was present in both squamous and columnar epithelium of BE patients. Compared to the squamous epithelium of BE patients their columnar epithelium displayed a 2.3-fold (p=0.02) increase in FXR mRNA. Also IBABP (2.2-fold; p=0.0029), SHP (2.7-fold; p=0.007), IL-8 (1.5-fold; p=0.04), and MIP3α (1.7-fold; p=0.019) transcription were increased. Exposure of esophageal cell-line TE7 to deoxycholic acid resulted in a similar induction. Induction was abolished by the FXR antagonist guggulsterone.

Conclusions: Expression of the bile acid receptor FXR, the bile acid metabolism genes IBABP and SHP and the chemokines IL-8 and MIP3α, are increased in Barrett's epithelium. The in vitro induction of FXR by deoxycholic acid suggests that bile acids can actively induce the inflammatory response in BE by recruiting immune cells.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition of the esophagus that is characterized by the replacement of normal squamous epithelium by specialized intestinal epithelium containing goblet cells. Although BE itself is a asymptomatic condition, it is associated with an increased risk for the development of esophageal adenocarcinoma (EAC) (1). Although the exact pathogenesis is unknown, BE is thought to develop from gastro-esophageal reflux disease, and is almost always accompanied by a chronic inflammation of the esophageal lining (2).

Epithelial cells play an important role in the induction of inflammatory responses. The exposure to refluxate components such as gastric acid and bile stimulates epithelial cells to produce chemokines that promote the influx of immune cells, and thus induce an inflammatory response (3, 4). Recently we have shown that exposure of epithelial cells to certain bile acids induces the production of the chemokines MIP3α, and IL-8 (5). However the exact molecular pathways that are involved in the expression of these chemokines remains unclear.

The Farnesoid X receptor (FXR) is a nuclear receptor that regulates the expression of bile acid synthesis, transport, and absorption. The main role of FXR is to limit intracellular bile acid overload and toxicity, by acting as a bile acid sensor that activates export and absorption mechanisms (6-8). In accordance with these functions FXR is highly expressed in the liver and small intestine. Recently FXR was found to be present in BE but not in normal squamous epithelium of the esophagus (9), the authors show a role of FXR in enhanced apoptosis seen in BE indicating that FXR has a functional role in pathobiology of BE. The functional expression of FXR in BE is combined with a recent report that FXR is also expressed by human immune cells (10), suggests a role for FXR in the inflammatory response observed in BE, e.g. by its action on transport of bile acids into Barrett's epithelial cells and a stimulatory effect on the inflammatory response. This prompted us to test for the putative role of FXR in the initiation and maintenance of the inflammatory response in BE. In order to test this we have compared the relative expression levels of FXR and two important FXR regulated bile acid metabolism factors, i.e., small heterodimer partner (SHP) and ileal bile acid binding protein (IBABP) (6-8, 11) and the chemokines IL-8, and the macrophage inflammatory protein 3α (MIP3α) in normal healthy squamous epithelium and in columnar Barrett's epithelium. These two chemokines have been selected as they are important promoters of the influx of neutrophils (IL-8) and B-cells (MIP3α) (12), cells that are abundantly present in BE (13). Our findings confirmed that bile acid can indeed induce the transcription of these genes as exposure of the esophageal TE7 cell line to bile acids not only affect the transcription of FXR and IBABP but also induces IL-8 and MIP3α transcription. This chemokine induction was absent when cells were exposed to bile acids in the presence of the FXR inhibitor guggulsterone (14-16).

Table 1. Patient characteristics

	Healthy controls (n=7)	BE patients (n=15)
Age (years)	36 ± 11	63 ± 14
Male (%)	4 (57)	10 (66)
Length BE segment (cm)	-	5.2 ± 1.7
Proton pomp inhibitor medication (%)	1 (14)	11 (73)

MATERIALS AND METHODS

Patients

Patient characteristics are outlined in Table 1. Paired biopsy samples were collected from 15 patients with a known history of BE. As controls, we collected paired esophageal biopsies from seven control subjects who underwent endoscopy for an unrelated cause, did not display any endoscopic signs of reflux and/or Barrett's, had no reflux-related complaints, odynophagia, or problems with passage of food through the esophagus. From each BE patient, two-paired biopsy sets were obtained; two adjacent samples from normal squamous epithelium, and two adjacent samples from the Barrett's epithelium. From each healthy control, a single paired set was obtained from squamous epithelium approximately 2 cm above the squamo-columnar junction. Endoscopic evaluation, biopsy collection, and histopathologic assessment were performed using routine standard procedures. One sample of each set was used for the experimental analysis as described below, the other, adjacent sample was analyzed by an expert GI pathologist (HvD), who evaluated haematoxyline and eosin stained sections for the presence of specialized intestinal metaplasia characteristic of BE (17). The study was approved by the local ethical review board of the Erasmus MC - University Medical Center Rotterdam and informed consent was obtained from all patients prior to endoscopy.

Semi-quantitative RT-PCR

Total RNA was isolated from the biopsies using TRIzol-reagent (Invitrogen, Groningen, The Netherlands) and remaining traces of chromosomal DNA were eliminated using a DNA-free RNA kit (Zymo, Orange, CA, USA). cDNA was synthesized with Avian Myeloma Virus reverse transcriptase according to the instructions of the manufacturer (Promega, Madison, WI, USA) as previously described (18). PCR reactions were performed with GoTaq (Promega) according to the manufacturer instructions. All primers are listed in Table 2 and were designed with aid of primer designer software (Clone manager, version 8, Scientific and Educational Software, Cary, NC, USA) using sequences from the NCBI database (http://www.ncbi.nlm.nih.gov). PCR conditions were 35 cycles consisting of a 30s denaturing step at 94°C; a 30s extension step at 50-60°C (depending on primers used; see Table 2); and a 30s elongation step at 72°C. After completion of these 35 cycles a final

Table 2. PCR primers

Gene	Forward primer	Reverse primer	T[a]
FXR	5′CTGGAAGTGGAACCATACTC3′	5′GTTACAGGCATCTCTGCTAC3′	59°C
SHP	5′GGAATATGCCTGCCTGAAAG3′	5′CTCCAATGATAGGGCGAAAG3′	55°C
IBABP	5′CAGGATGGGCAGGACTTCAC3′	5′ CATAGGTCACGCCTCCGATG3′	60°C
IL-8	5′GTGGCTCTCTTGGCAGCCTTCTGAT3′	5′TCTCCACAACCCTCTGCACCCAGTTT3′	55°C
MIP3α	5′ATGTCAGTGCTGCTACTC3′	5′TGTCACAGCCTTCATTGG3′	50°C
GAPDH	5′CCTGCACCACCAACTGCTTA3′	5′GCCTGCTTCACCACCTTCTT3′	56°C

[a] Temperature used for annealing these primers

5 min extension step was performed at 72°C, with the exception of GAPDH for which only 25 cycles were used. PCR products were size separated on a 2% agarose gel and visualized under UV light upon staining with ethidium bromide. Band size and intensities were determined by densitometry with Kodak 1D version 3.5 software (Kodak, Rochester, NY, USA) and data were normalized using the housekeeping gene GAPDH, as described previously (18). RT-PCR densitometric data are presented as mean ± standard error of the mean.

Statistical analysis

Statistical analyses were performed using the Mann-Whitney U-test of the SPSS 11.0 software (SPSS, Chicago, Illinois, USA). A two-sided p-value < 0.05 was considered statistically significant.

Immunohistochemistry

Paraffin-embedded biopsy specimens were serially sectioned at 4 µm, mounted on adhesive slides and dried overnight at 37°C. Specimens were deparaffinized, and antigen retrieval was performed for 15 minutes at 100°C in a microwave oven in 10 mmol/L Tris-EDTA buffer, pH 9.0. After cooling to room temperature, samples were blocked with non-immune serum for 20 minutes. The sections were stained using the primary antibody FXR/NR1H4 (R&D Systems, Oxon UK; clone A9033A) in a 1:100 dilution. After washing, bound antibodies were visualized with Envision (Dako B.V., Haverlee, Belgium). The sections were subsequently counterstained with Mayer haematoxyline and evaluated under a light microscope (Zeiss, Axioskop, Sliedrecht, The Netherlands). As a positive control normal human ileum and colon was used, as negative controls we used an isotype matched primary antibody and performed stainings without the addition of the primary antibody.

Cell culture

The esophageal cell line TE7 (19) was kindly provided by dr. George Triadafilopoulos, Stanford University, Palo Alto, CA., USA. This is an internationally accepted and widely used epithelial cell line as a representation of the esophagus epithelium. The cell line

was cultured in RPMI1640 (pH 7.4) supplemented with 2 mM L-glutamine (Bio Whittaker, Verviers, Belgium), 10% Fetal Calf Serum (Hyclone, Logan, UT, USA), and 20 units/ml penicillin/ streptomycin. Cells were routinely maintained as a subconfluent monolayer in a 75

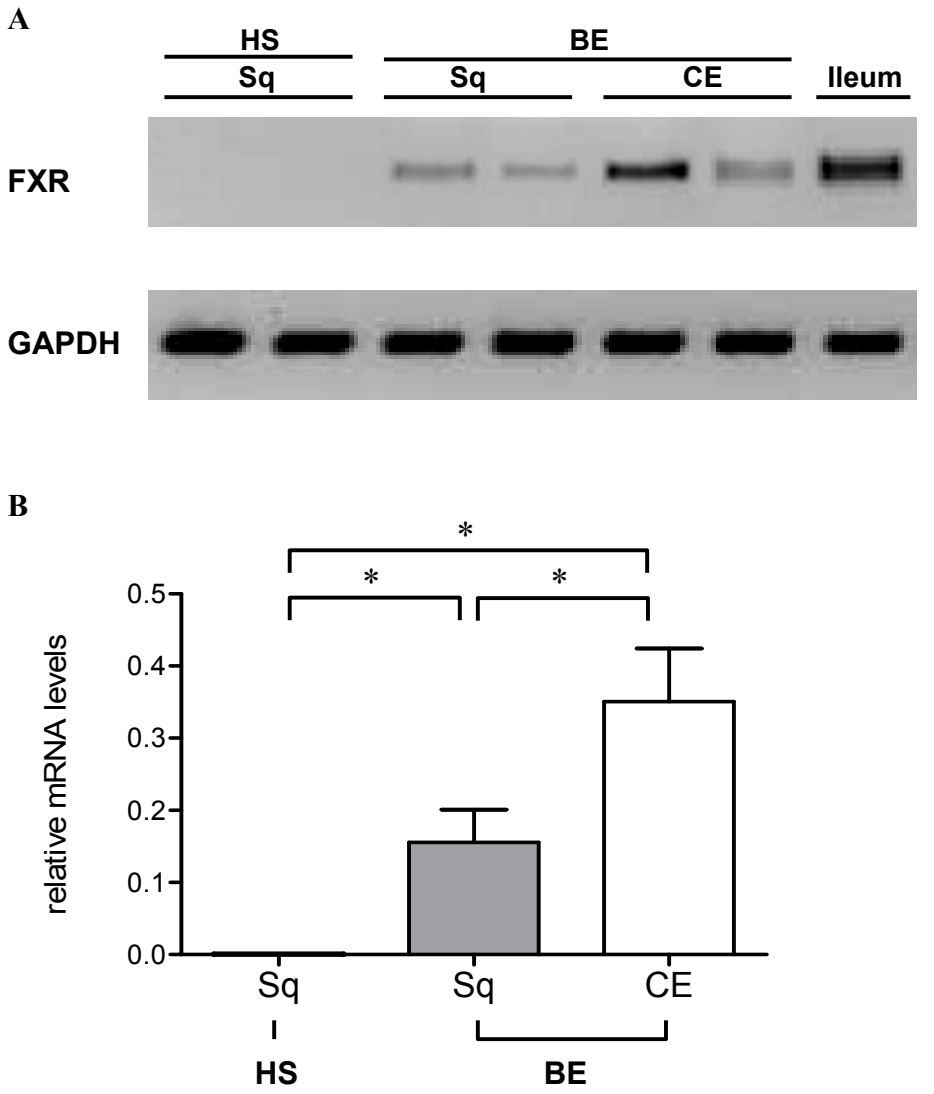

Figure 1: FXR mRNA expression in esophageal squamous epithelium (Sq) of healthy subjects (HS; n=7), and paired samples of squamous epithelium (Sq) and columnar epithelium (CE) from Barrett's (BE) patients (n=15), (* $p<0.05$). (A) Representative RT-PCR results of FXR mRNA levels in HS, 2 paired samples of Sq and BE epithelium, and as a positive control ileum. (B) Relative amounts of FXR mRNA in HS and paired samples of squamous epithelium and columnar epithelium from Barrett's patients. Amounts of FXR were normalized against the housekeeping gene GAPDH. The mean FXR levels values in CE were significantly higher than those seen in Sq ($p=0.02$).

cm^2 tissue culture flask in a humidified incubator with 5% CO_2 at 37°C, for a maximum of 20 generations.

RNA analysis of cells incubated with DCA with or without guggulsterone

Cells were seeded at 0.4 x 10^6 cells/well in a 12-well plate and incubated overnight at 37°C. Subsequently, cells were exposed to different concentrations of (0-200 μM) guggulsterone (Calbiochem, VWR International, Amsterdam, The Netherlands) for 18 h, followed by incubation with deoxycholic acid (DCA) to a final concentration of 0-200 μM for a period of 0, 1, 3 and 6 h. Cells were also incubated with similar concentrations of DCA without guggulsterone using parallel wells for each time point. Prior to harvesting cells were washed once with phosphate buffered saline (pH 7.4) and lysed in TRIzol. RNA isolation and RT-PCR was performed as described above.

RESULTS

Increased mRNA levels of FXR and FXR regulated genes in Barrett's epithelium

Figure 1 shows the relative mRNA expression level of FXR mRNA in squamous epithelium of healthy subjects, and paired biopsy samples of squamous and columnar Barrett's epithelium. Relative levels were calculated from RT-PCR data by normalization against the housekeeping gene GAPDH. No expression of FXR mRNA could be detected in esophageal squamous epithelium of healthy subjects. In contrast, in squamous epithelium of BE patients significant levels of FXR mRNA were observed. The highest levels of FXR mRNA levels were observed in the columnar epithelium of the Barrett's patients, where levels were on average 2.3-fold (p=0.02) higher than in the corresponding squamous epithelium of these patients.

To investigate whether FXR expression in columnar Barrett's epithelium had a functional role, expression levels of the FXR regulated bile acid metabolism genes SHP and IBABP were determined (Figure 2A).

While SHP mRNA levels were below detection limit and only low levels of IBABP mRNA were observed in healthy esophageal squamous epithelium (Figure 2A), a clear signal was observed in the epithelium (both squamous and columnar) of BE patients. As observed with FXR expression, the relative mRNA levels of SHP and IBAP were significantly higher in columnar Barrett's epithelium than in squamous epithelium of BE patients (2.7-fold ± 0.88 (p=0.007) for SHP and 2.2-fold ± 0.55 (p=0.0029) for IBABP).

To determine the possible role of FXR in inducing the inflammatory response, the expression levels of two important chemokines were determined. While MIP3α mRNA levels were below the detection limit and only low levels of IL-8 mRNA were observed in healthy squamous epithelium (Figure 2B), significant mRNA levels for these genes were found in the esophageal samples from BE patients. Again the highest levels for these

genes were present in the columnar BE epithelium compared to squamous epithelium of BE patients (1.7-fold ± 0.59 (p=0.019) for MIP3α and 1.5-fold ± 0.45 (p=0.04) for IL-8).

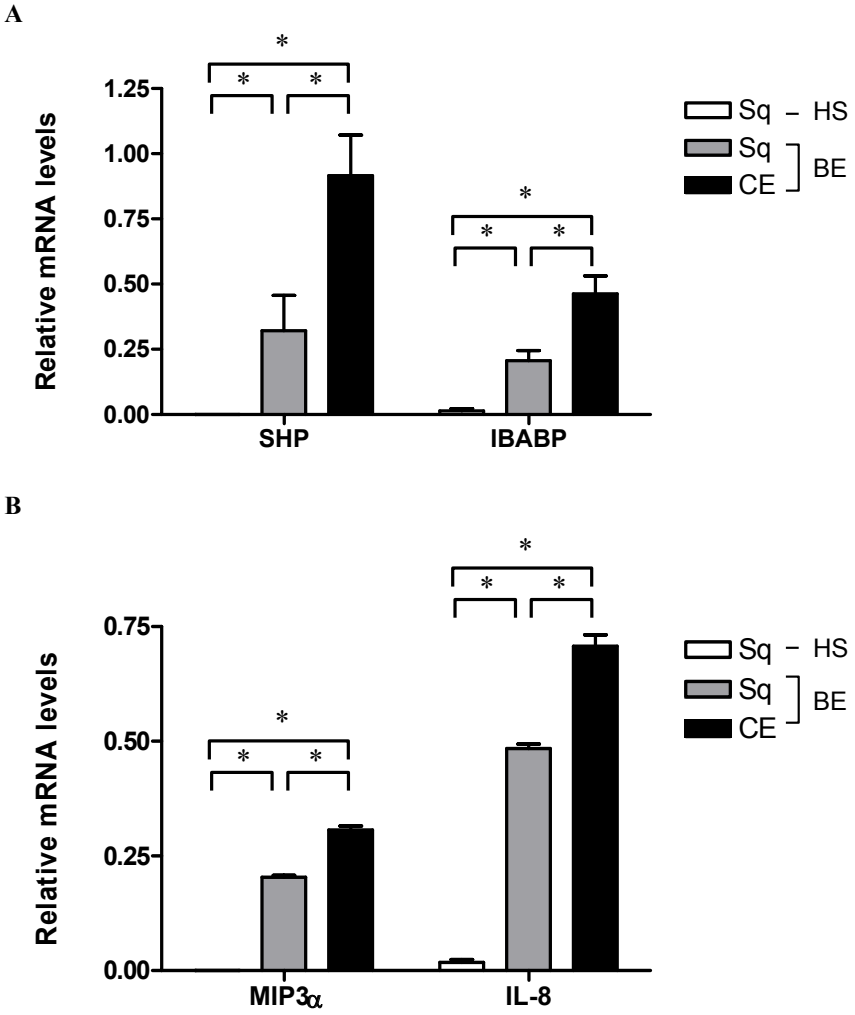

Figure 2: mRNA levels of SHP, IBABP, MIP-3α and IL-8 in healthy subjects and BE patients

A: mRNA levels of SHP and IBABP in esophageal squamous epithelium (Sq) of healthy subjects (HS, n=7), and paired samples of squamous epithelium (Sq) and columnar epithelium (CE) from Barrett's patients (BE; n= 15), (* p<0.05). Mean mRNA values of SHP and IBABP were significantly higher in CE compared to Sq epithelium (IBABP p=0.0029; SHP p=0.007). mRNA levels were normalized against the housekeeping gene GAPDH.

B: mRNA levels of MIP3α and IL-8 in esophageal squamous epithelium (Sq) of healthy subjects (HS; n=7), and paired samples of squamous epithelium (Sq) and columnar epithelium (CE) from Barrett's patients (BE; n=15), (* p<0.05). Mean mRNA values of MIP3α and IL-8 were significantly higher in Barrett's epithelium compared to squamous epithelium (MIP3α p=0.019; IL-8 p=0.04). mRNA levels were normalized against the housekeeping gene GAPDH.

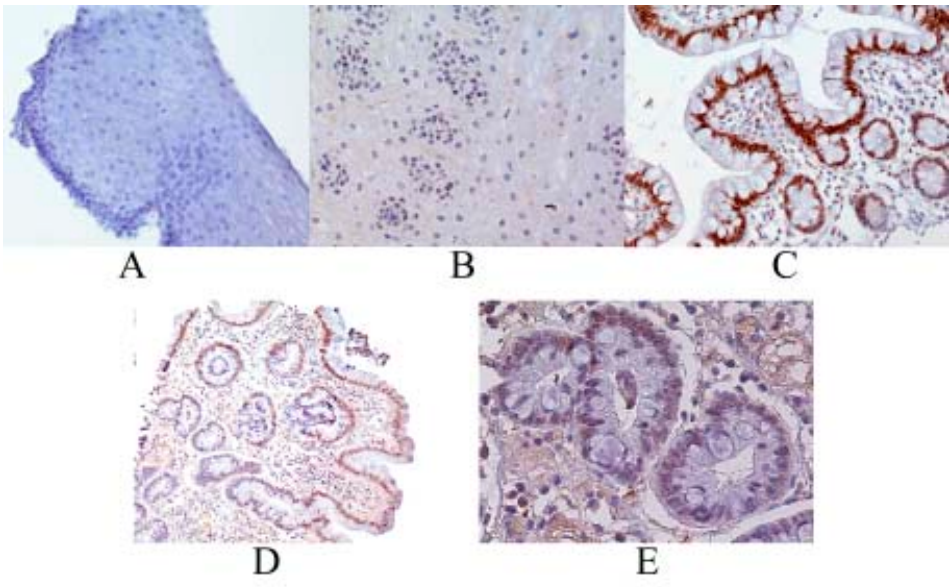

Figure 3 FXR protein expression in squamous esophageal epithelium of a healthy subject (A), squamous epithelium of a Barrett's patient (B), Barrett's epithelium (D), together with an magnification (E) and a positive control; ileum (C), visualized by immunohistochemistry using a monoclonal antibody against FXR.

Increased protein levels of FXR in Barrett's epithelium

To investigate where FXR is located in various tissues and whether the observed increase in FXR mRNA levels also results in an increase of FXR protein we performed immunohistochemical stainings with an FXR specific antibody. While esophageal squamous epithelium of healthy controls did not show FXR staining (Figure 3A), both squamous epithelium (Figure 3B) and columnar epithelium (Figure 3D and E) of BE patients showed a strong FXR staining (Figure 3D and E). The staining was predominantly present in the nuclei of epithelial cells. Remarkably, some metaplastic regions did not show any FXR specific staining. These negative areas seemed randomly distributed throughout the crypts and were histologically indistinguishable from those that did stain. Figure 3C shows FXR staining of the ileum, which was used as a positive control.

DCA induces IBABP and the chemokines IL-8 and MIP3α in a FXR specific way

To determine whether bile acids can activate FXR expression, TE7 cells were treated with DCA in the absence or presence of the FXR antagonist guggulsterone. A clear dose- and time-dependent increase in FXR expression levels was found after treatment with physiological levels (100-300μM) of DCA (20). This response could be completely inhibited in a dose- (Figure 4) and time-dependent (not shown) manner by pre-treatment with guggulsterone.

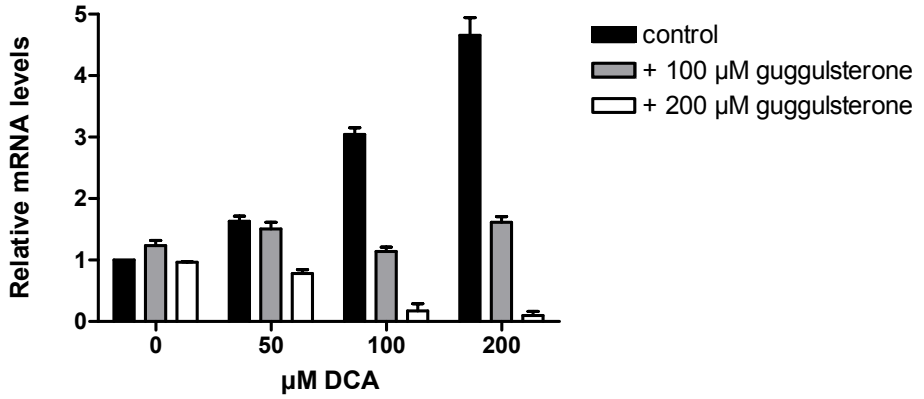

Figure 4: mRNA levels of FXR in TE7 treated with DCA for 6 h in the presence or absence of guggulsterone.

Based on these results we selected a single time point (6 h), DCA concentration (200 µM), and guggulsterone concentration (200 µM) and used these to investigate the putative involvement of FXR in the transcription of chemokines. The two chemokines IL-8 and MIP3α showed a significant increase in mRNA levels after exposure to DCA; (3.3-fold ± 0.8; p=0.007, and 19.2-fold ± 1.5; p<0.0001, respectively). Pre-treatment with guggulsterone

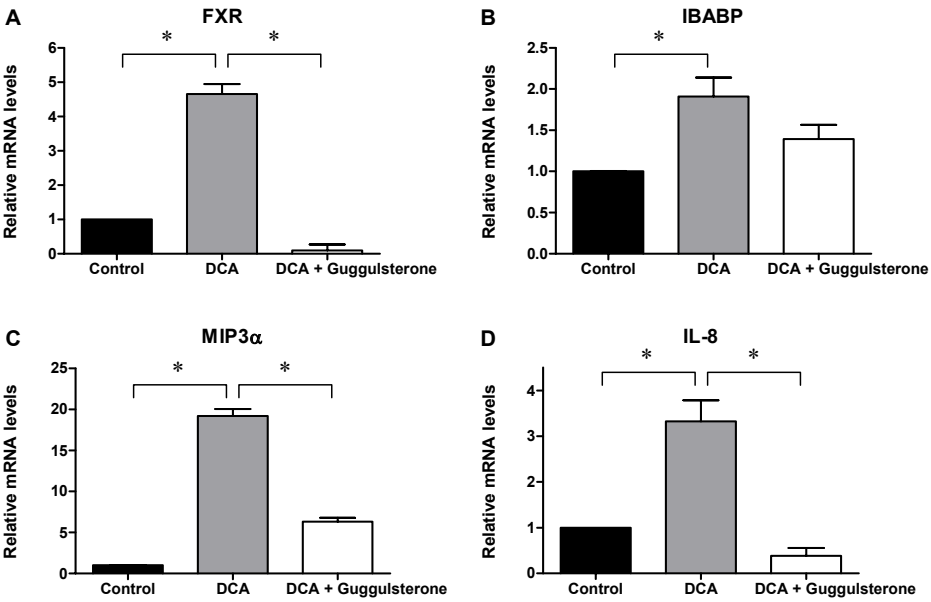

Figure 5: TE7 cells treated with DCA in the presence or absence of guggulsterone. mRNA levels of FXR (A), IBABP (B), MIP3α (C) and IL-8 (D) were determined, control levels were set on 1. The mean mRNA levels of genes increased significantly after DCA exposure compared with unexposed cells. When the cells were pre-treated with guggulsterone, an inhibitor of FXR, all genes showed a clear decrease in mRNA levels. mRNA levels were normalized against the housekeeping gene GAPDH, * p<0.05.

resulted in a decreased ability of DCA to stimulate IL-8 (2.9-fold ± 0.3; p=0.005) and MIP3α (3.0-fold ± 0.8; p=0.0002) (Figure 5C and D). The mRNA levels of FXR increased 4.7-fold ± 0.5 (p=0.002) as a result of DCA treatment (Figure 5A). No DCA mediated increase in FXR levels was observed (p=0.001) when the cells had been pre-treated with guggulsterone. As a control of FXR specific activity we investigated the transcription of IBABP. The DCA mediated induction of FXR was indeed accompanied by a 1.9-fold ± 0.4 (p=0.017) increase in the expression of IBABP (Figure 5B), which was reduced upon guggulsterone treatment (1.4-fold ± 0.3; p=0.148).

DISCUSSION

BE is a premalignant condition resulting from chronic gastroesophageal reflux. Bile acids play an important role in the development of BE, since bile acids are an important toxic component of the refluxate. The nuclear bile acid receptor FXR is involved in the regulation of bile acids by regulating the synthesis, absorption and excretion of bile acids. Given the fact the FXR is also expressed on various immune cells where it is thought to be involved in the activation of the immunological processes (10, 21), we postulated that FXR plays a role in the pathogenesis and the inflammation seen in BE. We therefore tested the expression levels of genes involved in the bile acid metabolism (i.e. FXR) and the immune system, (i.e. IL-8 and MIP3α), in biopsies from control subjects and BE patients. The observed mRNA levels indicate a role for FXR in the induction of chemokines, which was supported by in vitro assays.

In the present study we did not observe FXR expression in the healthy esophagus, a clear expression was found in the esophageal lining of BE patients, both in the squamous epithelium and in the columnar BE epithelium. These results confirm those of a recent study, in which an increased FXR expression was found along with progression from normal esophagus towards BE (9). While these authors reported a low FXR expression in normal esophagus, we could not detect any FXR above the detection level in our healthy controls. This discrepancy may be explained by the fact that the esophageal samples in the previous study were actually obtained from patients with reflux symptoms (9), while our controls did not have any signs of reflux at all, indicated by the limited use of proton pump inhibitors in the control group (Table I). This suggests that reflux induces the expression of FXR in healthy squamous epithelium, which is in line with our findings that squamous epithelium of patients with BE and squamous epithelium of patients with reflux esophagitis (data not shown) FXR is expressed, albeit at low levels. The samples for immunohistochemistry used in this study were from routine patient materials and were not controlled. We therefore used these used these data primarily to provide an indication as to where FXR is located in the various tissues and if an increase in expression levels could be detected at a protein level.

A relationship between FXR expression and reflux is further confirmed by our in vitro studies, which show that exposure to DCA results in increased FXR expression, and FXR specific induction of chemokines. These experiments can however be criticized by our experimental set up, in which a relative long-term exposure to guggulsterone was required, and the fact that (in order to ensure cell viability), these experiments had to be performed at a neutral pH. Obviously, in patients with gastro-esophageal reflux, the distal esophagus is exposed to refluxate for short periods of time (minutes) and this occurs in a acidic environment (22). This drawback could be overcome in future studies where the role of FXR is tested in an animal model for the development of BE (23), using FXR KO mice (24).

That the increase in FXR levels does have a physiological role in BE is supported by the fact that target genes of FXR involved in cellular bile acid metabolism, i.e., SHP and IBABP, both were increased in squamous epithelium and in Barrett's epithelium of BE patients (Figure 2). This implies the presence of a functional bile acid transport system, suggesting an adoption of the esophagus to the potentially toxic exposure of bile acids in BE patients.

On the other hand the ectopic expression of FXR might well enhance the inflammatory reaction seen in BE patients. These chemokines have been shown to promote the influx of immune cells, such as neutrophils (IL-8) and B-cells (MIP3α) (12). Both these chemokines (Figure 2) and cell types (13) are abundantly present in BE. In addition, our in vitro experiments clearly showed that exposure to DCA resulted in a significant increase in mRNA levels of IL-8 and MIP3α (Figure 5). This increase was inhibited by guggulsterone, a specific inhibitor of FXR. It is understandable that guggulsterone inhibits the synthesis of mRNA of genes regulated by FXR, but this same pattern was unexpectedly also observed for the mRNA levels of FXR itself. Nevertheless our results suggests a direct involvement of FXR in the chronic inflammation observed in BE patients.

Our results suggest an important role for biliary reflux in the initiation and maintenance of the inflammatory response in Barrett's esophagus. Additionally a pathogenic effect of FXR in the development of BE towards adenocarcinoma is further supported by the recent findings that the FXR antagonist guggulsterone also leads to an increase in apoptosis (9). FXR could therefore be a potential target for future therapies preventing the development of BE and esophageal adenocarcinoma.

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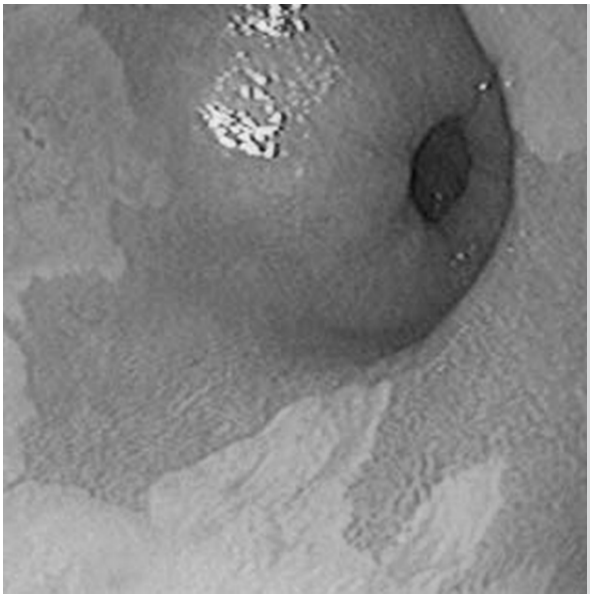

Chapter IX

General discussion and future aspects

L.M.G. Moons

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder in western populations, and its incidence is rising in the last decades. This also concerns its major complication; esophageal adenocarcinoma (EAC) [1]. EAC is a highly lethal cancer, which develops secondary to gastroesophageal reflux, and is associated with a 5-year survival of only 13%. Survival of EAC can presently only be improved by early tumor detection, which increases the chance of successful endoscopic ablation or surgical resection. The major risk factor for the development of EAC is the presence of a Barrett's esophagus (BE). BE is an asymptomatic condition closely associated with the presence of gastroesophageal reflux [2], and is associated with a 30-60 fold excess risk of EAC when compared to the overall population [3,4]. As early tumor detection in BE is the most successful approach to EAC, screening for the presence of BE in the GERD population and custom made surveillance of patients with BE are needed. In this thesis, it was investigated whether new markers for the presence or development of BE, or progression to EAC could by identified by studying the etiology of BE.

SURVEILLANCE

Patients with BE undergo an upper endoscopy every three years in order to detect neoplastic progression in a subset of patients [5]. It is still being debated whether current surveillance strategies are cost-effective. As only 1 of every 200 patients with BE will progress to an adenocarcinoma during their lifetime [6], this approach is expensive and results in increased consumption of public health resources. In addition, repeated endoscopies are experienced as inconvenient by patients, and introduce health risks to patients who will often not develop EAC [7]. Current research is therefore directed at finding new methods for stratifying patients into high- and low risk patients in order to achieve a custom-made surveillance for individual patients.

COX-2 gene polymorphisms are involved in progression to EAC

Individual susceptibility for a specific disease phenotype is partially determined by genetic make-up. Single nucleotide polymorphisms in inflammation related genes associated with different protein levels or enzyme activity, may alter the host response to ongoing exposure to gastroesophageal reflux, and thereby the host susceptibility to progression to EAC. In chapter V, we have reported an association between gene polymorphisms in the gene encoding for cyclooxygenase 2 (COX-2) and reflux esophagitis, BE, and EAC. In the gene encoding for COX-2, PTGS2, several polymorphisms have been described. Two of these polymorphisms are associated with different COX-2 enzyme lev-

els or activity. The C-residue at position –765 is associated with increased enzyme activity resulting in increased prostaglandin (PG) E_2 and PGD_2 levels [8]. The A-residue at position –1195 is associated with increased expression of COX-2 [9]. We observed that the COX-2 CA-haplotype (C_{-765} & A_{-1195}) was more frequent in EAC patients (21%) than in patients with reflux esophagitis (12%) and BE (12%; $p<0.001$). Homozygosity of the CA-haplotype was even associated with a 3.8-fold (95%CI 1.1-14.6) increased risk of EAC as compared to patients with BE. The COX-2 CA-haplotype is associated with increased levels of PGE_2, which suggests a relation between increased levels of PGE_2 and an increased risk of malignant progression in BE. This is in line with current knowledge on COX-2 and PGE_2, as 1) a significant increase in the local levels of the COX-2 enzyme [10] and its product PGE_2 is observed during the progression of reflux esophagitis to BE and EAC [11,12], 2) modulation of the immune system through the administration of COX-2 inhibiting agents such as aspirins or non-steroidal anti-inflammatory drugs (NSAIDs) resulted in a reduction of EAC formation in animals[13,14], and was associated with a lower incidence of EAC in retrospective human studies [15-19], and 3) high levels of PGE_2 were associated with more aggressive tumor behavior [20]. It is therefore likely that the signaling cascade including PGE_2, and its binding to one of its four known receptors (EP1-4) is of importance for esophageal carcinogenesis [21]. We were unable to detect a difference in COX-2 haplotype distribution between patients with reflux esophagitis and BE, suggesting that PGE_2 signaling is especially important in the progression of BE towards EAC.

It can not be concluded from this study whether COX-2 CA haplotyping could be used for differentiation between high or low risk patients, and future studies are necessary to address this question. There are however serious limitations on using COX-2 polymorphisms for surveillance purposes, as the number of patients with a COX-2 CA-haplotype was low, even in the group of EAC patients (21%). A larger group of EAC patients did not had the CA-haplotype, and would therefore be incorrectly excluded from more intensive follow-up. If used in clinical decision making at all, it has to be used in combination with other parameters. To my opinion, the relevance of the association between the COX-2 CA-haplotype and the increased risk of EAC is therefore not its use in clinical practice, but its biological indication that PGE_2 signaling via EP receptors might be part of an important signaling cascade in esophageal neoplastic progression (Figure 1). Testing for the presence and extent of this signaling cascade in biopsies taken from the BE-segment may be promising, as this may indicate the presence of a pathway leading to malignant progression. It also indicates that blocking of this COX-2/PGE_2 signaling pathway by COX-2 inhibitors, or in the near future with PGE_2 receptor blockers (supposed less cardiovascular side effects), may be useful for chemoprevention in patients with BE.

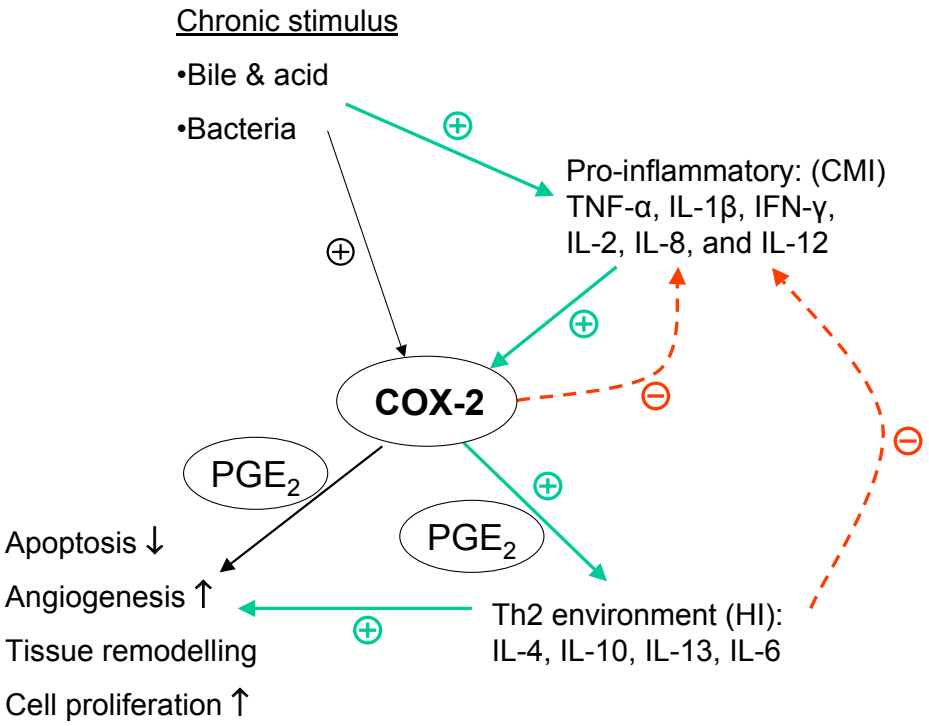

Figure 1: Both reflux components as well as pro-inflammatory cytokines induce the expression of COX-2 in esophageal epithelial cells. Increased expression of COX-2 will result in increased generation of the main carcinogenic metabolite PGE_2. PGE_2 in its turn decreases apoptosis and promotes angiogenesis and epithelial proliferation.[55] In addition, PGE2 induces a shift towards humoral immunity (HI). A HI results in remodeling of the microenvironment, thereby even enhancing angiogenesis and epithelial proliferation, but also inhibition of cellular mediated anti-tumor immunity (CMI) resulting in escape of immune surveillance.

SCREENING

More than 90% of the patients presenting with EAC had no prior diagnosis of BE at an upper endoscopy before detection of their EAC [22-24]. Improving surveillance techniques should therefore be complemented by screening of the GERD population for the presence of BE. Currently, it is impossible to identify those patients who would benefit from screening endoscopy, as there are no clinical parameters available which reliably discriminate between high- and low risk groups [25,26]. It is therefore necessary to gain more insight in the etiology of BE, as this may provide new targets for intervention.

Chronic esophageal inflammation may be such an important target. Reflux-induced inflammation of the esophagus precedes the development of BE, and severity of inflammation correlates with the development of BE and EAC in animal models [13,14]. Furthermore, the endoscopic diagnosis of reflux esophagitis was associated with a nine-

time higher risk of EAC as compared to patients with reflux-related symptoms who did not [27]. Use of anti-inflammatory drugs such as non-steroid anti-inflammatory drugs (NSAIDs) and aspirin have been shown to diminish mucosal inflammation and to be associated with a lower incidence of EAC and BE in both humans and animals [15-19,28]. Severe chronic mucosal inflammation precedes BE development as was demonstrated in animal models, but has not been proven in humans yet. This is partially due to the fact that progression from reflux esophagitis towards BE is only infrequently observed in the clinical situation [29], and the majority of patients already have developed BE at first endoscopic examination. In order to test whether different host immune responses could predispose people exposed to gastroesophageal reflux to the development of BE, we have tested IL-1β, IL-1Ra, IL-8, IL-6, IL-2, IL-10 and IL-12 gene polymorphisms in 255 patients with BE and 240 patients with reflux esophagitis in chapter IV, V, and VI.

SEVERE MUCOSAL INFLAMMATION IS A DRIVING FORCE FOR PROGRESSION TOWARDS BE

In Chapter IV, we report on a link between the IL-12 C-residue at position +1188, which is associated with increased expression of IL-12p70 [30,31], and an increased risk of BE in a population with gastroesophageal reflux disease (OR 1.8 95%CI 1.17- 2.69; p=0.007). These findings implicate a role for high IL-12p70 levels in the development of BE. IL-12p70 has a pro-inflammatory function by favoring and maintaining Th1 differentiation, and inducing Th1 cytokine synthesis (mainly IFN-γ) [32]. IL-12p70 is therefore believed to be a major link between innate immunity and the recruitment and polarization of adaptive immunity towards a cellular immune response (Th1). The finding of an increased prevalence of the IL-12p70-high genotype (IL-12B C-allele) in BE, indicates that a strong host cellular immune response may be involved in the pathogenesis of BE in humans, as was suggested from observations in animal models [13,14]. The association between the IL-12B C-allele and BE, was modified by the presence of a hiatal hernia, which increased the risk of BE (OR 2.9 95%CI 1.32-6.58; p=0.008). This supports the hypothesis that an increasing severity of esophageal inflammation induced by increased exposure to the gastroesophageal refluxate and a pro-inflammatory host response predisposes to development of BE.

We also observed that the IL-10_{-1082} GG genotype interacted with the IL-12B polymorphism, and modified the risk of BE associated with the IL-12B C-allele (OR 1.0 95%CI 0.5-1.9; p=0.1). Patients with a IL-12B AA genotype (low IL-12p70 expression) even had a significantly lower risk of BE when this genotype was present in combination with the IL-10_{-1082} GG genotype (OR 0.6 95%CI 0.34-0.99; p=0.011). As the IL-10_{-1082} GG genotype is associated with higher IL-10 expression [33], and IL-10 inhibits IL-12p70 expression by ac-

tivated dendritic cells, the IL-10_{-1082} GG genotype may decrease the IL-12 p70 production, and in this way the risk of BE. This is also evidenced by a recent observation that IL-10 genotypes interacted with IL-12 genotypes on the level of IL-12p70 expression [34]. The IL-10_{-1082} GG genotype was shown to decrease IL-12p70 expression by LPS stimulated dendritic cells in spite of the presence of a IL-12p70-high genotype [34]. These findings further support the notion that increased expression of IL-12p70 might be important in the pathogenesis of BE. These findings in a human population suggest that, like in animal models, severe mucosal inflammation precedes the development of BE. In addition, BE is associated with a strong inflammatory response with higher numbers of immune cells in the mucosa of patients with BE than in the mucosa of patients with reflux esophagitis (Chapter III). This finding is in agreement with the observations made in the genetic association studies, and further strengthens the hypothesis that a strong inflammatory host response is involved in the development of BE.

Progression towards BE is paralleled by a switch to a more humoral dominated immune response.

In chapter III, the immune response in BE was demonstrated to differ from the immune response in reflux esophagitis. BE was associated with higher numbers of IgG^+ and IgE^+ plasma cells and IgE bearing mast cells, where reflux esophagitis was associated with higher numbers of macrophages and cytotoxic T cells. This suggested that BE is associated with a more predominant Th2 type immune response, and reflux esophagitis is associated with a more Th1 predominated immune response. This is supported by a study of Fitzgerald et al. who showed that BE was associated with increased expression of IL-4 and IL-10 (Th2 cytokines) and reflux esophagitis was associated with higher levels of IFN-γ, IL-1 and IL-8 (Th1 cytokines) [35]. We therefore conclude that the progression of reflux esophagitis towards BE, is associated with severe mucosal inflammation and is paralleled by a switch from a cell mediated immune response to a more humoral dominated immune response.

Chronic Th2 inflammation and esophageal carcinogenesis

The development of a Th2 dominated immune response in BE has consequences for the increased tendency for neoplastic progression associated with BE. Recent studies have shown an association between the presence of a Th2 immune response and the development of a (pre-)malignant disorders in the presence of chronic inflammation [36-38]. In a Th2 dominated microenvironment tumor development is more likely to occur, as this th2 dominated microenvironment facilitates tumor survival by several pathways including increased proliferation and angiogenesis, decreased apoptosis, and decreased tumor surveillance [36,37]. The latter comes from studies were immune compromized mice were shown to be more susceptible for the development of spontaneous intestinal epithelial carcinomas [39,40], and that the development of a Th2 microenvironment

could aggravate neoplastic growth [41]. Enhanced anti-tumor immune activity was noticed in B cell deficient mice [42,43], and it is only recently that two mechanisms of B cell promoted carcinogenesis have been identified. B cells were shown to be responsible for suppression of natural killer cell and T cell mediated anti-tumor immunity, by influencing IFN-γ production at time of activation. In a HPV16 skin cancer model, products secreted by activated B cells were shown to be responsible for tumor development by recruitment of innate immune cells, inducing angiogenesis and proliferation, and remodeling of the extracellular matrix. Interruption of these pathways resulted in a significant delay in carcinogenesis [44]. BE is associated with increased proliferation and angiogenesis[45], altered tissue remodeling[46], decreased apoptosis[47], and decreased tumor surveillance[48]. Whether the increased number of IgG^+ B cells observed in the mucosa of BE patients play a role in these mucosal alterations needs to be investigated, but in the light of recent findings it seems conceivable that they do.

Mechanisms involved in the recruitment of a mucosal immune response

Following from the observations that progression of reflux esophagitis towards BE is preceded by a strong inflammatory host response, and that BE is associated with a more humoral dominated immune response, the questions emerge which pathways are involved in the recruitment of an inflammatory response, and which factors are involved in the regulation and polarization of the immune response in BE. It seems likely that luminal components such as acid, pepsin, bile acids, food products, and bacteria, continuously interact with epithelial cells and the underlying stromal- and immune cells, since reflux and BE are associated with an increased permeability of the epithelial barrier[49,50]. In addition, as the use of acid-inhibiting medication does not influence mucosal inflammation in BE [35,51] (Chapter III), it seems likely that an increased sensitivity to immune activation and reflux independent pathways are part of the inadequate inflammation in BE. In the following section I will discuss the several immune characteristics observed in our studies, which may provide important clues to identifying the factors involved in recruitment and regulation of the esophageal immune response.

Recruitment of an adaptive immune response

IL-1B (-511 and +3953), IL-1RN (Chapter VI), IL-2, IL-6, and IL-8 genotypes (Chapter IV) were not differently distributed between patients with reflux esophagitis and BE. This indicates that the genetic regulation of IL-2, IL-6, IL-8, IL-1β, and IL-1Ra expression levels is not likely to play a decisive role in the progression of reflux esophagitis to BE. Increased IL-12p70 is mainly believed to form a crucial intermediate between the innate and adaptive immune response. IL-6, IL-8, IL-1β, and IL-1Ra function predominantly in the innate immune response. It might well be that the recruitment of an adaptive immune response is more important for the progression of reflux esophagitis towards BE, while the innate

immune response is only marginally involved. This may provide a logical explanation as to why not all patients with severe reflux esophagitis develop BE.

Antigen driven response

The majority of plasma cells in the mucosa of BE patients are IgG bearing, indicating that these plasma cells are not merely a reflection of changed immune tissue homeostasis caused by the different epithelial lining, but are actively recruited to this specific site of inflammation (Chapter III). It has also been shown that T lymphocytes become more numerous in tissues where metaplastic foci develop [52], and that persistent BE after endoscopic ablation therapy is associated with a lymphocyte infiltrate, which is absent in the neosquamous islands [53]. These findings suggest that the recruitment of an adaptive immune response in BE is not only secondary to gastroesophageal injury or loss of epithelial integrity, but that their presence may also be stimulated by reflux-independent factors. In chapter III we have described the presence of isolated lymph follicles (ILFs). ILFs were observed in the mucosa in 30% of the patients with BE, but not in the mucosa of patients with reflux esophagitis. As ILFs are potent structures for antigen presentation by activated myeloid cells, this further suggests the presence of a local antigen. Whether this antigen is related to specific protein expression on metaplastic tissue itself, or exposure to luminal antigens needs further research.

Luminal components modulate esophageal mucosal inflammation

Bile acids are a component of gastroesophageal reflux and are thought to be important mediators of the mucosal immune response. In chapter VIII we have investigated whether bile acids were able to modulate the immune response in the esophagus. Conjugated bile acids have recently been shown to modulate anti-microbial immune responses by binding to the nuclear Farnesoid X receptor (FXR) in intestinal epithelium [54]. By binding to FXR, bile acids were able to induce expression of several kinds of anti-microbial agents, and prevent bacterial translocation by promoting mucosal integrity [54]. FXR is most importantly known for its role in bile acid metabolism in the hepatocyte [55], but this study demonstrated that bile acid signaling via FXR has a role in the balance between the microbial flora and immune activation. We therefore tested whether FXR could also play an important role in BE, as bile acids are involved in the etiology of BE. FXR is expressed in specialized intestinal epithelial cells and in squamous epithelium of patients with BE, but not in non-inflamed squamous epithelium of healthy controls (Chapter VIII|). Stimulation of FXR by conjugated bile acids induced expression of FXR, I-BABP, and SHP, and induced the expression of the chemokines IL-8 and MIP-3α. The finding of increased levels of FXR gene targets I-BABP and SHP suggests that FXR signaling is present in specialized intestinal epithelial cells in BE, and is thereby the first report which shows the presence of receptor-dependent cellular signaling by conjugated bile acids in metaplastic epithelial cells. Secondly, FXR activation also resulted in the increased transcription of MIP-3α and

IL-8 by epithelial cells. IL-8 was already shown to be induced by conjugated bile acids [56], but esophageal MIP-3α expression was not observed before. MIP-3α expression is induced in the presence of epithelial damage, and is involved in the leucocyte recruitment of T cells and immature dendritic cells. Its up-regulation is associated with intestinal inflammation, and neutralizing antibodies were able to reduce 2,4,6-trinitrobenzene sulfonic acid induced mucosal inflammation. From this study it can be concluded that FXR is expressed in BE, and that binding to FXR by conjugated bile acids can modulate the immune response by inducing chemokine expression by epithelial cells (Chapter VIII).

Intestinal differentiation in the esophagus

CDX2 is believed to be a key regulator of intestinal differentiation, as ectopic gastric expression of CDX2 resulted in development of complete intestinal metaplasia in the stomach [57,58]. We demonstrated that CDX2 is being expressed in specialized intestinal epithelium of patients with BE, but not in metaplastic gastric type epithelium or squamous epithelium. We also showed that Cdx2 mRNA was present in biopsies taken from the squamous epithelium of the esophagus just above the neo-squamocolumnar junction. This suggests that CDX2 might indeed be expressed at an early stage of the disease and that induction of its expression may precede the change in mucosal lining. It has recently been established that CDX2 expression can be induced by bile acids and by the pro-inflammatory agents IL-1β and TNF-α[59-64]. This induction of CDX2 is dependent on the level of methylation of the Cdx2 promoter[59,61]. In a state of hypermethylation, CDX2 is not being expressed, which is the case in normal squamous epithelium [65]. When the level of methylation is reduced transcription factors were able to induce Cdx2 transcription. The switch in mucosal lining may thus be initiated by the surrounding environment such as bile acids, inflammatory agents, and microbial flora, by inducing CDX2 expression and thereby the cell fate of esophageal progenitor cells.

FUTURE ASPECTS AND HYPOTHESIS

That Th2 inflammation might be able to influence local epithelial differentiation follows from the observation that stem cell differentiation or transdifferentiation is greatly influenced by signals arriving from stromal cells and the extra cellular matrix [66]. Epithelial cells can be induced to produce mucins via stimulation of the IL-4 receptor-α by Th2 cells [67]. Exposure of mice airway to IL-4 and IL-13 were shown to result in goblet cell metaplasia positive for Alcian-Blue staining [68,69]. Mice infected with Trichinella spiralis show a two phase response, with first a strong Th1 dominated response followed by switch to a Th2 dominated response at day 8. After day 8, goblet cell hyperplasia is observed which was shown to be induced by Th2 cells [70]. In another study, Th2 cells were shown to induce intestinal villus atrophy with goblet cell metaplasia, which was mainly medi-

ated by IL-4 [71]. The association between ectopic specialized intestinal differentiation and the presence of chronic inflammation also follows from the observations that goblet cell metaplasia, with intestine specific expression of MUC2 and CDX2, is also observed in other parts of the body such as in the stomach induced by infection with Helicobacter pylori, in the nasal sinus by repeated exposure to wooddust [72], and in the gallbladder induced by chronic cholecystolithiasis [73]. These are all inflammatory conditions, however with different provoking stimuli and surrounding environment.

Development of SIE could therefore be a defensive tissue response to severe mucosal damage. BE is associated with higher levels of inflammation induced oxidative damage than reflux esophagitis, suggesting that an increased level of mucosal inflammation due to increased gastroesophageal reflux in the presence of a pro-genetic background is involved in the progression of reflux esophagitis towards BE. The ongoing exposure to reactive oxygen species may lead to epithelial mutagenesis (tumor initiation; Figure 2). It seems likely that severe inflammation and severe oxidative stress trigger a pathway

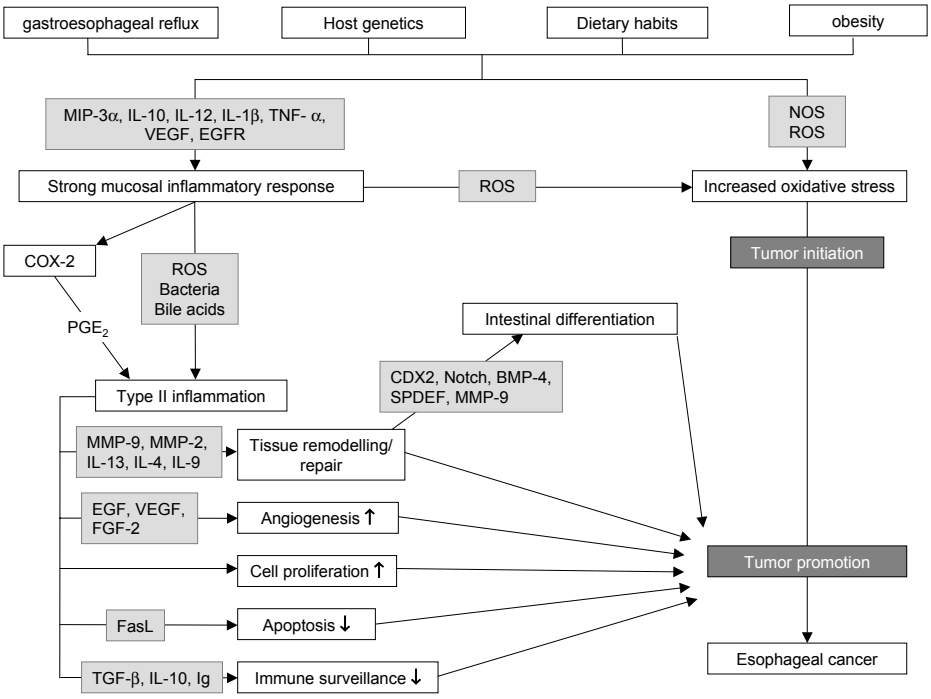

Figure 2: Schematic overview of a hypothetic model for inflammation promoted esophageal carcinogenesis. Gastroesophageal reflux and all factors influencing its intensity or composition such as host characteristics, dietary habits and obesity, results in mutagenic changes in esophageal epithelium (tumor initiation). The transformed cells can however survive and develop into an invasive tumor in a tumor facilitating microenvironment such as in the presence of a type II dominated mucosal immune response (tumor promotion), as this response is associated with increased proliferation, angiogenesis and tissue remodeling, and decreased apoptosis and tumor surveillance. A strong pro-inflammatory immune response, prostaglandin (PG) E2 and other yet unidentified factors (perhaps bile acids, bacteria and reactive oxygen species (ROS)) trigger this shift to a type II inflammation.

directed at lowering the negative effects on epithelial integrity. This tissue response includes the development of a Th2 immune response resulting in increased angiogenesis, tissue remodeling, and proliferation, but decreased apoptosis and immune surveillance (tumor promotion) (Figure 2). Although lowering the damaging effect on the mucosa, this mucosal environment facilitates transformed cells in their survival, growth, and metastases. This change in mucosal environment could therefore provide an explanation for the increased tendency in BE for neoplastic progression.

Concerning the development of BE; it could be that this defensive tissue response alters the microenvironment of the esophageal stem cell, thereby changing the direction of epithelial differentiation by providing different signals to esophageal stem cells (Figure 2). The development of BE could therefore be the epiphenomenon of the altered microenvironment in the esophagus and just be a reflection of a tumor promoting environment facilitating growth and metastases of transformed cells. If this will prove to be right, future studies should be directed at detection of the level of mucosal inflammation and its characteristics to obtain new targets for primary or secondary chemoprevention, and identification of those patients who will most benefit from screening and surveillance.

CONCLUSION

This thesis supports the hypothesis that the development of BE is preceded by the presence of a strong pro-inflammatory immune response. This pro-inflammatory immune response is induced by prolonged, intensive, frequent episodes of reflux of gastroduodenal content, but is also partly determined by a host pro-inflammatory genetic make-up. This triggers a defensive tissue response characterized by a humoral dominated immune response which promotes tumorigenesis by facilitating a tumor supportive microenvironment characterized by increased proliferation, increased angiogenesis, decreased apoptosis, tissue remodeling and decreased tumor surveillance. This hypothesis is supported by the finding that COX-2 polymorphisms associated with increased PGE_2, are linked to an increased risk of developing EAC. Reflux components such as bile acids, and bacteria may be important factors influencing the development of this adaptive tissue response, by modulating epithelial and stromal cell behavior by interacting via receptors such as FXR, and perhaps inducing expression of CDX2.

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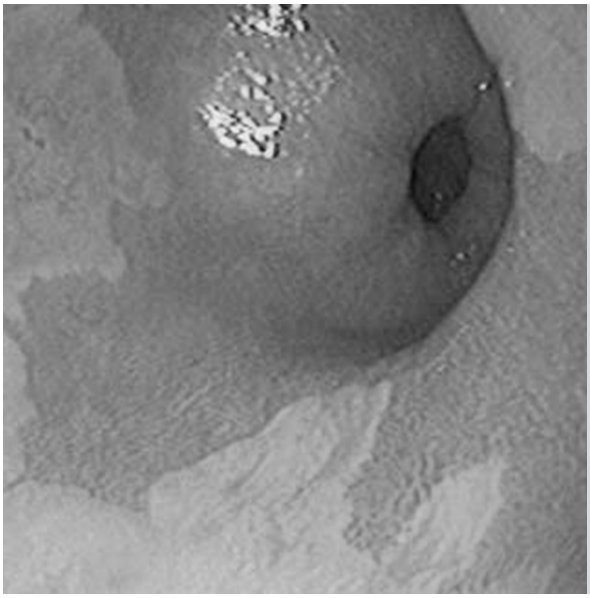

Chapter

X

Nederlandse samenvatting

Reflux ziekte is een veel voorkomende aandoening in de westerse samenleving, en ontstaat door blootstelling van de slokdarm aan "teruggestroomde maaginhoud", ook wel gastroesofageale reflux genoemd. Het voorkomen van reflux ziekte in de bevolking neemt met de jaren toe. Dit geld ook voor de belangrijkste complicatie van reflux ziekte; het adenocarcinoom van de slokdarm (ACS) [1]. ACS ontstaat als gevolg van langdurige, frequente, intense episodes van gastroesofageale reflux. ACS is moeilijk te genezen, en is vaak op het moment van detecteren al uitgezaaid naar buiten de slokdarm. De vijfjaarsoverleving van ACS van 13% is daarom laag. Overleving van ACS kan op dit moment alleen worden verbeterd met vroege detectie van de kanker, zodat radicale verwijdering van de tumor door operatief ingrijpen of endoscopische ablatie mogelijk is. De belangrijkste risicofactor voor het ontstaan van ACS is de aanwezigheid van een Barrett oesofagus (BO). BO is een asymptomatische aandoening die secundair ontstaat aan gastroesofageale reflux [2]. De aanwezigheid van BO is geassocieerd met een 30-60 keer hoger risico op ACS dan de rest van de Nederlandse bevolking [3,4]. Omdat vroege detectie van ACS geassocieerd is met de beste overleving, is het nodig nieuwe methoden te ontwikkelen die patiënten met een verhoogd risico op ACS (hoog-risico BO patiënten) kunnen identificeren.

SURVEILLANCE

Patiënten met BO ondergaan elke drie jaar een gastroscopie om een eventueel aanwezig ACS in een vroeg stadium te kunnen detecteren [5]. Het is echter nog steeds onduidelijk of deze strategie kosteneffectief is. Slechts 1 op de 200 patiënten met BO ontwikkelt ACS [6], wat deze benadering duur maakt, en leidt tot een toegenomen gebruik van de capaciteit van de gezondheidszorg. Tevens vinden patiënten het frequent ondergaan van een gastroscopie onprettig, en bestaat er een toegenomen kans op complicaties zoals bloeding, aspiratie en perforatie. Zeker voor patiënten die nooit een ACS zullen krijgen is dit een evident nadeel [7]. Op dit moment wordt veel onderzoek verricht naar nieuwe methoden of technieken die mensen met een hoog risico op ACS kunnen identificeren. Het vervolgen van patiënten op basis van een risicoprofiel maakt het mogelijk de nadelen voor de groep die er geen baat bij heeft te verminderen, en het succespercentage van de surveillance te verhogen.

COX-2 gen polymorfismen zijn betrokken bij de progressie naar ACS

De aanleg om een bepaalde ziekte te krijgen is voor een deel bepaald door de genetisch opmaak van een individu. Single nucleotide polymorfismen (SNP) in ontstekingsgerelateerde genen die resulteren in verschillen in eiwitconcentraties of enzym activiteit, kunnen de reactie van het individu op voortdurende blootstelling aan reflux in belangrijke mate beïnvloeden. Dit zou mogelijk kunnen resulteren in een verhoogde kans op het

krijgen van ACS. In hoofdstuk V, laten wij zien dat er een relatie bestaat tussen genetische polymorfismen in het gen (PTGS2) coderend voor het eiwit cyclooxygenase 2 (COX-2) en de aanwezigheid van ACS. In het gen PTGS2 zijn verschillende polymorfismen aangetoond. Twee van deze polymorfismen resulteren in verschillende COX-2 enzym concentraties en activiteit. Het C-residu op positie –765 is geassocieerd met toegenomen enzym activiteit, resulterend in verhoogde productie van prostaglandine (PG) E_2 en PGD_2 [8]. Het A-residu op positie –1195 is geassocieerd met een toegenomen concentratie van het eiwit COX-2 zelf [9]. Het COX-2 CA-haplotype (C_{-765} & A_{-1195}) werd meer frequent waargenomen in patiënten met ACS (21%) dan in patiënten met reflux oesofagitis (12%) en BO (12%; $p<0.001$). Homozygoot CA-haplotype was zelfs geassocieerd met een 3.8 keer (95%CI 1.1-14.6) toegenomen risico op ACS ten opzichte van patiënten met BO. Omdat het COX-2 CA-haplotype leidt tot toegenomen productie van PGE_2, lijkt er een relatie te bestaan tussen toegenomen productie van PGE_2 en een verhoogd risico op progressie naar kanker. Dit wordt ondersteund door bevindingen in ander onderzoek waarbij 1) een significante toename van het COX-2 enzym [10] and PGE_2 is waargenomen gedurende de progressie van reflux oesofagitis naar BO en ACS [11,12], 2) modulatie van het immuunsysteem door remming van COX-2 met aspirines of non-steroide anti-inflammatoire middelen (NSAID) resulteerde in een afname van het ontstaan van ACS in diermodellen [13,14] en het gebruik van deze middelen leek te beschermen tegen het ontstaan van ACS in humaan retrospectief epidemiologisch onderzoek [15-19], en 3) hoge productie van PGE_2 is geassocieerd met aggressiever tumor gedrag [20]. Het lijkt daardoor waarschijnlijk dat PGE_2, en de 4 receptoren (EP1-4) waaraan PGE_2 bindt, van belang zijn bij het ontstaan van ACS [21]. Er was geen verschil in het voorkomen van het CA-haplotype tussen patiënten met een relfux oesofagitis en BO. Dit suggereert dat PGE_2 vooral belangrijk is tijdens de progressie van BO naar ACS, en minder tijdens de progressie van reflux oesofagitis naar BO.

Op basis van deze studie kan niet worden geconcludeert dat het testen van COX-2 haplotypen gebruikt kan worden voor het herkennen van hoog-risico BO patiënten. Er zijn namelijk enkele belangrijke beperkingen in het gebruik van COX-2 haplotypen. Slechts 21% van de patiënten met een ACS is drager van het CA-haploptype. Een deel van de ACS patiënten zou daarom onterecht niet als hoog-risico patiënt worden aangemerkt. Als de COX-2 haplotype bepaling gebruikt zou worden voor surveillance dan zou dit gepaard moeten gaan met andere parameters. Het is eerder de biologische consequentie, dat verhoogde productie van PGE_2 en binding van PGE_2 aan zijn receptoren een belangrijke rol speelt in de carcinogenese. Het testen op de aanwezigheid en mate van PGE_2 acitiviteit in de slokdarm zou mogelijk veel meer aanvulling kunnen opleveren voor de dagelijkse praktijk, daar het een aanwijzing geeft voor het bestaan van een pathologische signaal transductie pathway. Daarnaast kan PGE_2 een therapeutisch aangrijpingspunt zijn, waarbij specifieke PGE_2 receptorremmers zouden kunnen worden toegepast ter chemopreventie. Dit zou mogelijk minder vasculaire bijwerkingen kunnen hebben dan het gebruik van de huidige COX-2 remmers.

SCREENING

Bij meer dan 90% van de patiënten die zich presenteren met een ACS op de endoscopieafdeling, was niet eerder de diagnose BO gesteld [22-24]. Het verbeteren van de huidige follow-up van patiënten met BO zou daarom moeten worden aangevuld met screening naar BO bij mensen met gastroesofageale reflux ziekte. Op dit moment is het echter onmogelijk om de patiënten met BO goed te kunnen identificeren in de groep van patiënten met reflux ziekte, zodat een veel te grote groep patiënten een endoscopie zou moeten ondergaan om een nieuwe BO patiënt te ontdekken [25,26]. Het is daarom belangrijk meer inzicht te krijgen in de etiologie van BO, omdat dit mogelijk nieuwe aangrijpingspunten kan opleveren voor screening en interventie.

Chronische ontsteking van de slokdarm zou zo'n aangrijpingspunt kunnen zijn. In diermodellen gaat de reflux geïnduceerde ontsteking van de slokdarm (oesofagitis) vooraf aan het ontstaan van BO. Daarnaast correleert de ernst van de ontsteking met het ontstaan van BO en ACS [13,14]. De aanwezigheid van reflux oesofagitis bij endoscopie van de slokdarm is geassocieerd met een negen maal hoger risico op ACS ten opzichte van patiënten met reflux gerelateerde klachten maar zonder reflux oesofagitis bij endoscopie [27]. Het gebruik van ontstekingsremmers zoals NSAID's en aspirine resulteerde in een verminderde ontsteking, wat weer was geassocieerd met een verminderd voorkomen van ACS [15-19,28]. Dat een ernstige chronische slijmvliesontsteking in diermodellen vooraf gaat aan het ontstaan van BO is reeds duidelijk aangetoond, maar dit is echter niet bewezen voor mensen. Dit komt deels door het feit dat progressie naar BO zelden wordt gezien in de praktijk, en dat bij het merendeel van de patiënten, BO reeds aanwezig is bij de eerste gastroscopie [29]. Om te testen of verschillen in de ontstekingsreactie de uitkomst van blootstelling aan gastroesofageale reflux beïnvloedt, zijn genetische polymorfismen in de genen coderend voor IL-1β, IL-1Ra, IL-8, IL-6, IL-2, IL-10 en IL-12 bepaald in 255 patiënten met BO en 240 patiënten met reflux oesofagitis.

Ernstige ontsteking van de slokdarm is een drijvende kracht achter het ontstaan van een Barrett oesofagus.

In hoofdstuk IV, laten wij zien dat de aanwezigheid van het C-residu op positie +1188 van het IL-12B gen, welke bekend is met een verhoogde productie van IL-12p70 [30,31], is geassocieerd met een verhoogd risico op BO (OR 1.8 95%CI 1.17- 2.69; *p*=0.007). Deze bevinding impliceert een rol voor hoge IL-12p70 productie in het ontstaan van BO. IL-12p70 stimuleert de differentiatie van naïeve T cellen naar T cellen met een Th1 fenotype, en stimuleert de expressie van Th1 cytokinen [32]. Het wordt daarom aangenomen dat IL-12p70 een belangrijke brug vormt tussen de aspecifieke immuun respons en het aantrekken van een Th1 dominante adaptieve immuun respons. Het frequenter voorkomen van het IL-12 genotype, suggereert dat een sterke gastheer-bepaalde immuunrespons betrokken is bij de pathogenese van BO [13,14].

De associatie tussen het IL-12B C-allele en BO, wordt gemodificeerd door de aanwezigheid van een hiatus hernia. De aanwezigheid van een hiatus hernia gecombineerd met de aanwezigheid van het IL-12B C-residu verhoogde het risico op BO (OR 2.9 95%CI 1.32-6.58; *p*=0.008). Dit ondersteunt de hypothese dat een toegenomen ernst van de ontsteking predisponeert voor het ontstaan van BO.

We namen ook waar dat het IL-10_{-1082} GG genotype een interactie aanging met het IL-12B polymorfisme. In aanwezigheid van het IL-10_{-1082} GG genotype veranderde de associatie tussen het IL-12B C-allele en het ontstaan van BO (OR 1.0 95%CI 0.5-1.9; *p*=0.1). Patiënten met een IL-12B AA genotype (lage IL-12p70 expressie) hadden zelfs een lager risico op het krijgen van BO wanneer aanwezig in combinatie met het IL-10_{-1082} GG genotype (OR 0.6 95%CI 0.34-0.99; *p*=0.011). Omdat het IL-10_{-1082} GG genotype is geassocieerd met hogere IL-10 expressie [33], en IL-10 de expressie van IL-12p70 door geactiveerde dendritische cellen remt, is het goed mogelijk dat aanwezigheid van het IL-10_{-1082} GG genotype leidt tot een verminderde expressie van IL-12p70. Dat dit mogelijk het geval kan zijn wordt ondersteund door een recente observatie door Peng et al.[34]. In deze studie verlaagde de aanwezigheid van het IL-10_{-1082} GG genotype de IL-12p70 expressie door LPS gestimuleerde dendritische cellen ondanks de aanwezigheid van het met hoge IL-12p70 expressie geassocieerde IL-12B genotype [34]. Dit suggereert dat verhoogde expressie van IL-12p70 een belangrijke rol speelt in het ontstaan van BO. Omdat BO is geassocieerd met een toegenomen aantal ontstekingscellen in het slijmvlies ten opzichte van reflux oesofagitis (hoofdstuk III). suggereert dit verder dat, overkomend met de diermodellen, een ernstige ontsteking van slokdarm vooraf gaat aan het ontstaan van BO.

Progressie naar BO gaat gepaard met een verandering naar een meer humoraal gedomineerde immuun respons

In hoofdstuk III wordt beschreven dat de immuun respons in BO verschilt van de immuun respons in reflux oesofagitis. BO was geassocieerd met een hoger aantal IgG^+ en IgE^+ plasma cellen, en IgE+ mestcellen, waar reflux oesofagitis juist was geassocieerd met een toegenomen aantal macrofagen en cytotoxische T cellen. Dit suggereert dat BO wordt gekenmerkt door een dominante Th2 respons, en reflux oesofagitis door een meer dominante Th1 respons. Dit wordt ondersteund door de studie van Fitzgerald et al. die aantoonde dat BO was geassocieerd met een toegenomen transcriptie van IL-4 and IL-10 (Th2 cytokinen), en reflux oesofagitis was geassocieerd met toegenomen transcriptie van IFN-γ, IL-1 en IL-8 (Th1 cytokinen) [35]. Hieruit concluderen wij dat de progressie van reflux oesofagitis naar BO is geassocieerd met een ernstige ontsteking van de slokdarm en dat deze progressie gepaard gaat met het ontstaan van een Th2 gedomineerde immuun respons.

Chronische Th2 ontstekingen en het ontstaan van kanker

Het ontstaan van een Th2 gedomineerde immuun respons in BO kan consequenties hebben voor de verhoogde neiging tot het ontwikkelen van kanker. Recente studies tonen aan dat de aanwezigheid van een Th2 dominante immuun respons is geassocieerd met het ontstaan van kwaadaardige aandoeningen in de aanwezigheid van een chronische ontsteking [36-38]. In een Th2 gedomineerd micromilieu ontwikkelt zich eerder een tumor, omdat de groei van tumorcellen wordt gefaciliteerd door toegenomen celdeling en angiogenese, en afgenomen apoptose en tumor immunosurveillance [36,37]. Dit laatste komt uit studies waarin immuun gecompromitteerde muizen gevoeliger bleken voor het spontaan ontwikkelen van gastrointestinale tumoren [39,40]. Tevens werd aangetoond dat de aanwezigheid van een Th2 gedomineerde immuun respons dit juist deed toenemen [41].

De humorale immuun respons blijkt mogelijk een belangrijke rol te spelen in het verhoogd risico op kanker. Toegenomen immuun activiteit tegen de tumor werd waargenomen in muizen die deficiënt zijn voor een adequate B cel respons [42,43]. B cellen blijken in staat natural killer cellen en T cellen gericht tegen de tumor te kunnen remmen door de INF-γ productie te beïnvloeden op moment van activatie van deze cellen. In een HPV16 huidkankermodel[44], bleken geactiveerde B cellen eiwitten te produceren die het ontstaan van huidkanker stimuleren door ondersteuning van de angiogenese, celdeling, en verandering van de extracellulaire matrix. Remming van de B cellen leidde tot een aanzienlijke vertraging van het ontstaan van huidkanker [44].

BO is geassocieerd met een toegenomen celdeling, angiogenese [45], en veranderingen in de extracellulaire matrix[46], en afgenomen apoptosis [47], en tumor immunosurveillance [48]. Of de vele IgG+ plasma cellen in de lamina propria van BO een rol spelen bij deze veranderingen in de slokdarm zal moeten blijken uit aanvullend onderzoek, maar in het licht van de huidige bevindingen is dit niet onwaarschijnlijk.

Mechanismen betrokken bij het aantrekken van een immuun respons

Als inderdaad progressie van reflux oesofagitis naar BO vooraf wordt gegaan door een heftige genetische bepaalde immuun respons, en deze immuun respons wordt gekenmerkt door humorale eigenschappen, dan is het belangrijk te weten welke mechanismen de ernst en de samenstelling van de ontsteking bepalen. Daar reflux zo'n prominente positie inneemt in de etiologie is het zeer goed mogelijk dat luminale factoren zoals zuur, pepsine, galzuren, voedsel antigenen en bacteriën een rol spelen bij de inductie van een immuun respons. Omdat reflux is geassocieerd met een toegenomen doorlaatbaarheid van de wand, is het voor de hand liggend dat deze bestanddelen in contact kunnen komen met de ontstekingcellen die direct onder het epitheel zijn gelegen [49,50]. Dit zou dan weer kunnen leiden tot immuun activatie in aanwezigheid van een door reflux veroorzaakt stress signaal. Een toegenomen gevoeligheid tot immuun activatie zou dan ook een belangrijk probleem kunnen zijn in de slokdarm.

Luminale factoren beïnvloeden de immuun respons in de mucosa van de slokdarm.

Galzuren zijn een belangrijk onderdeel van het refluxaat, en van galzuren wordt gedacht dat zij de immuun respons kunnen beïnvloeden. In hoofdstuk VIII hebben we onderzocht of galzuren inderdaad in staat zijn de immuun respons in de slokdarm te beïnvloeden. Het is recent aangetoond dat geconjugeerde galzuren een rol spelen in de antimicrobiële immuun respons door te binden aan de farnesoid X receptor in intestinaal epitheel(FXR) [54]. Door te binden aan FXR bleken galzuren in staat de expressie van verschillende antimicrobiële bestandelen te induceren en de translocatie van bacteriën te verhinderen door de integriteit van de mucosale barrière te versterken [54]. FXR wordt tot expressie gebracht door het gespecialiseerd intestinaal type epitheel van de slokdarm, en in plaveiselepitheel van patiënten met BO. FXR wordt echter niet tot expressie gebracht in het plaveiselepitheel van gezonde controles zonder reflux oesofagitis. Stimulatie van FXR door geconjugeerde galzuren induceerde de expressie van FXR zelf, maar ook van FXR respons eiwitten zoals I-BABP en SHP. Dit is daarmee de eerste studie die een receptor afhankelijk beïnvloeding van epitheelcellen door galzuren aantoont. Daarnaast leidde stimulatie van FXR ook tot de expressie van de chemokines IL-8 en MIP3α. Van IL-8 was al eerder bekend dat de expressie kon worden geïnduceerd door galzuren. Dit is echter nieuw voor MIP-3α [56]. Expressie van MIP-3α wordt geïnduceerd in de aanwezigheid van schade aan het epitheel en is betrokken bij het aantrekken van T cellen en dendritische cellen in de darm. De inductie van MIP3α speelt een belangrijke rol in 2,4,6-trinitrobenzene sulfonic acid geïnduceerde schade aan de dunne darm, omdat neutraliserende antilichamen tegen MIP3α het ontstaan van een ontstekingsreactie bleke te remmen. Hieruit concluderen wij dat FXR tot expressie wordt gebracht in BO, en dat binding aan FXR door geconjugeerde galzuren de immuun respons in de slokdarm kunnen beïnvloeden (hoofdstuk VIII).

Intestinale differentiatie in de slokdarm

Van CDX2 wordt aangenomen dat het een belangrijke regulator is van intestinale differentiatie, daar ectopische expressie van CDX2 in de maag resulteerde in het ontstaan van intestinale metaplasie in de maag [57,58]. In deze thesis wordt aangetoond dat CDX2 tot expressie wordt gebracht in BO, maar niet in maagtype epitheel en in plaveiselepitheel. Tevens is aangetoond dat *Cdx2* mRNA aanwezig was in 1/3 van de biopten genomen uit het plaveiselepitheel van patiënten met BO. Dit suggereert dat CDX2 inderdaad vroeg tot expressie wordt gebracht en dat inductie van CDX2 mogelijk vooraf gaat aan het ontstaan van intestinale metaplasie. Het is recent aangetoond dat expressie van CDX2 kan worden geïnduceerd door galzuren en door pro-inflammatoire cytokinen zoals IL-1β en TNF-α [59-64]. Deze inductie van CDX2 is afhankelijke van de mate van de *Cdx*2 promotor methylatie [59,61]. In een staat van hypermethylatie wordt CDX2 niet tot expressie gebracht, zoals in normaal plaveiselepitheel [65]. Wanneer de mate van methylatie afneemt kan

transcriptie van *Cdx*2 worden geïnduceerd. De verandering van de mucosale bekleding van de slokdarm kan dus worden geïnitieerd door signalen uit de omgeving zoals door galzuren, cytokinen, en de bacteriële flora door de expressie van CDX2 te induceren of de methylatie status van de *Cdx*2 promotor te veranderen.

HYPOTHESE EN TOEKOMSTIG ONDERZOEK

Dat Th2 gedomineerde micromilieus in staat kunnen zijn de locale epitheliale differentiatie te beïnvloeden volgt uit de bevindingen dat stamcel differentiatie sterk wordt beïnvloedt door signalen afkomstig van naast gelegen stromale cellen en uit de extracellulaire matrix [66]. Epitheelcellen kunnen aangezet worden tot de expressie van mucinen door stimulatie van de IL-4 receptor door Th2 cellen [67]. Blootstelling van luchtwegen van muizen aan IL-4 en IL-13 resulteerde in het ontstaan van slijmbekercel metaplasie [68,69]. Muizen geïnfecteerd met *Trichinella spiralis* vertonen een twee fasen respons. Allereerst een sterke Th1 gedomineerde respons gevolgd door een overschakeling naar een Th2 gedomineerde respons op dag 8. Na dag 8 ontstaat een sterke slijmbekercel hyperplasie welke wordt geïnduceerd door Th2 cellen [70]. In een andere studie werd aangetoond dat Th2 cellen villus atrofie en slijmbekercel hyperplasie konden induceren, voornamelijk gemedieerd door IL-4 [71]. De relatie tussen het ontstaan van ectopisch CDX2+ en MUC2+ intestinaal type epitheel en de aanwezigheid van een chronische ontsteking wordt verder benadrukt door de observatie, dat deze weefselverandering ook wordt waargenomen bij chronische ontsteking van de maag ten gevolge van chronische infectie met *Helicobacter pylori*, in de sinus nasalis bij frequente blootstelling aan houtstof [72], en in de galblaas bij chronische ontsteking van de galblaas door chronische cholecystolithiasis [73]. Dit betreft allemaal chronische ontstekingen, maar echter met verschillende uitlokkende stimuli. Het lijkt dus eerder de ontsteking zelf die een rol zou kunnen spelen bij het ontstaan van intestinale metaplasie.

De ontwikkeling van intestinale metaplasie zou dus heel goed een defensieve respons kunnen zijn tegen ernstige weefselbeschadiging. Dit wordt ondersteund door de bevinding dat BO is geassocieerd met de hoogste mate van ontsteking gemedieerde oxidatieve schade. De aanhoudende blootstelling aan reactieve zuurstof radicalen kan weer resulteren in genetische mutagenese. Het is mogelijk dat een ernstige ontsteking met toegenomen oxidatieve stress een adaptieve respons uitlokt, welke is gericht op beperking van de schade op het epitheel en de mucosale integriteit. Deze adaptieve respons betreft o.a. het ontstaan van een Th2 gedomineerde immuun respons. Dit leidt dan weer tot een toegenomen proliferatie en angiogenese, en afgenomen apoptose en tumor immunosurveillance (tumor promotie). Alhoewel het direct beschadigende effect op de mucosa wordt opgevangen leidt deze verandering op den duur tot de facilitatie van tumor groei. Dit zou een mogelijke verklaring kunnen geven voor de verhoogde

neiging tot maligne ontsporing in BO. Deze adaptieve immuun respons kan tevens het micromilieu van de stamcel sterk veranderen en daarbij de differentiatie richting beïnvloeden. De ontwikkeling van BO zou daarom meer een epifenomeen van het veranderde micromilieu kunnen zijn. Dit behoeft echter nader onderzoek.

CONCLUSIE

Deze thesis ondersteunt de hypothese dat de ontwikkeling van BO wordt vooraf gegaan door een sterke mate van ontsteking van de slokdarm. Deze ontsteking wordt geïnduceerd door aanhoudende, intense en frequente blootstelling van de slokdarm aan gastroesofageale reflux, maar wordt ook voor een belangrijk deel bepaalt door genetische eigenschappen van het individu. In enkele gevallen lokt dit een adaptieve weefsel respons uit welke wordt gekarakteriseerd door het ontstaan van een dominant humorale immuun respons. Deze Th2 gedomineerde immuun respons faciliteert tumor groei door toegenomen proliferatie en angiogenese, en afgenomen apoptose and tumor immunosurveillance. Deze hypothese wordt ondersteund door de bevinding dat COX-2 polymorfismen geassocieerd met een hogere productie van PGE_2, zijn geassocieerd met een verhoogd risico op ACS. Reflux bestanddelen zoals galzuren en bacteriën kunnen belangrijke factoren zijn in het ontstaan van zo'n adaptieve immuun respons, daar zij het gedrag van epitheel- en stromale cellen kunnen beïnvloeden via interactie met receptoren zoals FXR, en mogelijk door de inductie van CDX2.

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Curriculum vitae

Leon Moons is geboren in Gouda op 10 november 1974. Hij heeft zijn V.W.O diploma gehaald op het Sint Antonius College in 1993. In hetzelfde jaar is hij gestart met de studie geneeskunde aan de universiteit van Antwerpen. In 1994 zettte hij deze studie voort aan de medische faculteit van de Erasmus Universiteit te Rotterdam. Gedurende deze periode heeft hij zich ingezet voor onderzoek naar het isoleren van humaan DNA uit faeces aangebracht op FOBT kaartjes ten einde non-invasieve screening naar colorectaal carcinoom te kunnen ondersteunen. Hij heeft dit onderzoek verricht onder supervisie van Prof. dr. Lindemans and dr. R.N van Schaik. In 2002 heeft hij zijn studie geneeskunde afgerond, waarna hij in hetzelfde jaar is gestart met promotieonderzoek naar de etiologie van een Barrett slokdarm onder begeleiding van prof. dr. P.D. Siersema en dr. J.G. Kusters op de afdeling Gastroenterologie en Hepatologie van het Erasmus MC (afdelingshoofd Prof. dr. E.J. Kuipers). Op 1 december 2005 is hij gestart met zijn vooropleiding Interne Geneeskunde in het Ijsseland Ziekenhuis onder supervisie van de opleider dr. H. Van der Wiel. Op 1 december 2007 is hij begonnen aan zijn opleiding tot Maag-, Darm-, en Leverarts in het Erasmus MC onder supervisie van de opleiders dr. R. de Man en Prof. Dr. E.J. Kuipers. Op 26 mei 2006 is hij in het huwelijk getreden met Margriet E.B. Lems, en is sinds 3 mei 2007 de trotse vader van zijn dochter Veerle.