Barrett's Esophagus: a Molecular Characterization

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Barrett's Esophagus: a Molecular Characterization

Barrett-oesofagus: een moleculaire karakteristiek

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Chapter 1

General introduction

BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

Barrett's esophagus is a premalignant condition that is most likely caused by gastroesophageal reflux. In the western world, about 30% of adults have reflux complaints, such as heartburn, and about 10% of reflux patients will develop Barrett's esophagus [1]. Barrett's esophagus is characterized by chronic inflammation, and like other chronic inflammatory lesions, it is associated with cancer development. Patients with Barrett's esophagus have a 30 times increased risk for the development of esophageal adenocarcinoma compared to the general population [2]. Barrett's esophagus can progress to esophageal adenocarcinoma through the intermediate stages low-grade dysplasia and high-grade dysplasia [3]. Esophageal adenocarcinoma has a poor prognosis, the overall survival is only 15-20% [2].

Endoscopical and histological diagnosis
During upper gastrointestinal (GI) endoscopy, Barrett's esophagus is clearly visible as salmon-red appearing mucosa in the distal esophagus. Histologically, Barrett's esophagus is defined as the presence of columnar epithelium of the intestinal type containing goblet cells, which is called intestinal metaplasia. In addition to intestinal metaplasia, gastric mucosa of the cardiac or fundic type is also frequently present in the esophagus, but this type of mucosa is not premalignant and therefore not regarded as Barrett's esophagus [2]. Patients with Barrett's esophagus are invited to enter surveillance-programs to screen for dysplasia and adenocarcinoma development. They undergo upper GI endoscopy every two years, during which at every 2 cm of the columnar segment, 4-quadrant biopsies are taken [4]. These biopsy sets are routinely evaluated by a pathologist for the presence of low-grade dysplasia, high-grade dysplasia or esophageal adenocarcinoma.

Maintenance and treatment
Once established, Barrett's esophagus cannot regress to normal squamous epithelium unless the epithelium is completely removed. Therefore, patients receive therapies with the intention of minimizing the risk of adenocarcinoma development. In order to reduce acid reflux, patients receive proton-pump inhibitor therapy [5]. In patients with severe reflux complaints, surgical nissen fundoplication can partly restore the function of the upper esophageal sphincter [6]. To eliminate high-grade dysplasia endoscopically, patients are often treated by photodynamic therapy and/or endoscopic mucosal resection [7]. Patients with esophageal adenocarcinoma are only only considered for curative surgery when they have no or only loco-regional lymph node metastases [8]. Patients who are not considered for surgery, undergo palliative treatment, usually brachytherapy or stent-placement [9].

Molecular biology of Barrett's esophagus and esophageal adenocarcinoma
The differentiation of normal squamous epithelium into intestinal metaplasia, and the progression through the Barrett's esophagus - dysplasia - adenocarcinoma sequence is mediated by molecular alterations that are probably induced by the

chronic inflammation present in Barrett's esophagus [10]. The transformation from a normal cell into a tumor cell is thought to require four to seven alterations, each of them leading to the induction of proteins involved in tumorigenesis or downregulation of proteins protecting the cell [11]. These alterations are usually comprised of genetic lesions or altered methylation patterns of genes, resulting in changes in mRNA and protein expression. In the Erasmus MC a wide range of expertise on molecular biological research on Barrett's esophagus and esophageal adenocarcinoma is present in several departments. The research described in this thesis connects to previously published Erasmus MC theses on molecular biology of esophageal adenocarcinoma [12, 13].

Aim of this thesis

The aim of this thesis is to characterize the expression of several genes involved in intestinal differentiation, chronic inflammation, and mucosal protection during intestinal differentiation in Barrett's esophagus, and progression to dysplasia and esophageal adenocarcinoma.

Outline of the thesis

In chapter 2, an overview of the existing literature about molecular alterations in intestinal differentiation and carcinogenesis in the esophagus is given. In chapter 3, the expression of the intestinal transcriptionfactor CDX2 in Barrett's esophagus is assessed. In chapter 4 and chapter 5, the question whether or not CDX2 is present in gastric type mucosa of the esophagus is discussed. In chapter 6, the expression of seven principal mucins in Barrett's esophagus, dysplasia and esophageal adenocarcinoma is investigated. In chapter 7, genes involved in high-grade dysplasia development are identified with the use of transcription profiling analysis (microarray). In chapter 8, the influence of bile acids on chemokine expression is investigated in an esophageal cell-line model, and chemokine transcription is also assessed in reflux esophagitis, Barrett's esophagus, high-grade dysplasia, and esophageal adenocarcinoma. In the final chapter, chapter 9, the results described in this thesis are summarized and discussed.

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Chapter 2

Molecular alterations during development of esophageal adenocarcinoma

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SUMMARY

The incidence of esophageal adenocarcinoma has risen significantly over the last decades. During esophageal carcinogenesis many molecular alterations occur that disrupt essential cellular processes, directing the cell to a rapidly proliferating, immortal state. The chronic inflammation that is present in Barrett's esophagus creates an environment in which such molecular alterations are both induced and tolerated. Here, the novel insights in the molecular mechanisms that underlie the development of esophageal adenocarcinoma are reviewed, focussing on the role of inflammation, angiogenesis, apoptosis inhibition, loss of cell cycle control and loss of cell-cell adhesion. These novel developments will open new perspectives for diagnosis, treatment, and prevention of esophageal adenocarcinoma.

INTRODUCTION

Esophageal adenocarcinoma is generally believed to develop from Barrett's esophagus in a step-wise progression involving low-grade dysplasia and subsequently, high-grade dysplasia. This is based on the observation that the different stages are often observed adjacent to each other in esophageal biopsies. In addition, many patients with low-grade and high-grade dysplasia progress to adenocarcinoma [1, 2]. The progression through this sequence is mediated by molecular alterations that are thought to be induced by the chronic inflammation present in Barrett's esophagus [3]. The transformation from a normal cell into a tumor cell is thought to require four to seven alterations, each of them leading to the induction of proteins involved in tumorigenesis or downregulation of proteins protecting the cell [4]. These alterations are usually comprised of genetic lesions or altered methylation patterns of genes, resulting in changes in mRNA and protein expression. In this review the focus will be on the molecular alterations that affect the subsequent processes during the metaplasia-dysplasia-carcinoma sequence: inflammation, intestinal metaplasia development, angiogenesis, cell proliferation, apoptosis, cell-cycle regulation, and cell-cell adhesion.

INFLAMMATION AND OXIDATIVE STRESS

Barrett's esophagus is characterized by a chronic inflammation that is most likely the result of repeated gastroesophageal reflux. As with other chronic inflammatory lesions, such as Helicobacter pylori induced gastritis and inflammatory bowel disease, Barrett's esophagus also predisposes to cancer [1, 5-8]. The carcinogenic role of inflammation in Barrett's esophagus is illustrated by the observation that anti-inflammatory drugs, such as aspirin, are able to (partially) protect against esophageal adenocarcinoma [9]. The major components of gastroesophageal reflux are gastric acid, bile, and pancreatic enzymes. Their repetitive combined presence in the esophagus induces cell damage [10]. Damaged epithelial cells usually start to produce inflammatory mediators such as cytokines and chemokines, which leads to the attraction of inflammatory cells. These inflammatory cells produce reactive oxygen species and other free radicals to eliminate the exposed antigen, in this case the damaged cell [11]. The reactive oxygen species and other free radicals can damage the cell-membrane, which alters the membrane-permiability and the activity of membrane receptors. Moreover, these reactive oxygen species do not only affect the damaged cell but are also able to cause damage in the surrounding cells. DNA mutations caused by reactive oxygen species occur at random and can be lethal. However, surviving cells can develop mutations that lead to the induction of genes involved in carcinogenesis (Figure 1) [12]. Increased amounts of reactive oxygen species have been observed in Barrett's esophagus [13], and animal studies suggests that oxidative stress plays a role in the formation of an esophageal adenocarcinoma [14, 15].

Several enzymes and other factors mediate scavenging of toxic oxygen and

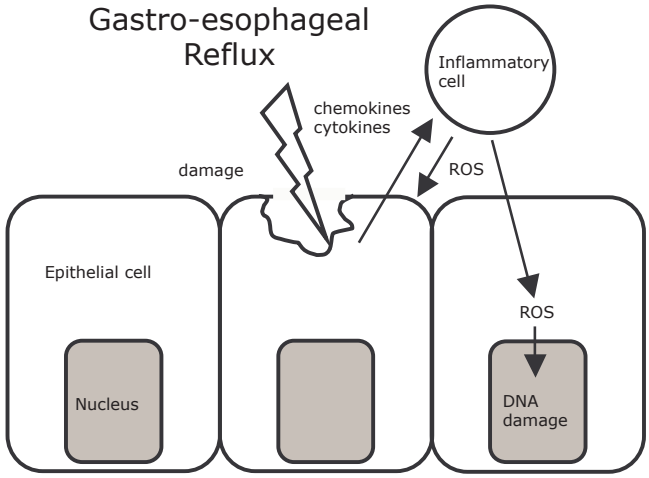

Figure 1 Schematic representation of the effect of gastroesophageal reflux on esophageal cells. The components of gastroesophageal reflux damage the epithelial cells, as a consequence these cells will start to secrete inflammatory mediators such as cytokines and chemokines that attract inflammatory cells. These inflammatory cells produce reactive oxygen species (ROS) that can cause DNA damage in undamaged cells, thereby inducing tumor promoting mutations.

other radicals. Enzymes such as gluthatione-S transferase, gluthatione peroxidase, superoxide dismutase, catalase, vitamin C, and vitamin E catalyze a reaction in which a toxic radical is converted into a less toxic compound. This catalysis prevents DNA damage and cancer cell formation. The glutathione-S transferase family of radical detoxifying enzymes comprises many isoforms, whose expression is gradually downregulated during the malignant progression of Barrett's esophagus [16, 17]. Furthermore, inhibition of glutathione peroxidase secretion has been observed in this disorder [18]. The expression of superoxide dismutase was not changed during neoplastic progression [17], and catalase levels in Barrett's esophagus and esophageal adenocarcinoma are not yet determined. Plasma and tissue vitamin C levels, but not vitamin E levels were decreased in Barrett's esophagus compared with normal squamous epithelium [19]. Although not all enzymes involved in detoxification of radicals are decreased in Barrett's esophagus and adenocarcinoma, overall scavenging capacity seems diminished during esophageal carcinogenesis.

INFLAMMATORY MEDIATORS

Upon injury caused by gastroesophageal reflux, the damaged esophageal cells will start to secrete inflammatory mediators such as cytokines and chemokines, and this leads to the migration of inflammatory cells to the damaged tissue. NF-κB is a transcription factor that is responsible for the upregulation of many pro-inflammatory cytokines and chemokines, and it has also been linked to several oncogenic functions such as proliferation, metastasis and angiogenesis [20]. NF-κB is activated in response to the bile acid deoxycholic acid in a esophageal cell-culture model [21, 22]. Indeed activated NF-κB was present in about half of Barrett's metaplasia and in most adenocarcinoma samples [21], indicating that reflux induced NF-κB activation contributes to esophageal carcinogenesis.

The NF-κB regulated pro-inflammatory chemokine interleukin-8 (IL-8) attracts neutrophils, which are the most potent producers of reactive oxygen species. Using in vitro experiments it was demonstrated that expression of IL-8 is induced in esophageal cells by a brief exposure to the bile acid deoxycholic acid [22]. Expression of IL-8 but also of cytokine IL-1β is increased in Barrett's esophagus [23]. This effect was most pronounced near the squamocolumnar junction. Distally, where most adenocarcinomas occur, expression of the anti-inflammatory cytokines IL-4 and IL-10 was observed [23, 24]. This suggest a tight balance between pro-inflammatory and anti-inflammatory agents in cancer development [3, 25], but the exact chemokine and cytokine profiles in esophageal adenocarcinoma still have to be determined. Increased understanding of the role of chemokines and cytokines in esophageal adenocarcinoma is required for novel therapeutic interventions controlling expression of these chemoattractants.

Neutrophils are frequently observed inflammatory cells in Barrett's esophagus. These cells are attracted by the NF-κB induced chemokine IL-8, but the calprotectin complex functions also in chemotaxis of these cells. The calprotectin complex has been implicated in chronic inflammatory diseases, such as inflammatory bowel disease. In the esophagus, both subunits of the complex, calgranulin A and B have been shown to be associated with the development of high-grade dysplasia in Barrett's esophagus [26] implicating a role for calprotectin and for neutrophils in the onset of neoplastic progression.

Cyclooxygenase 2 (COX-2) is the rate-limiting enzyme in the conversion of arachidonic acid into prostaglandins. Prostaglandin E2 (PGE2) has many tumor-promoting properties, such as inhibiting apoptosis, increasing proliferation and angiogenesis, and inducing the production of matrix metalloproteinases (see paragraph on cell-cell adhesion and Figure 2). COX-2 is inducible by a variety of factors, such as proinflammatory cytokines (TNF-α, TGF-β) and growth factors

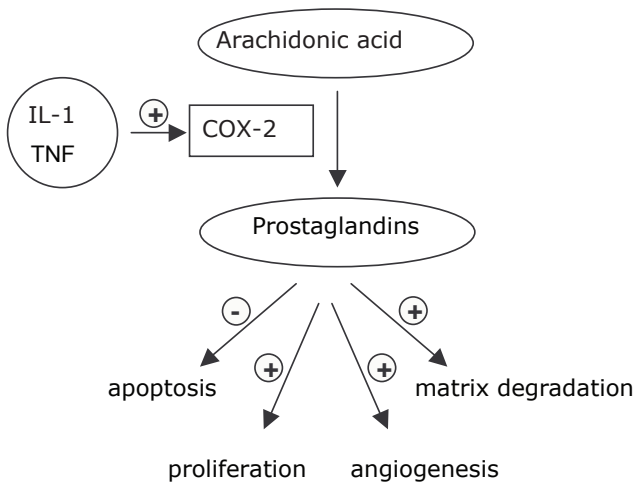

Figure 2 The role of COX-2 in malignant progression. Pro-inflammatory cytokines such as IL-1 and TNF-α induce cyclooxygenase 2 (COX-2), which converts arachidonic acid into prostaglandins. These prostaglandins are capable of inhibiting apoptosis and inducing cell proliferation, angiogenesis and loss of cell-cell adhesion by means of matrix metalloproteinase (MMP) upregulation.

[27, 28]. It has been demonstrated using *in vitro* as well as in *ex-vivo* models for Barrett's esophagus that COX-2 expression is induced by bile and acid [29, 30], suggesting that gastroesophageal reflux induces expression of this enzyme, thereby promoting carcinogenesis. Indeed, increased COX-2 levels were observed in Barrett's esophagus and adenocarcinoma [31]. Since COX-2 induces tumorigenesis via several pathways, it is suggested that this enzyme is one of the key factors in esophageal carcinogenesis.

INTESTINAL PROTEINS

Barrett's esophagus is characterized by the presence of intestinal metaplasia, and only this type of metaplasia predisposes to esophageal adenocarcinoma [32]. Several proteins that are normally expressed exclusively in intestinal tissue, are also present in Barrett's esophagus. An example is CDX2, a transcription factor involved in intestinal differentiation, which functions in the development of intestinal epithelium [33]. As Barrett's esophagus is defined as a replacement of normal esophageal epithelium by intestinal-like epithelium, CDX2 has been suggested to play an important role in the development of this lesion [34, 35]. Although a tumor-suppressor function has been suggested for CDX2 [36], in the esophagus this is unlikely since loss of CDX2 expression was only observed in few adenocarcinomas [35].

CDX2 is responsible for the transcription of several intestinal-specific genes, such as MUC2. Mucins are large glycoproteins that are key components of the mucus layer on epithelium. This layer protects the epithelium from mechanical and chemical damage. Mucins are divided into two groups; i) the membrane bound, and ii) the secreted mucins [37]. Each tissue type has its own characteristic mucin pattern and this pattern changes during metaplastic and neoplastic progression [38]. The predominant mucins in Barrett's esophagus are MUC2, MUC5AC and MUC6 [39, 40]. In high-grade dysplasia, MUC4 expression is upregulated [41], whereas in adenocarcinoma MUC1 expression is upregulated and MUC2, MUC5AC and MUC6 expression is downregulated [39]. The role of these changes in mucin expression is unknown. However, it can be hypothesized that the carbohydrate structures of overexpressed MUC1 and MUC4 mask tumor antigens, thereby inhibiting the recognition and clearance of tumor cells by the immune system [42].

ANGIOGENESIS

Barrett's esophagus is highly vascularized tissue, and the presence of microvessels gives the Barrett's tissue its characteristic salmon pink color. This vascularization is necessary for the recruitment of immune cells. However, the formation of new blood vessels, angiogenesis, is also essential in tumors for nutrient delivery, since rapidly proliferating cells need an excess of nutrients, which cannot be provided by the surrounding tissue. Without the supply of nutrients, the tumorcells

cease proliferation [43]. Furthermore, the proximity of abundant blood and lymph vessels enables tumorcells to rapidly metastasize.

Vascular endothelial growth factor (VEGF) is an important mediator of blood vessel growth. VEGF expression has been observed in goblet cells in Barrett's esophagus [44] and increases during the development of esophageal adenocarcinoma [45]. Furthermore, mRNA levels of fibroblast growth factor (FGF), another growth factor involved in microvessel formation, also increased during adenocarcinoma development [46]. COX-2 metabolites have many roles in angiogenesis. Prostaglandins E2 and I2 induce VEGF and FGF, regulate branch formation, and enhance the expression of MMPs that degrade the extracellular matrix, which promotes the growth of vessels [47].

The induction of angiogenesis is probably an relative early process in esophageal adenocarcinoma formation, since most precursor lesions are already highly vascularized. This means that reducing angiogenesis is a good approach for the prevention of adenocarcinoma development.

INCREASED PROLIFERATION

Tissue damaged by gastroesophageal reflux will start to proliferate in order to replace the damaged cells by new ones. When this proliferation gets out of control, neoplastic lesions will appear. Proliferation is mediated by three groups of proteins: extracellular growth factors, their receptors, and the proteins involved in intracellular signalling that will eventually lead to proliferation.

Tissue damage results in the induction of growth factors. These factors will bind to their receptors and transduce the growth signal to an intracellular signalling pathway that will eventually lead to proliferation. Aberrant expression of epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) has been reported in Barrett's esophagus, dysplasia and esophageal adenocarcinoma [48, 49]. Another growth factor receptor that resembles EGFR is c-ErbB-2, which is considered to be an orphan receptor since no ligand has yet been identified [50]. Aberrant c-ErbB-2 expression has been observed in approximately 10% of low-grade dysplasia, 20% of high-grade dysplasia and 30% of adenocarcinoma samples [51, 52]. These growth factors and their receptors in the damaged esophageal epithelium probably function in wound healing, but an increase in their levels could also contribute to the uncontrolled proliferation that is involved in neoplastic transformation.

Many oncogenes are responsible for the intracellular signalling that will lead to proliferation. Wnt-signalling is involved in the transcription of oncogenes such as c-myc and cyclin D1. This signalling pathway is outlined in Figure 3. The main component of the Wnt-pathway is β-catenin. Normally, β-catenin forms a complex with E-cadherin (see paragraph on cell-cell adhesion). Cytosolic β-catenin is captured in a complex with APC, axin, and other proteins.This results in the phosphorylation of β-catenin leading to its proteolytic degradation. Binding of Wnt to the frizzled receptor inhibits the complex formation, and free β-catenin is allowed

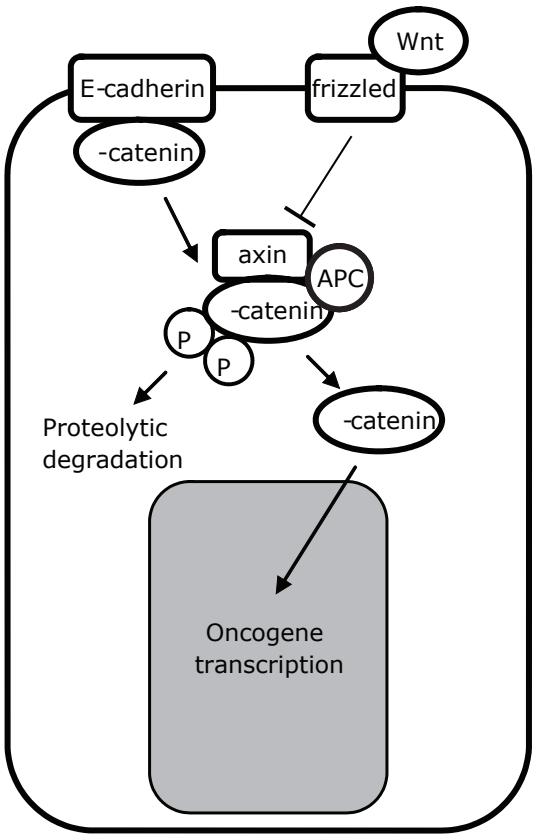

Figure 3 Schematic representation of the Wnt-pathway. Normally, β-catenin forms a complex with E-cadherin. Cytosolic β-catenin is captured in a complex with APC and axin, and other proteins. This results in phosphorylation of β-catenin leading to its proteolytic degradation. Binding of Wnt to the frizzled receptor inhibits the complex formation, and free β-catenin is allowed to enter the nucleus where it enhances transcription of several oncogenes.

to enter the nucleus where it enhances transcription of several oncogenes [53]. Defects in the proteins that form the inhibitory complex will lead to increased nuclear translocation of β-catenin.

In Barrett's esophagus and esophageal adenocarcinoma, hypermethylation of the APC promoter occurs [54]. This probably leads to decreased APC transcription. Loss of axin expression has not been observed in Barrett's esophagus and esophageal adenocarcinoma [55]. This means that hypermethylation of the APC promoter seems the most important mechanism for β-catenin induction and nuclear translocation. Indeed, nuclear translocation of β-catenin was observed in dysplastic Barrett's esophagus and adenocarcinoma [56, 57]. Increased nuclear β-catenin levels enhance expression of the c-myc oncogene. C-myc overexpression was observed in Barrett's esophagus and adenocarcinoma [58, 59], and overexpression of other genes, thereby preventing apoptosis and increasing proliferation. Clearly components of the Wnt-pathway are involved in esophageal carcinogenesis, and prevention of nuclear β-catenin translocation may form a good option in order to prevent neoplastic progression.

ESCAPE OF APOPTOSIS

Apoptosis is a mechanism of programmed cell-death that eliminates injured cells or cells that otherwise function abnormally. Regulation of apoptosis requires a delicate balance between different groups of proteins. First of all, apoptosis is accomplished through a cascade of several caspase proteins. This cascade is regulated by different stimuli, which are all activated through specific pathways. A range of specific death receptors, all having their own ligands, function to activate these caspases. Examples of these death receptors are Fas (CD95) and Fas-ligand, and TNF receptor and TNF-α [60]. Furthermore, the Bcl family of proteins play important roles in the induction and inhibition of apoptosis. Well-known members of this family are Bax (pro-apoptotic), Bcl-2 and Bcl-XL (anti-apoptotic). Bax induces mitochondrial cytochrome C release, which triggers the caspase cascade. Induction of Bax is mediated by the tumor-suppressing protein p53, whereas it is inhibited by Bcl-2 and Bcl-XL (Figure 4)[61].

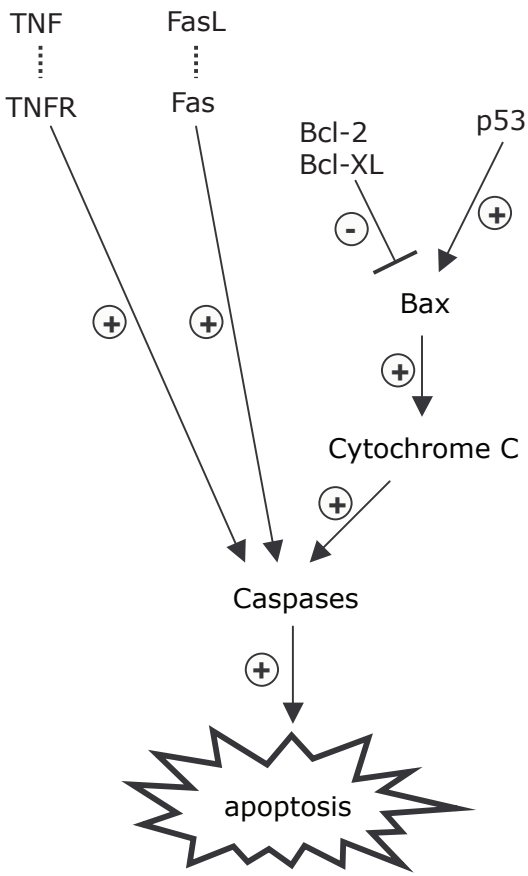

Figure 4 Signals involved in apoptosis. Binding of ligands TNF-α and FasL to their specific death receptors TNF Receptor (TNFR) and Fas, respectively, induce a caspase cascade that will drive the cell into apoptosis. This cascade is also activated by cytochrome C, which is regulated by members of the Bcl family proteins such as Bax, Bcl-2, and Bcl-XL, and p53.

Disruption of these pathways will lead to prolonged survival of damaged cells, which will lead to tumorigenesis. However, the role of apoptosis inhibition in adenocarcinoma development is pivotal. The Bax and Bcl-2 ratio is pro-apoptotic during malignant progression [41, 62], whereas Bcl-XL protein is increased along the metaplasia-dysplasia-adenocarcinoma pathway [63, 64], and thus has an anti-apoptotic effect. In contrast, Fas-ligand is overexpressed in dysplasia and adenocarcinoma, which leads to induction of apoptosis [65, 66]. However Fas-ligand could also be produced to kill lymphoid cells and thereby prevent the immune system from recognizing and eliminating the tumor cells [67]. Fas expression seems to compensate for this proapoptotic effect by showing decreased expression in dysplasia and adenocarcinoma [68, 69]. Although these data are conflicting, it was recently suggested that Barrett's esophagus is relatively resistant to apoptosis [70], which can promote the outgrowth of mutated esophageal cells.

Another way for a tumor cell to escape apoptosis is to overexpress the enzyme telomerase, which is a ribonucleoprotein that prevents telomere shortening. Telomere shortening limits the number of times that a cell can divide. Inhibition of this process extends the life-span of a cell and represses apoptosis [71]. Telomerase expression increases during the different developmental stages of esophageal adenocarcinoma [72, 73], thus preventing the cell to go into apoptosis.

LOSS OF CELL-CYCLE CONTROL

A replicating cell progresses through the cell-cycle, consisting of the G1-phase, the S-phase (DNA synthesis), the G2 phase, and the M-phase (mitosis) (Figure 5).

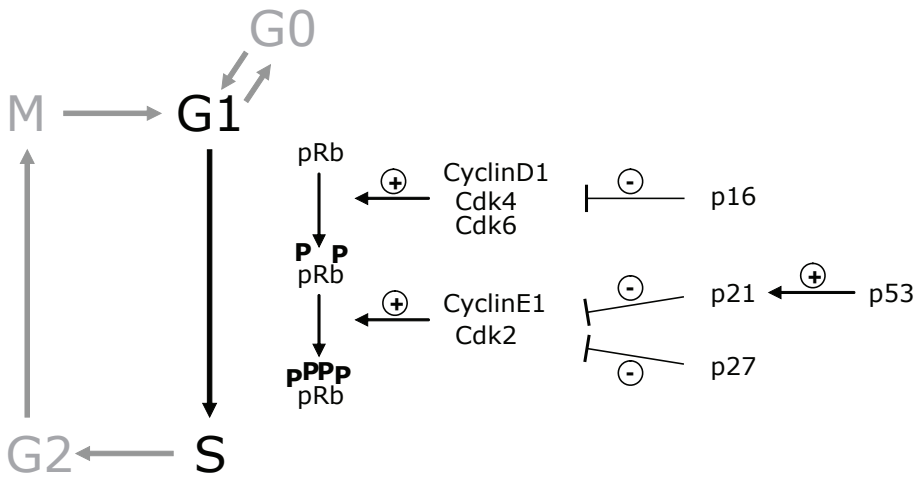

Figure 5 Schematic representation of the cell-cycle (left) and factors involved in progression of the cell-cycle to the S phase (right). One of the key regulators in this process is the Retinoblastoma protein (pRb). Complexes of cyclin D1 with CDK4/6, and Cyclin E with CDK2 phosphorylate pRb. These complexes are inhibited by p16 (CDKN2), p21, p27kip, and p53. Phosphorylated pRb enables the cell to progress to the S-phase, and is thus crucial for tumor development.

Most cells escape from the cell-cycle in the G1 phase and are in a resting phase (G0). For proliferation the cell has to progress from the G1 to the S-phase. The progression of the cell cycle is regulated by a variety of proteins, such as cyclins and cyclin dependent kinases (Figure 5). Various cyclin dependent kinases (Cdks) form complexes with cyclins. Progression to a new stage in the cell cycle requires the action of one or more cyclin-Cdk-complexes. One of the key regulators in this process is the Retinoblastoma protein (pRb). Unphosphorylated pRb binds to the E2F family of transcription factors, thereby inhibiting the transcription of genes leading to progression to the S-phase, such as cyclin A and cyclin E [74]. The cyclin D1 and CDK4/6, and cyclin E and CDK2 complexes both phosphorylate pRb. p16 (CDKN2), p21, and p27kip are small proteins that inhibit these CDK/cyclin complexes, which will decrease the pRb phosphorylation (Figure 5).

For uncontrolled proliferation to occur, it is required that the cell progresses to the S-phase, so abnormalities in proteins that control this progression are frequently observed in carcinogenesis. Accumulation of mutant pRb during the development of adenocarcinoma has been reported [75], there is however no information on the exact pRb phosphorylation state during malignant progression of Barrett's esophagus. There may however be an increased proliferation state in Barrett's esophagus, as increased cyclin D1 expression has been reported in Barrett's esophagus and adenocarcinoma [76]. Furthermore, inactivating alterations in the inhibitors of pRb phosphorylation, p16, $p27^{kip}$, and p21 will lead to increased pRb phosphorylation and progression of the cell cycle. Inhibition of p16 by promoter hypermethylation was a frequently occurring event [77, 78], and cytoplasmic staining of abnormal $p27^{kip}$ was observed in adenocarcinoma, while in non-dysplastic Barrett's esophagus and in dysplasia staining was predominantly nuclear [79]. Furthermore, in $p27^{kip}$ knockout mice that underwent gastrectojejunostomy to induce columnar-like epithelium in the esophagus, adenocarcinoma was observed more frequently than in wild-type mice [80].

The extensively studied tumor-suppressor gene p53 is involved in the inhibition of many tumor-promoting processes, such as DNA repair and apoptosis, and is sometimes called "the guardian of the genome". p53 is an activator of p21 and thus inhibiting pRb phosphorylation (Figure 5). Accumulation of mutant p53 protein occurs frequently in high-grade dysplasia and adenocarcinoma, but also in non-dysplastic Barrett's esophagus and low-grade dysplasia, although less frequently [81-83]. Several reports suggest that p53 is actively involved in the progression from dysplasia to esophageal adenocarcinoma [81, 84]. Interestingly, while p53 is involved in the transactivation of p21, p21 itself seems to be upregulated in a p53-independent manner in Barrett's esophagus and esophageal adenocarcinoma [85, 86]. These alterations in this intricate network of interacting factors suggest that during esophageal adenocarcinoma development, cells progressively lose cell cycle control. However, it is still unclear which factors initiate this process.

LOSS OF CELL-CELL ADHESION

Inflammatory cells need to penetrate into damaged tissue in order to allow them to contact and eliminate their targets. This means the tightly associated epithelial cells should be loosened to allow passage of the inflammatory cells. However, intimate cell-cell contact also plays an important role in preventing the development of malignancies, as contact with a neighbouring cell also serves to inhibit proliferation. When cell-cell interactions are reduced, this contact inhibition will decrease and the cell is allowed to proliferate in an uncontrolled fashion. Moreover, when the cells are relatively loosely attached to each other, it is easier for tumor mass to invade neighbouring tissues, and for tumor cells to metastasize via lymph or blood vessels.

The main proteins involved in cell-cell adhesion are E-cadherin and β-catenin [87]. These proteins form a complex in which in which β-catenin attaches to the cytoskeleton, and E-cadherin is located on the cell-surface so that it can bind to the E-cadherin molecules on the surface of neighbouring cells. The pro-inflammatory cytokine TNF-α is produced by inflammatory cells and mediates phosphorylation of the β-catenin-E-cadherin complex. These phosphorylated complexes have a decreased affinity for E-cadherin molecules on other cells and phosphorylation enhances its degradation [88]. In Barrett's esophagus, E-cadherin levels decrease along the metaplasia-dysplasia-carcinoma sequence [56, 57, 89]. Moreover, increased phosphorylation of β-catenin will result in higher nuclear levels of this protein, which induces the wnt-signalling pathway, thus enhancing proliferation rates (see paragraph on increased proliferation).

Other proteins that promote cellular detachment are the matrix metalloproteinases (MMPs). These are enzymes that degrade the extracellular matrix, thereby allowing for the influx of immune cells, but also enhancing the migration and metastasis of tumor cells [90]. MMPs are induced by prostaglandin E2, a compound that is also induced by inflammation (Figure 2). Increased MMP-7 and MMP-12 expression is associated with Barrett's esophagus, whereas increased MMP-1, MMP-2, and MMP-9 expression with esophageal adenocarcinoma [91, 92].

In a Barrett's adenocarcinoma metastasis is a frequently occuring and early process. It is suggested that the chronic inflammation present in Barrett's esophagus influences this process, thus any therapeutic option that would decrease the ongoing inflammation might prove beneficial in the prevention of esophageal adenocarcinoma.

CLINICAL IMPLICATIONS

Understanding the molecular alterations that occur during development of esophageal adenocarcinoma will provide new insights for the diagnosis, prevention and treatment of this neoplasm. Various molecular alterations that mark the presence of dysplasia or adenocarcinoma have been suggested as molecular markers for the early detection of these lesions. However, p53 accumulation is until now

the only alteration that has been associated with progression to a more malignant phenotype in the sequence Barrett's esophagus, dysplasia and finally adenocarcinoma [93].

The standard therapy for the prevention of adenocarcinoma development in Barrett's esophagus is treatment with proton-pump inhibitors, which mediates acid suppression. In biopsies of Barrett's esophagus, proliferation was reduced, while differentiation was induced after treatment with a proton pump inhibitor [94]. However, it is still debatable if acid suppression alone can prevent the development of adenocarcinoma, since bile is also an important cause of reflux-induced damage to the esophagus [95].

Commonly used drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), aspirin and COX-2 inhibitors have been suggested to prevent the development of esophageal adenocarcinoma [9], but large-scale clinical trials have not yet been performed. NSAIDs, aspirin, and COX-2 inhibitors are involved in the reduction of inflammation. They suppress NF-κB activation, thereby inhibiting the expression of NF-κB regulated cytokines and cyclin D1 [93]. COX-2 is also involved in several oncogenic processes. COX-2-inhibitors reduced proliferation in Barrett's esophagus [96, 97], decrease microvessel formation [98], and inhibit the nuclear translocation of β-catenin thereby suppressing the transcription of several oncogenes [53]. Therefore, COX-2 inhibitors have been suggested to be the most promising chemopreventive agents for the prevention of esophageal adenocarcinoma development.

Currently, several new agents for the treatment of esophageal cancer are under development. Iressa is a potent inhibitor of EGFR that shows promising results in the treatment of small-cell lung cancer [99]. Furthermore, the telomerase inhibitor PPA has been demonstrated to induce growth arrest and apoptosis in an esophageal adenocarcinoma cell line [100]. These inhibitors can provide new therapeutic options for the treatment of esophageal adenocarcinoma.

CONCLUSIONS

The accessability of esophageal tumors by endoscopy makes it relatively easy to study the molecular changes that occur during carcinogenesis. However, the lack of well-described *in vitro* or animal models for esophageal adenocarcinoma complicates the conduction of functional studies. Many of the molecular alterations have been described, but they have not been connected to a functional sequence in which they occur, and it is still not clear which alteration at what time will initiate tumogenesis. Most likely, various molecular changes occurring at the same time eventually determine whether adenocarcinoma will occur. The abnormalities that are identified fit in the well-known models of carcinogenesis, describing increased proliferation, loss of cell cycle control, escape of apoptosis, loss of cell-cell adhesion, and angiogenesis as the main processes that will lead to tumor formation [43]. However, excessive activation of the immune system also plays an important role in carcinogenesis. The chronic inflammation present in Barrett's esophagus,

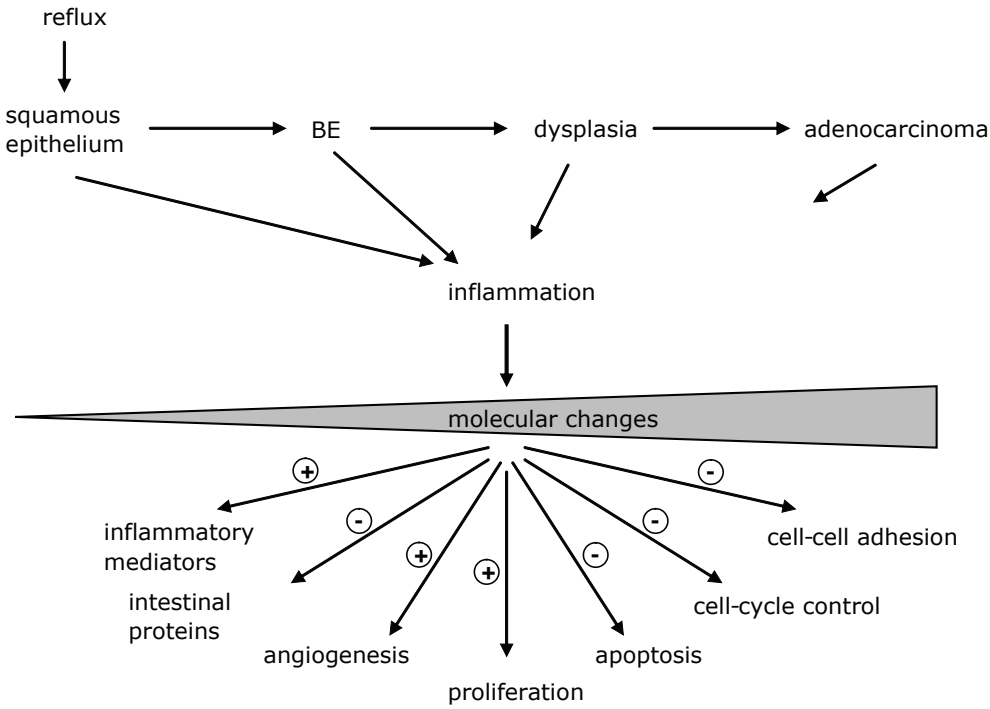

Figure 6 The effect of inflammation on molecular changes in esophageal adenocarcinoma development. Under influence of gastroesophageal reflux the normal squamous epithelium is damaged and replaced by columnar epithelium of the intestinal type, which is called Barrett's esophagus. The damaged cells secrete inflammatory mediators such als cytokines and chemokines that will attract inflammatory cells. These cells produce reactive oxygen species that can cause DNA damage. This DNA damage leads to molecular alterations and the tissue can progress to dysplasia and esophageal adenocarcinoma. These molecular alterations are involved in inflammation, intestinal protein expression, angiogenesis, proliferation, apoptosis, cell-cycle control, and cell-cell adhesion.

which is already metaplastic tissue, can form the trigger for further damage leading to the development of dysplasia. As severity of dysplasia progresses, the damaging effect of inflammation will be less inportant, because the molecular alterations present at that stage will cause further changes, that eventually will result in the development of esophageal adenocarcinoma (Figure 6).

The understanding of the molecular processes underlying the development of esophageal adenocarcinoma will provide markers for the detection of early malignant lesions, such as high-grade dysplasia. New molecular techniques, such as microarray analysis will allow the identification of abnormal gene expression for many genes at once. Furthermore, with the elucidation of molecular changes during esophageal carcinogenesis, new targets for anti-tumor therapy have been identified. This will lead to the development of new therapeutic possibilities that will increase the survival of patients with esophageal adenocarcinoma.

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Chapter 3

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

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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 CDX2 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 ($p<0.001$). CDX2 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 mRNA are strongly associated with the presence of SIE in the esophagus. As CDX2 mRNA was also present in one-third of BE patients with endoscopically normal appearing squamous epithelium, expression of CDX2 may precede morphological changes observed in BE. Therefore, pathways involved in the induction of CDX2 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.

Table 1. Patient characteristics

Patient characteristics		patients	biopsies	age mean (SD)	male %
First group					
IHC[a]	Adenocarcinoma	4	19	78.5 (2.7)	75%
	Barrett's esophagus	16	79	70.8 (14)	66%
	Reflux esophagitis	20	40	61.8 (11.6)	71%
Second group					
RT-PCR[a]	Adenocarcinoma	6	12	68.9(11.5)	57%
	Barrett's esophagus	19	76	65.1(15.1)	55%

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

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 CDX2 mRNA. The study was approved by the Central Committee on Research Involving Human Subjects in The Netherlands in 2002.

Histological analyses

Sections from the biopsies and part of the biopsies taken for RNA analysis, were stained with haematoxylin 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 streptavidin-alkaline phosphatase complex (DAKO, Glostrup, Denmark). A red color was developed using new-fuchsin 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 CDX2 5'-CCCAGCGGCCAGCGGCGAAACCTGT / 5'-TATTTGTCTTTTGTCCTGGTTTTCA and MUC2 5'-CAGGATGGCGCCTTCTGCTA / 5'-ATGCTGCTCCAAGCTGAGGT. Levels of CDX2 and MUC2 mRNA were standardized to levels of β-actin using the primers β-actin 5'-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 four 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 four BE patients showing a grade 1, seven BE patients a grade 2, and five 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

Table 2. Histological classification of inflammation

Reflux esophagitis		Barrett's esophagus		
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]

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.

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).

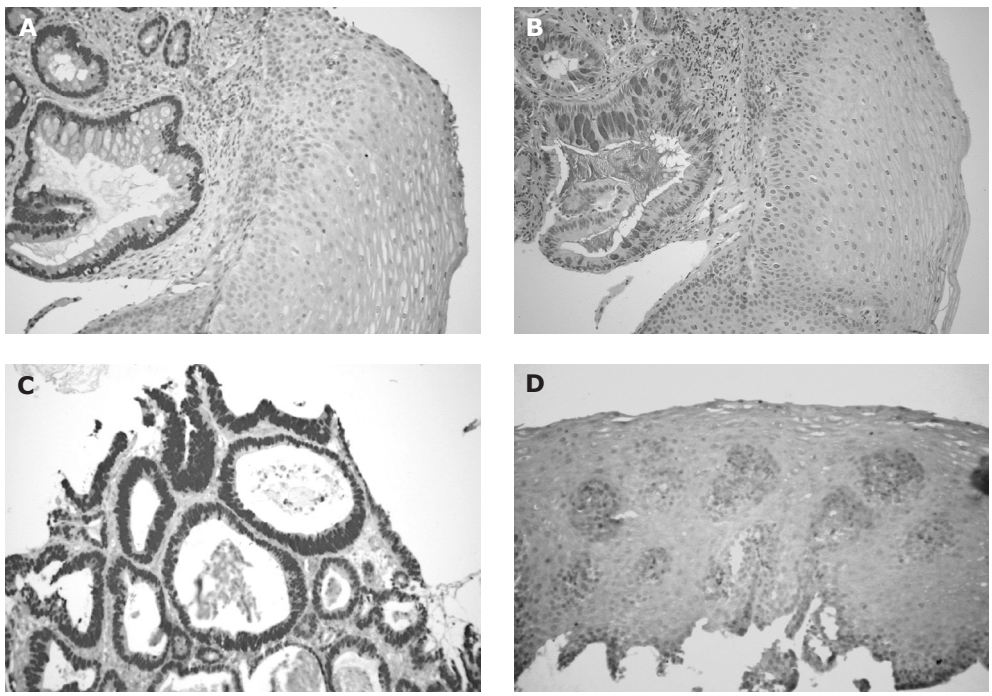

Figure 1 CDX2 protein in Barrett's esophagus, esophageal adenocarcinoma, and reflux esophagitis. (A) A positive nuclear stain (red) for CDX2 was observed in 16/16 samples of Barrett's esophagus. A representative slide of patient 10 is shown. (B) The presence of CDX2 was associated with goblet cells, 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). A color version of this figure is printed on page 113.

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 patients, the presence of SIE was confirmed in the biopsies taken adjacent to those used in the transcriptional analysis. CDX2 mRNA was detected in 13/19 BE segments and in 4/6 esophageal adenocarcinomas (Figure 2b). The levels of CDX2 mRNA did not significantly differ between BE and esophageal adenocarcinoma (p=0.9) (Figure 2b).

In order to determine if expression of CDX2 precedes the morphological changes

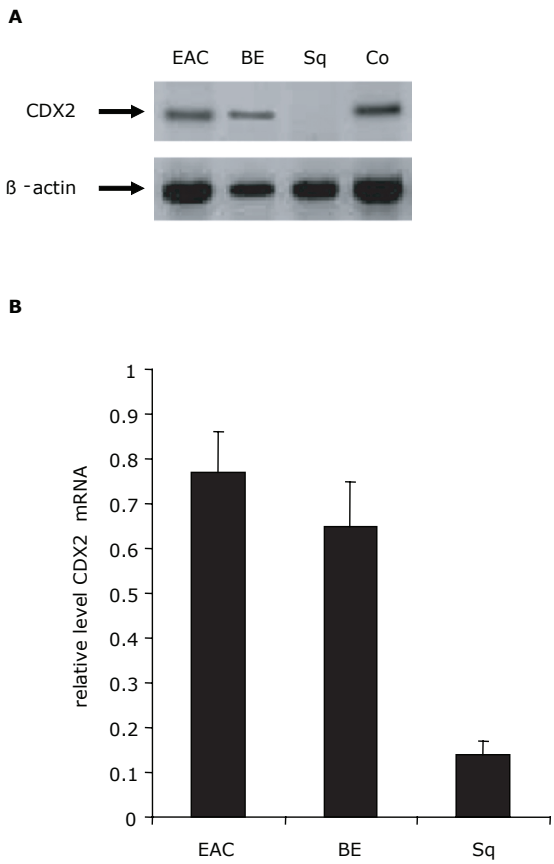

Figure 2 CDX2 mRNA in Barrett's esophagus, squamous epithelium and esophageal adenocarcinoma. (A) CDX2 mRNA was detected by RT-PCR (95 bp fragment) in 4/6 esophageal adenocarcinoma (ADC) samples, in 13/19 Barrett's esophagus (BE) samples, in 6/19 squamous epithelium (Sq) samples and in colon control tissue (Co). (B) Relative mRNA levels of CDX2 in squamous epithelium, BE and esophageal adenocarcinoma. Levels of CDX2 mRNA were normalized against β-actin. The mean CDX2 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 CDX2 did not differ significantly between BE (n=13) and esophageal adenocarcinoma (n=4) (NS: p<0.1).

seen in BE, levels of CDX2 mRNA were also determined in squamous epithelium biopsies obtained five cm above the neosquamous-columnar junction of patients with BE (Figure 3). Low levels of CDX2 mRNA were present in 6/19 (32%) samples of squamous epithelium (Figure 3). The relative levels of CDX2 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 MUC2 mRNA. CDX2 mRNA was

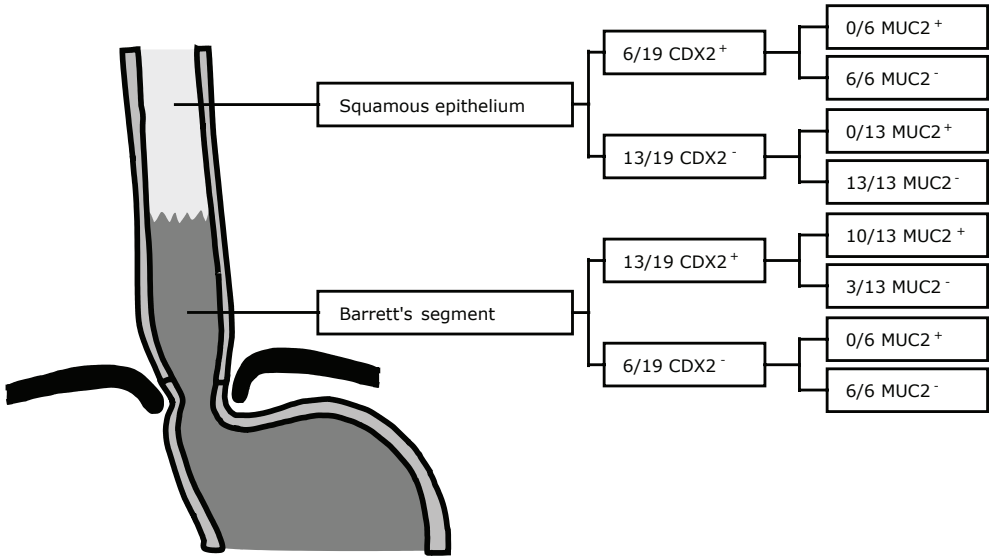

Figure 3 Schematic overview of the distal esophagus, summarizing the transcription of CDX2 mRNA and goblet cell-specific MUC2 mRNA in biopsies taken from the Barrett's segment and the squamous epithelium of patients with Barrett's esophagus.

present in all MUC2 positive samples. Furthermore, samples without the presence of CDX2 mRNA did not have MUC2 transcription. In only three BE samples with CDX2 transcription, MUC2 mRNA was absent (Figure 3). MUC2 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 CDX2 mRNA were however detected in approximately one third of the squamous epithelium samples of patients with BE. The presence of CDX2 mRNA also correlated with goblet cell specific MUC2 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, 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 CDX2 mRNA was determined showed SIE, CDX2 mRNA was not detected in 6/19 (32%) BE segments. In the biopsies taken from these segments, MUC2 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 CDX2 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, CDX2 mRNA levels were also determined in reflux-exposed squamous epithelium of patients with BE. Low levels of CDX2 mRNA were indeed observed in 6/19 (32%) of the squamous epithelium samples tested (Figure 3). In addition, transcription of MUC2 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-columnar junction 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 CDX2 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], CDX2 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 duodenogastro-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 4

CDX2 expression in columnar metaplasia of the remnant esophagus in patients who underwent esophagectomy

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submitted

ABSTRACT

Background Patients who have undergone esophagectomy with gastric tube reconstruction often have complaints of gastroesophageal reflux. A subset of these patients will develop columnar epithelium in the remnant esophagus, which can be of the gastric or intestinal type (Barrett's esophagus).
Aim To determine whether gastric-type mucosa in the esophagus is a precursor stage of intestinal metaplasia.
Patients and Methods The medical records of 613 patients having undergone esophagectomy were reviewed for the endoscopic presence of segments with columnar mucosa in the remnant esophagus. Of them, 45 patients underwent endoscopic follow-up. The presence of intestinal metaplasia was determined histologically by haematoxylin and eosin, alcian blue, and periodic acid-Schiff staining in archival biopsy samples. Intestinal characteristics were identified by immunohistochemical staining for CDX2, MUC2 and cytokeratins 7 and 20. CDX2 transcription was assessed by RT-PCR.
Results Eighteen of 45 patients (40%) had histologically proven columnar epithelium in the remnant esophagus, and were included in this study. Histological analysis revealed gastric-type mucosa in all samples. Eight samples with gastric-type mucosa also had foci of intestinal metaplasia. CDX2 and MUC2 expression was observed in the regions with intestinal metaplasia, and in two patients, CDX2 expression was observed in gastric-type glands at a distance of intestinal glands. CDX2 transcription was identified in two patients without intestinal metaplasia. Diffuse cytokeratin 7 and superficial cytokeratin 20 expression was identified in both gastric-type mucosa and intestinal metaplasia.
Conclusion In the majority of patients, expression of CDX2 and MUC2 was only detectable in intestinal metaplasia, but CDX2 was also observed in four cases in gastric-type mucosa. This could indicate that part of the gastric-type mucosa and the intestinal metaplasia may share a common pathway, eventually leading to the development of specialized intestinal epithelium.

INTRODUCTION

Barrett's esophagus (BE) is a chronic inflammatory condition in which the normal squamous epithelium of the lower oesophagus is replaced by specialized columnar epithelium of the intestinal type. This condition is most likely caused by gastro-esophageal reflux [1]. Patients with BE have an increased risk of developing esophageal adenocarcinoma.

One of the histologic characteristics of BE is the presence of goblet cells. In addition to intestinal metaplasia (IM), gastric-type mucosa (GM) is also frequently observed in the distal esophagus, but this type of epithelium is probably not associated with an increased risk of esophageal adenocarcinoma [2]. It has been hypothesized however that IM could evolve from this gastric-type epithelium [3].

The presence of IM is associated with the expression of intestinal proteins, such as CDX2, MUC2, and a specific expression pattern of cytokeratins (CK), i.e. CK7 and CK20 [4-6]. CDX2 is a transcription factor involved in intestinal differentiation [7, 8], and overexpression leads to the formation of IM in the stomach of mice [9]. Since low mRNA levels have been observed in the esophageal squamous epithelium of patients with BE [10], CDX2 may be responsible for skewing esophageal epithelial differentiation towards an intestinal phenotype and thus have an important role in intestinal metaplasia development. The exact pathogenetic pathway of the development of columnar epithelium and IM in the esophagus has not yet been identified. It is generally accepted that components of gastroesophageal reflux - mainly acid, bile and pancreatic juice - are the cause of this condition. The role of CDX2 in this process is still under investigation, but it has been observed that CDX2 is upregulated in response to acid in an in vitro model [11], which implicates that components of gastroesophageal reflux can induce intestinal differentiation in the exposed epithelium.

Patients who underwent esophagectomy with gastric tube reconstruction are prone to develop reflux in the remnant esophagus, as the previous protection by the lower esophageal sphincter is no longer present. Prospective studies have shown that 50-75% of these patients will develop a segment of columnar epithelium in the remnant esophagus and that in 25% of these segments IM is present [12-14]. These patients after esophagectomy and gastric tube reconstruction are therefore a human in vivo model to study the development of BE, as the onset of esophageal damage and the progression to IM can be closely monitored.

The aim of this study was to determine whether GM and IM are different entities or consecutive stages of metaplastic progression. Therefore, we investigated if early intestinal differentiation, by means of intestinal marker expression, was present in GM. If present, this could indicate that GM is indeed a precursor lesion for IM in the esophagus.

PATIENTS AND METHODS

Patients and material

The medical records of 613 patients who underwent esophagogastroscopy after esophagectomy with gastric tube reconstruction between 1994 and 2004 in our centre were examined. For resection all anastomoses between the remnant esophagus and the gastric tube were created monolayer handmade with absorbable suture and situated in the neck. Fourty-five patients (7.3%) received endoscopic follow-up a half year or longer after esophageal resection. Eightteen of these patients (40%) were identified with histologically proven columnar epithelium in the remnant esophagus. At the time of endoscopy, which was conducted after a mean of 58.7 months, there were no signs of recurrent high-grade dysplasia, adenocarcinoma or squamous cell carcinoma. Furthermore, dysplasia or carcinoma was not present in these segments. Archival paraffin embedded biopsies were available for all eightteen patients. For five patients, follow-up archival biopsies were obtained. This means that a total of 23 biopsies was analyzed histologically. For three patients frozen biopsies were available for RNA analysis.

Histology and immunohistochemistry

Ten subsequent sections from one sample were mounted on adhesive slides, dried overnight at 37°C, and deparaffinized with xylene. One of serially sectioned slides (4 μm) was stained with haematoxylin and eosin (H&E) and evaluated for the presence of GM and/or IM. Alcian Blue (AB) and periodic acid-Schiff (PAS) staining in consecutive slides was performed to facilitate the detection of mucin-producing goblet cells. All slides were evaluated by a specialized GI pathologist who was blinded for the origin of the samples. For immunohistochemistry, antigen retrieval was performed by boiling the deparaffinized samples in a 10 mM monocitric acid buffer (pH 6.0) in a microwave for 15 min, and slowly cooling down to room temperature (CDX2, MUC2, and cytokeratin 7) or by treating the slides 10 minutes with 0.1% pronase and 10 minutes with cold TBS buffer (cytokeratin 20). The samples were incubated in TBS buffer containing 10% rabbit non-immune serum (DAKO, Glostrup, Denmark), and 10% normal human plasma (DAKO) for 30 minutes. Primary antibodies used were anti-CDX2 (clone 392M, Biogenex, San Ramon, CA, USA) in a 1:100 dilution, anti-MUC2 (clone Ccp58, Novocastra, Newcastle upon Tyne, UK) in a 1:100 dilution, anti-cytokeratin 7 (clone ov-TL 12/30, Biogenex) in a 1:50 dilution, and anti-cytokeratin 20 (clone KS 20-8, DAKO) in a 1:160 dilution. Detection was performed with a biotin-labeled rabbit anti-mouse antibody (DAKO), followed by the addition of a streptavidine-alkaline phosphatase complex (DAKO) using new fuchsine as substrate.

CDX2 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). Reverse transcription (RT)-PCR was performed using intron-spanning primers for CDX2 5'-CCCAGCGGCCA-

GCGGCGAAACCTGT / 5'-TATTTGTCTTTTGTCCTGGTTTTCA and housekeeping gene β-actin –5'-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 through 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 GoTaq Taq polymerase (Promega). PCR conditions were 35 cycles at 94°C (30 s); 55°C (30 s) and 72°C (1 min). Southern blotting confirmed the identity of the CDX2 PCR products.

RESULTS

Histological analysis for columnar epithelium

To identify regions with GM and IM, subsequent H&E, AB, and PAS stained slides were evaluated. GM was observed in all biopsy samples. Focal IM as characterized by the presence of goblet cells, was observed within the regions of GM in eight samples of seven patients (Table 1). The indication for resection in the 18 patients with a newly developed segment of columnar epithelium was high-grade dysplasia within BE (n=4) esophageal adenocarcinoma within BE (n=6), adenocarcinoma of the gastro-esophageal junction without visible Barrett's epithelium (n=4), squamous cell carcinoma (n=3), and congenital atresia (n=1). Incidence rates of GM and IM were not significantly different between these groups.

Table 1. Patient characteristics

Indication for resection *	n	columnar epithelium	available biopsies	biopsies with GM	biopsies with IM**	Mean time after resection (months)***
BE + HGD	20	4 (20%)	5	5/5	1/5	50.0 (10-81)
BE + EADC	139	6 (4%)	7	7/7	2/7	38.2 (6-118)
EADC	223	4(2%)	4	4/4	2/4	80.5 (31-108)
SCC	184	3 (2%)	5	5/5	2/5	52.3 (11-132)
other	47	1 (1%)	2	2/2	1/2	148
total	613	18 (3%)	23	23/23	8/23	58.7 (6-148)

*Indication for resection: BE: Barrett's esophagus, HGD: high-grade dysplasia, EADC: esophageal adenocarcinoma, SCC: squamous cell carcinoma, other: other indications, such as strictures or perforations
**IM was observed within regions of GM
***Mean interval between resection and the detection of columnar epithelium in the remnant esophagus after resection

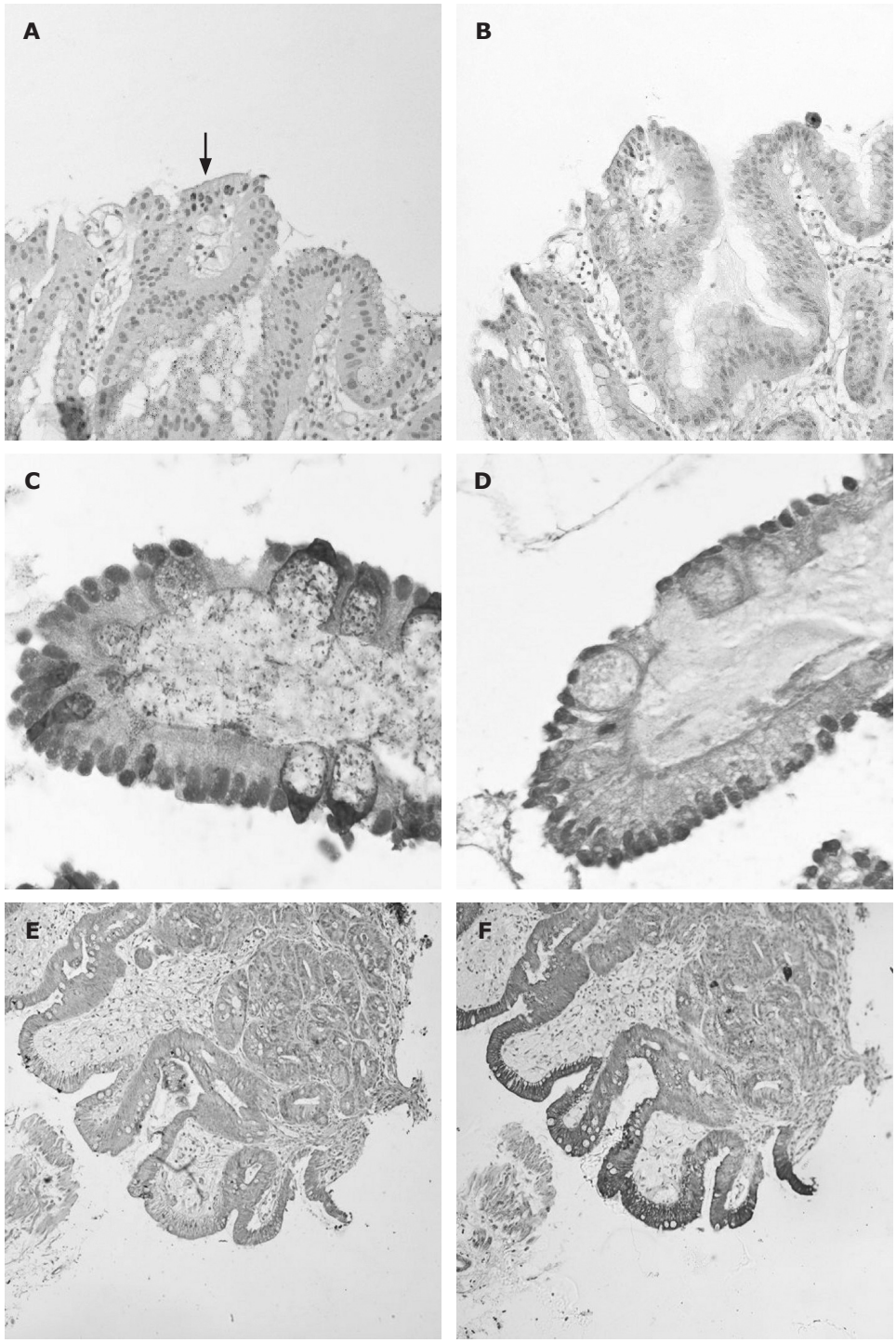
A
B
C
D
E
F

Table 2. Immunohistochemistry results

protein	GM	IM
CDX2	2/23	8/8
MUC2	0/23	8/8
Cytokeratin 7	23/23	8/8
Cytokeratin 20	23/23	8/8

Intestinal marker expression

Expression of the intestinal proteins CDX2 and MUC2 was evaluated by immunohistochemistry. Nuclear CDX2 expression was observed in all 8 samples with IM. In 6/8 biopsies, expression was only observed in regions with IM, but in two patients expression was also present both in IM and in GM at a distance of a region of IM (Figure 1a-b). These gastric glands were of the cardia-type. MUC2 expression was not detected in these latter glands. Both these patients underwent a resection for esophageal adenocarcinoma within Barrett's esophagus. MUC2 expression was observed in the cytoplasm of goblet cells of all patients with IM (Figure 1c-d). Co-localization of MUC2 expression and CDX2 expression was observed in all MUC2 positive samples.

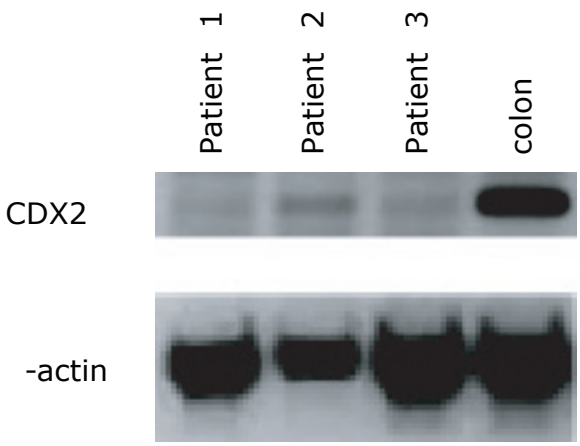

Figure 2 RT-PCR analysis of CDX2 and housekeeping gene β-actin transcription in three biopsies from the newly formed columnar segment of patients that underwent esophagectomy. Histological analysis of adjacent biopsy samples revealed IM in patient 2 and only GM in patient 1 and 3. Colon was used as a positive control.

Figure 1 Histological and immunohistochemical detection of intestinal characteristics. **A** Nuclear CDX2 expression in gastric-type glands at a distance of IM (arrow)(200x magnification). **B** The same region. Alcian Blue staining in adjacent gastric-type glands does not detect goblet cells (200x magnification). **C** cytoplasmic MUC2 staining in goblet cells (400x magnification). **D** Haematoxylin and Eosin staining in the same region (400x magnification). **E** Diffuse cytokeratin 7 staining in IM (100x magnification). **F** Superficial cytokeratin 20 staining in the same region (100 x magnification). A color version of this figure is printed on page 114.

Cytokeratin 7/20 pattern
Cytokeratin staining was evaluated as described by Ormsby et al [15]. A moderate to strong diffuse cytokeratin 7 cytoplasmic staining and strong superficial cytokeratin 20 cytoplasmic staining was considered as a "Barrett-like" staining pattern. This staining pattern was however observed in all samples, i.e. in GM as well as in IM (Figure 1e-f).

CDX2 transcription
Frozen biopsy samples from three patients were available for RNA analysis. In the biopsy samples of all three patients, CDX2 mRNA was present, albeit in low amounts (Figure 2). Patient 2 had histologically proven IM, and patient 1 and 3 only had GM. β-actin transcription was indicative for the amount of input RNA.

DISCUSSION

In the present study IM was observed in 7/18 patients who had developed a segment of columnar epithelium in the remnant esophagus after esophagectomy with gastric tube reconstruction. In all seven patients CDX2 expression was observed. CDX2 expression was also observed in gastric-type glands at a distance of IM (Figure 1a). Furthermore, CDX2 transcription was observed in two samples without IM (Figure 2). We therefore propose that the expression of CDX2 in gastric-type glands could be indicative of early intestinal differentiation in columnar epithelium and that GM is related to IM. In a few repeated prospective studies, the development of IM from GM has been reported [14, 16].

It is hypothesized that Barrett's esophagus arises from a pluripotent stem cell, that is normally capable of both gastric and intestinal differentiation [17] as well as differentiation into squamous epithelium [18]. In the esophagus, these cells will normally differentiate into squamous epithelium, but under influence of reflux-induced chronic inflammation this differentiation may be directed towards formation of columnar epithelium [19]. It could well be that these stem cells initially will differentiate into GM, but a stimulus such as acid or inflammatory mediators is able to induce the expression of CDX2 in this epithelium, which will also lead to the expression of other intestinal proteins, leading to an intestinal phenotype.

MUC2 expression was only observed in goblet cells in IM, and not in the columnar cells of GM. MUC2 is a CDX2-regulated mucin [20], but co-localization with CDX2 was only present in IM and not in adjacent GM. This indicates that despite its CDX2 regulation, MUC2 seems not to be involved in early differentiation. PAS staining was observed throughout the biopsy samples and this was only indicative for the presence of goblet cells. Alcian Blue staining was also observed in goblet cells and in some samples in other mucin producing columnar cells. However, it has been reported previously that these alcian blue positive columnar non-goblet cells are not indicative for the presence of intestinal differentiation [21].

The cytokeratin pattern of GM and IM was also determined in this study. The

expression of cytokeratins 7 and 20 has been reported to provide a pattern that is unique for BE, and therefore could be able to distinguish BE from GM and from IM of the cardia [15, 22]. The present study, but also other studies [23], did not confirm this observation as similar cytokeratin 7/20 patterns were observed in both GM and IM.

Until now there has been only a single study that has reported on the expression of markers in IM of the remnant esophagus, i.e. cyclooxygenase 2 and binding of the antibody DAS-1 [24]. The current study is the first to report for CDX2 expression in the newly formed IM in the remnant esophagus.

It has been reported that 50-75% of patients who have undergone esophagectomy with gastric tube reconstruction will develop a segment of columnar epithelium in their esophageal remnant after a mean of 38-61 months after esophagectomy [12-14]. However, these studies were performed on selected patient groups. In retrospective studies with an unselected population, an incidence of 10% has been reported [24, 25]. Our frequency of columnar epithelium was 40% in patients that had endoscopic follow-up. We do not routinely perform follow-up upper GI endoscopies in patients who have undergone esophageal resection. In this study, only 7.3% of esophagectomy patients underwent gastroscopy after resection, but less than half of the patients survived longer than two years after esophagectomy and thus only in a small selection of patients newly formed columnar epithelium can be detected. Patients who were diagnosed with newly formed columnar epithelium underwent upper GI endoscopy for other indications, mostly dilation of strictures or suspicion for recurrent carcinoma, which was however not present in any of these patients.

GM and IM were not only observed in patients who underwent esophageal resection for adenocarcinoma, but also in patients who had had squamous cell carcinoma. This is not new as others also reported this finding [12, 13, 24]. It does however indicate that BE is more likely an acquired, i.e. induced, than an inherited, i.e. preexistent, condition.

In conclusion, CDX2 expression is present in IM, and in four patients, CDX2 was observed in GM at a distance of IM or without IM. This study was performed in a relatively small group of patients that developed a segment of columnar epithelium in the remnant esophagus, but nevertheless, it provides evidence that GM and IM share a common pathway, eventually leading to the development an intestinal phenotype.

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Chapter 5

CDX2 is an early marker of epithelial intestinal differentation in columnar epithelium of the esophagus

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submitted

ABSTRACT

Barrett's esophagus is defined as the presence of columnar epithelium with intestinal metaplasia in the esophagus. However, non-metaplastic gastric-type mucosa, which can be of the fundic or cardia-type, is also frequently present in the esophagus. The aim of this study was to investigate whether CDX2, an early marker for intestinal differentiation, was present in gastric-type mucosa in patients with endoscopic Barrett's esophagus and to characterize the expression of CDX2 regulated MUC2 in this epithelium. Biopsies taken from columnar epithelium of the distal esophagus were collected from 61 patients at two different endoscopies. Haematoxylin and eosin, alcian blue and periodic acid-Schiff staining was performed to detect the presence of goblet cells, which are specific for intestinal metaplasia. CDX2 and MUC2 expression was determined by immunohistochemistry. Intestinal metaplasia was present in 55/122 samples. In all 55 samples with intestinal metaplasia CDX2 and MUC2 expression was detected. Furthermore, CDX2 expression was also observed in gastric-type glands adjacent to or at distance of intestinal metaplasia, which were all of the cardia-type. In addition, CDX2 was detected in cardia-type mucosa in 23/67 samples without intestinal metaplasia. In thirteen of these biopsies MUC2 positive goblet-like cells that were negative for alcian blue were detected. In conclusion, it was shown that in addition to intestinal metaplasia and cardia-type mucosa, a hybrid of these two epithelium types that has several intestinal characteristics but does not contain fully differentiated goblet cells is present. These results implicate that intestinal metaplasia and cardia-type mucosa in the esophagus are related to each other.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition that is characterized by the replacement of the normal squamous epithelium of the esophagus by columnar epithelium of the intestinal type, intestinal metaplasia (IM). Gastroesophageal reflux is generally accepted as the major causative agent of BE [1]. BE can progress to esophageal adenocarcinoma via intermediate stages low-grade dysplasia and high-grade dysplasia [2].

In addition to IM, gastric-type mucosa (GM) is also frequently observed in the columnar lined esophagus [3], however only IM is supposed to be associated with adenocarcinoma development [4]. GM can be subdivided in fundic-type mucosa and cardia-type mucosa (CM). The distribution of IM in the columnar segment is usually patchy, but it is mainly localized at the proximal end of the columnar segment [5]. Due to sampling error, IM can be missed and patients with only patchy IM could falsely be excluded from surveillance programs [6]. Several authors have suggested IM and GM are related to each other [7, 8], however, at present there is no biological evidence that supports this theory.

The homeobox protein CDX2 is a transcription factor involved in intestinal differentiation [9]. It regulates transcription of several intestinal proteins, such as MUC2 and sucrase-isomaltase [10, 11]. CDX2 is involved in the development of IM, since CDX2 transgenic mice have developed IM in the gastric antrum [12]. CDX2 expression has also been observed in BE [13, 14], and CDX2 mRNA has been detected in the squamous epithelium of patients with BE [15]. This indicates that CDX2 is also involved in intestinal differentiation in Barrett's esophagus. It has been reported that CDX2 is also present in GM outside regions with in the esophagus [13]. However it is not clear if this epithelium has other intestinal characteristics.

The aim of this study is to determine if early intestinal differentiation in GM, by means of CDX2 expression, is present in a population of patients with endoscopic BE in which IM had not been observed at all endoscopies, and if present, to characterize other phenotypic characteristics of this epithelium.

PATIENTS AND METHODS

Patients and material

Sixty-one patients that had had at least two follow-up endoscopies with biopsies were selected for this study. The biopsies were selected for i) the presence of IM in both biopsies, ii) the presence of IM in one biopsy sample and only GM in the other, and iii) the presence of only GM in both biopsies, based on the pathology reports. From all patients, archival paraffin embedded biopsy sets were available. Based on the presence of IM, the patients were divided into three groups (Table 1): group I: patients with IM in biopsy sets from both endoscopies, group II: patients with IM in biopsy sets from one endoscopy, but with no IM in biopsy sets from the other endoscopy, and group III: patients with no IM in biopsy sets from

the first and the second endoscopy. This study was approved by the local ethical review board and informed consent was obtained prior to endoscopy.

Histology and immunohistochemistry

Four subsequent sections (4 μm) from one sample were mounted on adhesive slides, dried overnight at 37°C, and deparaffinized with xylene. One of these serially sectioned slides was stained with haematoxylin and eosin (H&E) to evaluate for the presence of GM or IM. Alcian Blue and periodic acid-Schiff (PAS) stainings in consecutive slides were performed to facilitate detection of mucin producing goblet cells. All slides were evaluated by a specialized GI pathologist. For immunohistochemistry, antigen retrieval was performed by boiling the deparaffinized samples in a 10 mM monocitric acid buffer (pH 6.0) for 15 min, and slowly cooling down to room temperature. Endogenous peroxidase activity was inhibited by using a 0.5% solution of H_2O_2 in phosphate-buffered citric acid for 15 minutes. The samples were incubated in TBS buffer containing 10% rabbit non-immune serum (DAKO, Glostrup, Denmark), and 10% normal human plasma (DAKO) for 20 minutes. Sections were incubated for 16 hours at 4°C with primary antibody anti-CDX2 (clone 392M, Biogenex, San Ramon CA, USA) in a 1:100 dilution or anti-MUC2 (clone Ccp58, Novocastra, Newcastle upon Tyne, UK) in a 1:100 dilution. Detection was performed with a biotin-labeled rabbit-anti-mouse antibody (DAKO), followed by the addition of a streptavidin-horseradish peroxidase complex (DAKO) using 3-amino-9-ethylcarbazole as substrate. Slides were analyzed for nuclear CDX2 and cytoplasmic MUC2 staining by two independent researchers that were blinded for presence of IM. Statistical analysis was performed using the chi-squared test using SPSS software (version 11.0 SPSS inc). A p-value < 0.05 was considered significant.

RESULTS

The presence of IM

IM was defined as the presence of goblet cell containing glands. Consecutive H&E, alcian blue, and PAS stained slides were evaluated for the presence of IM. In 17% of the biopsy sets there was disconcordance between the result of histological evaluation of these slides and the routine analysis performed at the biopsy set. In half of our samples IM was observed whereas the diagnosis of the routine pathologist was negative and in the other half IM was not observed whereas the diagnosis of the routine pathologist was positive. An explanation for this disconcordance is probably the fact that the slides used in this study were cut from a region that was at least 0.1 mm deeper located in the paraffin blocks than the slides that were used for routine histology. In addition routine analysis was mostly performed by general pathologists, viewing only H&E stained slides, while we used additional alcian blue and PAS stained slides, and these were analyzed by a specialized pathologist (HvD). The presence of IM based on the sections of this study was taken as starting point for further analyses for which consecutive slides were used.

In total, IM was observed in 55/122 biopsies (45%). 41/55 IM-positive biopsies contained both IM and GM and 14/55 IM positive biopsies contained only IM. Based on the presence of IM, group I (IM - IM) contained 15 patients, group II (IM - No IM) contained 25 patients, and group III (No IM - No IM) contained 21 patients (Table 1). Mean age at first endoscopy, length of the columnar segment, interval between the two endoscopies, and use of proton-pump inhibitors were not statistically significant between these three groups.

Table 1. patient characteristics

	I	II	III
1st endoscopy	IM	IM/No IM	No IM
2nd endoscopy	IM	IM/No IM	No IM
patients	15	25	21
mean age at first endoscopy *	58 (28-82)	55 (27-78)	54 (27-74)
mean length columnar segment (cm) *	3,87 (2-8)	3.37 (2-7)	2,56 (2-5)
interval between endoscopies (years) *	3 (0-13)	3 (1-9)	2 (0-10)
use of PPI*	18/23	15/20	10/18

*p=NS

CDX2 expression

CDX2 expression was defined as the presence of clear red coloration of at least four adjacent positive staining nuclei in the same gland. CDX2 expression was observed in 100% of IM positive biopsies in group I (Figure 1a -b). In group II, all IM positive biopsies were also positive for CDX2 (Table 2). CDX2 expression was also observed in 23/67 biopsies without IM (Figure 1c-d), i.e. 13 in group II, and 10 in group III (Table 3). In 4/10 CDX2 positive samples of group III, IM had previously been observed in either one or both blocks during routine evaluation by a pathologist. However, in this study IM was observed in none of the two sets of biopsies. In one patient in group III, both IM negative biopsies were positive for CDX2.

In samples with both IM and GM, CDX2 expression was not restricted to IM, but it was also observed in gastric-type glands that were located adjacent to the regions with IM (Figure 1e-f). In all cases, these gastric glands were of the cardia-type. Moreover, in some cases CDX2 expression was observed in cardia-type glands at a distance of the region with IM, on the other side of the biopsy sample (Figure 1g-h). These results are shown in Table 3. Strikingly, CDX2 expression in adjacent cardia-type glands was observed more often in group II compared with group I (34% vs. 24%), while in group I expression was mainly restricted to IM regions compared with group II (63% vs 17%). These differences were not significant ($p = 0.1$), however, a trend was seen towards increased CDX2 expression outside regions with IM in the groups that had IM in only one set of biopsies.

Table 2. CDX2 staining in all groups

	I	II	III
biopsies	30	50	42
first endoscopy			
IM	15	16	0
CDX2 *	15	24	5
MUC2	15	20	3
second endoscopy			
IM	15	9	0
CDX2 *	15	14	5
MUC2	15	14	1
total			
IM	30	25	0
CDX2	30	38	10
MUC2	30	34	4

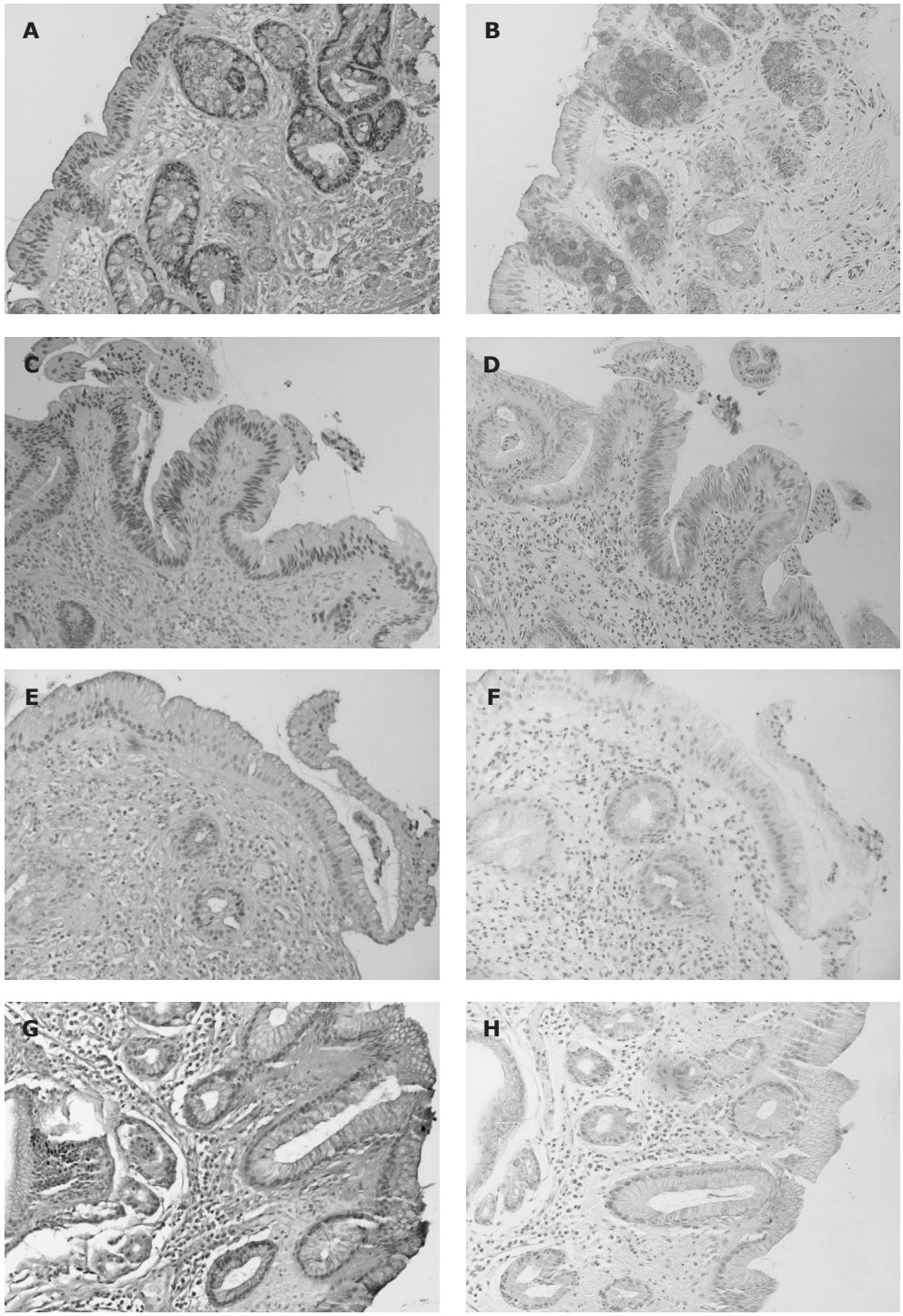
*p<0.001

Table 3. Location of CDX2 and MUC2 staining

location	I	II	III
CDX2			
total CDX2 positive biopsies	30	38	10
only in IM part of biopsies	19 (63%)*	13 (34%)	0
in IM and in adjacent GM	5 (17%)*	9 (24%)	0
in IM and in GM at distance of IM	6 (20%)	3 (8%)	0
no IM present in biopsies	0	13 (34%)	10 (100%)
MUC2			
total MUC2 positive biopsies	30	34	4
only in IM part of biopsies	23 (77%)	15 (44%)	0
in IM and in adjacent GM	6 (20%)	6 (18%)	0
in IM and in GM at distance of IM	1 (3%)	4 (12%)	0
no IM present in biopsies	0	9 (26%)	4 (100%)

*p=0.1

Figure 1 Immunohistochemical and histological analyses of CDX2 staining. a-b CDX2 and alcian blue staining in IM, 200 x magnification. c-d CDX2 and alcian blue staining of GM in samples without IM, 200 x magnification. e-f CDX2 and alcian blue staining in gastric-type glands adjacent to IM, the region with IM is not visible in this picture, but located on the left sight of this picture. 200 x magnification. g-h CDX2 and alcian blue staining in gastric-type glands at distance of IM, the region with IM is not visible in this picture, but is located on the other (left) site of this picture. 200 x magnification. A color version of this figure is printed on page 115.


A
B
C
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MUC2 expression

Cytoplasmic MUC2 staining in goblet cells was observed in all regions with IM. Staining of the extracellular mucus layer was not observed, since the antibody that was used only recognizes an epitope on the immature, not completely glycosylated form of MUC2. Furthermore in most cases in which CDX2 was observed in adjacent and distant CM, MUC2 was also expressed in this regions (Table 3). Moreover, MUC2 was also expressed in CM in 13/67 samples without IM. In this epithelium, MUC2 was expressed in goblet-like cells, that however did not stain positive with alcian blue (Figure 2).

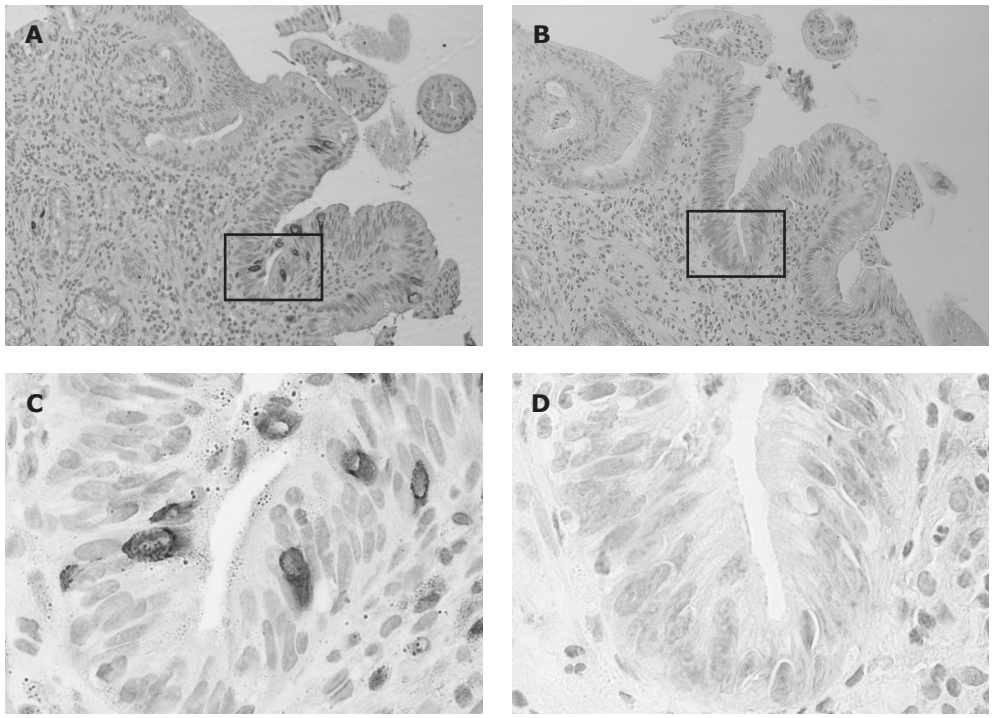

Figure 2 Immunohistochemical and histological analyses of MUC2 staining. a-b MUC2 and alcian blue staining of GM in samples without IM. The same region as Figure 1c-d is shown. 200x magnification. c-d Magnified view of MUC2-staining goblet-like cells in gastric-type glands.1000x magnification. A color version of this figure is printed on page 116.

DISCUSSION

Esophageal GM (CM or fundic-type epithelium) and IM often co-exist in the columnar-lined esophagus [3]. This study provides the first biological evidence that there is a intermediate type of epithelium is histologically similar to CM, but has CDX2 and in some cases MUC2 expression. Expression of the intestinal transcription factor CDX2 was not only observed in IM, but also in adjacent and distant CM and even in CM in some samples without IM.

During embryonic intestinal development, low CDX2 levels are present in the ear-

liest stages of intestinal formation [16]. These levels increase with the formation of intestinal columnar epithelium [9]. This indicates that CDX2 is an early marker for intestinal development. CDX2 expression in GM in the esophagus, as observed in this study, may represent early intestinal differentiation in this epithelium.

CDX2 could reveal early intestinal differentiation prior to morphologic changes, such as the presence of goblet cells, that are detected by conventional histological methods. Although possible due to the two-dimensional analysis of the biopsies, it is however unlikely that goblet cells have been missed in the analysis, as large areas without goblet cells, but with CDX2 expression were frequently observed (Figure 1g-h). This makes it highly unlikely that IM was present in these areas.

CDX2 expression in adjacent CM has been reported previously [17], however the present study focussed on this type of epithelium and determined further characteristics. It was observed that in this some of these CM regions goblet-like cells were present that were positive for an immature form of MUC2, but did not stain with alcian blue. This suggest that the MUC2 apomucin is present, but that the proteins that regulate the glycosylation are not expressed in this epithelium.

It is thought that columnar epithelium in the esophagus arises from a pluripotent stem cell, that is normally capable of both gastric and intestinal differentiation [18] as well as differentiation into squamous epithelium [19]. In the esophagus these cells will normally differentiate into squamous epithelium, but under influence of reflux-induced chronic inflammation this differentiation can go towards fundic-type mucosa, CM or IM [20]. The results of this study implicate that the stem cells can also differentiate into a hybrid epithelium that resembles CM but has intestinal characteristics such as CDX2 expression and MUC2 positive goblet-like cells.

In most cases, the presence of this hybrid epithelium was associated with the presence of IM in the patient, either in the other biopsy set, at an other cross-section of the biopsy set used for routine histology, or in earlier biopsies that were not included in this study. The distribution of IM in the columnar segment is usually patchy [5], and due to sampling error, focal intestinal metaplasia can be easily missed, even when the four-quadrant biopsy protocol [21] is used. The detection of hybrid epithelium, by means of CDX2 staining, can probably reduce sampling error, since it can identify patients that have IM at an other site of the columnar segment.

Furthermore, sampling error can also occur when focal IM at only one side of a biopsy is missed when routine histology is based on one or two cross-sections of the biopsy sets. CDX2 expression was observed in four of the disconcordant biopsy sets with only GM in this study, but with IM at previous routine histology. This observation illustrates the value of CDX2 in the detection of intestinal differentiation.

In conclusion, it was shown that in addition to intestinal metaplasia and cardia-type mucosa, a hybrid of these two epithelium types is present that has several intestinal characteristics but does not contain fully differentiated goblet cells. These results implicate that intestinal metaplasia and cardia-type mucosa in the esophagus are related to each other.

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Chapter 6

MUC4 is increased in high-grade dysplasia in Barrett's esophagus and is associated with a pro-apoptotic Bax/Bcl-2 ratio

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ABSTRACT

Patients with Barrett's esophagus are at an increased risk of esophageal adenocarcinoma. During the process of neoplastic progression, changes occur in mucosal mucin expression. Mucin expression may thus represent a marker for the detection of dysplasia. Mucins protect epithelium from damage by foreign bodies, but have also been implicated in regulatory processes like apoptosis. The objective of this study was to determine the expression pattern of mucins in neoplastic Barrett's epithelium and correlate it with the expression pattern of apoptosis-markers Bax and Bcl-2. A total of 37 patients with Barrett esophagus were included. Sixteen patients had Barrett esophagus without dysplasia (BE), six patients had Barrett esophagus with high-grade dysplasia (HGD), and fifteen had an infiltrating adenocarcinoma (EAC). Biopsies were obtained from the squamous epithelium, BE, and, when present, from foci of suspected HGD or EAC. MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 as well as Bax and Bcl-2 mRNA were determined by semi-quantitative RT-PCR. Protein levels of MUC2, MUC5AC and MUC6 were determined by immunoblotting. The mucin pattern varied between different stages of neoplastic progression in Barrett's epithelium. All mucins except MUC4 had low mRNA levels in squamous epithelium, and were at least 4 times higher in BE and HGD ($p < 0.001$). Most mucin mRNA levels were less markedly increased in EAC. In HGD and EAC, MUC4 levels were significantly elevated compared to BE ($p = 0.037$). The Bax/Bcl-2 ratio was increased in HGD compared to BE (ratio 1.71 versus 0.83, $p = 0.04$). Protein levels of MUC2, MUC5AC and MUC6 were correlated well with mRNA data. Mucin expression varies during the development of EAC in Barrett's epithelium. In particular MUC4 could serve as a tumor marker in this process. In contrast to animal studies, upregulation of MUC4 in HGD is associated with increased apoptosis. This suggests that MUC4 plays a minor role in the regulation of apoptosis in BE.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition in which the normal squamous epithelium of the lower esophagus is replaced by specialized intestinal epithelium. Gastroesophageal reflux is believed to play an important role in causing BE [1]. Adenocarcinoma (EAC) is thought to develop in BE in a stepwise manner via low-grade dysplasia and high-grade dysplasia (HGD) [2]. EAC has a poor prognosis, as metastasis occur early and symptoms present lately in the course of the disease. Overall, 5-year survival is only 10-15% [3].

Early detection of malignant progression is the key factor for improving the outcome of esophageal EAC. Current guidelines recommend endoscopic surveillance to detect dysplasia and to diagnose carcinoma at an early treatable stage [4]. However, the efficacy of endoscopic surveillance is thwarted by several factors [5]. Sampling error is likely to occur in endoscopic random-biopsy sampling [6]. In addition, a correct pathological diagnosis of dysplasia is difficult and focal neoplastic changes can easily be missed in small biopsy samples. This is reflected by a considerable inter- and intraobserver variability among pathologists in the interpretation of epithelial changes in BE [7].

The use of molecular markers in addition to normal endoscopical and histological evaluation could significantly enhance the detection of neoplasia in BE, both *in vitro* and *in vivo*. Particularly markers that indicate the presence of HGD could be helpful in the identification of patients at risk of malignant transformation.

Mucins are large glycoproteins that are the main components of the gel-like mucus-layer on the surface of the intestine. This layer serves to protect the mucosa from mechanical damage. Each type of mucosa has a unique pattern of mucin expression, and this pattern changes during neoplastic progression. Therefore, mucins are putative molecular markers for the development of adenocarcinoma in BE. Currently, more than 10 MUC genes have been reported, the best-characterized being MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6. Of these only MUC1 and MUC4 are expressed in the normal squamous lining of the esophagus [8], whereas BE predominantly expresses MUC2 and to a lesser extent MUC5AC and MUC6 [9-11].

In addition to protection against mechanical damage, membrane-bound mucins MUC1 and MUC4 have also been described to be involved in oncogenic pathways. MUC1 has an ability to bind β-catenin, thereby altering its ability to bind E-cadherin which leads to a decreased cell-cell adhesion and uncontrolled proliferation [12, 13]. MUC4, a mucin that is upregulated in several tumor-types, acts as ligand for the receptor tyrosine kinase Erb-B2, thereby regulating the $p27^{kip}$ cell-cycle inhibitor [14]. MUC4 also inhibits apoptosis in a xenotransplanted melanoma in nude mice [15].

The objective of this study was to evaluate the expression patterns of MUC1-4, MUC5AC, MUC5B, and MUC6 in high-grade dysplasia and adenocarcinomas in Barrett's esophagus and determine the association with the apoptosis markers Bax and Bcl-2.

METHODS

Patients

A total of 37 patients with BE were included. Sixteen patients had BE without dysplasia, six patients had BE with high-grade dysplasia, and fifteen had an infiltrating adenocarcinoma. Patients were evaluated by standard esophagoscopy and high magnification chromendoscopy. Biopsies were obtained from the squamous epithelium, Barrett's epithelium, and, when present, from foci of suspected high-grade dysplasia or adenocarcinoma. Two expert gastrointestinal pathologists independently evaluated haematoxylin and eosin stained sections for the presence of BE, grade of dysplasia or EAC. Al patients with HGD were treated with endomucosal resection (EMR). Resected tissue was investigated histologically, and in none of 6 patients, invasive adenocarcinoma was found. Ten patients with adenocarcinoma were treated surgically and 5 patients received palliative treatment. All adenocarcinomas had infiltrated into the submucosa and 3 patients had local lymph node metastases. Eight tumors were well and 7 were poorly differentiated. This study was approved by the ethical committee of the Erasmus MC Rotterdam and informed consent was obtained prior to endoscopy.

RNA and protein isolation from biopsies

Biopsies were snap-frozen in liquid nitrogen. Total RNA was isolated using Trizol-reagent (Invitrogen, Groningen, The Netherlands) according to instructions of the manufacturer. Remaining chromosomal DNA was removed with the RNA-free DNA kit (Zymo, Orange, USA). Total RNA concentrations were estimated by RNA electrophoresis on an agarose gel and band intensity was compared with that of a marker band with a known concentration. From the same biopsy specimen, a protein fraction was isolated according to the instructions of the manufacturer and denatured in 1% sodium dodecyl sulphate. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, USA).

RT-PCR

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. cDNA was synthesized by incubation of the mRNA for 30 min at 42°C. PCR-reactions (total volume of 25 µl) contained 1 µl of the cDNA solution, 1 x PCR-core buffer (Promega), 2 mM magnesium chloride, 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 visualized on a 1% agarose gel and stained with ethidiumbromide. Band size and intensity were measured with Kodak 1D software (Kodak, Rochester, USA) version 3.5 by normalizing against the housekeeping gene β-actin, as described previously [16]. All primers are listed in Table 1.

Protein Cross-blot

Twenty microgram protein was loaded in the lanes of a reverse line blotter (Immu-

Table 1. PCR-Primers

Amplified gene	Forward primer	Reverse Primer
MUC1	5'TGCATCAGGCTCAGCTTCTA3'	5'ACGTCGTGGACATTGATGGT3'
MUC2	5'CAGGATGGCGCCTTCTGCTA3'	5'ÁTGCTGCTCCAAGCTGAGGT3'
MUC3	5'CCAGCCAGGATGTGAACAG3'	5'GACAGTCGATGGCGTTGTC3'
MUC4	5'TCAACGCCTCGGTGGCATAC3'	5'CTGTCACATCGCGCACGTCT3'
MUC5B	5'TTGACGGCACCTCTTACACC3'	5'GCCTGGAAGACTTGGCCATT3'
MUC5AC	5'CCGGAGGTGAACATTGAAC 3'	5'TCTGTGGCGGTATATGGTG3'
MUC6	5'GCAGGAGGAGATCACGTTCA3'	5'CGCCTCCTCTGTGGCTTCAT3'
Bax	5'GGCCCACCAGCTCTGAGCAGA3'	5'GCCACGTGGGCGTCCCAAAGT3'
Bcl-2	5'GTGGAGGAGCTCTTCAGGGA3'	5'ÁGGCACCCAGGGTGATGCAA3'
β-actin	5'GACAGGATGCAGAAGGAGAT3'	5'AGTCATAGTCCGCCTAGAAG3'

netics, Cambridge, UK) on a PVDS membrane (Roche, Mannheim, Germany). After one hour of incubation at room temperature the membrane was blocked in 5% Protifar non-fat milk (Nutricia, Cuijk, Netherlands) in phosphate buffered saline containing 0.1% Tween. The primary antibodies anti-MUC2 clone Ccp58 (Novocastra, Newcastle, USA), anti-MUC5AC clone 45M1 (Zymed, San Francisco, USA), and anti-MUC6 clone CLH5 (Novocastra) were loaded in an angle of 90 degrees on the protein samples. An alkaline phosphatase labelled Goat anti-mouse IgG antibody (Southern Biotech Associates, Birmingham, USA) was used as conjugate and 0.18 mg/ml 5-bromo-4-dichloro-3-indolyl phosphate (BCIP, Promega) and 0.35 mg/ml Nitroblue Tetrazolium (NBT, Promega) in 0.1M Tris-HCl pH 9.5, 0.1M NaCl, and 5 mM $MgCl_2$) were added as substrate. Immunohistochemical staining of gastric antrum and colonic mucosa confirmed that the primary antibodies detected the mature mucin protein in case of MUC5AC and MUC6, or a precursor peptide, in case of MUC2.

Statistical analyses

RT-PCR data are presented as mean ± SEM. Statistical analyses were performed with the Mann-Whitney U-test. A p-value <0.05 was considered statistically significant.

RESULTS

Mucin mRNA levels

The results of RT-PCR testing of different mucins in squamous epithelium, BE, HGD, and EAC are shown in Figure 1. The mean transcription levels of individual mucins relative to transcription of β-actin in the same sample are given in Figure 2. Overall, mucin transcription levels were low or absent in squamous epithe-

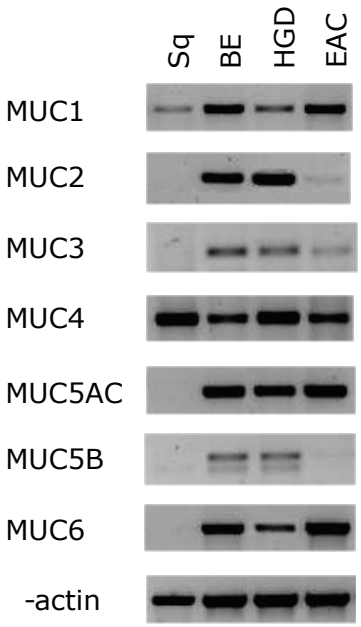

Figure 1 Representative examples of RT-PCR results. mRNA of MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B and MUC6 in squamous epithelium (Sq), Barrett's esophagus (BE), High-grade dysplasia (HGD), and adenocarcinoma (EAC). mRNA was isolated from biopsy samples of a patient with BE containing HGD and EAC. β-actin was used as control for RNA-input. PCR products were analyzed on a 1% agarose gel containing ethidiumbromide.

lium (relative transcription level < 0.2), except for MUC4 (relative transcription level 1.1). Compared with squamous epithelium, MUC4 was downregulated in BE (relative transcription level 0.62; $p = 0.01$), while other tested mucins were considerably upregulated in BE (relative transcription level > 0.4, $p<0.001$). In HGD, transcription levels of mucins resembled that of BE, except for MUC4, which was significantly higher in HGD than in BE (relative transcription levels 1.27 versus 0.62; $p = 0.037$). In most adenocarcinomas, mucin mRNA levels were lower compared to BE without dysplasia or with HGD. Amplification of residual chromosomal DNA was not found. Sequencing and comparison with previous published sequences confirmed the authenticity of the mucin PCR-products.

Mucin protein expression

Protein fractions from the same biopsies as the mRNA fractions were analyzed by protein cross-blot to determine the mucin patterns on a protein level (Figure 3). Commercial antibodies that recognize the denatured antigen were only available for MUC2, MUC5AC and MUC6. Cross-blot analysis revealed that in squamous epithelium expression was present for MUC2 in 2/32 patients, for MUC5AC in 9/32 patients, and MUC6 in 2/32 patients. In more than half of the BE samples, expression of MUC2 (17/30 patients), MUC5AC (28/30 patients), and MUC6 (26/30 patients) was found. In HGD, MUC6 was expressed in 15/30 samples, while expression of MUC2 and MUC5AC was comparable to that in BE (3/6 and 6/6 respectively). MUC5AC and MUC6 protein were found in 9/15 and MUC2 protein in 3/15 of the EAC samples (Figure 4). Protein data were found to correlate with the mRNA data.

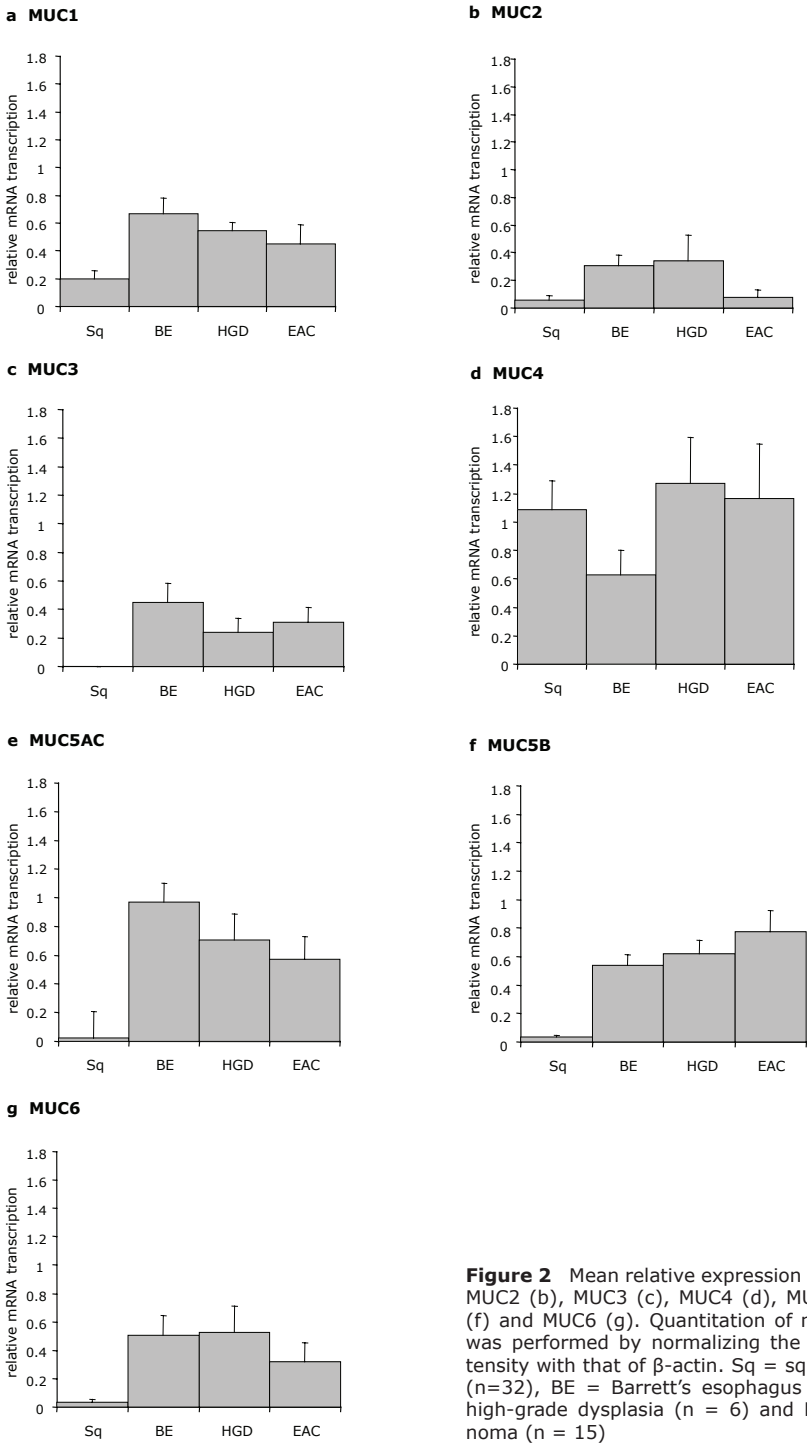

Figure 2 Mean relative expression levels of MUC1 (a), MUC2 (b), MUC3 (c), MUC4 (d), MUC5AC (e), MUC5B (f) and MUC6 (g). Quantitation of mucin mRNA levels was performed by normalizing the band-area and intensity with that of β-actin. Sq = squamous epithelium (n=32), BE = Barrett's esophagus (n = 30), HGD = high-grade dysplasia (n = 6) and EAC = adenocarcinoma (n = 15)

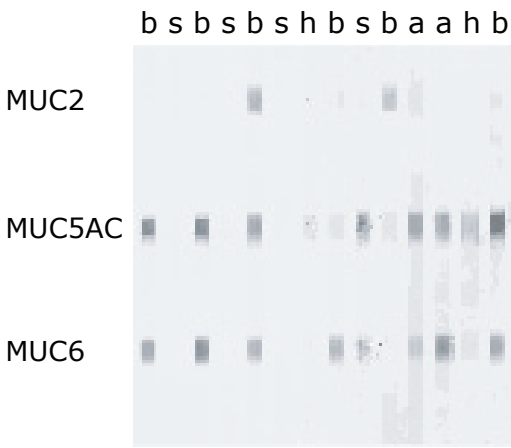

Figure 3 Representative example of protein cross-blot. Protein samples were spotted in lanes on a membrane. Primary antibodies were spotted in lanes in an angle of 90° and were detected with an alkaline phophatase labelled secondary antibody. s = squamous epithelium, b = Barrett's esophagus, h = high-grade dysplasia, a = adenocarcinoma

Bax and Bcl-2 mRNA levels in correlation with mucin mRNA levels

The correlation between mucin mRNA levels and the apoptosis-markers Bax and Bcl-2 was determined by calculating the ratio between mRNA-levels of the pro-apoptotic Bax and the anti-apoptotic Bcl-2. mRNA levels of Bax and Bcl-2 were determined by semi-quantitative RT-PCR. The results are shown in Figure 5. The Bax/Bcl-2 ratio was increased in HGD compared to BE (ratio 1.71 versus 0.83, p = 0.04). In EAC, the ratio was lower than in HGD (0.95), but this difference was not statistically significant (p > 0.22). The elevated Bax/Bcl-2 ratio in HGD was associated with an increase in MUC4 mRNA.

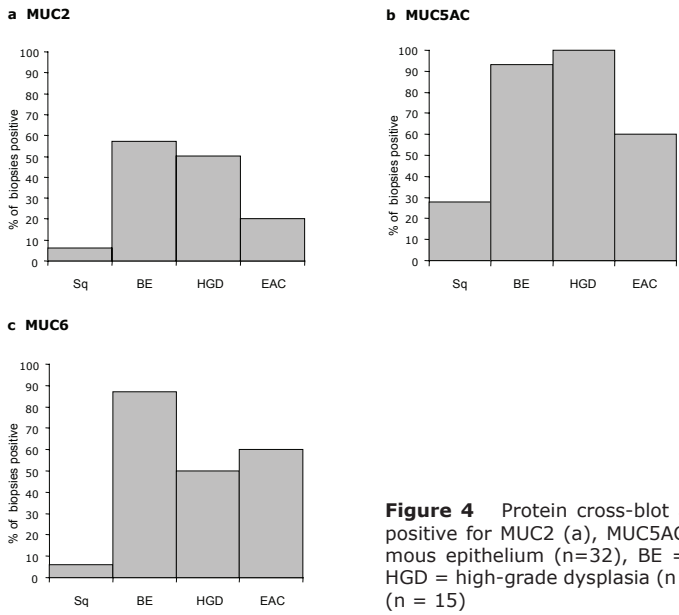

Figure 4 Protein cross-blot analysis: percentage of samples positive for MUC2 (a), MUC5AC (b) and MUC6 (c). Sq = squamous epithelium (n=32), BE = Barrett's esophagus (n = 30), HGD = high-grade dysplasia (n = 6) and EAC = adenocarcinoma (n = 15)

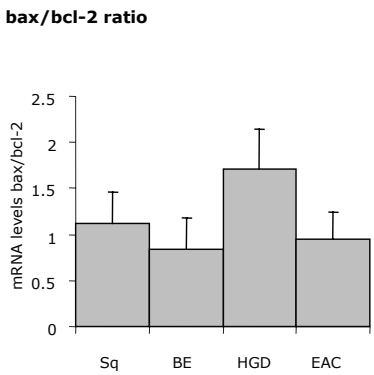

Figure 5 Summary of data of Bax/Bcl-2 ratios. Quantitation of Bax and Bcl-2 mRNA levels was performed by dividing the band-area and intensity of Bax by that of Bcl-2. Sq = squamous epithelium (n=9), BE = Barrett's esophagus (n = 8), HGD = high-grade dysplasia (n = 6) and EAC = adenocarcinoma (n = 4)

DISCUSSION

Changes in the expression pattern of mucins have been associated with the development of early carcinomas of the colon, gallbladder and the respiratory tract [17-20]. The expression pattern of mucins in esophageal adenocarcinoma has also been determined [9, 10, 21], however the mucin pattern in high-grade dysplasia, the lesion that is preceding infiltrating adenocarcinoma, has not been clarified. In this study, the transcription and expression of various mucins in biopsies of esophageal squamous epithelium, BE, HGD and EAC were determined. From each biopsy sample, both RNA and protein were purified, thus minimizing sample discordance between RNA and protein analysis. Most mucins were upregulated in BE compared to squamous epithelium. In adenocarcinomas, a greater variety with a trend towards lower mucin expression compared to BE was seen. Compared to non-dysplastic BE, HGD contained higher levels of MUC4 mRNA.

High expression of the secreted mucins MUC2, MUC5AC, MUC5B and MUC6 has previously been reported in BE [9, 21], as is their downregulation in esophageal adenocarcinoma of the esophagus [9, 10]. In contrast to these reports, low levels of these mucins were found in some squamous epithelium samples. Since RT-PCR is a very sensitive technique, these low levels could have escaped detection in immunohistochemical studies. Also the possibility that the squamous samples are contaminated with some intestinal glands or subepithelial proper glands can not be excluded. Adjacent biopsies were analyzed for purity of epithelium, since approaches designed for extraction of mRNA from fixated tissue that is used for histological analysis, are not validated for the quantitative comparison of mRNA-levels. Furthermore, the presence of MUC2, MUC5AC and MUC6 protein in the squamous epithelium could have been caused by reflux of gastric mucus components, including these mucins.

Most reports describe also high expression of MUC1 in squamous epithelium and low expression in BE [9, 10, 22], whereas the opposite is shown in the present study. An explanation for this could be that most studies used independent squamous epithelium samples, whereas in this study paired squamous/BE samples

from a single patient were used in which inflammation could have been altered the mucin pattern. The most striking result however is the upregulation of MUC4 in HGD compared to BE without dysplasia. This has not been reported before.

Duodenogastro-esophageal reflux has been implicated in the development of Barrett's esophagus and esophageal adenocarcinoma [23]. Recently it was found that in particular bile salts and their conjugates induce the transcription of MUC4 mRNA in esophageal cancer cells [24]. This could explain the observed increase of MUC4 in HGD.

Increased MUC4 expression has also been found in other early neoplastic lesions like early pancreatic carcinomas and dysplastic cervical lesions [25, 26]. When produced in normal quantities, MUC4 has been described to function as a steric barrier that limits the access of other cells and large molecules to the epithelium, thereby protecting it from damage [27]. On the other hand, overexpression of MUC4 disrupts the cell-cell and cell-matrix interactions, which could initiate the dissociation of tumor cells from the primary tumor [28]. Finally, overexpression of MUC4 may result in the masking of tumor antigens, thereby effectively suppressing tumor cell killing by cytotoxic lymphocytes [27]. Since esophageal adenocarcinoma often metastasizes in an early phase, loss of cell adhesion probably occurs already in an early stage of carcinogenesis. Increased MUC4 expression could attribute to the detachment of tumor cells from the primary adenocarcinoma.

MUC4 protein also acts as ligand for the receptor tyrosine-kinase Erb-B2, a proto-oncogene that is abnormally expressed in membranes of Barrett's epithelium and associated adenocarcinoma [29, 30]. The MUC4-Erb-B2 complex is involved in the inhibition of apoptosis [31], thereby promoting tumor growth. A strong suppression of apoptosis was observed in MUC4 overexpressing tumors in mice [15]. In this study, the mRNA levels of apoptosis markers Bax and Bcl-2 were also determined. Inhibition of apoptosis was not found in the HGD samples with increased MUC4 expression. In contrast, the Bax/Bcl-2 ratio was increased in HGD compared to BE. This might indicate that MUC4 has only a minor role in regulation of apoptosis in HGD in humans.

An increased apoptotic rate does not fit in the model of neoplastic progression [32]. However, reduced Bcl-2 expression during neoplastic progression of Barrett's esophagus has been reported before [33]. A recent report described clear Bax and Bcl-2 expression in both BE, HGD and EAC, but results were not quantitative [34]. Other apoptotic proteins, like Fas-ligand and caspases may be involved in an anti-apoptotic effect in BE with HGD, as compensation for the pro-apoptotic Bax/Bcl-2 balance.

The results of this study show that mucin expression is elevated in Barrett's esophagus. Membrane-bound mucin MUC4 is associated with the development of high-grade dysplasia in Barrett's esophagus, but not with a decrease in apoptosis. This suggests a role for MUC4 as early tumor marker in Barrett's esophagus. In combination with other tumor markers it could facilitate the diagnosis of an early adenocarcinoma both *in vitro* and *in vivo*. However, additional studies are necessary to verify this observation.

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Chapter 7

High-grade dysplasia in Barrett's esophagus is associated with increased expression of calgranulin A and B

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ABSTRACT

Background Patients with Barrett's esophagus are at increased risk of developing esophageal adenocarcinoma, a type of carcinoma that is preceded by dysplastic changes of the metaplastic mucosa. The aim of this study was to identify genes involved in the development of high-grade dysplasia in order to increase our understanding of the development of early neoplastic lesions in Barrett's esophagus.
Methods Seventy-six biopsies from 42 patients were included. Paired biopsy samples from non-dysplastic Barrett's esophagus and high-grade dysplasia from a single patient were split and used for histological evaluation and transcription profile analysis. Relative mRNA levels were tested for association with presence or absence of dysplasia. The other biopsy samples, including squamous epithelium, Barrett's esophagus, high-grade dysplasia and adenocarcinoma were used for testing the transcription profile by semi-quantitative RT-PCR and immunohistochemistry.
Results In high-grade dysplasia, 866 genes showed a >2-fold difference in mRNA levels compared to Barrett's esophagus. Of these, 33 displayed a >10-fold difference. Determination of mRNA levels by RT-PCR for 22 of these genes revealed two significantly upregulated genes in high-grade dysplasia, calgranulin A (S100A8; $p = 0.017$) and calgranulin B (S100A9; $p = 0.022$). Levels of calgranulin A and B protein were also increased in high-grade dysplasia. For the other 20 genes, differences in mRNA levels between high-grade dysplasia and Barrett's esophagus were observed, but none of these was significant.
Conclusion Calgranulin A and B are subunits of the calprotectin (S100A8/A9) complex that is involved in chemotaxis of neutrophils. This study reports for the first time that calprotectin may play a role in the development of neoplasia in Barrett's esophagus. In addition, this study shows that differentially expressed proteins in biopsies from Barrett's esophagus may be part of an array-like diagnostic test, that could facilitate the detection and diagnosis of advanced dysplasia in Barrett's esophagus.

INTRODUCTION

Barrett's esophagus is a premalignant lesion, which is associated with gastro-esophageal reflux [1]. Patients with Barrett's esophagus have an increased risk for the development of esophageal adenocarcinoma [2]. Adenocarcinoma is thought to develop in Barrett's esophagus in a stepwise manner via low-grade dysplasia (LGD) and high-grade dysplasia (HGD) [3].To date, the cellular and molecular mechanisms leading to neoplastic progression in Barrett's epithelium are still only partially understood [4].

HGD is generally seen as a precursor lesion for esophageal cancer, as a group of patients with HGD progresses to adenocarcinoma [5]. Several processes have been implicated in the transition of Barrett's metaplasia to HGD. For example, accumulation of p53 protein in HGD impairs apoptosis, reduced E-cadherin expression disturbs the cell-cell adhesion, and increased MUC4 expression induces tumor growth [6-8]. As none of these factors occurs in all HGD lesions, they do not seem to play a key role in the development of HGD, and other genes involved in this process still have to be identified. Several approaches to identify genes involved in neoplastic progression, such as immunohistochemical detection of known genes in tumor tissue, have been used, but these are restricted to genes that have already been implicated in cancer development.

Techniques like gene transcription profiling may aid in the identification of genes involved in neoplastic progression [9]. The biological processes underlying tumorigenesis will allow for improved detection and treatment of early neoplasms. In this study, an mRNA-based approach was used to identify genes whose transcription is affected by the transition from Barrett's esophagus without dysplasia to Barrett's esophagus with HGD. As the aim of this study was to identify major transcriptional changes in the esophageal tissue, it was decided to obtain RNA from complete routine clinical biopsy materials rather than using a method like laser capture microdissection to analyze these changes in a small group of specific cells. As a global gene profiling approach still requires significant amounts of RNA, we performed gene transcription profiling on material obtained from different locations of the esophagus from a single patient. Obtaining RNA from the various clinical stages of esophageal disease of a single, well characterized patient eliminates false positive results from interpatient differences. To test the general validity of the genes differentially transcribed, a semi-quantitative RT-PCR was performed on paired samples of a large panel of patients.

MATERIALS AND METHODS

Patients

A total of 76 biopsies from 42 patients with Barrett's esophagus without dysplasia (BE), high-grade dysplasia (HGD) or adenocarcinoma (EAC) were included (Table 1). Patients were evaluated by standard esophagoscopy and high magnification chromendoscopy. For microarray, jumbo biopsies were obtained from

Table 1. Patient characteristics

patientgroups	patients	Mean age (range)	% male	Biopsies obtained			
				Sq[d]	BE	HGD	EAC
Microarray							
HGD[a]	1	53	100	0	1	1	0
Total	1			0	1	1	0
RT-PCR							
BE[b]	13	63.8 (33-85)	46	12	13	0	0
HGD	2	62.5 (53-72)	50	2	2	2	0
EAC[c]	8	70.6 (57-81)	63	7	7	3	8
Total	23			21	22	5	8
IHC[e]							
BE	9	57.3 (36-85)	78	0	9	0	0
HGD	9	68.1 (53-79)	89	0	0	9	0
Total	18			0	9	9	0

[a] HGD: Barrett's esophagus with high-grade dysplasia
[b] BE: Non-dysplastic Barrett's esophagus
[c] EAC: Esophageal adenocarcinoma
[d] Sq: Esophageal squamous epithelium
[e] IHC: Immunohistochemistry

BE and HGD, and for semi-quantitative RT-PCR, biopsies were obtained from the squamous epithelium (Sq), BE, and, when present, from foci of suspected HGD, or adenocarcinoma. Eighty percent of the samples were paired. Adjacent biopsies were used for histology. Archival biopsies were obtained for the immunohistochemical analysis. All biopsies were analyzed by two expert pathologists, who independently evaluated haematoxylin and eosin-stained sections for the presence of intestinal metaplasia, grade of dysplasia, or EAC. This study was approved by the institutional review board of the Erasmus MC Rotterdam and informed consent was obtained from patients prior to endoscopy.

Microarray based transcriptional profiling

Total RNA was isolated from BE and HGD biopsy specimens with the nucleobond RNA kit (BD Clontech, Palo Alto, CA) according to the instructions of the manucfacturer. cDNA was synthesized and labeled using the Atlas Pure Total RNA labeling system (BD Clontech) according to the instructions of the manufacturer. cDNA was hybridized with the BD Atlas plastic human 12K array (BD Clontech) consisting of a chip with 11835 genes, including positive controls such as β-actin, GAPDH, and α-tubulin as well as negative controls and controls for cDNA synthesis. Microarray data were analyzed using Atlasimage 2.7 software (BD Clontech).

Table 2. Primers used for RT-PCR analysis

Gene products	Forward primer 5'…3'	Reverse primer 5'…3'	T[a]	Size[b]
Aldolase B	agcagaagaaggagctctca	agcacctccttggtctaact	55	311
CA11	gctggacttcttggagtctt	cttgctcaggtcatcgactt	50	378
Calgranulin A	cgtctaccacaagtactc	ccagtaactcagctactc	50	253
Calgranulin B	tggaacgcaacatagagacc	atgaactcctcgaagctcag	50	217
Cytokeratin 4	cctggacctggacagcatta	gccacggatacctgaagagt	53	249
Cytokeratin 13	ccgtggtgtctctacctgtt	ccagcctggcattgtcaatc	53	479
Fatty acid binding protein 1	gctctattgcaccatgagt	ttgtctccagctcacattcc	50	243
Fatty acid binding protein 2	ttggaaggtagaccggagtg	gttcagttccgtctgctagg	47	218
Gastric lipase	caacagccttgccttcattc	gcaacaggagctagagcata	50	309
H^+K^+ ATPase	gtgtggatcagcctgtacta	gaacttgcaggagaacttgg	50	351
Hydroxysteroiddehydrogenase	aacctggaggcttcctaaca	gcaaggcagatccacaagta	50	271
Integral membrane protein 2B	aagaagagcctggtgttg	ggacaggcacactgataa	47	257
Junction plakoglobin	cgtgtgctcaaggtgctatc	gtgtcaccagcgtcttgttc	53	295
MUC5B	ttgacggcacctcttacacc	gcctggaagacttggccatt	55	361
Pepsinogen A	gaacctggctccttcctgta	gatgtcgctctggatgttgg	53	387
Pepsinogen C	tcggcttgagtgagaatgag	atgccaatctgccagtagag	53	283
Prostate stem cell antigen	aagcccaggtgagcaacgagga	tggcgttgcacaagtcggtgtc	53	204
Psoriasin	gctgagaggtccataatagg	tagtctgtggctatgtctcc	47	242
Ribosomal protein P1	gtgacagtcacggaggataa	tcatcagactcctcggattc	50	257
TFF1	aatggccaccatggagaa	ggacgtcgatggtattagga	53	230
TFF2	aactgcggcttccctggaat	agaagcaccagggcacttca	53	238
Transthyretin	ctgccttgctggactggtat	atgcagctctccagactcac	50	202
β-actin	gacaggatgcagaaggagat	agtcatagtccgcctagaag	57	587

[a] Temperature used for annealing of primers
[b] PCR product length

Semi-quantitative RT-PCR

Total RNA was isolated from Sq, BE, HGD and EAC biopsies using Trizol-reagent (Invitrogen, Paisley, UK) and remaining traces of chromosomal DNA were eliminated using the DNA-free RNA kit (Zymo, Orange, CA). cDNA was synthesized with the use of Avian Myeloma Virus reverse transcriptase (Promega, Madison, WI). Primers were annealed by cooling down from 70°C to room temperature. cDNA was synthesized by incubation of the mRNA for 30 min at 42°C. PCR-reactions (total volume of 25 µl) contained 1 µl of the cDNA solution, 1x PCR-core buffer (Promega), 2 mM magnesium chloride, 0.4 µM forward and reverse primer, 200 µM of each nucleotide (Promega) and 0.02 U/µl Taq polymerase (Promega). All primers were designed with aid of primer designer software (version 5.10, scientific and educational software) and are listed in Table 2. PCR conditions were 35 cycles at 94°C (30 s) for denaturing; 47-57°C (30 s) for annealing, dependent on the primer couple used, and 72°C (1 min) for extention. PCR-products were visualized on a 2% agarose gel and stained with ethidiumbromide. PCR product identity was confirmed by sequencing. Band size and intensity were measured by

densitometry with Kodak 1D version 3.5 software (Kodak, New Haven, CT) by normalizing against the housekeeping gene β-actin, as described previously [8]. RT-PCR densitometric data are presented as mean +/- standard error of the mean. Statistical analyses were performed using the Mann-Whitney U-test. A *p*-value < 0.05 was considered statistically significant.

Immunohistochemistry

Paraffin-embedded biopsy samples were serially sectioned at 4 μm, 15-20 sections from one sample, mounted on adhesive slides, dried overnight at 37°C, and deparaffinized with xylene. First and last sections of each series were stained with haematoxylin and eosin, and evaluated for the presence of intestinal metaplasia and high-grade dysplasia. When intestinal metaplasia or high-grade dysplasia were present and adenocarcinoma was absent, the other sections were used for immunohistochemical analyses. Antigen retrieval was performed by boiling the deparaffinized samples in a 10 mM monocitric acid buffer (pH 6.0) in a microwave for 15 min, and slowly cooling down to room temperature. Subsequent samples were incubated in TBS buffer containing 10% rabbit non-immune serum (DAKO, Glostrup, Denmark), and 10% normal human plasma (DAKO) for 30 minutes. Primary antibodies used were anti-aldolase B (clone D18, Santa Cruz, Santa Cruz, CA) in a 1:200 dilution, anti-FABP1 (clone L2B10, Monosan, Uden, The Netherlands) in a 1:500 dilution, anti-cytokeratin 4 (clone 6B10, Monosan) in a 1:50 dilution, anti-cytokeratin 13 (clone 1C7, Monosan) in a 1:50 dilution, anti-calgranulin A (clone CF-145, Chemicon International,Temecula CA, USA) in a 1:50 dilution, and anti-calgranulin B (clone C-19, Santa Cruz) in a 1:40 dilution. Detection was performed with secondary biotin-labeled antibodies, rabbit-anti-goat (DAKO) and rabbit-anti-mouse (DAKO), followed by the addition of a streptavidine-alkaline phosphatase complex (DAKO) using fast blue as substrate.

RESULTS

Differential gene expression

Since it is difficult to obtain sufficiently large paired biopsy samples falling within our inclusion criteria, transcription profile analysis was limited to the material of four representative patients. Paired jumbo biopsies from BE, and HGD were obtained and immediately divided in three parts; the inner part was flashfrozen in liquid nitrogen and stored for subsequent use in transcription profiling analysis, while the two flanking parts were submitted for histological examination by two independent expert pathologists. Only for one of these four patients flanking parts matched all of our rigorous inclusion criteria: i.e. the BE sample did neither contain LGD or HGD, nor squamous epithelium, and in the HGD sample more than fifty percent of the epithelium was high-grade dysplasia and infiltrating adenocarcinoma was not present. The two frozen midsections of the two samples (BE and HGD) of this single patient were then used for RNA isolation and the differences in mRNA levels between these two samples were determined for all 11835 genes

present on the Atlas human 12K microarray. Briefly, 866 of 11835 genes (7.3%) displayed a significant (2-fold or greater) difference in mRNA levels between the two samples. As this is more than can be dealt with in a reasonable amount of time it was arbitrarily selected to further analyze only the 33 genes (0.3%) that showed a greater than 10-fold difference. The microarray results for these 33 genes are summarized in Table 3. The results for all tested genes will be made available as supplementary data.

Calgranulin A and B are differentially expressed in BE and HGD

A total of 22 genes predominantly involved in gastrointestinal processes and inflammation were selected to further analyze mRNA transcription. mRNA levels were determined by semi-quantitative RT-PCR in 21 BE and 5 HGD biopsies, as well as in 21 biopsies of the squamous epithelium 5 cm above the Z-line, and 8 advanced adenocarcinoma biopsies. Relative expression levels are listed in Table 4 and PCR results for a representative set of biopsies are shown in Figure 1a. Overall, both experimental setups displayed similar trends. Statistical analyses revealed a significant difference between BE and HGD for only 2 genes, calgranulin A and calgranulin B (Figure 1b and c). In other samples there was a trend towards up- or downregulation in HGD, but this did not reach significance. In the squamous epithelium calgranulin A and B mRNA levels were also significant increased compared to BE ($p<0.001$).

Differences in protein expression and localization

To determine the effect of the differential gene expression on both protein expression levels and location, an immunohistochemical analysis was performed on archival material of 9 patients with BE and 9 patients with HGD. The 6 genes, which showed largest differences between BE and HGD in RT-PCR were tested. In serially sectioned slides calgranulin A (S100A8), calgranulin B (S100A9), aldolase B, fatty acid binding protein 1 (FABP1), and cytokeratins 4 and 13 protein expression was determined. Adjacent Haematoxylin and Eosin stained slides were used to identify the regions with HGD and intestinal metaplasia. Calgranulin A expression was mainly observed in neutrophilic granulocytes. In the majority of patients, staining was also observed in the columnar epithelium, albeit less intense. In non-dysplastic columnar cells cytoplasmic staining was limited to the basal side of the cytoplasm, but in high-grade dysplastic columnar cells the entire cytoplasm was stained.

To exclude that the differences in mRNA levels were due to differences in inflammation, the number of infiltrating neutrophils was determined, and no significant difference was found ($p>0.05$). Calgranulin A was also intensely expressed in small islands of squamous epithelium present in some slides (Figure 2a and b). Calgranulin B expression was observed in the same areas as calgranulin A, but staining was more intense, probably due to aspecific background staining of the antibody (Figure 2c and d). Aldolase B expression (Figure 2e and f) was predominantly observed in the cytoplasm of columnar cells, but did not differ between BE and HGD. No staining was observed with either cytokeratin 4 or cytokeratin

Table 3. Genes with a more than 10-fold difference between BE and HGD in microarray performed on biopsy material from a 53-year old male patient*

	mRNA levels Microarray		
Genes	BE[a]	HGD[b]	Ratio BE/HGD
Gastric			
Pepsinogen A	2485	13	191
CA11	1902	14	136
TFF1	36222	692	52
Gastric lipase	13344	419	32
TFF2	1817	80	23
H+K+ ATP-ase	580	42	14
MUC5B	577	50	12
Pepsinogen C	4599	451	10
Intestinal			
Fatty acid binding protein 2	161	13	12
Hydroxysteroiddehydrogenase 2	315	28	11
Hepatic			
Fatty acid binding protein 1	1484	13	114
Aldolase B	1546	53	29
Pancreatic			
Chymotrypsinogen B1	4036	13	310
Colipase	2240	23	97
Elastase 3A	1603	18	89
Carboxypeptidase B1	780	13	60
Carboxylester lipase	838	30	28
Phospholipase A2	1249	52	24
Trypsin 2	16311	976	17
Tumor related			
Prostate stem cell antigen	1901	28	68
Chromogranin A	253	23	11
Ret proto-oncogene	535	52	10
Dual specificity phosphatase 1	47	536	0.088
Inflammatory			
Calgranulin B (S100A9)	77	799	0.096
Calgranulin A (S100A8)	199	5056	0.039
Psoriasin (S100A7)	17	1557	0.011
Miscellaneous			
Transthyretin	408	15	27
Integral membrane protein 2B	1652	126	13
Cytokeratin 13	43	438	0.098
Junction plakoglobin	185	2186	0.085
Thioredoxin	58	691	0.084
Cytokeratin 4	180	2257	0.080
Ribosomal protein P1	225	3581	0.063

[a] BE = Non-dysplastic Barrett's esophagus
[b] HGD = Barrett's esophagus with high-grade dysplasia
* A list of genes that showed a > 2-fold difference is published on www.gastrolab.nl

13 in BE or HGD. However, the squamous epithelium that was present in some slides always showed intensive cytokeratin 4 and 13 staining (data not shown). FABP1 expression was observed in the top of some villi. In BE, FABP1 protein was present in the entire top of the villus, while in HGD it was observed in only a few cells (Figure 2g and h).

Table 4. Relative mRNA levels of 22 differentially expressed genes determined by semi-quantitative RT-PCR

	mRNA levels Semi-quantitative RT-PCR				
Gene products	Sq (n = 21)[a]	BE (n = 22)[b]	HGD (n = 5)[c]	EAC (n = 8)[d]	p-value BE - HGD[e]
Gastric					
Pepsinogen A	0.01	0.09	0.07	0.52	0.9
CA11	0.01	0.02	0	0.05	0.48
TFF1	0.24	2.00	1.59	1.85	0.85
Gastric lipase	0.15	0.78	0.75	0.49	0.9
TFF2	0.35	2.21	1.86	1.98	0.71
H^+K^+ ATP-ase	0.01	0.15	0	0.09	0.2
MUC5B	0.03	0.54	0.62	0.77	0.83
Pepsinogen C	0.10	0.47	0.47	0.87	0.37
Intestinal					
Fatty acid binding protein 2	0.03	0.62	0.04	0.26	0.11
Hydroxysteroiddehydrogenase 2	0.04	0.91	0.74	0.47	0.9
Hepatic					
Fatty acid binding protein 1	0.02	1.09	0.77	0.35	0.57
Aldolase B	0.11	0.69	0.23	0.13	0.57
Tumor related					
Prostate stem cell antigen	1.74	1.82	1.29	0.89	0.32
Inflammatory					
Psoriasin	0.94	0.31	0.78	0.70	0.24
Calgranulin A	3.31	0.79	1.75	1.04	0.02*
Calgranulin B	4.01	0.82	1.81	1.06	0.02*
Miscellaneous					
Ribosomal protein P1	2.71	2.09	2.04	2.18	0.35
Cytokeratin 4	2.45	0.50	0.66	0.06	0.26
Junction plakoglobin	0.36	0.18	0.40	0.88	0.36
Cytokeratin 13	3.79	0.85	1.56	0.37	0.23
Transthyretin	0	0.05	0	0.05	0.49
Integral membrane protein 2B	1.39	1.19	1.09	1.59	0.67

[a] Sq = squamous epithelium
[b] BE = non-dysplastic Barrett's epithelium
[c] HGD = high-grade dysplasia
[d] EAC = esophageal adenocarcinoma
[e] Statistics were performed using the Mann-Whitney U-test. A p-value of < 0.05 was considered significant (*)

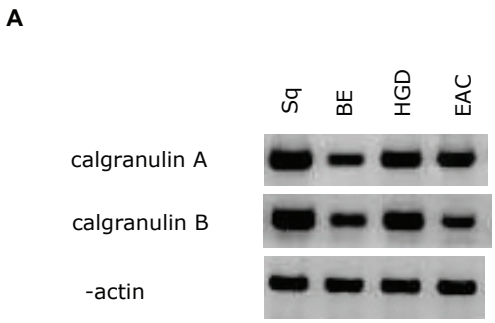
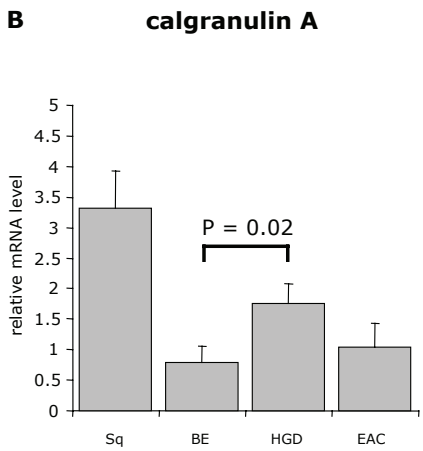
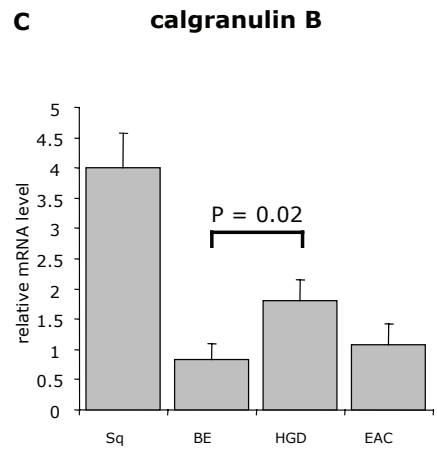


Figure 1 Representative RT-PCR results of calgranulin A and B on biopsies of squamous epithelium (Sq), non-dysplastic Barrett's esophagus (BE), high-grade dysplasia (HGD), and esophageal adenocarcinoma (EAC) from the same patient (A). Graphic representation of calgranulin A and B relative mRNA levels in Sq, BE, HGD, and EAC (B and C).

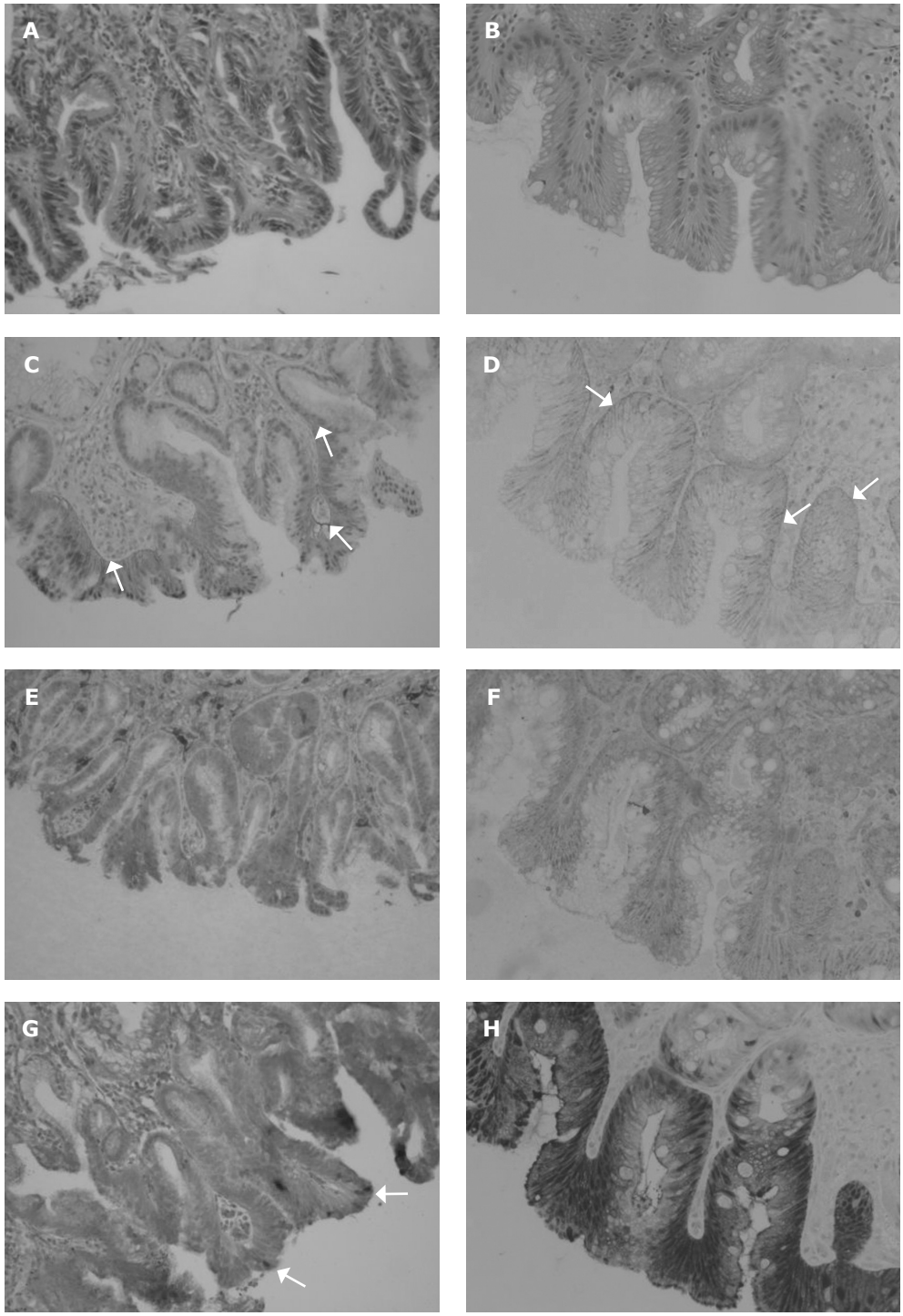




DISCUSSION

The identification of genes involved in the development of high-grade dysplasia in Barrett's esophagus can potentially help to increase our understanding of the development of early neoplastic lesions in Barrett's esophagus. In esophageal carcinogenesis, various proto-oncogenes, and tumor-suppressor genes have been studied and some of them, like p53, and MUC4 have indeed been implicated in the development of HGD [6, 10]. However, up to now, no genetic marker can be used to completely distinguish BE from HGD in 100% of patients [11]. This may indicate that there is probably not a single pathway involved in the development of HGD.

Figure 2 Haematoxylin and eosin stained control sections for high-grade dysplasia (HGD) (A) and non-dysplastic Barrett's esophagus (BE) (B). In HGD, calgranulin A is present in the entire cytoplasm (C, arrow), while in BE it was only observed on the basal site of the cell (D, arrow). Staining patterns for calgranulin B in HGD (E) and BE (F) resemble those of calgranulin A, except for an intense background staining probably due to aspecific staining of the polyclonal antibody. FABP1 is present in a small subset of cells in HGD (G, arrow), while it was observed in the entire top of the villus in BE (H). A color version of this figure is printed on page 117.


A
B
C
D
E
F
G
H

In this study differential gene expression analysis of a single rigidly selected sample was used to identify genes that have thus far not been implicated in the development of HGD. From this patient, paired biopsy samples containing either BE or HGD were obtained. The use of paired samples for transcriptional profiling analysis excludes interpatient variability thus generating more reliable data. As it proved impossible to obtain more large paired biopsy samples falling within our inclusion criteria within a reasonable time period we decided to test a selection of the most prominently differentially transcribed genes. Therefore two completely independent methods were used, i.e. semi-quantitative RT-PCR, and histology based comparison of protein expression and localization in a large set of biopsy samples, obtained from 41 Barrett's esophagus patients with or without dysplasia or adenocarcinoma.

The analysis of the transcription profiles of BE and HGD from this single patient revealed a 10-fold or greater difference in mRNA levels for 33 genes. To exclude false-positive results that are often generated by array techniques, mRNA levels of 22 of the identified genes were tested by semi-quantitative RT-PCR in paired samples of 23 patients, and by immunohistochemistry in 18 patients. Although the data (with regard to fold-increase) obtained by these two techniques can by no means be directly compared, one of the important findings was that the RT-PCR results revealed that calgranulin A and calgranulin B were significantly up-regulated in HGD compared with BE, and that none of the genes was significantly downregulated in HGD. For the 20 other tested genes, the same trends as in the microarray analysis were observed, however, the sample size was not big enough to achieve significant differences. Apart from increased mRNA levels, the immunohistochemical analysis revealed also an increase in cytoplasmic calgranulin A and B protein levels in HGD compared to BE.

Strikingly, both calgranulin A and calgranulin B exert most of their functions in a heterodimer, which is called calprotectin. They are members of the S100 family of calcium-binding proteins, and they are also known as S100A8 and S100A9, respectively. Calprotectin is mainly expressed in neutrophilic granulocytes and monocytes, where it is involved in cell trafficking to inflammation sites by influencing chemotaxis and endothelial adhesion, as are the undimerized subunits. Furthermore the complex is able to transport poly-unsaturated fatty acids such as arachidonic acid [12-14]. The expression of calgranulin A and B is however not limited to inflammatory cells, but it is also observed in keratinocytes and other epithelial cells such as squamous epithelium of the esophagus. In these tissue types, the dimer seems to have a role in wound healing [15, 16], thus linking this protein with proliferative activities.

In this study, calgranulin A and B expression was not only observed in neutrophilic granulocytes, but also in esophageal squamous cells and columnar cells of patients with Barrett's esophagus. Its presence in these cells may be caused by the presence of reactive oxygen species (ROS), since calgranulin A is induced by ROS and increased amounts of ROS have been demonstrated in esophageal squamous and columnar epithelium [17, 18]. Furthermore, calprotectin interacts with NADPH oxidase, which generates the superoxide anion, to transfer arachidonic

acid, a cofactor of this enzyme [19, 20].

Barrett's esophagus is not the only chronic inflammatory lesion in which calgranulin A and B are overexpressed. Increased amounts of the calprotectin complex have also been observed in Crohn's disease and the complex is currently used as diagnostic marker for the severity of inflammation [21]. Increased calgranulin expression has also been found in several malignancies, including gastric tumors, but its exact role in carcinogenesis is unknown [22, 23]. One explanation could be that in HGD increased amounts of ROS are present, resulting in an upregulation of calgranulin A. However, the fact that both subunits of the calprotectin heterodimer are upregulated in HGD and that the genes encoding calgranulin A and B are located in the same chromosomal region, suggest a common regulatory mechanism for both subunits. Since calgranulin A and B are not only expressed in neutrophilic granulocytes, but also in the columnar esophageal cells, the differences in expression could not only be due to an increase in influx of inflammatory cells, but also to elevated epithelial expression. Therefore we hypothesize that the calprotectin complex is involved in the proliferation of neoplastic cells, thus generating neoplastic growth in Barrett's esophagus. This hypothesis is supported by the proliferative role in wound healing [15, 16].

The identification of several differentially expressed proteins in this study demonstrate the value of new profiling techniques, such as microarray, in the elucidation of new molecular markers for early neoplastic lesions. Along with other differentially expressed proteins, such as p53, E-cadherin and MUC4 [6, 7, 10], the differentially expressed proteins may be part of an array-like diagnostic test, that can facilitate the detection and diagnosis of high-grade dysplasia. Such tests have already been developed for breast-cancer [9].

In conclusion, we have here reported on the differential gene expression between Barrett's esophagus without dysplasia and Barrett's esophagus with high-grade dysplasia. The fact that both subunits of the calprotectin complex, calgranulin A and B were elevated in HGD, suggests that calprotectin may play a role in the induction of proliferation of dysplastic columnar cells in Barrett's esophagus.

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Chapter 8

Differential, deoxycholic acid induced expression of chemokines in Barrett's esophagus and esophageal adenocarcinoma

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submitted

ABSTRACT

Barrett's esophagus is a premalignant condition that is characterized by chronic inflammation, initiated by gastroduodenal reflux. The inflammatory process is probably started and maintained by chemokines, which are chemotactic cytokines that control leukocyte trafficking during inflammation and neoplastic growth, and may attract neutrophils, T-cells, B-cells, and plasma-cells to the site of inflammation. In this study the effect of bile acids on the expression of key chemokines MCP-1 (CCL2), RANTES (CCL5), MIP-3α (CCL20), MEC (CCL28), and IL-8 (CXCL8) was determined in esophageal cell lines, as well as in 89 esophageal biopsies obtained from patients with reflux esophagitis, Barrett's esophagus and esophageal adenocarcinoma. Exposure of cultured epithelial cells (TE7 and OE21) to deoxycholic acid resulted in increased transcription and expression of MEC, MIP-3α, and IL-8, whereas transcription of MCP-1 and RANTES was not altered. In contrast, exposure to taurodeoxycholic acid did not result in increased chemokine mRNA levels. Similar transcriptional patterns were also found in biopsies obtained from patients with reflux esophagitis, Barrett's esophagus and esophageal adenocarcinoma. MCP-1 mRNA levels were increased in both reflux esophagitis and Barrett's esophagus, while MIP-3α and MEC mRNA levels were increased in Barrett's esophagus. MIP-3α mRNA levels increased with progressive histological stages of adenocarcinoma development. In conclusion, the expression of several key chemokines increases during the progression from metaplasia to dysplasia and adenocarcinoma, a process mimicked in vitro by incubation with deoxycholic acid. This suggests an important role for biliary reflux in the initiation and maintainance of the inflammatory response in Barrett's esophagus and esophageal adenocarcinoma.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition that is characterized by the replacement of the normal squamous epithelium of the lower oesophagus by specialized columnar epithelium of the intestinal type. Esophageal adenocarcinoma (EAC) is thought to develop in BE in a stepwise manner via low-grade dysplasia (LGD) and high-grade dysplasia (HGD) [1]. Gastroesophageal reflux is believed to play an important role in causing BE [2]. Upon exposure to the refluxate, the normal squamous mucosa of the esophagus will become inflamed, which is called reflux esophagitis (RE). In ten percent of these patients, the inflamed squamous epithelium will be replaced by columnar epithelium of the intestinal type, which is inflamed as well [3].

Epithelial cells play an important role in inducing an inflammatory response. The exposure of the esophageal mucosa to the refluxate can result in the epithelial production of inflammatory mediators that promote the influx of leukocyte subsets, thus inducing an inflammatory response. The specific chemokines that are secreted by the epithelial cells regulate the composition of the leukocyte influx by binding to the specific chemokine receptors on the cell surface of the leukocytes thereby promoting chemotaxis. Leukocytes that are abundantly present in the Barrett's epithelium are neutrophils, T-cells, and plasma-cells (Moons et al. in press). The composition of the secreted chemokine pool is often a good marker for monitoring and studying the inflammatory process in chronic inflammatory lesions, such as inflammatory bowel disease and chronic hepatitis C infection [4, 5]. Examples of key chemokines are MCP-1 (CCL2) and RANTES (CCL5), which both attract monocytes and T-cells, MIP-3α (CCL20), which attracts dendritic cells and activated B-cells; MEC (CCL28), which attracts plasmacells, and IL-8 (CXCL8), which mainly attracts neutrophils [6].

It is generally accepted that the main cause of inflammation in the esophagus is gastroesophageal reflux. The major components of the gastroduodenal refluxate are gastric acid and bile acids, which are irritants for the esophageal mucosa. Bile and gastric acid were demonstrated to have proliferative and anti-apoptotic effects on the esophageal mucosa *in vitro* and *ex vivo* [7, 8]. Of the bile acids, deoxycholic acid (DCA) and its conjugated forms are principal components of the gastroduodenal refluxate in patients with BE [9]. It has been reported that DCA is involved in the upregulation of inflammatory proteins such as cyclooxygenase-2 and IL-8 in BE [10, 11]. Since DCA is involved in reflux-induced cell irritation, and this irritation leads to the attraction of inflammatory cells, we hypothesize that deoxycholic acid could induce the expression of several chemokines.

Elevated levels of chemokines MCP-1, RANTES and IL-8 have been observed in RE compared to normal squamous epithelium of the esophagus [12], but despite of their crucial role in the induction and maintenance of the local immune response, the chemokine expression pattern in BE, or EAC has to our knowledge not been reported thus far.

The aim of this study was to test the hypothesis that bile acids drive the expression of key chemokines MCP-1, RANTES, MIP-3α, MEC, and IL-8, and to test

whether these chemokines are differentially expressed over the esophagitis-metaplasia-dysplasia-carcinoma sequence.

MATERIALS AND METHODS

Patients

A total of 89 biopsies from 50 patients with RE, BE without dysplasia, dysplasia or EAC were included. Patient and sample characteristics are outlined in Table 1. Endoscopic patient evaluation, and biopsy collection and histopathologic assessment were according to routine standard procedures. Biopsies were obtained from the normal squamous epithelium (Sq), RE, BE, and, when present, from LGD, HGD, or EAC. Adjacent biopsies were analyzed by an experienced GI pathologist, who evaluated haematoxylin and eosin stained sections for the presence of intestinal metaplasia, grade of dysplasia, or EAC. This study was approved by the local ethical review board of the Erasmus MC - University Medical Center Rotterdam and informed consent was obtained from patients prior to endoscopy.

Table 1. patient characteristics

patientgroups	patients	Mean age (range)	% male	Biopsies obtained					
				Sq[a]	RE[b]	BE[c]	LGD[d]	HGD[e]	EAC[f]
RE	11	52.8 (37-68)	64	0	11	0	0	0	0
BE	12	60.9 (42-85)	67	12	0	12	0	0	0
dysplasia	11	67.6 (53-79)	82	2	0	0	6	8	0
EAC	16	64.1 (44-79)	69	7	0	5	4	6	16
Total	50			21	11	17	10	14	16

[a] Sq: Normal squamous epithelium of the esophagus
[b] RE Reflux esophagitis
[c] BE: Non-dysplastic Barrett's esophagus
[d] Barrett's esophagus with low-grade dysplasia
[e] Barrett's esophagus with high-grade dysplasia
[f] EAC: Esophageal adenocarcinoma

Semi-quantitative RT-PCR

Total RNA was isolated from Sq, RE, ND, LGD, HGD and EAC biopsies using Trizol-reagent (Invitrogen, Paisley, UK) and remaining traces of chromosomal DNA were eliminated using the DNA-free RNA kit (Zymo, Orange, CA, USA) [13]. cDNA was synthesized with the use of Avian Myeloma Virus reverse transcriptase according to the instructions of the manufacturer (Promega, Madison, WI, USA), by incubating 10 ng total RNA with reverse trancriptase for 30 min at 42°C. PCR-reactions (total volume of 25 µl) contained 1 µl of the cDNA solution, 1x PCR buffer (Roche Diagnostics, Mannheim, Germany), 2 mM magnesium chloride, 0.4 µM forward and reverse primer, 200 µM of each nucleotide (Roche Diagnostics) and 0.02 U/µl

Table 2. PCR-Primers

Gene	Forward primer	Reverse Primer	T [a]
MCP-1	5'TCCTGTGCCTGCTGCTCATAG3'	5'TTCTGAACCCACTTCTGCTTG3'	50°C
RANTES	5'ATGAAGGTCTCCGCGGCA3'	5'CCTAGCTCATCTCCAAAGAGTTG3'	55°C
MIP-3α	5'ATGTCAGTGCTGCTACTC3'	5'TGTCACAGCCTTCATTGG3'	50°C
MEC	5'AGAAGCCATACTTCCCATTGC3'	5'AGCTTGCACTTTCATCCACTG3'	50°C
IL-8	5'GTGGCTCTCTTGGCAGCCTTCTGAT3'	5'TCTCCACAACCCTCTGCACCCAGTTT3'	55°C
β-actin	5'GACAGGATGCAGAAGGAGAT3'	5'AGTCATAGTCCGCCTAGAAG3'	55°C

[a] Temperature used for annealing of this primers

Taq polymerase (Roche Diagnostics). All primers were designed with aid of primer designer software (Clone Manager, version 5.10, Scientific and Educational software) using sequences from the NCBI database (www.ncbi.nlm.nih.gov), and are listed in Table 2. PCR conditions were 35 cycles at 94°C (30 s); 50-55°C (30 s) and 72°C (1 min). PCR-products were visualized on a 2% agarose gel and stained with ethidiumbromide. Band size and intensity were determined by densitometry with Kodak 1D version 3.5 software (Kodak, New Haven, CT, USA) and data were normalized using the housekeeping gene β-actin, as described previously [14]. RT-PCR densitometric data are presented as mean ± standard error of the mean. Statistical analyses were performed using SPSS software (version 11.0, SPSS Inc), using the Mann-Whitney U-test. A p-value < 0.05 was considered statistically significant.

Cell culture

The esophageal adenocarcinoma cell-line TE7 [15], and the esophageal squamous cell carcinoma cell-line OE21 [16] were kindly provided by dr. George Triadafilopoulos, Stanford University, Palo Alto, CA, USA. These cell-lines are internationally accepted and widely used tools for representation of the esophagus epithelium. Both cell-lines were cultured in RPMI 1640 with L-glutamine (Bio Whittaker, Verviers, Belgium) supplemented with 10% Fetal Calf Serum (Hyclone, Logan, UT, USA), and 20 units/ml penicillin/streptomycin. Cells were maintained as a subconfluent monolayer in a 75 cm^2 tissue culture flask in a humidified incubator with 5% CO_2 at 37°C.

RNA analysis of cells incubated with bile acids and acid

Cells were seeded at 0.4 x 10^6 cells/well in a six-well plate and incubated for 24h at 37°C. Subsequently, cells were incubated for 20 minutes with RPMI medium containing 10% fetal calf serum, L-glutamine and penicillin/streptomycin that was supplemented with 0.2 mM taurodeoxycholic acid (TDCA), 0.2 mM DCA, either at neutral pH or pH5.5, or control medium. Cells were harvested with a trypsin/EDTA solution (Gibco, Paisley, UK), cells were washed twice in control medium, and lysed in Trizol. RNA isolation and RT-PCR was performed as described above. Cell viability was determined by staining with tryphan blue. Only experiments with a

cell viability of 90% or more at the end of the experiment were included in the analyses. For both OE21 and TE7 cells three independent experiments were performed.

Chemokine protein level determination
TE7 cells were seeded at 0.4 x 10^6 cells/well in a six-well plate and incubated for 24h at 37°C. Subsequently, cells were incubated for 32 hours with RPMI medium containing 10% fetal calf serum, L-glutamine and penicillin/streptomycin that was supplemented with 0.2 mM DCA, or control medium. Aliquots of the culture medium were taken at t=0, t=1, t=2, t=4, t=8, t=12, t=24, and t=32 hours and were frozen until chemokine concentration determination. Protein concentrations of IL-8 and MIP-3α were determined by using standardized ELISAs for IL-8 (Biosource international, Nivelles, Belgium), and MIP-3α (R&D systems, Minneapolis, MN), according to the instructions of the manufacturer.

RESULTS

DCA induces chemokine transcription and expression in cell-lines
Gastroesophageal reflux contains both gastric acid and bile acids. To determine the effect of bile acids on chemokine transcription in the esophagus, an internationally well-accepted esophageal cell culture model was used. TE7 adenocarcinoma cells and OE21 squamous cell carcinoma cells were exposed to tissue culture medium supplemented with TDCA or DCA at predetermined physiological concentrations, or unsupplemented control medium, at a neutral pH. The bile acid concentrations and incubation times were deduced from the *in vivo* conditions during reflux episodes. Lower concentrations and shorter incubation times resulted in a less pronounced effect, while higher concentrations affected the cell viability. MCP-1 mRNA levels were below the detection limit in OE21 and TE7 cells. RANTES transcription was detected in OE21 cells, but not in TE7 cells, however TDCA and DCA did not induce RANTES mRNA levels. In contrast, MIP-3α, MEC,

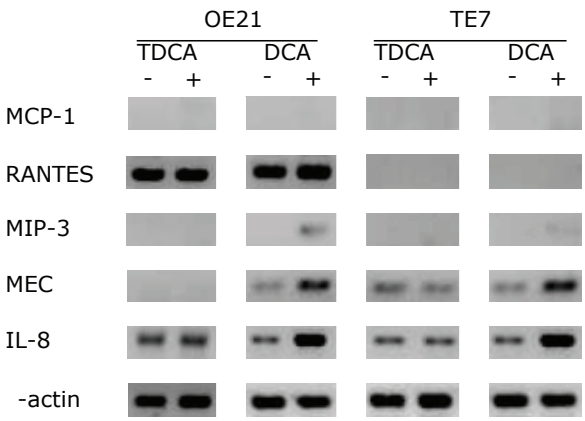

Figure 1 Representative RT-PCR results of bile acid assays. OE21 and TE7 cells incubated with 0.2 mM taurodeoxycholic acid (TDCA), 0.2 mM deoxycholic acid (DCA), or control medium for 20 min.

and IL-8 transcription was induced by DCA in both OE21 and TE7 cells. Incubation with equal concentrations of TDCA did not induce transcription of any of the tested chemokines (Figure 1).

Incubation of TE7 cells with DCA resulted also in increased expression of IL-8 and MIP-3α protein (Figure 2). After 4 hours of incubation, IL-8 production, and after 8 hours of incubation MIP-3α production of cells incubated with DCA was significant elevated compared to cells incubated with control medium ($p = 0.05$).

To determine the effect of pH, TE7 cells were cultured at pH7.4 and pH5.5 in the

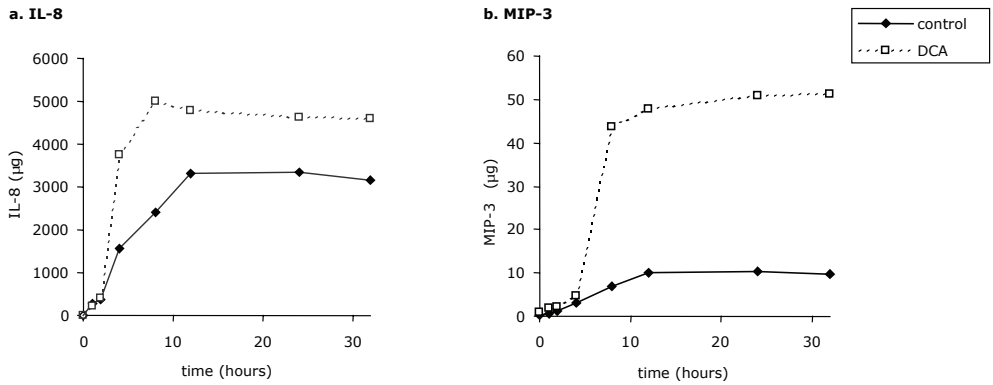

Figure 2 Graphic representation of A IL-8 and B MIP-3α production in culture supernatant of TE7 cells incubated with DCA (dotted line) or control medium (continuous line) at different time points during 32 hour of incubation.

presence and absence of DCA. Increased transcription of MEC was detected in cells cultured at pH7.4 with DCA and at pH5.5 without DCA. Surprisingly, DCA was not able to induce MEC transcription at pH5.5 (Figure 3). A pH lower than pH5.5 affected the cell viability.

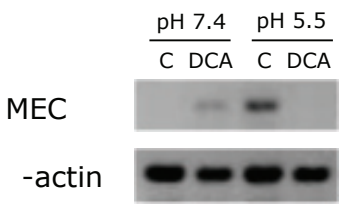

Figure 3 MEC transcription in TE7 cells incubated with either acid (pH5.5) and DCA alone, a combination of acid (pH 5.5) and bile, or control medium (C).

Chemokine mRNA levels in biopsies

To investigate whether the changes in chemokine transcription *in vitro* were matched *in vivo*, chemokine mRNA levels were determined in biopsies of the various esophageal disease stages. MCP-1 mRNA levels were significantly increased in RE ($p = 0.007$) and BE ($p = 0.001$) compared to Sq and levels were even more increased in EAC (Figure 4a). RANTES mRNA levels were not different between the

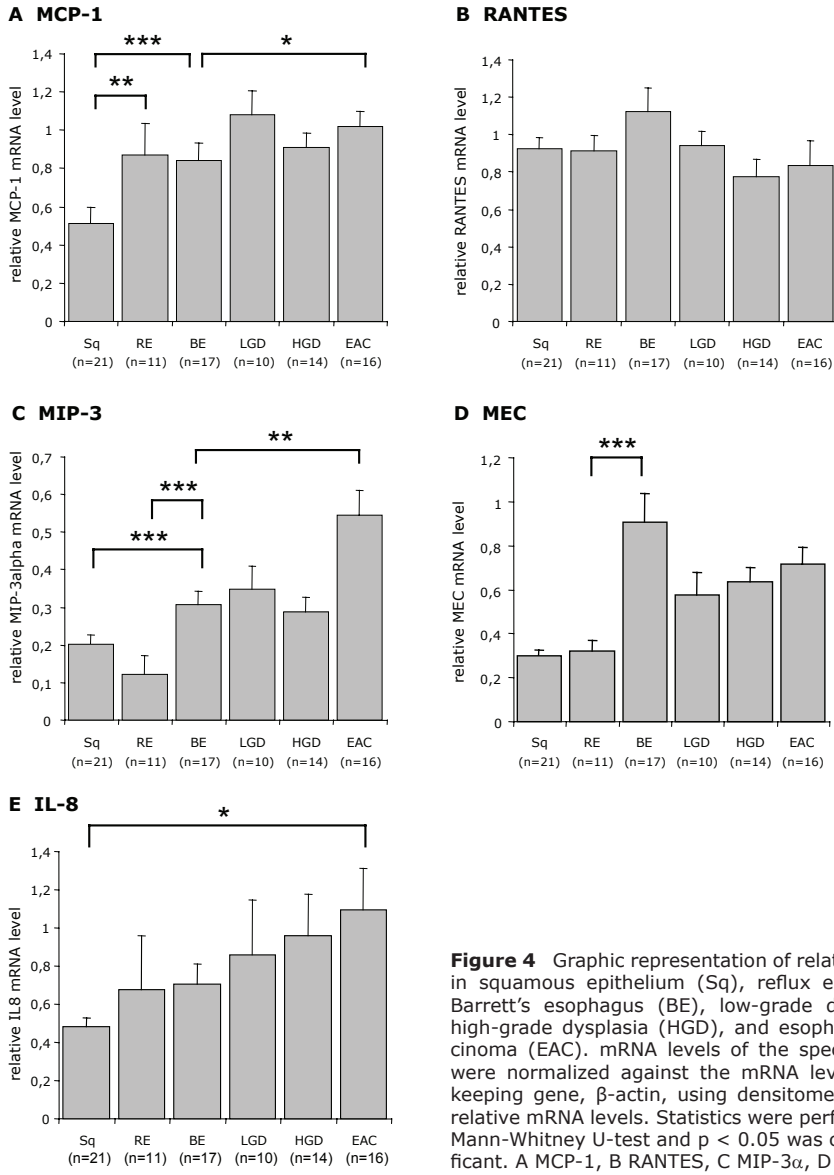

Figure 4 Graphic representation of relative mRNA levels in squamous epithelium (Sq), reflux esophagitis (RE), Barrett's esophagus (BE), low-grade dysplasia (LGD), high-grade dysplasia (HGD), and esophageal adenocarcinoma (EAC). mRNA levels of the specific chemokines were normalized against the mRNA levels of a housekeeping gene, β-actin, using densitometry, which gains relative mRNA levels. Statistics were performed using the Mann-Whitney U-test and $p < 0.05$ was considered significant. A MCP-1, B RANTES, C MIP-3α, D MEC, and E IL-8

several stages (Figure 4b), but MIP-3α was increased in BE compared to Sq and RE ($p < 0.001$) and increased even more in EAC ($p = 0.007$) (Figure 4c). MEC was significantly increased in BE compared to RE ($p < 0.001$). In contrast, in LGD, HGD, and EAC, MEC levels were decreased compared to BE, and increased compared to Sq and RE (Figure 4d). IL-8 was gradually increased along the Sq – BE – LGD – HGD – EAC sequence, but due to the large variation in the groups, only mRNA levels in EAC were significantly increased when compared to Sq (Figure 4e).

DISCUSSION

The chronic inflammation in BE and RE is most likely initiated and maintained by the exposure to the gastroduodenal refluxate, containing mainly gastric acid and bile. In this study an *in vitro* cell culture model was used to determine the effect of the secondary bile acid DCA and its taurine conjugate on chemokine transcription. It was observed that DCA, but not TDCA, induced the transcription and expression of IL-8, MIP-3α, and MEC, in both an esophageal squamous carcinoma cell-line and an adenocarcinoma cell-line. MCP-1 and RANTES were not induced by this bile acid. MCP-1 and RANTES are not secreted by all types of epithelial cells, which could explain the failure to detect RANTES in one and MCP-1 in both cell-lines. The presence of these chemokines in RE and BE could be explained by their secretion by inflammatory cells.

DCA is one of the most toxic components of the bile refluxate. It is generally believed that DCA is involved in carcinogenesis, since it can induce proliferation in animal models and since increased serum levels were observed in patients with colorectal cancer [17, 18]. DCA is able to induce DNA damage in esophageal cells [19]. DCA and TDCA have different effects on the transcriptionfactor NF-κB, which is probably involved in the transcription of these chemokines. It has been reported that DCA can activate NF-κB by degradation of one of its subunits, while TDCA acts mainly by phosphorylation of the protein [20]. This can explain the differences between these bile acids in the ability to induce chemokine transcription. Another explanation for this difference can be that TDCA is able to penetrate the membrane, while DCA may act on a membrane bound receptor, probably the epidermal growth factor receptor [21], thereby initiating a signalling cascade that probably leads to the transcription of chemokines.

The effect of acid (pH5.5) was the same as that of DCA. However, the combination of acid and bile failed to induce transcription of the chemokine MEC. An antagonistic effect of acid on induction of inflammatory mediators such as prostaglandins and COX2 has been reported before [7, 22]. An explanation for this phenomenon could be that a lower pH alters the binding capacity of DCA to a membrane receptor through which it exerts its effect.

Although the cell-line model with immortalized cancer cells is a limited representation of the real RE and BE, these results imply that DCA plays a key role in the induction of an immune response in BE and RE. Moreover, they demonstrate the important role of epithelial cells in the recruitment of the immune system.

To study the role of chemokines MCP-1, RANTES, MIP-3α, MEC, and IL-8 *in vivo*, their transcription was determined in different esophageal disease stages of the development of RE, BE and EAC. It was observed that mRNA levels of MCP-1, MIP-3α, MEC, and IL-8 were elevated in BE compared to normal squamous epithelium. These chemokines attract a subset of inflammatory cells that are abundantly present in BE, while the normal squamous epithelium is not chronically inflamed. MIP-3α and MEC are also significantly elevated in BE compared to RE.

Although RE and BE are both chronic inflammatory lesions, the inflammatory respons is slightly different. Reflux esophagitis is characterized by the expression of

proinflammatory cytokines such as IL-1β and IFNγ, a T-helper 1 (Th1) response, while in BE anti-inflammatory cytokines IL-4 and IL-10 are induced, which is a Th2 profile [23]. The differences in chemokine levels may lead to an altered T-helper response. For instance, the the increase in MCP-1 may lead to Th2 skewing [24], thereby stimulating the humoral immune response, while Th1 cells, which are attracted by RANTES, are not increasingly recruited.

Chronic inflammation, and in particular the innate immune response has also an important role in cancer development [25]. Cells of the innate immune response, such as monocytes, macrophages and neutrophils infiltrate in tumor tissues where they can be involved in extracellular matrix degradation by the upregulation of matrix metalloproteinases [26]. Chemokines, i.e. MIP-3α and IL-8, can trigger the release of these compounds from the immune cells, suggesting an active role in angiogenesis, tumor growth and metastasis [27, 28] [29] [30]. In this study this was illustrated by the increased MIP-3α and IL-8 mRNA levels in EAC. Other tumor-promoting cells that are part of the innate immune response are tumor-associated dendritic cells. These cells are defective in their ability to stimulate anti-tumor T-cells. It has been reported that MIP-3α is involved in the attraction of these dendritic cells [31].

Recently it has been shown that the adaptive immune system has also a role in carcinogenesis. It has been reported that the humoral immune response has an important role in tumor formation in chronically inflamed tissue [32]. The main cells in the humoral immune response are B-cells that are activated and proliferate to antibody secreting plasmacells. These cells are attracted by MIP-3α and MEC. Increased levels of these chemokines can lead to increased influx of B-cells and plasmacells and the antibodies they produce into the tumor environment, thereby promoting tumor formation.

MIP-3α seems to have many roles in carcinogenesis. EAC is not the only tumor type in which increased levels of this chemokine has been demonstrated. Elevated levels of MIP-3α has also been reported in pancreatic and hepatocellular carcinoma [33, 34]. Moreover, MIP-3α was observed in other premalignant chronic inflammatory lesions such as inflammatory bowel disease and chronic hepatitis [35, 36].

Chemokines have only recently been discovered as chemotactic agents involved in inflammation and tumorigenesis, but are already considered promising targets for therapeutic intervention. Analogous to the anti-cytokine based therapies that are currently under investigation for chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis, antibody based therapies against chemokines, such as IL-8 are under development and have promising results in preliminary pilot studies [37]. Anti-IL-8 based therapy does not only have anti-inflammatory effects, but it induces also tumor regression in mice [38]. The current study shows that the targets for anti-chemokine therapy are present and that in the future this therapy may have perspectives for the prevention and treatment of BE and EAC. Furthermore, non-steroidal anti-inflammatory drugs have been shown to protect against tumor development in chronically inflamed tissue [39]. These drugs have also been shown to protect against EAC [40].

In conclusion, the expression of several key chemokines increases during the progression from metaplasia to dysplasia and adenocarcinoma, a process mimicked in vitro by incubation with deoxycholic acid. This suggests an important role for biliary reflux in the initiation and maintainance of the inflammatory response in Barrett's esophagus and esophageal adenocarcinoma.

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Chapter 9

Summary and Conclusions

Barrett's esophagus is a common disorder in the western world. It is generally believed that at least 1% of the inhabitants of the western world suffer from Barrett's esophagus, but its incidence may well be 5% [1]. Esophageal adenocarcinoma is thought to develop from Barrett's esophagus in a step-wise manner, via low-grade and high-grade dysplasia [2]. Gastroesophageal reflux is generally believed to be the major cause of Barrett's esophagus. The reflux components, mainly gastric acid and bile acids, are harmful to the epithelium and it will become irritated. As a reaction on this irritation the normal squamous epithelium will be replaced by columnar epithelium that resembles the intestinal mucosa in a subset of patients. This epithelium, called Barrett's esophagus, is thought to be better adapted to exposure to bile and acid, but it is not completely insensitive to its action, as acid and bile apparently still induce the release of inflammatory mediators such as cytokines and chemokines from the epithelial cells, leading to the attraction of inflammatory cells. The continuing reflux insults establish the permanent presence of these inflammatory cells that results in chronic inflammation.

The function of the inflammatory cells is to remove the cells that are lethally damaged. One of the manners in which the damaged cells are killed by inflammatory cells is by the secretion of reactive oxygen species. These compounds, mostly radicals, will cause DNA damage that sends the damaged cells into apoptosis. However, a few cells will survive the reflux irritation and damage by immune cells. The DNA damage caused by reactive oxygen species can cause mutations in genes involved in several processes that gives the cell a growth advantage above other cells and (pre)neoplastic lesions will appear. The literature that underlies this theory and that describes the processes that will cause esophageal adenocarcinoma is reviewed in **chapter 2**.

THE DEVELOPMENT OF BARRETT'S ESOPHAGUS

Barrett's esophagus is characterized by the presence of intestinal metaplasia, a type of metaplasia known to often progress to esophageal adenocarcinoma [3]. Barrett's metaplasia resembles intestinal epithelium, both on the histological and molecular level. CDX2 is a homeobox protein that is specifically expressed in intestinal tissues and is involved in intestinal differentiation [4]. CDX2 is thus involved in the development of intestinal epithelium. Barrett's esophagus resembles the intestinal mucosa, and therefore we hypothesized that CDX2 expression was present in Barrett's esophagus. This hypothesis was proved in **chapter 3**. In this chapter, CDX2 was not only observed in intestinal metaplasia, but also in the squamous epithelium of patients with Barrett's esophagus, albeit in low amounts. This important finding indicates that CDX2 can mark cells that are differentiating to an intestinal phenotype, but do not yet have other intestinal characteristics.

This theory was investigated further in **chapter 4** and **chapter 5**, in which intestinal metaplasia was compared to another type of columnar epithelium that is frequently observed in the esophagus, gastric-type mucosa. This gastric-type mucosa has been regarded as not related to intestinal metaplasia and not meta-

plastic. CDX2 expression was again observed in intestinal metaplasia, but also in gastric-type mucosa adjacent to it and even in one third of samples that contained only gastric-type mucosa and no intestinal metaplasia. Since this was mainly observed in patients that had small segments of intestinal metaplasia, and large segments of gastric-type mucosa, we suppose that in these patients a hybrid epithelium is present that has characteristics of both intestinal and gastric-type mucosa. This supports the theory that intestinal metaplasia and gastric-type mucosa are related to each other.

Mucins are glycoproteins that protect the epithelial cells from mechanical and chemical damage. Every mucosa-type has a unique pattern of mucin expression. The expression patterns of the main mucins in Barrett's esophagus are described in **chapter 6**. Mucins that are secreted and form a gel-like mucus layer are normally expressed in the stomach and intestine. This mucus layer protects the epithelium against the chemical damage of enzymes that are secreted to digest the food particles. These mucins, MUC2, MUC5AC, and MUC6, were also observed in Barrett's esophagus. On the other hand, mucins that are membrane bound and form a protective layer on the cell-surface, MUC1 and MUC4, are expressed in the normal esophageal squamous epithelium, but were absent in Barrett's esophagus. These results show that Barrett's epithelium has been adapted to the harmful effect of reflux components.

The effect of one of the principal components of reflux, bile, on the Barrett's epithelium was investigated in **chapter 8.** In an esophageal cell-line model cells were exposed to the bile acid deoxycholic acid. Exposure to deoxycholic acid, one of the principal and most toxic components of reflux, resulted in the enhanced expression of the chemokines MIP-3α, MEC, and IL-8. Chemokines are chemoattractive cytokines and MIP-3α, MEC, and IL-8 attract a subset of inflammatory cells that have been observed in Barrett's esophagus (Moons et al. in press). For instance, IL-8 attracts neutrophils, and these cells are potent producers of reactive oxygen species. This bile induced chemokine production illustrate that the irritation caused by reflux leads to the attraction of inflammatory cells, and the reactive oxygen species that they produce can induce further damage.

THE DEVELOPMENT OF DYSPLASIA AND ADENOCARCINOMA IN BARRETT'S ESOPHAGUS

Barrett's epithelium is metaplastic, which means that it consists of cells that are newly formed at a place where they do not belong. Furthermore these cells have to be replaced frequently due to the reflux induced irritation. Taken these two things together this means that this epithelium has a higher proliferative capacity than normal esophageal epithelium. Genetic abnormalities can be induced by the reactive oxygen species produced by inflammatory cells, and cells that carry this abnormalities are allowed to grow out, and a more active proliferating, mutation bearing epithelium appears. Histologically, this epithelium is also slightly different from the normal Barrett's epithelium, and therefore it is called dysplasia, subdi-

vided in low-grade dysplasia and high-grade dysplasia. The development of esophageal adenocarcinoma in Barrett's esophagus is often preceded by low-grade and high-grade dysplasia [2]. High-grade dysplasia seems a point of no return, as half of the patients will develop esophageal adenocarcinoma [5].

Mucin expression often changes during neoplastic progression. The mucin pattern in high-grade dysplasia is described in **chapter 6.** Increased MUC4 expression was observed in high-grade dysplasia and also in esophageal adenocarcinoma. MUC4 is normally expressed in esophageal squamous epithelium. Its overexpression in high-grade dysplasia and adenocarcinoma indicates that the epithelium is dedifferentiating, a process that is often observed during carcinogenesis. Overexpressed MUC4 has also several oncogenic properties. It may enhance tumorigenesis by masking tumor-antigens and promoting cellular detachment. It should also inhibit apoptosis, but this was not observed in this chapter, since it was associated with a pro-apoptotic balance of apoptosis proteins Bax and Bcl-2. Increased apoptosis is not a feature of carcinogenesis, however, high-grade dysplasia is still not completely derailed and by increasing apoptotic rates it may try to compensate for the other tumor-promoting changes. In adenocarcinoma the bax/bcl-2 balance shifted to a more anti-apoptotic phenotype.

Calgranulin A and B are also associated with high-grade dysplasia development, which is described in **chapter 7**. These proteins form together the calprotectin complex, that is a involved in the attraction of neutrophils that are, as stated before, potent producers of reactive oxygen species. These can cause more tumor-promoting mutations so that adenocarcinoma can develop. Moreover, calprotectin is also expressed in normal squamous epithelium, where it functions in proliferation. This supports the concept of dedifferentiation in high-grade dysplasia and may implicate that overexpressed calprotectin increases the proliferation in high-grade dysplasia.

The presence of tumor-promoting mutations in high-grade dysplasia can itself lead to more mutations, for instance by defects in DNA repair genes. This leads to the development of esophageal adenocarcinoma, a rapid growing and quickly metastasizing type of cancer. Chemokines can increase the metastatic potential of cells. MIP-3α and MEC induce the expression of matrix metalloproteinases, enzymes that degrade the extracellular matrix, by inflammatory cells. This enables the cancer cells to migrate to lymph or blood vessels and metastasize. In **chapter 8** we tested chemokine expression in adenocarcinoma samples, and increased MIP-3α and MEC levels were observed in esophageal adenocarcinoma. This indicates that in addition to a role in chemotaxis of inflammatory cells these chemokines may also function in promoting metastasis.

CLINICAL IMPLICATIONS

Understanding the role of inflammation and the molecular alterations that occur during development of Barrett's esophagus will provide new insights for the prevention and treatment of this lesion. Moreover, the molecular changes that mark

the presence of dysplasia and adenocarcinoma can be used to diagnose the presence of malignant cells.

Inflammation is a target for many drugs, such as NSAIDs and steroids. It is also an aria in which many new drugs are under development. The identification of inflammatory mediators that play a role in the pathogenesis of Barrett's esophagus provide new targets for anti-inflammatory drugs. Commonly used drugs such as NSAIDs interfere with inflammatory pathways thereby decreasing inflammation. With the elucidation of the inflammatory profile of Barrett's esophagus, targets for commonly used inflammatory compounds will be identified. This opens new perspectives for the use of these drugs in the prevention of adenocarcinoma and treatment of Barrett's esophagus.

In a segment of columnar epithelium in the esophagus the distribution of intestinal metaplasia is usually patchy. Therefore, regions with intestinal metaplasia are often missed, which is called sampling error. Since CDX2 marks the presence of hybrid epithelium that is associated with the presence of intestinal metaplasia, CDX2 could function as a marker for intestinal differentiation during routine histology, to reduce sampling error.

Although Barrett's esophagus can be easily distinguished from normal esophageal mucosa, both on the endoscopical and histological level, the diagnosis of

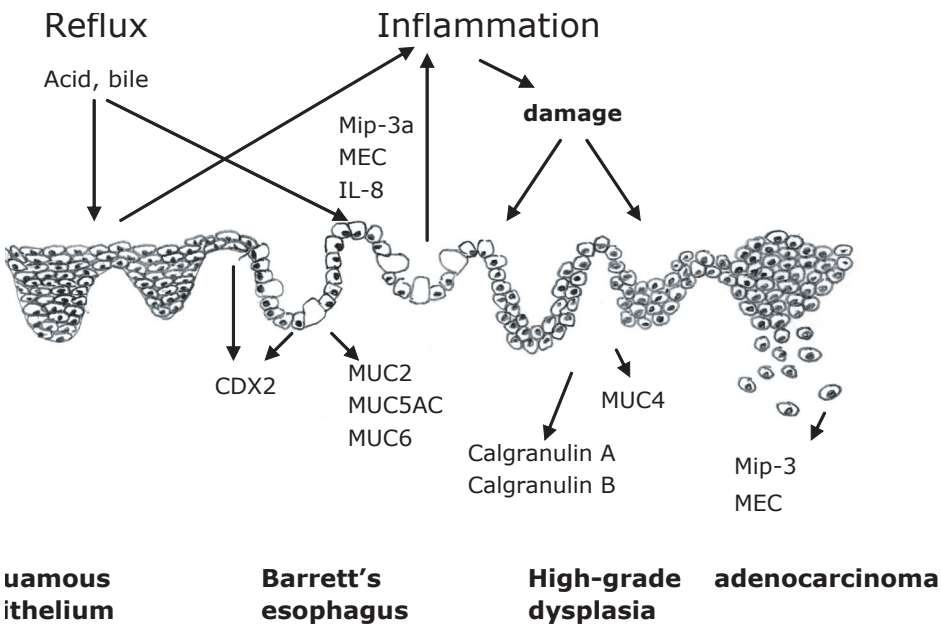

Figure 1 The development of Barrett's esophagus, dysplasia, and esophageal adenocarcinoma from squamous epithelium- and the molecular alterations described in this thesis that occur during this sequence. The components of the gastro-esophageal refluxate damage the epithelial cells, that will release inflammatory mediators, such as MIP-3α, MEC, and IL-8. These compounds attract inflammatory cells that will cause DNA damage in the epithelium. Some mutations will cause growth advantage and these cells will grow out to dysplasia, which can eventually progress to esophageal adenocarcinoma.

dysplasia is more complex. It is difficult to distinguish high-grade dysplasia from low-grade dysplasia and non-dysplastic Barrett's esophagus. Along with other findings, MUC4 and calgranulin A and B may function as molecular marker in the early detection of high-grade dysplasia and esophageal adenocarcinoma. Furthermore, insights in the processes that regulate the development of high-grade dysplasia can provide tools for the prevention of this process.

CONCLUDING REMARKS

The irritation of the esophageal epithelium by gastroesophageal reflux results in chronic inflammation and causes the induction of molecular and cellular alterations. Figure 1 gives a schematic overview of the molecular events that are characterized in this thesis. These events represent only a small proportion of all molecular changes that occur, some of them are described and many other have not yet been discovered. Despite the small proportion of alterations described in this thesis, the results that are described provide new insights in the development of Barrett's esophagus, dysplasia, and adenocarcinoma. Moreover, all these results will have clinical implications for the diagnosis, prevention, and treatment of these lesions.

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Appendix

CHAPTER 3

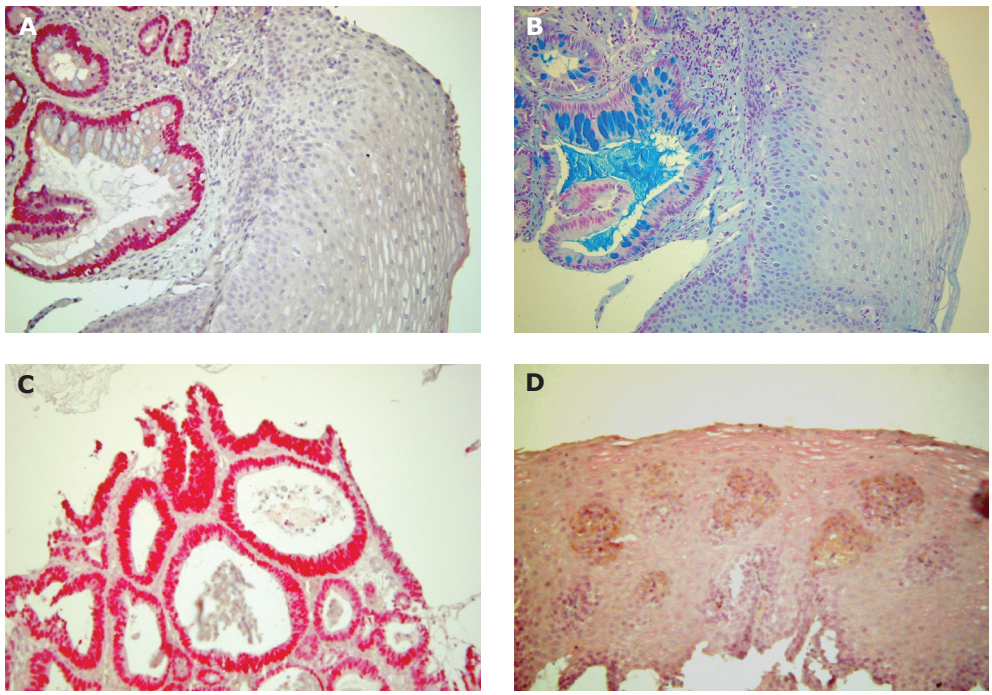


Figure 1 CDX2 protein in Barrett's esophagus, esophageal adenocarcinoma, and reflux esophagitis. (A) A positive nuclear stain (red) for CDX2 was observed in 16/16 samples of Barrett's esophagus. A representative slide of patient 10 is shown. (B) The presence of CDX2 was associated with goblet cells, 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).

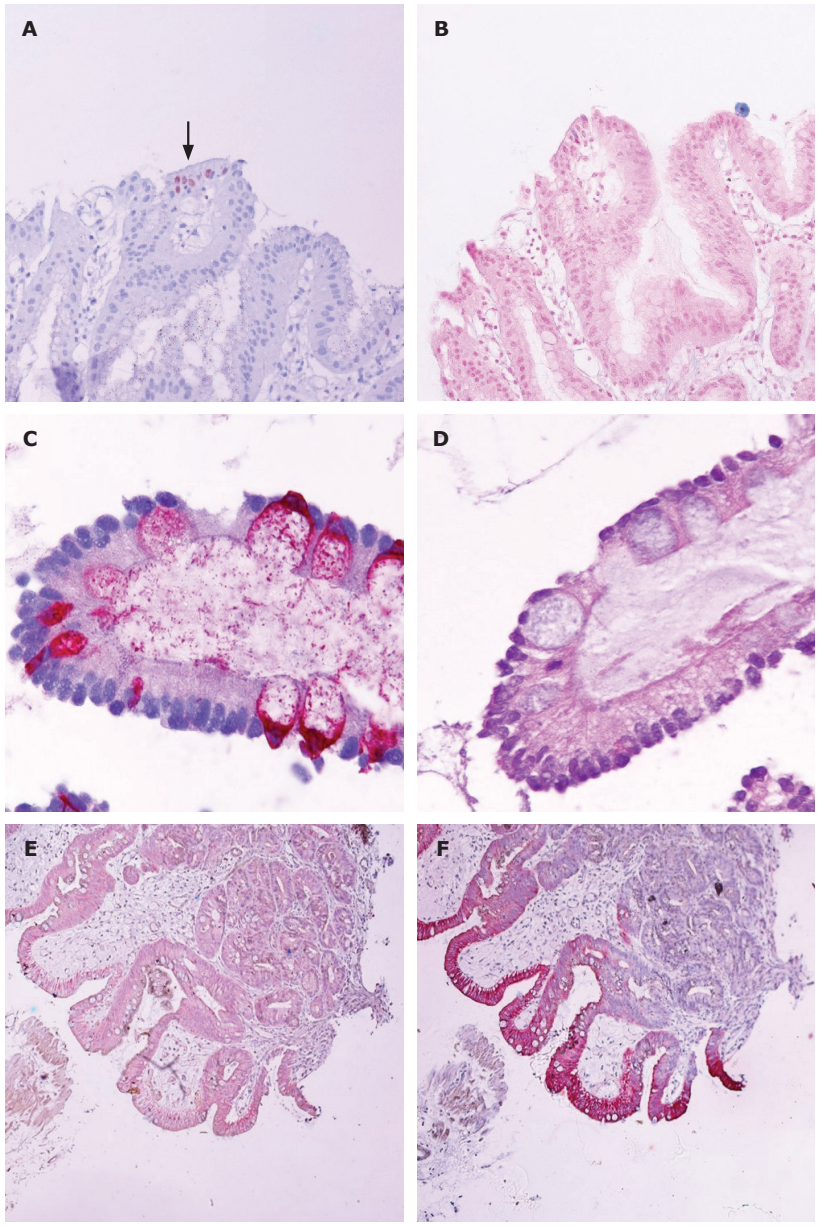


Figure 1 Histological and immunohistochemical detection of intestinal characteristics. **A** Nuclear CDX2 expression in gastric-type glands at a distance of IM (arrow)(200x magnification). **B** The same region. Alcian Blue staining in adjacent gastric-type glands does not detect goblet cells (200x magnification). **C** cytoplasmic MUC2 staining in goblet cells (400x magnification). **D** Haematoxylin and Eosin staining in the same region (400x magnification). **E** Diffuse cytokeratin 7 staining in IM (100x magnification). **F** Superficial cytokeratin 20 staining in the same region (100 x magnification).

CHAPTER 5

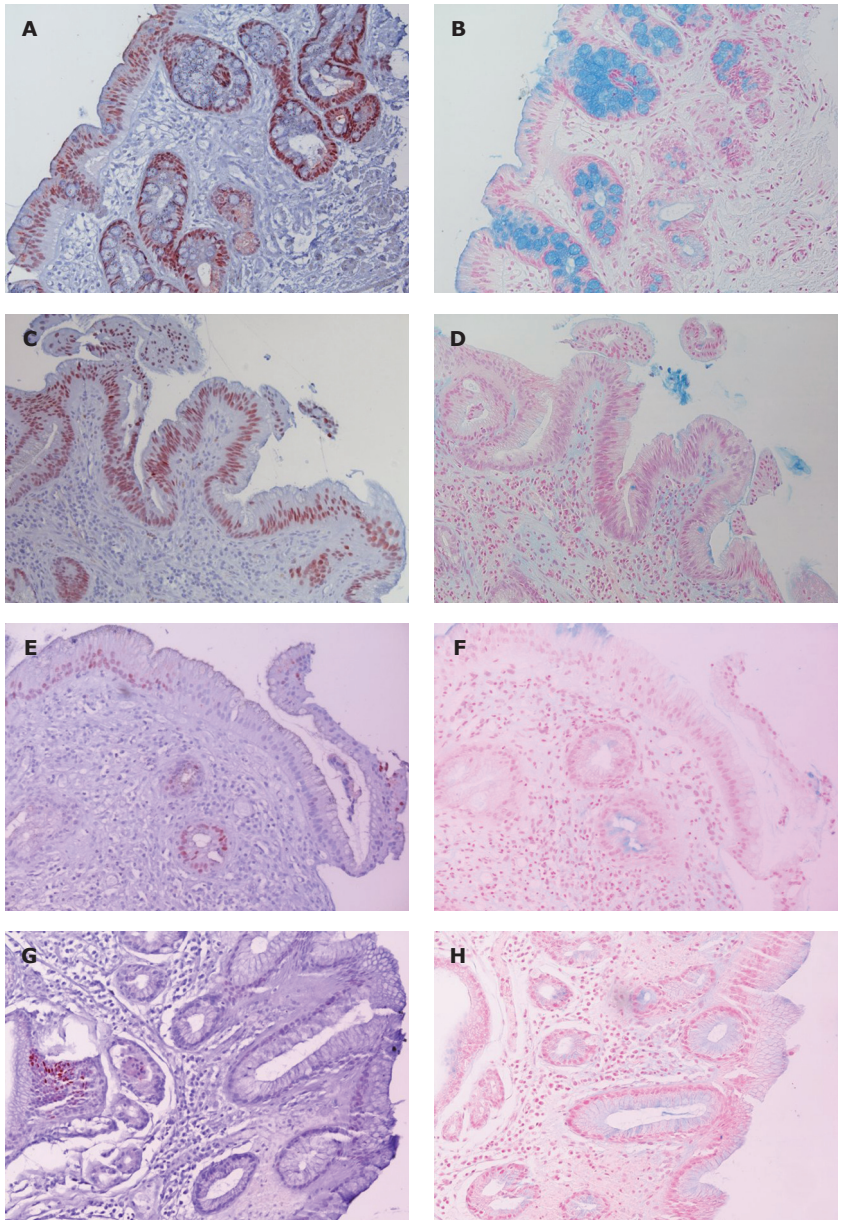


Figure 1 Immunohistochemical and histological analyses of CDX2 staining. a-b CDX2 and alcian blue staining in IM, 200 x magnification. c-d CDX2 and alcian blue staining of GM in samples without IM, 200 x magnification. e-f CDX2 and alcian blue staining in gastric-type glands adjacent to IM, the region with IM is not visible in this picture, but located on the left sight of this picture. 200 x magnification. g-h CDX2 and alcian blue staining in gastric-type glands at distance of IM, the region with IM is not visible in this picture, but is located on the other (left) site of this picture. 200 x magnification.

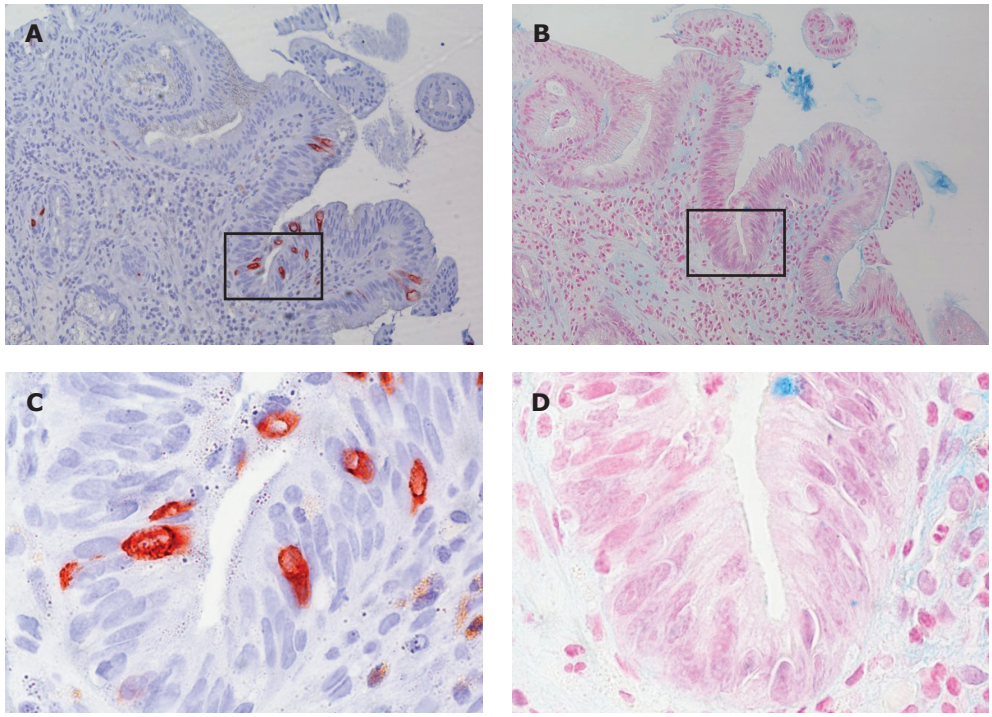


Figure 2 Immunohistochemical and histological analyses of MUC2 staining. a-b MUC2 and alcian blue staining of GM in samples without IM. The same region as Figure 1c-d is shown. 200x magnification. c-d Magnified view of MUC2-staining goblet-like cells in gastric-type glands.1000x magnification.

CHAPTER 7

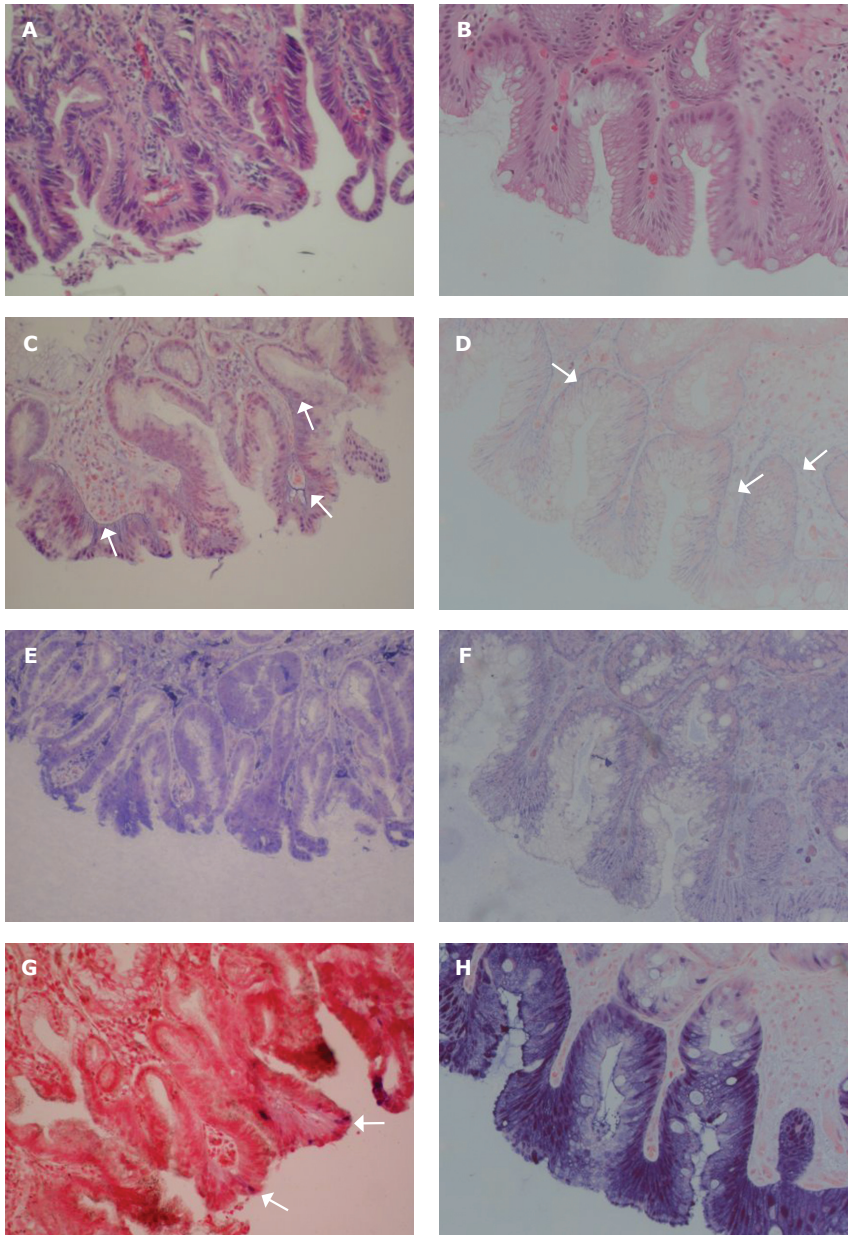


Figure 2 Haematoxylin and eosin stained control sections for high-grade dysplasia (HGD) (A) and non-dysplastic Barrett's oesophagus (BE) (B). In HGD, calgranulin A is present in the entire cytoplasm (C, arrow), while in BE it was only observed on the basal site of the cell (D, arrow). Staining patterns for calgranulin B in HGD (E) and BE (F) resemble those of calgranulin A, except for an intense background staining probably due to aspecific staining of the polyclonal antibody. FABP1 is present in a small subset of cells in HGD (G, arrow), while it was observed in the entire top of the villus in BE (H).

Samenvatting voor niet-ingewijden

Barrett-slokdarm is een afwijking die veel voorkomt, vooral in de westerse wereld. Waarschijnlijk heeft 1-5% van de inwoners van Noord-Amerika en Europa deze afwijking. Hoewel de meeste patiënten met een Barrett-slokdarm geen klachten hierdoor hebben, geeft de aanwezigheid van een Barrett-slokdarm toch een dertig keer grotere kans op het ontstaan van één van de vormen van slokdarmkanker, namelijk het adenocarcinoom. De ontwikkeling van een adenocarcinoom in de slokdarm gaat meestal geleidelijk, via de tussenstadia laaggradige en hooggradige dysplasie. Om de aanwezigheid van een adenocarcinoom in een vroeg stadium op te kunnen sporen, worden patiënten eens in de twee jaar endoscopisch onderzocht.

Barrett-slokdarm wordt waarschijnlijk veroorzaakt door het oprispen van maagzuur en gal uit de twaalfvingerige darm. Dit oprispen wordt reflux genoemd. Het maagzuur en de galzuren zijn schadelijk voor het normale slijmvlies van de slokdarm, dat door de blootstelling hieraan geïrriteerd raakt. Door deze irritatie zal in ongeveer tien procent van de mensen die last hebben van reflux, het normale slijmvlies van de slokdarm vervangen worden door slijmvlies dat lijkt op dat van de darmen, Barrett-slijmvlies. Het Barrett-slijmvlies is beter bestand tegen het schadelijke effect van maagzuur en galzuren, maar het is er niet helemaal ongevoelig voor. De blootstelling aan het maagzuur en de galzuren kan het slijmvlies aanzetten tot het produceren van eiwitten die ontstekingscellen aantrekken. Deze eiwitten worden chemokines en cytokines genoemd. Het voortdurend oprispen van zuur en gal leidt uiteindelijk tot de permanente aanwezigheid van ontstekingscellen, waardoor er een chronische ontsteking van het slijmvlies ontstaat.

Eén van de normale functies van de ontstekingscellen is het verwijderen van slijmvliescellen die ernstig beschadigd zijn. Om deze beschadigde cellen te doden, worden radicalen uitgescheiden. Radicalen zijn reactieve moleculen die DNA kunnen beschadigen. Cellen met DNA schade gaan normaalgesproken dood, maar sommige cellen zullen de DNA schade overleven. Door DNA schade kan kanker ontstaan. Wanneer de DNA schade optreedt in een gen dat tumorgroei afremt, en als het eiwit waarvoor het gen codeert door deze schade niet goed meer functioneert kan een cel ongeremd gaan delen waardoor de tumor groeit. Datzelfde geldt als er schade optreedt die ervoor zorgt dat een niet actief gen dat betrokken is bij celdeling, aangezet wordt. Omdat deze cellen hogere delingssnelheid hebben dan normale cellen, kunnen ze uitgroeien tot een tumor. Een uitgebreidere beschrijving van deze theorie en de literatuur die eraan ten grondslag ligt, wordt beschreven in **hoofdstuk 2**.

HET ONTSTAAN VAN BARRETT-SLOKDARM

Barrett-slijmvlies lijkt op het slijmvlies van de darmen, zowel microscopisch als moleculair-biologisch. Net als in darmslijmvlies zijn in Barrett-slijmvlies slijmbekercellen, grote, slijmproducerende cellen, aanwezig. CDX2 is een eiwit dat betrokken is bij het ontstaan van darmslijmvlies. Het is een transcriptiefactor, een eiwit dat andere genen "aanzet". CDX2 zet genen aan die specifieke darmfuncties

hebben, zoals darmenzymen en eiwitten die de slijmlaag op het darmslijmvlies vormen. Omdat Barrett-slijmvlies op darmslijmvlies lijkt, hebben we de hypothese geformuleerd dat CDX2 ook aanwezig is in Barrett-slijmvlies. Deze hypothese werd getest en bevestigd in **hoofdstuk 3**. CDX2 werd niet alleen gevonden in het Barrett-slijmvlies, maar ook in het naastliggende normale slijmvlies van de slokdarm, alhoewel in kleine hoeveelheden. Dit kan betekenen dat CDX2 een bepaald soort weefsel dat al wel bepaalde intestinale eiwitten produceert, maar dat nog niet echt een intestinaal fenotype heeft, kan aantonen. Deze theorie werd verder onderzocht in **hoofdstuk 4** en **hoofdstuk 5**. In deze hoofdstukken werd gekeken naar de expressie van CDX2 in maag-type slijmvlies in de slokdarm. Dit type slijmvlies lijkt op Barrett-slijmvlies, maar er zijn geen slijmbekercellen in aanwezig. Daarom worden de twee slijmvliessoorten als aparte soorten gezien, zeker ook omdat de aanwezigheid van maag-type slijmvlies geen groter risico op het ontwikkelen van slokdarmkanker vormt. In hoofdstuk 4 en 5 werd gevonden dat CDX2 expressie ook in sommige gebieden met maag-type slijmvlies in de slokdarm aanwezig is. In deze gebieden werd ook expressie van een ander darmeiwit, MUC2, gevonden. Dit betekent dat het maagtype slijmvlies waarin CDX2 en MUC2 voorkomen een soort kruising is tussen normaal maag-type slijmvlies in de slokdarm en Barrett-slijmvlies. Maag-type slijmvlies en Barrett-slijmvlies zijn dus waarschijnlijk verwant aan elkaar.

Het darmeiwit MUC2 behoort tot de familie van de mucines. Er zijn twee groepen mucines. Eén groep wordt uitgescheiden door het slijmvlies en vormt een beschermende, slijmlaag die het slijmvlies beschermt tegen mechanische schade door voedselbrokken die door het maag-darmkanaal passeren, en tegen chemische schade door stoffen zoals gal en verteringsenzymen. De andere groep blijft aan het celoppervlak gebonden en vormt zo op de cel een beschermende laag. Elk type slijmvlies heeft een specifiek patroon van de mucines die geproduceerd worden. In **hoofdstuk 6** wordt het mucine-patroon van Barrett-slijmvlies en normaal slokdarmslijmvlies beschreven. In Barrett-slijmvlies worden voornamelijk mucines die door het slijmvlies uitgescheiden worden geproduceerd, terwijl in normaal slokdarmslijmvlies juist mucines die gebonden zijn aan het membraan aanwezig zijn. Het veranderde mucinepatroon in Barrett-slijmvlies is waarschijnlijk het gevolg van het aanpassen van het slijmvlies aan de irriterende werking van het opgerispte zuur en gal.

Omdat gal een van de belangrijkste componenten van het oprispte refluxaat is, werd in **hoofdstuk 8** het effect van galzuren op Barrett-slijmvlies en normale slijmvlies van de slokdarm onderzocht. Hiervoor werd een model voor deze twee slijmvlies-types gebruikt, waarbij gekweekte kankercellen werden blootgesteld aan het galzuur deoxycholzuur, een van de belangrijke componenten van de opgerispte gal. Het blootstellen van de gekweekte cellen aan deoxycholzuur leidde tot een toename van de expressie van een aantal chemokines. Chemokines zijn eiwitten die meestal door slijmvliescellen worden uitgescheiden om ontstekingscellen aan te trekken. De chemokines die in dit model toenamen, trekken specifieke ontstekingscellen aan, waarvan is aangetoond dat ze aanwezig zijn in een Barrett-slokdarm. Dit betekent dat in een Barrett-slokdarm de irritatie, veroorzaakt door

het oprispen van gal, kan leiden tot een toename van het aantal ontstekingscellen, die voor verdere schade aan het slijmvlies kunnen zorgen.

HET ONTSTAAN VAN DYSPLASIE EN KANKER IN EEN BARRETT-SLOKDARM

Barrett-slijmvlies is metaplastisch, wat betekent dat het bestaat uit cellen die zijn gevormd op een plek waar ze niet thuishoren. Omdat deze cellen door de irritatie door zuur en gal waarschijnlijk beschadigd raken, moeten ze regelmatig vervangen worden. Deze twee dingen betekenen dat Barrett-slijmvliescellen sneller kunnen delen dan normale slijmvliescellen en een toegenomen celdelingssnelheid is een risicofactor voor het ontstaan van kanker. Verder kunnen de ontstekingscellen die zorgen voor de chronische ontsteking die aanwezig is in Barrett-slijmvlies verdere schade aan het slijmvlies veroorzaken, doordat ze radicalen produceren die het DNA van de slijmvliescellen kunnen beschadigen. Ook DNA schade, zoals mutaties in genen die de delingssnelheid van cellen reguleren, is een risicofactor voor het omvormen van een normale cel naar een kankercel. De aanwezigheid van deze risicofactoren in Barrett-slijmvlies betekent dat de slijmvliescellen kunnen veranderen in sneller delende, mutatie-bevattende cellen. Dit slijmvlies is echter nog niet compleet ontspoord en wordt daarom gezien als een voorstadium van kanker, dysplasie genoemd. In een Barrett-slokdarm wordt onderscheid gemaakt tussen laaggradige en hooggradige dysplasie. Wanneer hooggradige dysplasie aanwezig is, is de kans dat er kanker zal ontstaan erg groot.

Het mucinepatroon verandert vaak gedurende de ontwikkeling van kanker. Het mucinepatroon in hooggradige dysplasie in Barrett-slijmvlies is beschreven in **hoofdstuk 6**. Hier wordt beschreven dat de expressie van MUC4, een mucine dat is gebonden aan het celmembraan, is verhoogd in hooggradige dysplasie en in een adenocarcinoom in de slokdarm. MUC4 is waarschijnlijk direkt betrokken bij het kankerproces. Kankercellen hebben op het celoppervlak vaak tekens die herkend worden door het immuunsysteem, waardoor de kankercellen gedood kunnen worden. MUC4 heeft net als alle andere mucines veel suikerketens op het oppervlak, en deze kunnen de kankerceltekens verbergen, waardoor het immuunsysteem de cellen niet herkent als kankercellen, en waardoor de cellen niet opgeruimd worden. Verder is ook beschreven dat MUC4 is betrokken bij het remmen van geprogrammeerde celdood (apoptose), een proces dat ervoor zorgt dat beschadigde cellen doodgaan, waardoor ze plaats kunnen maken voor niet-beschadigde cellen. Verminderde apoptose is één van de kenmerken van kanker. Wij vonden echter juist een toename in apoptose in hooggradige dysplasie. Toegenomen apoptose is beslist geen kenmerk van kanker ontwikkeling, echter hooggradige dysplasie is nog niet compleet ontspoord en door meer apoptose zou het slijmvlies kunnen compenseren voor andere kanker-inducerende veranderingen. In adenocarcinomen van de slokdarm, die veel verder ontspoord zijn, werd wel een sterke afname in apoptose gemeten.

In **hoofdstuk 7** worden twee andere eiwitten beschreven die verhoogd tot expressie komen in hooggradige dysplasie. Deze eiwitten, calgranulin A en B vormen

samen het calprotectine complex, dat net als chemokines ontstekingscellen aantrekt, in dit geval neutrofiele granulocyten. Neutrofiele granulocyten produceren veel radicalen die DNA schade kunnen veroorzaken, waardoor de slijmvliescellen verder kunnen ontsporen. Daarnaast wordt calprotectine ook geproduceerd in het normale slijmvlies van de slokdarm, waar het waarschijnlijk betrokken is bij de celdeling.

De aanwezigheid van tumorgroei-stimulerende mutaties in hooggradige dysplasie kan leiden tot meer mutaties, bijvoorbeeld wanneer er een mutatie optreedt in een gen dat zorgt voor het opsporen en repareren van DNA schade. Deze mutaties kunnen leiden tot de ontwikkeling van een adenocarcinoom in de slokdarm. Chemokines kunnen bijdragen aan tumorgroei door ontstekingscellen aan te zetten tot het produceren van stoffen die de structuren buiten de cel afbreken. Deze structuren zorgen ervoor dat de cellen op hun plaats blijven. Wanneer deze afgebroken worden, kunnen tumorcellen makkelijk ingroeien in andere organen, of uitzaaien via de lymfe- of bloedvaten. In **hoofdstuk 8** werd de chemokine expressie in Barrett-slokdarm monsters vergeleken met een aantal dysplasie en adenocarcinoom monsters. Er werd gevonden dat de expressie van een aantal chemokines verhoogd was in de adenocarcinoom monsters. Dit kan betekenen dat deze chemokines, naast hun rol in het aantrekken van ontstekingscellen, ook een rol spelen bij het ontstaan van slokdarmkanker.

KLINISCHE BETEKENIS VAN DIT PROEFSCHRIFT

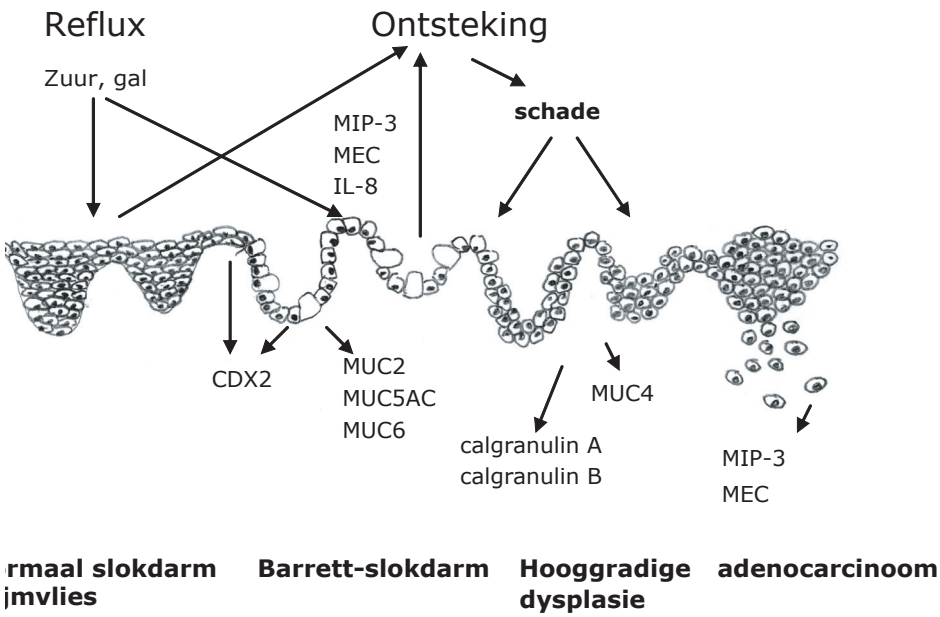
Het is belangrijk dat de rol van ontsteking en de moleculaire veranderingen die optreden tijdens de ontwikkeling van een Barret-slokdarm beter begrepen wordt, want hierdoor kunnen er nieuwe aangrijpingspunten gevonden worden voor mogelijke nieuwe geneesmiddelen. Deze kunnen gebruikt worden om Barrett-slokdarm te voorkomen en te behandelen.

Er zijn veel middelen, zoals NSAID's (niet-steroide anti-inflammatoire middelen) op de markt die ontsteking kunnen remmen. Ook worden er op het gebied van ontstekingsremmers nog steeds veel nieuwe middelen ontwikkeld. Het beschrijven van de cytokines en chemokines die een rol spelen bij de ontwikkeling van een Barrett-slokdarm, zoals in dit proefschrift in hoofdstuk 8 is gebeurd met een aantal chemokines, is belangrijk, omdat juist deze stoffen een belangrijk doel zijn voor ontstekingsremmers. Zo zou kunnen blijken dat ontstekingsremmers die tot nu toe werden gebruikt bij andere aandoeningen, ook helpen bij het verminderen van de ontsteking in een Barrett-slokdarm. Behandeling met deze ontstekingsremmers zou mogelijk ook kunnen leiden tot een verminderde kans op het ontstaan van een adenocarcinoom.

Verder kunnen ook de moleculaire veranderingen die optreden tijdens de ontwikkeling van dysplasie en kanker in een Barrett-slokdarm gebruikt worden om een betere diagnose te stellen. Het eiwit CDX2 zou mogelijk gebruikt kunnen worden om een nauwkeurigere diagnose van een Barrett-slokdarm te krijgen. In een gebied wat endoscopisch gezien allemaal Barrett-slijmvlies lijkt te zijn, blijkt

na microscopisch onderzoek het echte Barrett-slijmvlies vaak in de minderheid te zijn, omdat er ook veel maagslijmvlies aanwezig is. Wanneer er in de biopten alleen maar maag-slijmvlies aanwezig is komt de patiënt niet in aanmerking voor de tweejaarlijkse controle op dysplasie en kankerontwikkeling. De diagnose van Barrett-slijmvlies wordt gesteld op een aantal biopten die uit een willekeurig gedeelte van de slokdarm worden genomen, kan het zijn dat kleine regio's met echt Barrett-slijmvlies worden gemist tijdens het biopteren. Wanneer er CDX2 aanwezig blijkt te zijn in het maag-slijmvlies, is dat een goede aanwijzing dat er elders echt Barrett-slijmvlies aanwezig is.

Het verschil tussen Barrett-slijmvlies en het normale slijmvlies van de slokdarm is endoscopisch heel goed te zien, maar het zien van dysplasie is veel lastiger. Ook microscopisch is het lastig om dysplasie te onderscheiden van normaal Barrett-slijmvlies. Het gebruik van een aantal "markers", eiwitten in een bepaald stadium aanwezig zijn en in een ander stadium niet, zou de diagnose kunnen vergemakkelijken. Samen met eiwitten die al eerder in de literatuur zijn beschreven zouden MUC4 en Calgranulin A en B kunnen helpen bij het vroege opsporen van hooggradige dysplasie.



Figuur 1 De ontwikkeling van een Barrett-slokdarm, dysplasie en een adenocarcinoom vanuit normaal slokdarmslijmvlies en de moleculaire veranderingen die beschreven zijn in dit proefschrift, die optreden tijdens dit proces. Gal en zuur, de componenten van reflux, beschadigen de slijmvliescellen waardoor deze ontstekingsfactoren zoals MIP-3α, MEC en IL-8. Deze stoffen trekken ontstekingscellen aan en die kunnen zorgen voor DNA schade. Wanneer deze schade optreedt in bepaalde genen, kunnen de cellen harder gaan groeien. Hierdoor groeien ze uit tot dysplasie, die uiteindelijk kan overgaan in een adenocarcinoom.

CONCLUSIE

Het oprispen van zuur en gal kan irritatie van het slokdarmslijmvlies veroorzaken. Door deze irritatie raakt het slijmvlies chronisch ontstoken. De ontsteking kan leiden tot allerlei moleculaire veranderingen in de cel. De moleculaire veranderingen die zijn beschreven in dit proefschrift (Figuur 1) vormen slechts een klein gedeelte van alle veranderingen die optreden, sommige reeds beschreven, maar andere nog niet ontdekt. Ondanks dat het aantal veranderingen dat hier beschreven is, niet erg groot is, kunnen de beschreven veranderingen toch leiden tot nieuwe inzichten in het ontstaan van een Barrett-slokdarm, dysplasie en slokdarmkanker. De resultaten beschreven in dit proefschrift zullen in de toekomst mogelijk leiden tot nieuwe mogelijkheden voor de diagnose, preventie en behandeling van deze aandoeningen.

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CURRICULUM VITAE

Dorine Bax werd op 16 februari 1979 geboren in Dordrecht en groeide op in Hendrik-Ido-Ambacht. Zij behaalde in 1997 het VWO diploma aan het Develsteincollege te Zwijndrecht. In datzelfde jaar startte ze met de studie Medische Biologie aan de Vrije Universiteit in Amsterdam. Tijdens deze studie deed ze een onderzoekstage op de afdeling Medische Microbiologie en Infectiepreventie van het VUMC. Onder begeleiding van Monique Gerrits en Hans Kusters werd er onderzoek gedaan naar het moleculaire mechanisme van metronidazol-resistentie van Helicobacter Pylori. Een tweede onderzoeksstage werd gedaan op de afdeling Klinische Viro-Immunologie van het Centraal Laboratorium van de Bloedtransfusiedienst in Amsterdam (CLB/Sanquin). Onder leiding van Tim Beaumont en Hanneke Schuitemaker werd er onderzoek gedaan naar de neutralisatiegevoeligheid van het HIV-1 virus. In 2001 behaalde ze het doctoraal examen. Aansluitend startte ze haar promotieonderzoek op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC in Rotterdam naar moleculaire veranderingen die optreden gedurende de ontwikkeling van een Barrett-slokdarm en een slokdarm adenocarcinoom. Onder begeleiding van Hans Kusters, Peter Siersema en Ernst Kuipers werd het onderzoek verricht dat in dit proefschrift beschreven is.

Dankwoord

DANKWOORD

Nog niet zo lang geleden werd mij door een collega verteld dat hoe meer mensen je bedankt in een dankwoord, hoe meer je er ook vergeet. Toch is het niet mijn stijl om me er hier met een kort stukje vanaf te maken. De afgelopen jaren ben ik door talloze mensen geholpen om voor elkaar te krijgen dat er nu een proefschrift ligt. Natuurlijk was dit onderzoek niet mogelijk geweest zonder de medewerking van vele patiënten die materiaal hebben afgestaan en daarvoor wil ik hen graag bedanken.

Zes jaar geleden kwam ik als verlegen, stille studente voor het eerst binnenwandelen bij Hans Kusters, toen nog op de afdeling Medische Microbiologie en Infectiepreventie van de Vrije Universiteit in Amsterdam. Hans krabbelde in twintig minuten enthousiast alle onderzoekslijnen van de afdeling op een leeg A4-tje en een half uur later stond ik helemaal in de war weer buiten. Toch ben ik mijn eerste stage bij hem (en bij Simone en Monique) gaan lopen. Een periode die zes maanden had moeten duren, maar het werden er uiteindelijk tien, want ik had het geweldig naar mijn zin. Het was ook geen moeilijke beslissing om na mijn afstuderen in Rotterdam bij Hans promotieonderzoek te gaan doen. Beste Hans, heel veel dank dat ik promotieonderzoek bij je mocht doen. De afgelopen vier jaar hebben we heel fijn samengewerkt. Je bleef me begeleiden met het enthousiasme dat je al had tijdens ons eerste gesprek. Zonder jou had dit boekje er niet gelegen!

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