The development of new treatment strategies for oesophageal cancer
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Histopathologic evaluation of an animal model for Barrett's oesophagus and/or carcinoma is spontaneously induced by duodenal-(gastro-) oesophageal reflux for Barrett's oesophagus could provide better understanding of the distal oesophagus of the pathogenesis of the metaplasia-dysplasia-carcinoma sequence and would create the possibility to investigate new treatment strategies for this aggressive disease.
INTRODUCTION

The development of a Barrett's oesophagus is clinically associated with longstanding (duodeno)-gastro-oesophageal reflux, which replaces the normal squamous mucosa of the oesophagus by columnar mucosa. This Barrett's epithelium is generally considered as a pre-malignant condition, because a stepwise progression from intestinal metaplasia into dysplasia and finally into adenocarcinoma has clearly been observed. The specialized, intestinal-type, columnar epithelium is characterized by a change in morphology to an intestinal phenotype with the presence of goblet cells and a change from neutral to acid mucins (sialomucins and sulphomucins).

Immunohistochemically, Barrett's oesophagus resembles incomplete intestinal metaplasia of the gastric mucosa. It differs from complete intestinal metaplasia (characterized by goblet cells, Paneth cells and nonsecretory absorptive cells), by the presence of mucous secreting columnar cells, which contain both neutral and acid mucins. Incomplete intestinal metaplasia is more frequently associated with the development of dysplasia and adenocarcinoma than the complete type. During this stepwise progression of malignant degeneration, an accumulation of genetic changes is observed. Frequently described alterations in the intermediate steps of low-grade and high-grade dysplasia are an increase in the proliferation markers Ki67 and PCNA and mutation of the tumour suppressor gene p53.

An animal model of Barrett's oesophagus and adenocarcinoma would allow to study the various steps during malignant degeneration and would create the possibility to investigate new prevention and treatment strategies for this type of oesophageal cancer. There have been several attempts to develop animal models for Barrett's oesophagus and/or oesophageal adenocarcinoma. In some studies different types of oesophageal tumours (i.e. squamous cell carcinoma, adenocarcinoma and mixed tumours) were seen after the administration of exogenous carcinogens such as 2,6-dimethylnitrosomorpholine. The carcinomas which develop in these models do not resemble human Barrett's carcinomas. However, recent reports describe several surgical animal models in which Barrett's oesophagus and/or adenocarcinomas of the oesophagus develop without the administration of such exogenous carcinogens. Duodeno-forestomach anastomosis, pancreatico-oesophageal anastomosis, oesophagoduodenostomy, and oesophagojejunostomy with or without gastrectomy have all been reported in the literature. Unfortunately, these studies have shown contradictory results, and the histogenesis of the columnar epithelium and adenocarcinoma in these models is not clear.
The aim of the present study was to investigate two rat models for the development of Barrett's oesophagus and adenocarcinoma of the oesophagus. To analyse the histogenesis of the various morphological changes in these models, histopathologic evaluation with immunohistochemical stainings was performed.

MATERIALS AND METHODS

Approval
Both experiments were approved by the Animal Ethics Committee of the Academic Medical Center at the University of Amsterdam. Due to the expected high dropout rates, both studies were also carefully monitored by the 'animal study co-ordinator' of the Animal Ethics Committee.

Animals
In both experiments, 8-week-old male Sprague Dawley rats weighing 250-300 g were used (Harlan CBP, Austerlitz, The Netherlands). The rats were housed under standard laboratory conditions with a 12 h light-dark cycle and five animals per cage. Before use, the animals were allowed to acclimatize for at least one week. All animal handling was carried out by experienced biotechnicians. The animals were fed a regular diet (a commercially available rodent food) and had free access to tapwater, without exposure to carcinogens. Solid food was withdrawn the day prior to surgery, and for one day after surgery. All animals were weighed on a daily basis for the first two months, afterwards on a weekly basis. To analyse morphological changes, animals were scheduled to be killed 4 and 12 months after surgery, or sacrificed earlier if indicated. Reasons for early termination were weight loss of 30% of the pre-operative body weight, severe regurgitation and/or aspiration not recovering within 24 hrs.

Surgical Techniques

EXPERIMENT I
Forty-four rats underwent a end-to-side oesophagojejunostomy with gastrectomy (Figure 1A). After premedication with Buprenorfine 0.09 mg and Depomycine 0.15 cc, inhalation anaesthesia was induced (Isoflurane, N2O and O2). Via a midline laparotomy the oesophagus was transected at the oesophago-gastric junction and anastomosed end-to-side to the jejunum with a one layer, running suture (7/0 prolene). Care was taken to include the oesophageal mucosa to ensure adequate mucosal to mucosal apposition. The stomach was resected, as a previous study had shown that gastric
FIGURE 1A
End-to-side anastomosis of the oesophagus and the jejunum with gastric resection (Experiment I)

FIGURE 1B
Side-to-side anastomosis (oesophago-gastro-jejunostomy) with gastric preservation (Experiment II).
perforation frequently occurs when the stomach is left in situ. In this way, duodeno-oesophageal reflux (without gastric acid) was established. Immediately post-operatively, the rats received 2 ml of glucose 10% subcutaneously.

**Experiment II** Side-to-side oesophago-gastro-jejunostomy without gastrectomy was performed in 30 animals. After premedication with Buprenorfine 0.09 mg and Depomycine 0.15 cc, inhalation anaesthesia was induced (Isoflurane, N₂O and O₂). After a median laparotomy, the oesophagus, stomach and duodenum were identified, and the jejunum was anastomosed side-to-side with the distal oesophagus, with an anastomotic diameter of one cm (Figure 1b). A one layer running 7/0 prolene suture was used for the anastomosis, ensuring an accurate mucosal to mucosal apposition. Care was taken to avoid damage to the glandular stomach. In this way duodeno-gastro-oesophageal reflux was established, while leaving the stomach in place. Immediately post-operatively, the rats received 2 ml of glucose 10% subcutaneously.

**Histological examination**

Animals were sacrificed under general anaesthesia and subsequent exsanguination. The thoracic and abdominal cavities were inspected, especially for the presence of metastases, and the oesophagus, stomach and jejunum were excised en bloc, including the anastomosis. The oesophagus was opened longitudinally, examined macroscopically, and curled up from proximal to distal (Swiss roll), since with this technique the full length of the oesophagus could be examined. The specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin (H&E). An experienced gastrointestinal pathologist (FJWtK) reviewed all slides. The oesophagus was examined for the presence of hyperkeratosis, squamous hyperplasia, oesophagitis, ulcerations and finally intestinal metaplasia, dysplasia and carcinoma. Microscopically, intestinal metaplasia was defined as the presence of columnar mucosa with characteristic goblet cells. Dysplasia, arising in either squamous or columnar epithelium, was diagnosed using the Hamilton criteria. Carcinomas were classified in two groups: adenocarcinomas and squamous cell carcinomas.

**Immunohistochemistry**

To identify the histogenesis of pathologic changes seen after 4 and 12 months, immunohistochemical stainings were performed on 5 µm paraffin slides from tissue blocks. Intestinal metaplasia was demonstrated with high-iron diamine-alcian blue (HID-AB) staining, which is indicative of sulfomucin and sialomucin, and is characteristic for the presence of goblet cells.
To discriminate between complete and incomplete intestinal metaplasia, alcian blue pH 2.5 / periodic acid Schiff (AB-PAS) was used to distinguish blue acid mucins (AB-positive) from magenta neutral mucins (PAS-positive). Complete intestinal metaplasia was characterized by the presence of goblet cells secreting acid mucins and adjacent non-secretory columnar cells resembling normal absorptive enterocytes. Goblet cell metaplasia in association with secretory columnar neutral mucin cells, rather than mature absorptive cells, was considered indicative of incomplete intestinal metaplasia. To search for dysplastic and/or malignant changes immunohistochemically, the anti-rabbit-PCNA antibody at a dilution of 1:200, and the anti-rabbit-p53 antibody at a dilution of 1:500 were used (both Novocastra Laboratories, Newcastle, UK). The peroxidase reaction was developed with the use of diaminobenzidine. Cells were considered positive for PCNA and p53, when evident nuclear staining could be identified.

RESULTS

Histopathologic findings experiment I

Of the 44 operated animals, 33 (75%) died before the intended end of the experiment. Five (11%) due to peroperative complications, 15 (34%) from early anastomotic leakage, 5 (11%) from malnutrition due to late anastomotic strictureing and 8 (18%) from pulmonary complications due to massive aspiration. The body weight curve of the remaining eleven animals is depicted in Figure 2. From this curve it becomes clear that after the initial drop in weight immediately postoperatively, the surviving animals did not gain weight. The eleven surviving animals all had a very poor general condition, and it was decided to sacrifice all remaining animals after a median of 79 days (range 57 – 106) postoperatively. In all animals, the oesophagus was markedly dilated with a diameter varying from 3 to 5 mm. They all showed severe oesophagitis, with ulcerations extending into the proper muscular layer. There were small islands of regenerating squamous epithelium with a strong proliferation of the basal cell layer. In all animals this basal cell hyperplasia could be identified, together with papillary elongation and hyperkeratosis.

Columnar epithelium was seen in all rats at the site of the anastomosis and in the lower end of the oesophagus (Figure 3A). The length of this segment was always less than 2 mm. It was in continuity with the columnar epithelium of the jejunum in most of the cases, but in three cases the columnar cells moved upwards under the squamous epithelium of the oesophagus, to break through the covering squamous epithelium into the luminal surface more proximally.
In four of the eleven animals (36%) there were macroscopically visible tumours near the anastomosis, consisting of circumscribed areas of atypical tubular glands with large lakes of extracellular mucin (Figure 3B). The median diameter of these tumours was 5 mm (range 3 to 9 mm). On first sight these findings were described as well-differentiated mucinous adenocarcinomas, with large amounts of mucus, resembling the tumours described by Chen et al.\textsuperscript{13} and Clark et al.\textsuperscript{17} However, a closer look did not reveal convincing malignant characteristics. Although there was some apoptotic necrosis, and some ruptured tubules with mucinous dissection, nuclear polymorphism was rare. There were also very few mitotic figures in the outer cell layers of these tumours. The tumours were situated in the submucosa, covered by normal squamous mucosa, and did not infiltrate the proper muscular layer. There was no visible tumour growth on the luminal surface of the oesophagus or jejunum. In addition, there were no precursor lesions (dysplasia) found in the covering mucosa. The histological findings are summarized in Table 1.

**Histopathologic findings experiment II**

Six rats (20%) died perioperatively, one immediately after induction of anaesthesia and five from anastomotic leakage. Two additional (7%) animals were sacrificed at 2 months due to persistent weight loss and failure to thrive. The median body weight of the remaining animals over time is shown in Figure 2. These animals with a side-to-side anastomosis without gastric resection gained significantly more weight than those with total gastrectomy (experiment I). Ten animals were sacrificed according to protocol at 4 months. Pathology results of these animals showed a moderate oesophagitis with histological changes.
**TABLE 1:**
Histological findings 2-3 months after end-to-side oesophago-jejunostomy (ETS) with gastrectomy (experiment I), and 4 and 12 months after side-to-side oesophago-gastro-jejunostomy (STS) with gastric preservation (experiment II).

<table>
<thead>
<tr>
<th>Histological Findings</th>
<th>ETS 2-3 months</th>
<th>STS 4 months</th>
<th>STS 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=11)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>11</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Squamous hyperplasia</td>
<td>11</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Oesophagitis</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Ulcerations</td>
<td>11</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Columnar epithelium (median length)</td>
<td>1-2 mm</td>
<td>1-2 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>Dysplasia in Barrett</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumours histologically resembling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oesophagitis cystica profunda</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Tumours histologically resembling adenocarcinoma</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Tumours immunohistochemically resembling adenocarcinoma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous dysplasia</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Similar to the animals in experiment I (i.e. a 1-2 mm segment of columnar metaplasia at the anastomotic site, Table 1). Two additional animals were sacrificed at six months due to recurrent weight loss and failure to thrive. Pathology results were the same as in those rats sacrificed at 4 months. Interestingly, in the animals surviving one year, oesophagitis, ulcerations or erosions could hardly be identified anymore. Apparently, the previously damaged mucosa had been replaced by columnar epithelium and/or hyperplastic squamous epithelium. Histologic characteristics are summarized in Table 1. A segment of columnar epithelium was found in 9 out of 10 animals with a median length of 10 mm (range 0-22 mm) (Figure 3C). In this columnar epithelium, neither low-grade nor high-grade dysplasia could be identified. A large mucinous tumour (median diameter 12 mm, range 9 to 20 mm) could be found in seven out of ten animals (Figure 3D). These tumours were also located at the site of the anastomosis (often under normal squamous epithelium), and always limited to the submucosa, without involvement of the mucosa or proper muscular layer. In two cases the tumour was directly adjacent to the liver, but ingrowth rather than mechanical displacement could not be demonstrated.
FIGURE 3
Haematoxylin-eosin stained histology of the intestinal wall after oesophago-jejunostomy with (experiment I: panel A+B) and without (experiment II: panel C+D) gastric resection.
Experiment I: A: columnar epithelium at the anastomosis with severe ulcerative oesophagitis after 4 months. B: 'oesophagitis cystica profunda' after 2-3 months. The tumour is well-differentiated and is confined to the submucosa. There is no involvement of either the muscular layer or the mucosa. Experiment II: C: segment of columnar epithelium with goblet cells after 12 months. D: Tumour histologically resembling oesophageal adenocarcinoma after 12 months. There is glandular disruption with the formation of mucin lakes, some nuclear polymorphia, a few mitoses, and apoptotic necrosis. Magnification = 10x.

In comparison to the histological results at 4 months these tumours were significantly larger, and did have more malignant characteristics like cytonuclear polymorphia and disrupted and disorganised tubules. Therefore, these lesions were histologically resembling well-differentiated adenocarcinomas.

In four animals one or more squamous cell carcinomas were found in the middle or distal oesophagus at some distance from the anastomosis. These tumours ranged in diameter from 3 to 6mm (median 5 mm). In two other animals atypical, dysplastic squamous epithelium was found, which was considered as carcinoma in situ.
Immunohistochemical stainings

The columnar cells found above the anastomosis after 4 and 12 months were positive for acid mucins and the HID-AB staining showed typical goblet cells as demonstrated by the presence of sialomucins and sulphomucins. This was clearly different from the adjacent jejunal epithelium without HID-AB-positivity indicating that the columnar segments were resembling the specialized columnar epithelium of Barrett's oesophagus in humans (Figure 4A). With AB/PAS staining, acid mucins were identified in the absence of magenta neutral mucins, which is indicative of complete type intestinal metaplasia (with a supposedly lower malignant potential as compared to incomplete intestinal metaplasia) (Figure 4B). Immunohistochemically, there was neither evidence for dysplastic characteristics in the columnar mucosa after 4 months nor after 12 months. Expression of PCNA was predominantly restricted to the basal layer and normal maturation of columnar cells was seen (Figure 4C). Interestingly, areas with papillary hyperplasia showed stronger expression of PCNA than the columnar metaplastic segments. Also, p53 staining was consistently negative. In the tumours at the anastomosis resembling well differentiated adenocarcinomas, the HID-AB and AB/PAS staining showed a mucin profile which was comparable to the more proximal columnar epithelium.
(Figure 4D and E) after 4 and 12 months. After 12 months, there was a slight increase in intensity and number of PCNA positive cells in comparison to the surrounding normal and metaplastic epithelium (Figure 4F). However, p53 immunohistochemistry did not confirm any conversion towards malignant degeneration. Based on these immunohistochemical results, the diagnosis of reactive lesions fitting the diagnosis oesophagitis cystica profunda was established rather than well differentiated adenocarcinomas.

DISCUSSION

To investigate the histogenesis of Barrett's oesophagus and oesophageal adenocarcinoma, and to develop novel treatment strategies, the availability of an animal model would be of great value. This study demonstrates that duodeno-(gastro-)oesophageal reflux in a rat can induce columnar epithelium with immunohistochemical characteristics resembling human specialized intestinal metaplasia of a Barrett's oesophagus.

Although many studies demonstrated the presence of a short segment of columnar epithelium directly proximal to the anastomosis, most segments did not exceed 2 mm. The origin of this epithelium has been previously discussed in several publications. One hypothesis suggests stitching of jejunal mucosa to the oesophageal wall during surgical reconstruction with subsequent proximal migration of columnar epithelium. The other hypothesis suggests a true metaplastic process with the 'Barrett's oesophagus' originating from a pluripotent undifferentiated stem cell. We also found a 1-2 mm short columnar epithelial segment surrounded by squamous mucosa at the anastomotic site after 2 to 4 months in both models. Although the presence of squamous epithelium distal to the area of columnar epithelium as found in a minority of rats is suggestive for a true metaplastic event, it is still doubtful whether this short segment is not mechanically displaced columnar duodenal mucosa. After 12 months, a segment of columnar epithelium of ca. 10 mm was identified in 90% of the animals. These data are consistent with those of Van den Boogert et al. who described a long segment of metaplastic mucosa in the oesophagus (i.e. 6 mm of Barrett's mucosa after 8 months, and 16 mm after 12 months). Such a large area of columnar epithelium is not likely to develop solely due to mechanical forces. In addition, clear immunohistochemical differences were found from adjacent jejunal mucosa (especially the presence of typical goblet cells. From these observations we conclude that longstanding duodeno-gastro-oesophageal reflux is able to induce a Barrett's oesophagus in rats.
However, histological characteristics of dysplasia or immunohistochemical evidence of genetic changes associated with malignant degeneration were not found within the mucosa of any Barrett's segment. This is consistent with other animal studies that never demonstrated dysplastic epithelium in a short segment or long segment of columnar epithelium. This is in line with our observation that the intestinal metaplasia was predominantly of the complete type, which is in humans more frequently present at the oesophago-gastric junction and which is associated with a lower malignant potential in comparison with the incomplete type.

Unfortunately, the question whether this animal model of longstanding reflux without administration of exogenous carcinogens is also able to induce adenocarcinomas is not answered by this study. The question remains whether these tumours are true reflux-induced mucinous adenocarcinomas, or that they should be considered as mechanically induced inclusion bodies with changes fitting the diagnosis 'oesophagitis cystica profunda'. This description parallels the diagnosis of proctitis and gastritis cystica profunda in humans.\(^{25-27}\) Proctitis cystica profunda is a rare, benign disease of the rectum, most often seen in patients with rectal prolaps, but also in combination with inflammatory bowel disease or radiation therapy.\(^{25-26}\) It is characterized by the presence of mucinous cysts in the colonic or rectal wall. These cysts are found in the submucosa, sometimes reaching the tunica muscularis. The cysts are lined by a single layer of benign epithelial cells of variable appearance, ranging from normal-appearing mucosal elements to nondescript, atypical, cuboidal or flattened cells. The tumours described in the present experiments and previously described by others, fit this description. In a rat-model of radiation induced colitis cystica profunda, Geisinger described 'the insinuation of glands herniating from the mucosa through narrow gaps in the muscularis mucosae into the superficial submucosa'.\(^{25}\) Cells lining these herniating glands had normal appearances, and mitotic activity was also not noted. It is conceivable that the construction of an oesophago-jejunal anastomosis induces herniation of individual glands from the jejunum into the oesophageal submucosa, or that mechanical forces such as surgical stitches transpose these glands into the submucosa. Interestingly, in many of our tumours, stitches were present.

In man these 'cystica profunda' tumours do not develop into malignancy. The tumours observed after 4 months in the present study did not show characteristics of malignancy, and were considered as benign or reactive tumours due to mechanical forces. At one year however, the tumours had increased significantly in size, and the glands had become more disrupted with increasing nuclear atypia. In the lakes of mucin, single-floating cells could be observed.
Therefore, these tumours were histologically resembling adenocarcinomas. However, all tumours remained confined to the submucosa and did not infiltrate into deeper layers or adjacent structures. In addition, immunohistochemical evidence for malignancy could not be demonstrated, since p53 mutations were absent and only a slight increase in proliferation activity was seen. In most previous studies performing oesophagojejunostomy with gastric preservation, there has also been no definite evidence for a malignant nature of these tumours. Most so-called adenocarcinomas are well differentiated tumours limited to the submucosa with histological characteristics similar to the lesions described in the present study. The observation that selective COX-2 inhibition prevents the development of reflux induced ‘adenocarcinomas’ in rats by Butter et al., is in our opinion also not conclusive for the origin of these tumours, since COX-2 inhibition could also prevent reactive inflammatory lesions. However, Nishijima et al. demonstrated that a biliary diversion procedure, in which the end-to-side oesophagojejunostomy with gastrectomy was converted to Roux-en-Y oesophagojejunostomy after 20 or 30 weeks, prevented the development of oesophageal adenocarcinoma. This observation can not be explained if the lesions resembling oesophageal adenocarcinomas are solely mechanically induced inclusion bodies. Therefore, further studies into the true nature of the tumours found in these animal experiments are needed. Surprisingly, one or more squamous cell carcinomas were found in the middle or distal oesophagus at some distance from the anastomosis in four animals after 12 months. This finding has never been described in previous reports. However, since dysplastic squamous epithelium with a PCNA and a p53 increase was found to accompany the tumours, these lesions were considered to be true malignant degeneration of normal squamous oesophageal epithelium.

In conclusion, this study demonstrates that it is possible to induce a long segment Barrett’s oesophagus in the rat by performing a side-to-side oesophago-gastro-jejunostomy. After one year, ca. 1 cm of Barrett’s mucosa is present. Within 3 to 4 months tumours appear at the anastomotic site, which develop histological signs of malignancy after one year. However, these tumours are always confined to the submucosa, and since precursor lesions or immunohistochemical characteristics of malignant degeneration can not be identified, these tumours are likely to develop via mechanical forces (such as stitches) transposing mucosal glands into the submucosa. These transposed glands subsequently develop into large well-differentiated cyst-forming tumours fitting the diagnosis of ‘oesophagitis cystica profunda’. It seems to be unlikely that these tumours are induced by duodeno-gastro-oesophageal reflux. This animal model is, therefore, not suited for the study of true adenocarcinoma developed in a Barrett’s segment.
REFERENCES


