Carcinogenesis and treatment of adenocarcinoma of the oesophagus and gastric cardia
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Chapter 12

An animal model for Barrett’s oesophagus but not for Barrett’s cancer

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Abstract

Introduction. Barrett’s oesophagus and adenocarcinoma of the oesophagus are related to long-standing duodeno-gastro-oesophageal reflux. The development of an animal model in which duodeno-gastro-oesophageal reflux would be able to induce Barrett’s oesophagus and/or Barrett’s carcinoma would enable us to study the events taking place during the metaplasia-dysplasia-carcinoma sequence.

Methods. Two rat-models were applied. In the first experiment (44 animals) an oesophagojejunostomy with gastric resection was performed, to ensure duodeno-oesophageal reflux without gastric acid. In the second experiment (30 animals) an oesophagojejunostomy was performed, ensuring duodeno-oesophageal reflux. In both experiments animals were to be sacrificed at four and 12 months.

Results. In the first experiment, only 11 animals survived the early post-operative period. These animals had to be sacrificed at a median of 11 weeks due to persistent weight loss and failure to thrive. In all animals a two mm segment of metaplastic epithelium was found at the anastomosis. In four animals a large, well-differentiated, mucinous tumour fitting the diagnosis oesophagitis cystica profunda was observed.

In the second experiment, eight animals died post-operatively. Of the remaining animals, 12 were sacrificed at 4-6 months. In these animals, a short (two mm) segment of metaplastic epithelium was observed, without dysplasia. The ten remaining animals survived one year. At one year, in nine animals a Barrett segment with a length of 10 mm was observed. Also, in four animals one or more squamous cell carcinomas, surrounded by dysplastic squamous epithelium, were found. Finally, in seven animals a tumour with cytologic characteristics of a well-differentiated mucinous adenocarcinoma was found. However, these tumours originated in the submucosa, and did not reach either the luminal surface or the muscular layer. Although they showed cytological characteristics of malignancy, the definitive criteria of malignancy, i.e. infiltrative growth and/or dissemination, were not met.

Discussion/Conclusion. This is only the second study reporting the development of a long Barrett segment in an animal reflux model. Although tumours develop, these are not reflux induced. The true nature of these tumours remains to be elucidated.
Introduction

Adenocarcinoma of the oesophagus is one of the most lethal solid tumours, and its incidence is rapidly rising. Due to persistent duodeno-gastro-oesophageal reflux, the normal squamous epithelium of the oesophagus may be replaced by columnar epithelium, characterised by the presence of goblet cells, which are normally found in the small and large intestines. This intestinal metaplasia, or Barrett’s metaplasia, may be considered a pre-malignant condition, as there can be stepwise progression from intestinal metaplasia into dysplasia and finally into adenocarcinoma.\textsuperscript{1,2}

There have been several investigations into the (im)possibilities of animal models for Barrett’s oesophagus and/or oesophageal carcinoma. The results of these studies have been contradictory. In many studies different oesophageal tumours (\textit{i.e.} squamous cell carcinoma, adenocarcinoma and mixed tumours) were seen after the administration of exogenous carcinogens such as 2,6-dimethylnitrosomorpholine.\textsuperscript{3,4} Recent reports suggest that Barrett’s oesophagus and/or adenocarcinomas of the oesophagus may also develop in animals without the administration of exogenous carcinogens.\textsuperscript{5-8} Such a model would enable the study of carcinogenesis in a Barrett’s oesophagus without the confounding unphysiological effects of nitrosamines used as initial carcinogen.

In the present study we investigate two rat models for the development of Barrett’s oesophagus and adenocarcinoma of the oesophagus and the role of gastric juice and that of bile and pancreatic juice.

Materials and Methods.

Approval

Both experiments were approved by the Animal Ethics Committee of the Academic Medical Center/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 300 gr male Sprague Dawley rats (Harlan CBP, Austerlitz, The Netherlands) were used. A 12 hr light cycle was used. Rats were kept in normal cages, with five animals in each cage. All animal handling was carried out by experienced bio-technicians. Before use, the animals acclimatised for at least one week. The animals were fed a regular diet (a commercially available
rodent food) and had free access to tapwater. 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.

Surgical Techniques

Experiment I.

Forty-four rats underwent an oesophagojejunostomy after total gastrectomy. (fig 1) After pre-medication with Buprenorfine 0.09 mg and Depomycine 0.15 cc, general anaesthesia was induced using inhalation-anaesthesia (Isoflurane, N₂O and O₂). Via a midline laparotomy the oesophagus was transected at the oesophago-gastric junction and anastomosed end to side (a one layer, running 7/0 prolene suture) to the jejunum. Care was taken to include the oesophageal mucosa to ensure adequate mucosal to mucosal apposition. The stomach was resected, as a previous study has shown that gastric perforation occurs frequently when the stomach is left in situ. In this way, jejuno-oesophageal reflux (without gastric acid) was established. Immediately post-operatively, the rats received two ml of glucose 10% subcutaneously. Animals were to be killed after 12, 16, 20 and 24 weeks post-operatively, or sacrificed earlier on indication. 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.

Experiment II

Side to side oesophago-jejunostomy without gastrectomy was performed in 30 animals. After pre-medication with Buprenorfine 0.09 mg and Depomycine 0.15 cc, general anaesthesia was induced using inhalation-anaesthesia (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. Care was taken not to include the oesophago-gastric junction (fig. II) 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 jejuno-gastro-oesophageal reflux was established, while leaving the stomach in place. Immediately post-operatively, the rats received two ml of glucose 10% subcutaneously. Animals were to be sacrificed at four months and one year post-operatively. Animals could also be sacrificed on clinical grounds as mentioned in experiment I.
Handling of specimens

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, the circumference was measured, and the specimen was curled up from proximal to distal (Swiss roll) and fixed in formalin. Animals dying before the intended end of the experiment were also handled as above.

Histological examination

Specimens (Swiss roles) were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E). In a Swiss roll format, the full length of the oesophagus could be examined. An experienced gastro-intestinal pathologist (FJWtK) reviewed all slides. The oesophagus was examined for the presence of squamous hyperplasia, oesophagitis, ulcerations and finally intestinal metaplasia, dysplasia and carcinoma. Intestinal metaplasia was defined as the presence of columnar epithelium with the characteristic goblet cells. Dysplasia was diagnosed using the Hamilton criteria, arising in either squamous or columnar epithelium. Carcinomas were classified in two groups: adenocarcinomas and squamous cell carcinomas.

Results

Experiment I

Of the 44 operated animals, 33 (75%) died before the intended end of the experiment. Five (11%) due to per-operative complications, 15 (34%) from early anastomotic leakage, 5 (11%) from malnutrition due to late anastomotic stricture and 8 (18%) from pulmonary complications due to massive aspiration.

The body weight curves of the remaining eleven animals are depicted in fig. III. From this curve it is clear that after the initial drop in weight immediately post-operatively the surviving animals did not gain weight. The surviving eleven animals all had a poor general condition, and it was decided to sacrifice all these eleven animals before the intended end of the study.

The eleven remaining animals survived a median of 79 days (range 57 – 106) post-operatively. In all cases there was a marked dilatation of the oesophagus varying from 3 to 5 mm. In all animals there was a severe oesophagitis, with severe ulcerations reaching into the proper muscular layer. In three
cases there was a complete sloughing of the mucosa, with no normal epithelium left apart from a few millimetres normal squamous epithelium at the gastro-oesophageal junction. There were small islands of regenerating squamous epithelium with a strong proliferation of the basal cell layers. In all animals this basal cell hyperplasia could be found, together with papillary elongation. Histological findings are summarised in table I.

Columnar epithelium was seen in all rats at the site of the anastomosis and in the lower end of the oesophagus. In some cases, columnar cells moved upwards under the squamous epithelium of the oesophagus, to break through the covering squamous epithelium into the luminal surface. In others, the metaplastic epithelium was continuous with the jejunal epithelium. However, the length of this segment was always less than two mm. It was in continuity with the columnar epithelium of the jejunum in most of the cases.

In four of the eleven animals (36%) there was a macroscopically visible tumour near the anastomosis, consisting of circumscribed areas of atypical tubular glands with large lakes of extracellular mucin. (fig IV) The median diameter of these tumours was five mm (range three to nine mm). On first sight these abnormalities were described as well-differentiated mucinous adenocarcinomas, with large amounts of mucus, resembling the tumours described by Chen and DeMeester. However, although there was some atypia, a closer look did not reveal convincing tumour characteristics. Although there was some apoptotic necrosis, and some ruptured tubules with glandular disruption, nuclear polymorphia was minimal. There were also very few mitotic figures. The tumours were situated in the submucosa, below 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. (fig IV) Also, there were no precursor lesions (dysplasia).

**Experiment II**

Six rats died peri-operatively, one immediately after induction of anaesthesia and five from anastomotic leakage. Two additional animals were sacrificed at two months due to persistent weight loss and failure to thrive. Pathology results of these two animals showed a massive oesophagitis with ulceration. The median body weight of the 22 surviving animals in this group is also shown in fig III for comparison with Experiment I. The animals with a side to side anastomosis without gastric resection gained significantly more weight than those with total gastrectomy. Although the animals surviving 12 months after a STS oesophagojejunostomy with gastric preservation were in better shape and gained weight during the year, they were still weak and well below the weight curves of normal animals.

Ten animals were sacrificed at four months, also showing oesophagitis as described in experiment I but without intestinal metaplasia except for a short segment (two - three mm) at the anastomotic site.
Two additional animals were sacrificed at six months due to recurrent weight loss and failure to thrive. Pathology results were the same as those sacrificed at four months. Interestingly, after 12 months oesophagitis, ulcerations or erosions could hardly be identified anymore. Apparently, all the previously damaged mucosa had been replaced by either columnar epithelium or hyperplastic squamous epithelium. In the animals surviving one year, Barrett’s mucosa characterised by the presence of intestinal metaplasia with goblet cells was found in nine out of ten. The median length of this Barrett’s segment was ten mm (range 0-22). In these Barrett’s segments, dysplasia was not identified. (fig. V) This epithelium did not resemble the adjacent jejunal mucosa, which showed villi and crypts.

In four animals one or more areas of squamous cell carcinoma could be found in the middle or distal part of the oesophagus at some distance from the anastomosis. These tumours ranged in diameter from three mm to six mm (median five mm). In two other animals atypical, dysplastic squamous epithelium was found, which was considered as carcinoma in situ. Histologic characteristics are summarised in table I.

Large mucinous tumours (median diameter 12 mm, range nine to 20 mm) could be found in seven out of ten animals after ten months. (fig. VI) These tumours were located at the site of the anastomosis and often under normal squamous epithelium. They were 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. Again there was little nuclear polymorhia, few mitoses, and no precursor lesions. However, in comparison to the histological results at four months these tumours were significantly larger, and did have more malignant characteristics like more cytonuclear polymorhia and disrupted and disorganised glandular structures. Also, a few single cells were found in the large lakes of mucin. Although it is possible that these lesions are reactive, and thus comparable with the oesophagitis cystica profunda described at four months, these lesions had rather to be characterised as well-differentiated adenocarcinomas, based on cytologic criteria. In contrast to the squamous cell carcinomas, no precursor lesions could be found in the vicinity of these mucinous tumours, and the adenocarcinomas were located under normal squamous epithelium at the site of the anastomosis. Hallmarks of malignancy, that is infiltration in the surrounding tissues, vasoinvasive growth and/or dissemination, were not found.

Discussion

In this study, ca. 10 mm of Barrett’s mucosa developed in rats one year after oesophago-jejunostomy with gastric preservation. At the anastomosis, a large mucinous tumour could often be found, increasing in size and showing signs of malignant degeneration through time.
Just as in man, in rats the development of (experimental) oesophageal cancer is supposedly promoted by reflux of gastro-duodenal contents. Reflux induces a massive oesophagitis, with an increased proliferation in the basal cell layer, as demonstrated by an increase in PCNA and Ki-67 expression.\textsuperscript{12,13,14}

Although many studies demonstrated the presence of a short segment of columnar epithelium directly proximal to an oesophago-jejunostomy, these segments did not exceed two millimetres. Such a short segment of Barrett’s mucosa may be due to jejunal mucosa being stitched into the oesophageal wall during the surgical reconstruction. We have also found a one to three millimetres short ‘Barrett’s segment’ surrounded by squamous epithelium at the anastomotic site after two to four months in both experiments. Although the presence of squamous epithelium distal to the area of columnar epithelium suggests a true metaplastic event, it is doubtful whether this segment of metaplastic epithelium represents a true Barrett’s oesophagus or mechanically displaced jejunal mucosa.

In the only previously published study describing a longer segment of metaplastic mucosa in the oesophagus, six mm of Barrett’s mucosa could be found after eight months, and 16 mm after 12 months, using the same model as described in our first experiment.\textsuperscript{8} Dysplasia or tumours of any kind were not described by these authors. In our second experiment, a Barrett’s segment of ca. 10 mm was found in 90% of the animals after 12 months. Such a large area of Barrett’s oesophagus can probably not develop solely due to mechanical forces. From these observations we conclude, that duodeno-oesophageal reflux is able to induce intestinal metaplasia in rats.

While some studies deny the possibility of carcinomas developing without exogenous carcinogen such as nitrosamines, others suggest that reflux of duodenal or gastro-duodenal contents without administration of exogenous carcinogens can also induce oesophageal carcinoma in rats.\textsuperscript{3,5,15,16} The second aim of the present study was to analyse the potential role of gastric reflux (acid) and duodenal reflux (bile and pancreatic juice).

The administration of nitrosamine induces the development of squamous cell carcinomas in rats. Surgically induced duodeno-oesophageal reflux increases the frequency of oesophageal tumours in nitrosamine treated rats, changing the histologic type of carcinomas from squamous cell into adenocarcinoma. Some studies suggest that pancreatic juice is the most potent component of the refluxate, while others show that gastric acid may even protect against the development of carcinoma.\textsuperscript{3,17,18} Ireland and others showed that the prevalence of oesophageal adenocarcinoma was 30% in rats with duodeno-gastro-oesophageal reflux and 87% in rats with reflux of duodenal juice alone.\textsuperscript{15} In another study the animals with only gastric reflux did not show an increased incidence of malignancy in comparison with the non-refluxing control animals.\textsuperscript{19}

Miwa and co-workers showed that 80% of rats suffering from gastro-duodeno-oesophageal or duodeno-oesophageal reflux developed a carcinoma 50 weeks after surgery. Seventy percent of these tumours were adenocarcinomas.\textsuperscript{7,20}
The first experiment of the present study was set up as an attempt to duplicate the results mentioned above. The initially high early postoperative dropout rates could be partly attributed to the learning curve. However, the surviving animals all failed to thrive and were generally in bad condition. Therefore we felt obliged to terminate the experiment. Others, using the same model, found similar weight curves, corresponding with similar failure to thrive, but decided to continue the experiment. (J. Van den Boogert and X. Chen, personal communication) Due to the failure of experiment I, the precise role of acid and duodenal contents could not be examined in the present study.

What is the true nature of the tumours observed? Are they true reflux-induced mucinous adenocarcinomas, or should they be considered as mechanically induced changes fitting the diagnosis “oesophagitis cystica profunda”? DeMeester’s group has recently suggested, contrary to their previous reports, that the “mucinous adenocarcinomas” do not arise from (either columnar or squamous) mucosa, as would be the case in true reflux induced oesophageal carcinomas, but are limited to the submucosa, just as in our experiments. These tumours may therefore be (mechanically) related to the surgical construction of an anastomosis between oesophageal squamous and enteric columnar mucosa. We hypothesise, that there is a parallel to proctitis/colitis or gastritis cystica profunda, and therefore have termed the disorder oesophagitis cystica profunda. This disorder has never been described before in other animal experiments.

Proctitis/colitis cystica profunda is a rare, benign disease of the colo-rectum, most often seen in patients with rectal prolaps, but also in combination with inflammatory bowel disease or radiation therapy. It is characterised by the presence of mucinous cysts in the colonic or rectal wall. These cysts are found in the submucosa, sometimes reaching the proper muscular layer. 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 our 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 from the mucosa through narrow gaps in the muscularis mucosae into the superficial submucosa”. Cells lining these herniating glands had normal appearances, and mitotic activity was also not noted. It is conceivable that performance of an oesophago-enteric anastomosis creates the possibility of 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. In many of our tumours, stitches were present. However, in man these cystica profunda tumours do not develop into malignancy, while in our experiments there is a strong suggestion of malignant degeneration.

The tumours observed at four months in experiment I and II show some characteristics of malignancy, but might still be benign or reactive tumours due to mechanical forces. At one year, the tumours had increased significantly in size, and the glands had become more and more disrupted with increasing...
nuclear atypia. In the lakes of mucin, single-floating cells could be observed. These tumours can morphologically be considered as carcinomas. However, the reason for this malignant degeneration is as yet unclear. It should be underlined, that although cytological characteristics suggest a malignant process, the ultimate clinical characteristics of malignancy, i.e. tumour invasion and dissemination, were not observed.

The tumours are confined to the submucosa, and are therefore not in direct contact with the refluxate. Also, these tumours were already present at four months, that is, before the development of Barrett’s epithelium and no precursor lesions could be identified. Therefore, these carcinomas are more likely to develop via a mechanical process than via reflux.

**Conclusion**

Barrett’s oesophagus can be induced in the rat by performing an oesophago-jejunostomy. After one year, ca. ten mm of Barrett’s epithelium is present, while morbidity and mortality are acceptable. Within three to four months tumours appear at the anastomotic site. The precise nature of these tumours is yet unclear. Three options still remain: 1) at four months there is already a malignant process which enlarges at one year, 2) at four months there is a reactive or mechanical process which subsequently develops into a malignancy, or 3) at one year the tumours may still be considered benign.

Based on morphologic criteria we are inclined to support the theory that due to mechanical forces a mucinous tumour develops, which at first resemble benign human tumours found in the colon and stomach, called proctitis or gastritis cystica profunda, although they already share some characteristics of malignancy. After one year malignant degeneration seems to occur, although some major criteria of malignancy, i.e. infiltrative growth, vaso-invasion and/or distant dissemination, are not met. Further investigation into the true nature of these tumours is still necessary.

As they are confined to the submucosa, and precursor lesions can not be found, these tumours are more likely to develop via mechanical forces (such as stitches) introducing mucosal glands into the submucosa, than via reflux. This model is therefore not suited for the study of true Barrett’s carcinoma.
References


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Figure 1: End to side anastomosis of the oesophagus and the jejunum with gastric resection (Experiment I)
Figure 2: Side to side anastomosis (oesophago-gastro-jejunostomy) with gastric preservation (Experiment II).
Figure 3: Time course of weight changes in both experimental groups. Points represent the median.
Figure 4: A photomicrograph of a hematoxylin-eosin slide showing a mucinous tumours process at four months after oesophago-jejunostomy with gastric resection. The normal oesophageal epithelium has disappeared, and there is a massive ulcerative oesophagitis. The tumour is well-differentiated and is confined to the submucosa. There is no involvement of either the muscular layer or the mucosa. There is glandular disruption with the formation of mucin lakes, some nuclear polymorphia, a few mitoses (although not on the border of the tumour) and apoptotic necrosis.

1: oesophageal lumen, 2: mucinous tumour
Figure 5: A photomicrograph showing a haematoxin-eosin slide of Barrett’s oesophagus of 10 mm one year after oesophago-gastro-jejunostomy with gastric preservation.

1: oesophageal lumen, 2: Barrett’s mucosa
Figure 6: A detail of a well-differentiated mucinous adenocarcinoma found one year after oesophago-gastro-jejunostomy with gastric preservation. Note the large mucin lakes, the increased polymorphia, and the increased number of mitoses. However, there is no visible ingrowth in the adjacent liver tissue.

1: oesophageal lumen, 2: mucinous tumour, 3: liver tissue
Table 1: Incidence of histological findings two to four months after end to side oesophagojejunostomy (ETS) with gastrectomy, and four and 12 months after side to side oesophagojejunostomy (STS) with gastric preservation.

<table>
<thead>
<tr>
<th>Histological Findings</th>
<th>ETS 2-4 months (n=11)</th>
<th>STS 4 months (n=10)</th>
<th>STS 12 months (n=10)</th>
</tr>
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<tr>
<td>Squamous hyperplasia</td>
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<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Oesophagitis</td>
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</tr>
<tr>
<td>Ulcerations</td>
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<td>6</td>
<td>4</td>
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<tr>
<td>Length of columnar epithelium</td>
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<td>1 mm</td>
<td>10 mm (range 0-22)</td>
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<td>Dysplasia in Barrett</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Oesophagitis cystica profunda</td>
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<td>5</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>6*</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

*There were six squamous cell carcinomas surrounded by dysplastic squamous epithelium in four animals. There were two additional animals with dysplastic squamous epithelium, but without squamous carcinoma.