Evaluation of the intestinal microflora in the rat model for esophageal adenocarcinoma

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SUMMARY. Surgically induced duodenal reflux results in cancer development in the rat esophagus. One proposed mechanism of carcinogenesis relies on the production of carcinogens in the presence of bacterial overgrowth. Against this background, intestinal microflora in the rat jejunum was analyzed before and after reflux-inducing surgery. Total gastrectomy and esophagojejunostomy were performed on Sprague–Dawley rats to produce esophageal reflux of duodenal juice (n = 12). Three days before surgery they were randomized into three groups: animals which received tap water; animals which received acidified water at pH 1.8; and animals subjected to oral decontamination with triple antibiotics. During surgery and at autopsy after 2 weeks, intestinal juice was aspirated and analyzed immediately for bacterial content. The physiologic microflora of the rat jejunum contained Lactobacillus spp. and Bacteroides spp., both of which were resistant to the antibiotic regimen. Bacterial overgrowth with fecal bacteria was found following surgery. Acidified water did not alter the intestinal microflora. Triple antibiotics eliminated Escherichia coli and Proteus spp. and reduced the concentration of Enterococcus spp. Bacterial overgrowth by bacteria of the fecal flora occurs in the rat model of esophageal adenocarcinoma with the potential to catalyze the production of carcinogens.

INTRODUCTION

For unknown reasons, the incidence of esophageal adenocarcinoma has risen dramatically in the past 20 years.¹² It is known that metaplastic specialized intestinal epithelium (Barrett’s esophagus) is associated with an increased risk for the development of esophageal adenocarcinoma.³⁴ Both clinical studies⁵–⁹ and animal models¹⁰–¹⁵ have implicated esophageal exposure to duodenal juice as a key factor in the genesis of Barrett’s esophagus and possibly the development of adenocarcinoma.

Traditional animal models for esophageal adenocarcinoma include administration of nitrosamines and surgically induced reflux of gastroduodenal juice.¹⁰,¹¹ Animal studies without carcinogen administration have further shown that duodenal reflux alone induces development of adenocarcinoma of the glandular stomach as well as the forestomach and the esophagus.¹²,¹⁴,¹⁶,¹⁷ For example, adenocarcinoma was induced in nearly half (48%) of the animals by duodenoesophageal reflux 16 weeks after esophagojejunostomy.¹⁵

A proposed mechanism for carcinogenesis in this model relies on the production of carcinogenic N-nitrosamides from physiologic bile acids in the presence of bacterial overgrowth.¹⁸,¹⁹ In humans, bacterial colonization occurs as a result of reduced gastric acidity during treatment with proton pump inhibitors, H₂-blockers, or after gastric surgery.²⁰,²¹ Bacterial catalysis has been described as a relevant mechanism for the production of potentially carcinogenic nitroso compounds.²²

Given this background, the intestinal microflora was analyzed in the rat model of esophageal adenocarcinoma.

METHODS

Study protocol

Intraluminal pH

Esophagojejunostomy with preservation of the stomach may result in alterations of the pH at
were aspirated orad to the jejunotomy before suturing gastrectomy was completed. Intraluminal contents was divided above the gastroesophageal junction and incision and exposition of the stomach, the esophagus and the posterior vagal nerve were preserved. All following experiments included gastrectomy to eliminate any influence of gastric acidity.

**Intestinal microflora**
The intestinal microflora was analyzed in three animals before and 2 weeks after surgery in order to estimate the expected bacterial counts in the jejunum. Based on these results, an experiment was designed with the aim of modifying the intestinal microflora (n=9). One group was given tap water (control group), the second group received acidified drinking water instead of tap water, and the third group received orally administered antibiotics. Acidified drinking water was prepared by adding 0.016 M hydrochloric acid (pH 1.8) and 68 mg/ml sucrose. The orally administered antibiotics, tobramycin (320 mg/l), polymyxin B (400 mg/l) and vancomycin (500 mg/l), were added to the drinking water as reported in the literature. The group that received orally administered antibiotics was designed to be positive control group with maximum reduction of the intestinal microflora. The treatment of all animals started 3 days before the operation. Esophagojejunostomy and total gastrectomy were performed on all animals as described below. Jejunal contents were analyzed during surgery and 2 weeks later.

**Source of contamination**
To identify the source of bacterial contamination, the drinking water and the food were analyzed for bacterial content. The bacterial contents of the feces were analyzed in all animals on the day before autopsy.

The study protocol was approved by the Institutional Animal Care and Usage Committee of the University of Southern California, Los Angeles, CA (intraluminal pH), and by the University of Würzburg, Germany (intestinal microflora).

**Animal care and surgical procedure**
Eight-week-old Sprague–Dawley rats were anesthetized with xylazine hydrochloride (12 mg/kg) and ketamine (75 mg/kg) i.m. A gastrectomy and end-to-side esophagojejunostomy were performed using sterile techniques: after a midline upper abdominal incision and exposition of the stomach, the esophagus was divided above the gastroesophageal junction and gastrectomy was completed. Intraluminal contents were aspirated orad to the jejunotomy before suturing the esophagojejunal anastomosis with eight interrupted full-thickness stitches of 7-0 polypropylene. One milliliter of 0.9% sodium chloride was instilled into the peritoneal cavity and buprenorphin was given s.c. before abdominal wall closure. Water was permitted when the rats awoke, and standard solid chow was provided the next day ad libitum. Rats were on a 12-h light-dark cycle at a temperature of 21.6°C.

**Microbiological evaluation**
A defined volume of jejunal juice was aspirated during surgery (10 μl) and at autopsy 2 weeks later (5 μl). The samples were put on ice and analyzed within 2 h. For quantitative analysis, the samples were diluted 1:5 and 1:500 in brain–heart infusion. Each dilution (100 μl) was inoculated onto 5% sheep blood agar, Sabouraud glucose agar, MacConkey agar, chocolate agar, KV blood agar and anaerobic blood agar. Bacteria were grown, counted and identified to species level or, where appropriate, to genus level, using a standard method. The drinking water, the food and the feces of the animals were semiquantitatively analyzed using standard microbiological techniques. All data are reported as median values.

**RESULTS**

**Intraluminal pH**
The results of the intraluminal pH measurements in animals with esophagojejunostomy and preservation of the stomach are summarized in Fig. 1. Although the gastric juice remained acidic, it was completely neutralized when it reached the anastomotic site.

**Intestinal microflora**
The microbiological evaluation of the intestinal microflora before and after surgery is shown in Fig. 2A–C. In animals receiving tap water (Fig. 2A), the bacterial flora of the jejunum contained *Lactobacillus* spp. and *Bacteroides* spp. *Escherichia coli* was detected at low concentrations. Two weeks after gastrectomy and esophagojejunostomy, bacterial overgrowth of the jejunum with bacteria of the fecal flora was observed. *Escherichia coli*, *Proteus* spp., and *Enterococcus* spp. were present in concentrations up to one million per ml. *Bacteroides* spp. were found in much higher concentrations than preoperative, whereas the concentration of the *Lactobacillus* spp. did not change significantly. Detailed data for this group are shown in Table 1. In animals receiving acidified drinking water (Fig. 2B), overgrowth of the jejunum with fecal bacteria was also observed. The spectrum of bacteria...
before and after surgery was similar to the spectrum found in the group receiving tap water. In contrast, in operated animals receiving antibiotics (Fig. 2C), *E. coli* and *Proteus* spp. were eliminated and the concentrations of *Enterococcus* spp. were significantly reduced. The *Lactobacillus* spp. and *Bacteroides* spp. were resistant to the antibiotic regimen and observed before and after surgery in concentrations similar to the other two groups.

The additional treatment with acidified water or oral antibiotics was well tolerated by all animals over the 18-day time course. In all animals receiving antibiotics a distended colon was noted on surgery and at necropsy.

**Sources of contamination**

Analysis of the drinking water and the food did not show any bacterial contamination. Table 2 lists the bacteria identified in the rat feces. The bacterial spectrum was identical to the spectrum in the jejunum after surgery. Therefore, the bacteria in the jejunum are likely to derive from the feces. There was no significant difference in the bacterial flora of the feces of the animals in the three groups, except for the complete elimination of *E. coli* in animals receiving antibiotics.

**DISCUSSION**

Evaluation of the intestinal microflora showed that *Lactobacillus* spp. and *Bacteroides* spp. constitute the jejunal flora that is regularly present in unoperated animals. Following surgery, bacterial overgrowth with bacteria of the fecal flora occurred in all animals. These bacteria are capable of catalyzing endogenous reactions to produce potential carcinogens and co-carcinogens. Hydroxylation of primary to secondary bile acids can be carried out by *Bacteroides* spp. and *Lactobacillus* spp. Secondary bile acids have been previously related to the development of colon cancer and are thought to act as co-carcinogens. *Bacteroides* spp. and *Enterococcus* spp. contain enzymes that reduce nitrate to nitrite. Bacterial cytochrome cd1-nitrite reductase, catalyzing nitrosation, has been characterized in *E. coli* and *Proteus* spp. These enzymes could catalyze the formation of nitroso compounds of bile acids, which are known carcinogens.

It has been well established that surgically induced duodenal reflux is carcinogenic in the rat esophagus. In this study, bacterial overgrowth was documented in all evaluated animals. As explained, bacterial overgrowth is likely to be a relevant factor in this model. A possible approach to evaluate this issue further is to modify the intestinal microflora. With this aim, the drinking water was modified in two ways: one group received acidified water, an approach that has been already evaluated in this tumor

**Table 1.** Bacterial content of the jejunum in the animals receiving tap water (control group)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time</th>
<th>Lactobacillus</th>
<th>Bacteroides</th>
<th>Proteus</th>
<th>Enterococcus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Before surgery</td>
<td>2 × 10⁵</td>
<td>8 × 10⁴</td>
<td>–</td>
<td>–</td>
<td>4 × 10³</td>
</tr>
<tr>
<td>2</td>
<td>Before surgery</td>
<td>8 × 10⁴</td>
<td>8 × 10³</td>
<td>–</td>
<td>–</td>
<td>2 × 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>Before surgery</td>
<td>2 × 10⁵</td>
<td>2 × 10⁴</td>
<td>–</td>
<td>–</td>
<td>3 × 10⁵</td>
</tr>
<tr>
<td>1</td>
<td>After surgery</td>
<td>–</td>
<td>4 × 10⁷</td>
<td>8 × 10⁵</td>
<td>8 × 10⁶</td>
<td>8 × 10⁶</td>
</tr>
<tr>
<td>2</td>
<td>After surgery</td>
<td>4 × 10⁵</td>
<td>2 × 10⁵</td>
<td>3 × 10⁵</td>
<td>–</td>
<td>3 × 10⁵</td>
</tr>
<tr>
<td>3</td>
<td>After surgery</td>
<td>4 × 10⁵</td>
<td>3 × 10⁵</td>
<td>2 × 10⁵</td>
<td>2 × 10⁶</td>
<td>2 × 10⁶</td>
</tr>
</tbody>
</table>

**Table 2.** Bacterial content of the rat feces 2 weeks after surgery and after 17 days of treatment. The antibiotic regimen eliminated *E. coli* and did not alter the other fecal flora

<table>
<thead>
<tr>
<th>Group</th>
<th>Lactobacillus</th>
<th>Bacteroides</th>
<th>Proteus</th>
<th>Enterococcus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Acidified water</td>
<td>+ +</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>+ +</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>
model;\textsuperscript{15} the other group received water containing an antibiotic regimen with a known effect on the microflora of the rat.\textsuperscript{23}

In the group receiving acidified water, no significant alterations of the intestinal microflora were observed. In the group receiving oral antibiotics, *E. coli* and *Proteus* spp. were eliminated and the *Enterococcus* spp. concentrations were significantly reduced. However, *Lactobacillus* spp. and *Bacteroides* spp. were not eradicated or reduced by this antibiotic regimen. As described above, these remaining bacteria are capable of altering the duodenal juice. Consequently, the described antibiotic regimen cannot be recommended for further testing of the importance of bacterial colonization in the pathogenesis of esophageal carcinoma. Only the use of germ-free animals would allow this issue to be studied reliably. Another approach for the evaluation of the effects of bacterial overgrowth would be the analysis of duodenal juice to detect bacterial products that are known carcinogens.

In conclusion, bacterial overgrowth with bacteria of the fecal flora was present in the rat model of esophageal adenocarcinoma. The observed bacteria have the potential to produce endogenous carcinogens. It has to be evaluated in further studies whether these carcinogens actually develop in this animal model.

References