Duodenogastric reflux and foregut carcinogenesis: analysis of duodenal juice in a rodent model of cancer

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The incidence of esophageal adenocarcinoma is increasing rapidly. In rats, surgically induced duodenoesophageal reflux is carcinogenic. One proposed mechanism of carcinogenesis is based on the reaction of physiological bile acids with nitrite to produce carcinogenic N-nitroso amides. To test this hypothesis, duodenal juice was analyzed for endogenously formed N-nitroso bile acids and its genotoxicity was determined. Esophagojejunostomy was performed on 15 Sprague–Dawley rats to produce duodenoesophageal reflux. At the time of surgery and 2 and 6 weeks later, duodenal contents were aspirated and analyzed immediately. High performance liquid chromatography coupled to tandem mass spectrometry was used to detect bile acids and their nitroso derivatives. Genotoxicity was assessed using a micronucleus test. The characteristic pattern of bile acid derivatives, with taurocholic acid (TCA) and glycocholic acid (GCA) as the predominant conjugates, was detected in all samples. However, even selective reaction monitoring experiments failed to demonstrate the presence of any N-nitroso-TCA or N-nitroso-GCA. In addition, other nitroso derivatives could not be detected in any of the samples by neutral loss experiments monitoring the loss of nitric oxide (detection limit 0.1% of the concentration of TCA). All samples were cytotoxic, but neither the preoperative nor the postoperative samples were genotoxic. Duodenal juice was cytotoxic but not genotoxic. Tumorigenesis of esophageal adenocarcinoma in the rodent model could not be linked to a specific carcinogen, especially not to nitroso bile acids. Chronic inflammation is likely to be the mechanism of carcinogenesis by duodenogastric reflux.

Introduction

The incidence of esophageal adenocarcinoma has risen dramatically in the past 20 years (1–3). This disease develops in a metaplasia–dysplasia–carcinoma sequence from Barrett’s esophagus as a complication of reflux disease (4,5). Barrett’s esophagus and esophageal adenocarcinoma are associated with increased exposure of the esophagus to duodenal juice (6–10). This observation has renewed interest in the importance of the components of duodenal juice for foregut carcinogenesis.

Reflex of duodenal juice is a prerequisite for the development of esophageal adenocarcinoma in rats treated with nitrosamines (11–13). Furthermore, recently published studies have shown that surgically induced duodenal reflux alone is carcinogenic to the esophagus in rats (14–17) and mice (18,19). One proposed mechanism of carcinogenesis relies on the reaction of physiologic bile acids with nitrite producing carcinogenic N-nitrosoamides (20–24). Bacteria that are capable of catalyzing endogenous reactions to produce nitroso compounds (25,26) have already been identified in this rat model (27).

The aims of this study were: (i) to analyze the duodenal juice of rats that had undergone surgery to see if N-nitroso bile acids—a marker for the endogenous formation of nitroso compounds—were present and (ii) to test the duodenal juice for genotoxicity.

Materials and methods

Study protocol

Fifteen 8-week-old Sprague–Dawley rats were subjected to esophagojejunostomy to produce duodeno-esophageal reflux. During surgery, samples of duodenal contents were aspirated and stored at –80°C. Two weeks (n = 12) and 6 weeks (n = 3) later, the esophagus and the attached jejunum were excised under anesthesia and luminal contents were aspirated. The anatomic region of the esophagus was fixed in formalin and evaluated after hematoxylin–eosin staining. The duodenal contents were analyzed <2 h after aspiration by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS). The genotoxicity of the aspirates was assessed using a micronucleus test. The study protocol was approved by the animal welfare committee of the University of Würzburg, Germany.

Animal care and surgical procedure

Using sterile techniques under anesthesia with ketamine (75 mg/kg) and xylazine hydrochloride (12 mg/kg) i.m., gastrectomy and end-to-side esophagojejunostomy were performed (Figure 1). A midline upper abdominal incision was made. The gastrectomy was performed, then intraluminal contents were aspirated through the jejunojejunostomy located 4 cm distal to the ligament of Treitz. The esophagojejunal anastomosis was sutured with eight interrupted full-thickness stitches of 7-0 polypropylene. Before abdominal wall closure, 1 ml of 0.9% sodium chloride was instilled into the peritoneal cavity and buprenorphin was given s.c. 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–12 h dark cycle at 21.6°C.

HPLC-MS analysis

Chromatographic separation was performed with an Applied Biosystems 140b pump. A SunChrom Triathlon autosampler (BAI, Bensheim, Germany) was used for sample injection (5 µl). HPLC-MS analysis was performed using a TSQ 7000 tandem mass spectrometer system equipped with an electrospray interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were conducted on a DEC 5000/33 workstation (Digital Equipment, Unterföhring, Germany) with the help of ICIS 8.1 software (Finnigan MAT).

Immediately before HPLC analysis, the duodenal samples were diluted with aqueous 10% (v/v) acetonitrile (typically 0.1 ml) and centrifuged to obtain a clear supernatant. Chromatographic separation for HPLC-MS analysis was performed on a Eurospher 100 C18 column (100 mm×2.0 mm i.d.; 5 µm diameter; Knauer, Berlin, Germany). Solvent A was 0.05% (v/v) trifluoroacetic acid (TFA) in water and solvent B was acetonitrile. For gradient elution the HPLC was programmed as follows: pressurization with 50% solvent B; equilibration for 10 min at 10% solvent B; linear gradient elution: 0 min, 10% solvent B; 18 min: 90% solvent B; 25 min: 90% solvent B. For pneumatically assisted electrospray ionization (ESI), the spray capillary voltage was set to 3.5 kV. The temperature of the heated inlet capillary was 240°C. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 units). Protonated molecules [M+H]+ were detected with a total scan duration of 1.0 s for a single spectrum (mass range m/z 150–800). Product ion scanning was performed at a collision gas pressure of 267 mPa Ar and collision energies of 15 eV (N-nitroso-TCA, N-nitroso-GCA) or 25 eV (TCA, GCA) with a scan duration of 1.0 s. The most abundant product ions were chosen for the selected reaction monitoring (SRM) experiments at a scan duration of 0.25 s.

Abbreviations: GCA, glycocholic acid; TCA, taurocholic acid.

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each: Selected ions for detection of \(N\)-nitroso-TCA: \(m/z\) 545 to \(m/z\) 514 and \(m/z\) 545 to \(m/z\) 509; selected ions for detection of \(N\)-nitroso-GCA: \(m/z\) 495 to \(m/z\) 464 and \(m/z\) 495 to \(m/z\) 459. To detect unknown \(N\)-nitroso derivatives, the neutral loss of NO \([M+H-30 \text{ u}]^+\) and HNO \([M+H-31 \text{ u}]^+\) was monitored scanning precursor ions from \(m/z\) 150 to \(m/z\) 1000 with a total scan duration of 1.0 s for a single spectrum. Commercially available bile acids and chemically synthesized \(N\)-nitroso derivatives of TCA and GCA (28) were used as reference substances.

**Fig. 1.** Surgical model for esophageal adenocarcinoma: induction of duodeno-oesophageal reflux following gastrectomy and reconstruction with end-to-side esophagojejunostomy.

**Micronucleus test**

The micronucleus test was applied to the samples obtained before surgery and 2 weeks after surgery. This test, a well established mammalian genotoxicity assay (29), is sensitive to various kinds of genotoxic lesion. Duodenal juice was added to 1.5 ml cultures of exponentially growing L5178Y mouse lymphoma cells in various concentrations. Etoposide (5 ng/ml) or ethyl methanesulfonate (400 µg/ml) served as positive controls. The chemical was replaced by washing out with medium after 4 h; the duodenal juice was left in the culture. Cytochalasin B (5 µg/ml) was added for the time of treatment. This compound prevents cell division after mitosis, resulting in binucleate cells. The analysis of micronucleus frequencies can thus be limited to the actively proliferating cell fraction. The proportion of binucleate cells can serve as an indicator for cytotoxic effects (inhibition of cell proliferation). After 20 h expression time, cells were harvested, placed on glass slides by cytopsin centrifugation and fixed with methanol (−20°C, 1 h). To stain nuclei and micronuclei, the slides were incubated in 0.00625% (w/v) acridine orange in Sörensen buffer (67 mmol/L of Na₂PO₄/KH₂PO₄, pH 6.8), washed twice in Sörensen buffer and mounted in buffer for microscopy. Using a 500× magnification, cells were analyzed for the number of nuclei and micronuclei. The proportions of cells containing one or two or more nuclei and the proportions of binucleate cells containing micronuclei were determined. Objects were classified as micronuclei if they appeared to be separated from the nucleus/nuclei, were round or oval, had an area of less than quarter of the area of a nucleus and showed staining characteristics similar to those of the nuclei. Results are listed as the mean of two slides from one experiment with at least 1000 cells analyzed for the number of nuclei and with 1000 binucleates analyzed for the presence of micronuclei per slide.

**Results**

Histological examination revealed severe esophagitis in all animals after 2 and 6 weeks. The esophagitis was characterized as hyperplastic when the squamous epithelium was thickened.
with normal maturation and hyperkeratosis, or as regenerative when the squamous epithelium showed increased height of lamina propria papillae, basal cell hyperplasia and absence of hyperkeratosis. Regenerative changes are identical to changes described for reflux esophagitis in humans. There was no columnar lining in the esophagus. In one animal exposed to reflux for 6 weeks, a small (1 mm diameter) tumor was found at the anastomotic site; this was identical to the mucinous adenocarcinoma described in previous studies (14–17).

The maximum volume of the aspirates was 25 µl during surgery and 15 µl at completion of the experiment. Aliquots of 2–6 µl were used for HPLC or the micronucleus test. The pH of the aspirates was between 6.6 and 7.3.

HPLC-MS analysis showed the characteristic pattern of bile acid derivatives, with TCA and GCA as the predominant conjugates, in all preoperative samples (Figure 2) and in the duodenal preparations collected 2 and 6 weeks after surgery (Figure 3, upper chromatogram). Identification of TCA and GCA was confirmed by comparing the retention times, molecular ions [M+H]+ and characteristic production spectra obtained from the animal samples with data from commercially available reference compounds.

Neither N-nitroso-TCA nor N-nitroso-GCA could be detected in any animal sample (Figure 3, bottom chromatogram). It was estimated that the lower limit of detection of N-nitroso-TCA or N-nitroso-GCA would be 0.1% of the signal intensities of the respective bile acids, TCA and GCA. Of note, N-nitroso-TCA and N-nitroso-GCA were reasonably stable at −20°C for >4 in diluted aqueous solution containing 2% (v/v) acetic acid, while the ESI mass spectrum of reference compounds demonstrated the lability of the N-nitroso bile acids under study. The absence of N-nitroso-TCA and N-nitroso-GCA in the animal samples was further substantiated by highly specific selective reaction monitoring experiments (data not shown). Formation of trace amounts of N-nitroso-TCA and N-nitroso-GCA in this esophageal cancer model can therefore be excluded. Finally, neutral loss experiments were applied to detect any N-nitroso compound present in the animal samples. Again, no nitroso derivatives were detected (data not shown).

The micronucleus assay detected a wide range of toxicity which was measured as a reduction of the number of binucleate cells. However, duodenal juice did not induce formation of micronuclei at any of the concentrations tested here. Two representative experiments are shown here (Table I). The results were consistently negative. A previous experiment that did not use cytochalasin B also yielded negative results. Thus we conclude that the duodenal juice obtained before and after surgery was not genotoxic in the in vitro micronucleus assay using the described protocol.

**Discussion**

In this animal study, the observed histological changes induced by reflux of duodenal contents were similar to those described in previous studies. Esophagitis developed in all animals and a tumor was observed in one animal after 6 weeks’ exposure. The experimental conditions were comparable to those in studies that demonstrated reflux-induced carcinogenesis (14–17).

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**Fig. 3.** HPLC-MS analysis of duodenal juice sampled 2 weeks after surgery. (Bottom) mass chromatogram for m/z 495 and m/z 545 ([M+H]+ of N-nitroso-GCA and N-nitroso-TCA); (Top) mass chromatogram for m/z 466 and m/z 516 ([M+H]+ of GCA and TCA). Note that the scaling of the two chromatograms differs by a factor of about 1:1000.

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The mechanisms accounting for carcinogenesis in this animal model are not yet clear. One likely explanation concerns the carcinogenic potential of N-nitroso compounds (21). In addition to supply from dietary and environmental sources, endogenous formation of N-nitroso derivatives has to be taken into consideration. For example, the chemical nitrosation reaction of bile acids has been reported in the presence of nitrite or nitrite sources (28). In this study, HPLC-MS analysis demonstrated the absence of N-nitroso bile acid conjugates in the animal model. The primary constituents of duodenal juice, TCA and GCA, were found in all samples. N-Nitroso TCA and N-nitroso-GCA have only moderate mutagenic and carcinogenic potential, as determined by testing them at ~0.12 mM in Salmonella typhimurium (23) and after application of 300 mg of these compounds in rats (24), so it appears very unlikely that endogenous nitrosation of bile acids contributes to reflux-induced carcinogenesis. It could be argued that analysis of only two N-nitroso bile acids cannot rule out the presence of other N-nitroso compounds or the relevance of these substances with regard to the observed carcinogenic effects. However, chemical nitrosation is a non-specific reaction, so N-nitroso-TCA and N-nitroso-GCA can serve as marker substances for endogenous nitrosation reactions in general. As no nitroso derivatives were formed from the primary constituents of duodenal samples, TCA and GCA, in this study, it seems unlikely that other nitroso compounds are formed in this model. This conclusion was further substantiated by neutral loss experiments monitoring the cleavage of NO for detection of any N-nitroso compound present in the animal samples. Again, no nitroso derivatives were detected, excluding the presence of substantial amounts of endogenously formed nitroso compounds. Previous reports on the synthesis of N-nitroso bile acid conjugates have mentioned the formation of nitrite esters besides N-nitroso amides. These bile acid nitrites could serve as an additional marker of nitrosation reactions. However, a prominent role of nitrites in this animal model can be excluded based on the mass chromatogram shown in Figure 3. No molecular ions of N-nitroso amides or any isobaric mononitrite esters derived from GCA and TCA were identified. The nitroso compounds of TCA and GCA have been shown to be genotoxic in in vitro tests (23), so the lack of genotoxicity noted here is further evidence that no relevant concentrations of nitroso compounds were present in the tested samples of duodenal juice. Moreover, there were no other components that showed genotoxicity, although a wide range of toxicity could have been detected using the amounts of sample applied. This is in accordance with the results of previous studies that identify bile salts as possible tumor promoters, but not as direct carcinogens (30).

Carcinogenesis by duodenogastric reflux has been shown in the rodent model in rats and mice (14–19). What were the mechanisms of carcinogenesis in this model, if carcinogenic nitroso compounds were absent and the duodenal juice was not genotoxic? On the basis of the available data, we believe that chronic inflammation is likely to be a major factor in tumor development in this model: The chronic inflammatory process increases oxidative stress (31). Markers for oxidative stress have already been documented in human esophagitis (32–34) and in this rat model (35,36). Furthermore, tumor yield was increased in rats receiving iron dextran (50 mg Fe/kg i.p.) (15), an oxidant that augments oxidative stress (31). It has been shown in subsequent studies that iron supplementation enhances inflammation and the production of reactive oxygen and nitrogen species in the esophageal epithelium (36,37). These processes could contribute to the development of esophageal adenocarcinoma. The concept of mutagenesis driven by mitogenesis for the induction of these tumors is further supported by the pattern of p53 mutation in Barrett’s carcinoma (38,39). The mutation pattern, with a predominance of G→C→A/T transitions, is similar to the mutations described for in vitro tests mimicking the situation of chronic inflammation (40).

In clinical studies, it has been shown that acid reflux and duodeno-esophageal reflux act synergically in the development of esophagitis, Barrett’s esophagus and dysplasia in Barrett’s esophagus (8,9). Acid reflux alone or with exogenous carcinogen does not induce esophageal adenocarcinoma in the rat model (12,14). Chemical analysis using the most sensitive methods and biological testing for genotoxicity failed to show the presence of nitroso bile acids, so carcinogenesis in this model is unlikely to be mediated by nitroso bile acids or related nitrosation products. There is strong evidence that chronic inflammation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample tested</th>
<th>Concentration</th>
<th>Binucleate cells (%)</th>
<th>Micronucleus-containing cells per thousand binucleate cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>400 µg/ml</td>
<td>79</td>
<td>4.5</td>
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<tr>
<td></td>
<td>ethyl methane sulfonate</td>
<td>2 µl/ml</td>
<td>73</td>
<td>3.7</td>
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<tr>
<td></td>
<td>before surgery</td>
<td>4 µl/ml</td>
<td>32</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>2 weeks after surgery</td>
<td>2 µl/ml</td>
<td>83</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 µl/ml</td>
<td>68</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 µl/ml</td>
<td>37</td>
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</tr>
<tr>
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<td>control</td>
<td>5 ng/ml</td>
<td>73</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>etoposide</td>
<td>2 µl/ml</td>
<td>36</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>before surgery</td>
<td>4 µl/ml</td>
<td>13</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>2 weeks after surgery</td>
<td>2 µl/ml</td>
<td>57</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 µl/ml</td>
<td>35</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The percentage of binucleate cells indicates cytotoxic effects (inhibition of cell proliferation). The number of micronucleus containing cells is a measure of genotoxicity. Ethyl methane sulfonate and etoposide served as positive controls for micronucleus induction. Duodenal juice concentrations are given as µl/ml cell culture.
is the main mechanism for foregut carcinogenesis by duodenogastric reflux. The specific relevance of duodenogastric reflux may be related to the development of secondary bile acids or DNA adducts (41).

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References


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