Association Between Duodenal Contents Reflux and Squamous Cell Carcinoma – Establishment of an Esophageal Cancer Cell Line Derived from the Metastatic Tumor in a Rat Reflux Model

KUAN-HAO CHEN, KEN-ICHI MUKAISHO, ZHI-QIANG LING, AKIHIKO SHIMOMURA, HIROYUKI SUGIHARA and TAKANORI HATTORI

Department of Pathology, Shiga University of Medical Science, Seta-tsukinowa-cho, Otsu, Shiga, 520-2192, Japan

Abstract. Squamous cell carcinoma (SCC) of the human esophagus has a multifactorial etiology involving several environmental and/or genetic factors. Recently, gastroesophageal reflux has been implicated as a causative factor in upper aerodigestive tract carcinogenesis. The development of esophageal squamous cell carcinoma (ESCC) in a duodenal contents reflux model without any known carcinogen present has been reported previously. In this study, the duodenal contents reflux model without gastrectomy was used. At 60 weeks post-operatively, all surviving animals had malignant lesions as follows: ESCC (40%), esophageal adenocarcinoma (EAC) (20%) and adenosquamous carcinoma (40%). In one subject, a well-differentiated ESCC was detected with thoracic dissemination and metastases in lymph nodes. A novel cell line, designated ESCC-DR, was established from the thoracic metastatic tumor at the 60th post-operative week. These cells were transplanted into nude mice, and the developed nodules represented a well differentiated ESCC, resembling that of the parent site. Duodenal contents reflux has a great potential for malignant initiation and plays a role in developing not only EAC but also ESCC.

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related mortality in the world (1). The overall 5-year survival rate for patients diagnosed with esophageal cancer is poor (e.g. 3%-10%) (2). This malignancy exists in two main forms with distinct etiological and pathological characteristics, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). The majority of esophageal cancer world-wide are ESCCs (1). It is widely accepted that ESCC is associated with smoking and alcohol (3-5). On the other hand, a risk of ESCC is reported not to be associated with gastroesophageal reflux. In addition, individuals with long-standing and severe symptoms of reflux display odds ratios of 43.5 for EAC and 1.1 for ESCC (6). However, recent studies have demonstrated that refluxed duodenal contents cause esophageal carcinoma in rats without exposure to carcinogens (7, 8). The histological spectrum of these carcinomas includes ESCC, EAC and adenosquamous carcinoma (7, 8). N-nitrosocompounds from multiple sources, including reflux of gastric juice, have been postulated as one of the causative factors of esophageal cancer (9, 10). Recently gastroesophageal reflux has also been implicated as a causative factor in upper aerodigestive tract carcinogenesis, with SCC as the most frequent histological type (11-15). To elucidate the causative factors underlying esophageal carcinomas including SCC, the histological features of a rat duodenal contents reflux model were investigated with slight modification, i.e., esophago-jejunostomy without gastrectomy. From a metastatic tumor in this model, a novel cell line was established, ESCC-DR, and its characteristics were determined. The high potential of duodenal contents reflux for malignant initiation and the association between duodenal contents reflux and ESCC are discussed.

Materials and Methods

All procedures complied with the ethical guidelines for animal experimentation on the care and use of laboratory animals at Shiga University of Medical Science, Japan.

Duodenal contents reflux model and histological examination of esophageal tumors. Male Wistar rats weighing approximately 250 g were used in this experiment. After fasting for 24 hours, a midline laparotomy incision was made under inhalation anesthesia using diethyl ether. Reflux of duodenal contents was induced according to previously reported procedures with a slight modification (16) in that the esophageo-gastric junction was transected, and the distal cut end closed, the proximal cut end was anastomosed end-to-side to the upper jejunum from about 2 cm anal to the origin.

Correspondence to: T. Hattori, Department of Pathology, Shiga University of Medical Science, Seta-tsukinowa-cho, Otsu, Shiga, 520-2192, Japan. Tel: +81 77 548 2166, Fax: +81 77 543 9880, e-mail: hattori@belle.shiga-med.ac.jp

Key Words: Esophagus, duodenal reflux, squamous cell carcinoma, cell line, rat reflux model.
Subsequently, to reduce the volume of duodenal contents reflux compared with the previously reported model, a serosal suture between the esophagus and the jejunum was added after esophago-jejunostomy as indicated.

Animals were allowed access to water 12 hours post-operatively and food 36 hours later, and were not treated using any known carcinogens. Animals were sacrificed using an overdose of diethyl ether at post-operative weeks 10 (n=16), 20 (n=16), 30 (n=16), and 60 (n=5). Resected esophagus were fixed in 10% formalin in phosphate-buffered saline (PBS) for 4 hours and embedded in paraffin. Serial 4-μm sections were stained with hematoxylin and eosin (HE). The histological features were classified into esophageal ulcer, squamous cell hyperplasia, squamous cell dysplasia, Barrett esophagus, Barrett dysplasia, squamous cell carcinoma, ESCC, adenosquamous carcinoma, and EAC.

Establishment of a cell line. A cell line from a thoracic metastatic tumor at the 60th week post-operative was established according to a previously reported procedure (17). In briefly, the tissue sample was rinsed using PBS (Nakalai Tesque, Kyoto, Japan) and then culture medium, and then minced using a sharp pair of scissors. Fragments were placed into 25 cm² plastic flasks (Corning, NY, USA) and these were left at an angle of 90° for 30 min before adding culture medium. The growth medium used for primary cultures was Dulbecco’s modified Eagle’s medium (DMEM; Nakalai Tesque, Kyoto, Japan) with 1% Antibiotic-Antimycotic solution (GIBCO, NY, USA), and 10% fetal bovine serum (FBS). Culture flasks were left undisturbed for 3 days in a humidified incubator containing 5% CO₂ at 37°C, then the culture medium (4 ml/flask) was changed twice a week. After 2 weeks, 3 flasks containing 2-3 colonies was kept. The colonies with small cell numbers by sterile cotton swab were excluded and only the largest colony in the same flask was kept. Then one of these flasks containing one colony was used. Confluent cultures were subcultured after 30 days from the start of culturing by replacing growth medium with PBS. After draining off the solution, 0.05% Trypsin/0.53 mM-EDTA (Nakalai Tesque) was added to transfer culture cells into a monolayer culture system. Cells were transferred at dilutions of 1:4 using 0.05% Trypsin/0.53 mM-EDTA solution to detach cells.

Backtransplantation. Five nude mice (BALB/cA Jcl-nu) were subcutaneously inoculated with 1x10⁶ ESCC-DR cells into the back skin. Developed tumors were removed 4 weeks after injection, fixed in 10% formalin in PBS for 4 hours and embedded in paraffin. Serial 4-μm sections were stained with HE.

Results

Histological findings in this model. All histological findings are summarized in Table I. In the esophagus of the present reflux model, we detected esophageal ulcers (93.8%), hyperkeratosis (80.9%) and dysplasia with mild atypia (12.5%) at 10 weeks post-operatively. At 20 weeks post-operatively, esophageal ulcers (100%) and dysplasia with moderate to severe atypia (56.3%) were detected. Barrett esophagus (31.3%) and Barrett dysplasia (0.82%) were also detected. At 30 weeks post-operatively, we detected Barrett esophagus (50.0%), squamous cell dysplasia (75.0%), ESCC (18.8%), and EAC (12.5%). No adenosquamous carcinoma was detected within 30 weeks post-operatively. At 60 weeks post-operatively, all animals had malignant lesions as follows; ESCC (40%), EAC (20%) and adenosquamous carcinoma (40%). When we opened the thoracic cavity of 1 of 5 surviving rats at 60 weeks post-operative weeks, we detected ESCC (40%), EAC (20%) and adenosquamous carcinoma (40%).

Table I. Histological findings at 10, 20, and 30 weeks post-operatively.

<table>
<thead>
<tr>
<th>Histological findings</th>
<th>10 weeks</th>
<th>20 weeks</th>
<th>30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal ulcer</td>
<td>15/16</td>
<td>16/16</td>
<td>16/16</td>
</tr>
<tr>
<td>Squamous hyperplasia</td>
<td>12/16</td>
<td>16/16</td>
<td>16/16</td>
</tr>
<tr>
<td>Squamous dysplasia</td>
<td>2/16</td>
<td>9/16</td>
<td>12/16</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0/16</td>
<td>0/16</td>
<td>3/16</td>
</tr>
<tr>
<td>Barrett esophagus</td>
<td>0/16</td>
<td>5/16</td>
<td>8/16</td>
</tr>
<tr>
<td>Barrett dysplasia</td>
<td>0/16</td>
<td>1/16</td>
<td>2/16</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0/16</td>
<td>0/16</td>
<td>2/16</td>
</tr>
</tbody>
</table>

Figure 1. Animal model. We established a duodenal contents reflux model, using esophago-jejunalostomy without gastrectomy. To reduce the volume of duodenal contents reflux compared with the previously reported model, a serosal suture between the esophagus and the jejunum was added after esophago-jejunalostomy as indicated.
post-operatively, we detected an increase in the wall thickness on the serosal side of esophagus, swelling of lymph nodes, and multiple small nodules on the surface of the lung, heart and peritoneum (Figure 2). There were deep ulcerations in the lower portion of the esophagus and an unevenly eroded surface in the middle portion apart from the anastomosis. We tried to establish the cell line using the metastatic site. Microscopic examination revealed the tumor was a well-differentiated SCC with thoracic dissemination and metastasis in mediastinal lymph nodes and the lung (Figure 3).

Establishment of a cell line. A cell line, ESCC-DR, was established from the metastatic site at 60 weeks post-operatively. This cell line maintained a doubling time of 24-hour through more than 30 passages from the initial culture (Figure 4).

Backtransplantation. Injection of 10⁶ cultured cells on the 15th passage produced tumors in 5 out of 5 mice. Macroscopically, all mice exhibited nodules, measuring about 1 cm in diameter, during the 4 weeks after injection (Figure 5). Histologically, nodules represented well differentiated ESCC, resembling those of the primary and metastatic sites (Figure 6).

Discussion

In a duodenal contents reflux model of rats, we detected ESCCs with thoracic dissemination and metastases in lymph nodes developed at 60 weeks post-operatively. Although many previous studies have reported that refluxed duodenal contents caused esophageal carcinomas in rats without exposure to carcinogens, this, to our knowledge, the first report that describes the development of esophageal carcinoma with thoracic dissemination and metastases in the reflux model without using any known carcinogens. This indicates that the duodenal reflux can induce the tumors of highly malignant behavior as well as of malignant histology.

Figure 2. Macroscopic findings of a case of thoracic disseminated case. a) Serosal side in the thoracic cavity: Swelling of lymph nodes, and multiple small nodules in the surface of lung, heart and peritoneum were detected. b) Mucosal side: Marked thickness of esophageal wall, deep ulceration and an unevenly eroded surface were detected.
A cell line from the metastatic tumor was also established. The cultured cells were transplanted into nude mice, and produced tumors similar to that of the parent site. These results have confirmed a high potential of duodenal contents reflux for malignant initiation.

However, little is known about the mutagenic potential of refluxed material on esophageal mucosa. Thissen et al. recently reported preliminary evidence of the mutagenic potential of bile reflux on esophageal epithelium, using Sprague Dawley/Big Blue F1 lacI transgenic rats which underwent esophagoduodenostomy to surgically create duodeno-gastric-esophageal reflux (18). On the other hand, many researchers have already addressed the role of bile acids and pancreatic juice in elucidating carcinogenic effects of duodenal contents (19, 20), and others referred to the presence of bacterial flora in duodenal juice that are capable of catalyzing endogenous reactions to produce nitroso compounds (21, 22). Recently, we evaluated the inhibitory effect of thiazolidine-4-carboxylic acid (thioproline) as a nitrite scavenger and as a most sensitive probe to detect N-nitroso compounds. Thioproline was found to prevent esophageal and remnant stomach carcinogenesis, thereby indicating the possible role of N-nitroso compounds in carcinogenesis (10, 23). In addition, extensive research in China has also suggested that N-nitroso compounds and their precursors are probable etiological factors for esophageal cancer in the areas of high incidence (24).

Although it is widely accepted that duodenogastric-esophageal reflux is directly linked to Barrett’s esophagus and to the development of EAC, a risk of ESCC is reported
not to be associated with gastroesophageal reflux (6). In contrast, results of several studies using rat duodenal contents reflux models have shown that development of esophageal carcinomas includes squamous-cell carcinoma (8, 10, 25). In this study, the incidence of pure adenocarcinoma is lower than that of squamous cell carcinoma. It is unclear what factors lead to the formation of carcinomas of specified histology. Miwa et al. suggested ESCC developed in places distant from the anastomosis compared to EAC (8). This means that histological features may depend on the volume of reflux contents; small amounts of reflux causes ESCC and a large volume of reflux causes EAC. In our modified model, we added a serosal suture between the esophagus and the jejunum after esophago-jejunostomy. This addition of a serosal suture may decrease the reflux of duodenal contents compared with other models, so that the incidence of ESCC was higher than that of EAC in this study.
Great interest has been focused on gastroesophageal reflux as an independent carcinogenic factor and a co-carcinogen of laryngeal SCC in association with smoking and alcohol assumption (11-15). Commarota et al. reported that the risk of laryngeal SCC increased more than 10-fold in 20 years or more after gastric resection in a retrospective case-control study of subjects admitted in the same hospital (14). This study included 828 consecutive patients with laryngeal cancer and 825 controls with acute myocardial infarction. The term of 20 years is quite long. Although they mentioned the carcinogenetic effect of duodenogastroesophageal reflux in laryngeal cancer, they also suggested that the distance of the larynx from the stomach and the presence of a low-grade, chronic stimulation could explain the long time needed for duodenogastroesophageal reflux to become dangerous. This hypothesis also supports the possibility that continuous small amounts of reflux contents might be associated with the development of SCC.

In conclusion, duodenal contents reflux has a great potential for malignant initiation, inducing not only EAC but also ESCC, and even developing highly malignant tumors with thoracic dissemination and metastases in lymph nodes and the lung. From the metastatic tumor, we have established the first culture model that was derived from the reflux-induced tumors. This cell line, designated ESCC-DR, could represent a suitable material for molecular analyses of ESCC and tests of chemotherapeutic agents against ESCC.

Acknowledgements

This work was supported in part by Grant-in-aid for Cancer Research from the Ministry of Health, Labour and Welfare.

References


Received May 29, 2006
Revised December 14, 2006
Accepted December 19, 2006