Oxidative damage in an esophageal adenocarcinoma model with rats

Xiaoxin Chen, Yu Wei Ding, Guang-yu Yang, Flordeliza Bondoc, Mao-Jun Lee and Chung S. Yang

Laboratory for Cancer Research, College of Pharmacy, Rutgers University, 164 Frelinghuysen Road, Piscataway, NJ 08854, USA

Introduction

Esophageal adenocarcinoma (EAC) has received considerable attention in recent years because of its rapid increase in incidence. Between 1976 and 1990, the incidence rate of EAC in the USA tripled, with a yearly increase of ~10%, which was the fastest increase of all the cancers (1,2). It now accounts for >50% of all the esophageal cancers, and affects about 10,000 people per year (3). EAC has extremely bad prognosis with a 5 year survival rate of ~10% (4); therefore, it is of great importance to understand the pathogenesis and develop strategies for the prevention of this deadly disease.

It is now clear that most of the EACs develop from a premalignant disease, columnar-lined esophagus (CLE), also known as Barrett’s esophagus, characterized by the replacement of squamous epithelium in the esophagus by columnar epithelium (3,5). Most CLE is preceded by reflux esophagitis, which is a commonly seen clinical identity in the western countries, with >30% of the general population experiencing its symptoms at least once every month (6). Approximately 10% of all the reflux esophagitis patients will eventually develop CLE (5,7). According to a large autopsy study, the incidence of CLE in the general US population was estimated to be one out of 80 (8). The risk of EAC for CLE patients has been estimated to be about one in 100 patient-years, 30–125 times higher than the general population (5,9).

Oxidative damage has been proposed to be closely related to reflux esophagitis, and a possible cause for CLE (10). Wetscher et al. (11) found that reactive oxygen species (ROS), as measured by chemiluminescence and lipid peroxidation, increased with the grade of esophagitis and were the highest in CLE. Anti-reflux surgery prevented the development of oxidative damage in the esophagus. Consistent with this idea is that β-carotene had been shown to prevent, even reverse, the progression of human CLE (12). Epidemiological studies also indicated an inverse association between the intake of β-carotene and the risk of EAC (13). The strong association between EAC and smoking may partially attribute to the fact that cigarette smoking stimulates endogenous pulmonary and vascular production of ROS, and depletes endogenous antioxidant defense mechanisms (14).

Esophagoduodenal anastomosis (EDA), also known as esophagogastro-duodenostomy with rats is the most commonly used surgical model to produce duodenogastric-esophageal reflux. This model produces CLE and low incidence of EAC, and carcinogenesis can be enhanced by treatment with nitrosamines (15–17). However, all the EDA rats developed iron-deficiency anemia due to iron malabsorption. Supplementation with iron dextran by i.p. injection in the rats prevented anemia after surgery, but greatly enhanced the incidence of EAC (16). Our previous study also showed that iron-supplemented EDA rats had significantly higher levels of inflammation, cell proliferation, inducible nitric oxide synthase (iNOS) and nitrotyrosine immunostaining, iron deposition, as well as EAC tumors in the distal esophagi than those without iron supplementation (18). Since iron is known to promote oxidative stress, this appears to be an excellent model to study the role of oxidative damage in esophageal adenocarcinogenesis.

In this study, we further determined the oxidative damage parameters and carcinogenesis in our EDA model with i.p.

© Oxford University Press
Iron supplementation. We were particularly interested in finding out the target cells of oxidative stress. Blood and tissue iron levels were determined to investigate how excess iron was deposited in the esophagus. The data presented here further supported our hypothesis that oxidative damage plays an important role in the formation of EAC.

Materials and methods

Animals and treatment

Six-week-old male Sprague–Dawley rats from Taconic Farms (Germantown, NY) were housed two per cage, given commercial rat chow and water ad libitum, and maintained on a 12 h light/dark cycle. They were allowed to acclimate for 2 weeks prior to surgery. Solid food was withdrawn 1 day before and for 1 day after surgery. EDA was performed according to the procedure described previously (16). This procedure was approved by the Animal Care and Facilities Committee, Rutgers University (protocol #04–017). The animals were given iron dextran i.p. (50 mg Fe/kg/month), starting 4 weeks after surgery and continuing for the duration of the experiment. Another group of animals was included as non-operated controls (Table I).

The animals were weighed weekly. Blood samples (200 µl) were taken from the orbital venous sinus in 10 animals of each group under anesthesia. DNA was extracted from esophageal epithelium with a Wako Extractor WB (Wako Chemical, Richmond, VA), hydrolyzed and analyzed by an HPLC method, using malonaldehyde bis(diethyl acetal) (MDA; Sigma) as the standard.

Table I. Histopathology of rat esophagi at 11, 30 and 35 weeks after EDA

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>No. of animals</th>
<th>Esophagitis (Hetzel grade&lt;sup&gt;ab&lt;/sup&gt;)</th>
<th>CLE</th>
<th>CLE with dysplasia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EAC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 11</td>
<td>I: non-operated control 5</td>
<td>–</td>
<td>2.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Week 30</td>
<td>II: EDA + 50 mg Fe/kg/month</td>
<td>7</td>
<td>3.0 ± 0.5</td>
<td>10</td>
<td>4 (40%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Week 35</td>
<td>I: non-operated control 4</td>
<td>–</td>
<td>3.3 ± 0.5</td>
<td>33</td>
<td>21 (63%)</td>
<td>20 (60%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The data represent means ± SD of all the animals with esophagitis. The Hetzel grading system is as follows: grade 0, normal appearing mucosa; grade 1, mucosa edema, hyperemia and/or friability of mucosa; grade 2, superficial erosions involving <10% of mucosal surface of the esophageal squamous mucosa; grade 3, superficial erosions/ulcerations involving 10–50% of the esophageal squamous mucosa; grade 4, deep peptic ulceration anywhere in the esophagus or confluent erosion of >50% of the esophageal squamous mucosa.

<sup>b</sup>Not significant among the three time points (P > 0.05).

<sup>c</sup>Week 35 significantly higher than week 11, but there is no significant difference between week 35 and week 30, and between week 30 and week 11.

<sup>d</sup>Week 35 and week 30 are significantly higher than week 11 (P < 0.05). No significant difference between week 30 and week 35.

Esophagitis and dysplasia, CLE was diagnosed when there was inflammation of the esophageal mucosa, usually with the partial loss of cell polarity and maturation, nuclear atypia, and an increase in mitotic figures in the CLE cells. EAC was diagnosed when dysplastic columnar epithelial cells invaded through the basement membrane.

Iron histochemistry and immunohistochemical staining

Ferric iron in the tissue sections was detected using the Prussian blue staining plus intensification with diaminobenzidine (DAB) (21). The avidin–biotin–peroxidase complex method (Elite ABC kit; Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining for transferrin receptor (TIR), heme oxygenase 1 (HO1), and metallothionein (MT). A monoclonal mouse anti-rat TR (10 µg/ml; Serotec, Raleigh, NC), a rabbit polyclonal anti-HO1 (1:100; Affinity Bioreagents, Golden, CO), and a mouse monoclonal anti-MT (2 µg/ml; Accurate, Westbury, NY) were used. Sections were pre-treated with target unmasking fluid (Pharmingen, San Diego, CA) or with trypsin. Negative controls were established by replacing the primary antibody with PBS and normal serum.

Determination of oxidative damage

Epithelium of the lower half of the frozen esophagus was stripped off and used for all the biochemical analyses. Lipid peroxidation was determined using an HPLC-based thiobarbituric acid reactive substance (HPLC-TBARS) method, using malonaldehyde bis(diethyl acetal) (MDA; Sigma) as the standard (22). Lipid peroxidation was expressed as nmol MDA/mg tissue protein.

For the analysis of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), genomic DNA was extracted from esophageal epithelium with a Wako Extractor WB kit (Wako Chemical, Richmond, VA), hydrolyzed and analyzed by an HPLC system equipped with a reverse-phase column (PPF column, 60A, 5 µm). The amount of 8-OH-dG residues per 10<sup>6</sup> 2′-dG residues was included.

Statistical analysis

The result on pathogenesis was analyzed by the χ² test. Other data were analyzed by the Student’s t-test using the computer software Statview 4.2.

Results

Fifty-five rats underwent EDA, five (9%) died: one due to anesthesia, three due to blockage (esophageal stricture), and one due to unknown reason. Animals were killed at 11, 30 and 35 weeks after EDA. The body weights of the EDA rats were significantly lower than the non-operated control rats throughout the experiment (P < 0.05) (Figure 1A).

Iron nutritional status

In the first 4 weeks after the surgery, iron nutritional status parameters (hemoglobin, total serum iron and transferrin saturation) dropped markedly, then rose after iron supplementation and gradually reached plateau levels (Figure 1B–D). Injection of iron dextran i.p. (50 mg Fe/kg/month) into the non-operated group significantly increased iron nutritional status parameters (hemoglobin, total serum iron and transferrin saturation) (Figure 1B–D).
Oxidative damage in rat esophageal adenocarcinoma model

Fig. 1. Average body weight and iron nutritional status of rats after EDA and i.p. iron supplementation (□, group I, non-operated control; ■, group II, EDA + 50 mg Fe/kg/month): average body weight (A), hemoglobin (B), total serum iron (C) and transferrin saturation (D), total serum iron immediately after the iron injection (E) and transferrin saturation immediately after the iron injection (F).

of 50 mg Fe/kg/month i.p. resulted in adequate hemoglobin levels, although the total serum iron and transferrin saturation were still significantly lower than those of the non-operated controls (P < 0.05). We also examined total serum iron and transferrin saturation immediately after the injection of iron dextran. Within 12–24 h after i.p. injection of iron, total serum iron and transferrin saturation values peaked at 900 µg/dl and 0.70, respectively, and then decreased to the levels prior to iron injection within 3 days (Figure 1E and F).

Histopathogenesis
The Hetzel grading system was used to quantify the reflux esophagitis in all the EDA rats (Table I). The Hetzel grade of Group II slightly increased from week 11 to week 30 and week 35, but the difference was not statistically significant. All the CLE occurred at the distal esophagus, extending upwards from the esophagoduodenal anastomosis. All the EAC were well-differentiated mucinous adenocarcinomas, arising in the squamocolumnar junction area. The incidence of EAC increased from week 11 to week 30 and week 35 (Table I). Some EAC tumors had remarkable sizes, as big as 1.5 cm in diameter. Small ulcers and necrotic foci were found on some tumors.

Iron deposition in the esophagus
Positive iron staining, as detected by Prussian blue staining with DAB intensification, was seen in macrophages not only in the submucous layer, but also those in the conical papillae of lamina propria (Figure 2A). Substantial amount of staining was seen in the distal esophagus, especially the squamocolumnar junction, where inflammation was most severe and all the EAC arose. Some of the iron-positive macrophages were immediately adjacent to the CLE cells (Figure 2B). Negative iron staining was observed in the esophagi of the non-operated controls. Interestingly, iron-positive cells were scarcely seen in the EAC, but mostly found along the edges of the tumors.

In addition to ‘free’ iron, we further localized TfR that binds transferrin, the carrier for iron transportation. It is known that proliferating cells assimilate iron by overexpressing TfR (24). Many CLE cells in the squamocolumnar junction were found to over-express TfR, suggesting that these cells could assimilate a lot of iron in transferrin-bound form. Less positive staining was seen in columnar cells away from the squamocolumnar junction (Figure 3A and B). Some EAC cells, macrophages and some columnar cells in the duodenal epithelial crypt and the crypt-villus junction were also found to express TfR. No TfR was observed in the superficial villus enterocytes of the duodenum, as reported previously (24). It was particularly interesting that most iron, either in the ‘free’ or ‘bound’ form, was localized in the squamocolumnar junction area, where all the EAC resided.

Oxidative damage in the rat esophagus
We measured three oxidative damage parameters in the lower part of the esophageal epithelia of EDA rats: TBARS for oxidative damage to lipid, 8-OH-dG for oxidative damage to DNA, and carbonyl contents for oxidative damage to protein (Table II). As compared with the non-operated controls, the EDA rats had significantly higher levels of TBARS, 8-OH-dG and carbonyl content at 11 and 30 weeks after surgery. However, we did not observe time-dependent increase of these parameters in the EDA group.

To localize the cells subject to oxidative damages, we examined the expression of two known oxidative stress-responsive genes, HO1 and MT, by immunohistochemistry.
Both CLE cells at the squamocolumnar junction, and EAC overexpressed both HO1 and MT (Figure 4A–D). Overexpression of MT in the basal cells of the esopagitic squamous epithelia was also observed.

**Discussion**

EDA in rats, which mimics the human situation by introducing mixed reflux of gastric and duodenal contents into the esophagus, is the most commonly used animal model to produce CLE and EAC. Iron malnutrition is a serious problem for EDA rats. Previous experience showed that many EDA rats could not survive a 30 week experiment without iron supplementation due to severe iron-deficiency anemia (16). After EDA, insufficient gastric acid resulted in the formation of insoluble ferric salt that is non-absorbable. Occult bleeding due to reflux esophagitis, and rapid passage of food through the duodenum where most iron was absorbed, may also contribute to the iron deficiency (26). Injection of 50 mg Fe/kg/month resulted in adequate hemoglobin and better health.

After i.p. injection of iron dextran, high short-term spikes of serum iron and transferrin saturation appeared at 12–24 h. When the esophageal epithelium was hyperemic and edematous under gastroesophageal reflux, the iron dextran may easily exude out of the dilated capillaries into the interstitial tissue. In the meantime, macrophages in reticuloendothelial system can incorporate some ‘free’ serum iron into siderophore, and carry them to the site of inflammation, where they are poorly re-used and deposited (27). This may explain why the EDA rats all had excess ‘free’ iron deposited in the esophagus, especially in the squamocolumnar junction area where the esophagitis was most severe, while their total serum iron and transferrin saturation values were yet lower than those of the non-operated control rats.

On the other hand, the metabolism of the bound form of iron is well regulated by the expression of TfR and ferritin.

**Table II.** TBARS, 8-OH-dG and carbonyl content in the rat esophageal epithelia after EDA

<table>
<thead>
<tr>
<th>Time point</th>
<th>Group</th>
<th>n</th>
<th>TBARSb (nmol MDA/mg protein)</th>
<th>8-OH-dG (per 10⁵ 2’-dG)</th>
<th>Carbonyl content (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 11</td>
<td>I: non-operated control</td>
<td>5</td>
<td>0.14 ± 0.01</td>
<td>1.45 ± 0.38</td>
<td>1.71 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>II: EDA + 50 mg Fe/kg/month</td>
<td>7</td>
<td>0.30 ± 0.03</td>
<td>2.42 ± 0.58²</td>
<td>3.44 ± 0.38²</td>
</tr>
<tr>
<td>Week 30</td>
<td>I: non-operated control</td>
<td>4</td>
<td>0.20 ± 0.04</td>
<td>1.61 ± 0.35</td>
<td>1.91 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>II: EDA + 50 mg Fe/kg/month</td>
<td>10</td>
<td>0.33 ± 0.04</td>
<td>3.08 ± 0.38²</td>
<td>5.86 ± 1.87²</td>
</tr>
</tbody>
</table>

*The data represent means ± SD.
²Significantly different from that of Group I of the same time point (P < 0.05). Not significantly different between week 11 and week 30.
After iron-binding transferrin gets into cells expressing TfR, the iron is normally stored in ferritin (28). Although incorporation of iron into transferrin and ferritin confers some immediate protection against iron-related toxicity, the iron in ferritin and transferrin are also readily released when stimulated (28,29). As a consequence, iron-related toxicity may still happen in cells where the iron was delivered and stored, and may even affect the adjacent cells (29). In the present study, many CLE cells, EAC cells and macrophages over-expressed TfR, suggesting that these cells assimilated a considerable amount of iron in transferrin-bound form and then stored it in ferritin (Figure 3A and B). The premalignant CLE cells appeared not only surrounded by excessive amount of exogenous iron, but also overloaded with endogenous iron.

It is likely that local iron overload is directly responsible for the enhancement of carcinogenesis. In the present study, the incidence of EAC in the EDA groups on 50 mg Fe/kg/month was as high as 60% at 30 weeks after surgery. EDA itself only induced 7% of EAC in rats at 30 weeks after surgery in one study (15), and no EAC in another two studies (30,31). It is interesting that all the EACs were seen at the squamocolumnar junction of the esophagus. A few factors may contribute to this phenomenon: (i) columnar cells at the squamocolumnar junction tend to be highly proliferating to replace the damaged squamous epithelium. The turnover rate of the columnar cells is approximately five times that of squamous cells, and the columnar epithelium is more resistant to damage from acid and bile acid than squamous epithelium (32). (ii) The squamocolumnar junction has long been predicted to be a site of epithelial stem cells, since graft-versus-host disease after bone marrow transplantation had predilection for the squamocolumnar junction in gut (33). (iii) Substantial amounts of iron were deposited in this region, and both iron and chronic inflammation are known to induce oxidative damage. Extensive oxidative damage on the proliferating stem cells could increase the chance of developing EAC. Our previous study showed overexpression of iNOS and nitrotyrosine in the rat esophagus after EDA and iron supplementation (18). In the present study, 8-OH-dG, lipid peroxidation and carbonyl content in the esophageal epithelia were significantly higher in the EDA rats than the non-operated controls. A strong correlation between oxidative damage and esophageal adenocarcinogenesis was suggested. Although we did not observe time-dependent increase of oxidative damage in the esophagus, it was quite possible that, at 11 and 30 weeks after surgery, oxidative damage had reached a plateau. In another oxidative damage-related carcinogenesis model, lipid peroxidation and 8-OH-dG were found to increase at a very early stage, and then reached their plateau levels (34). Recently, the concept of ‘persistent oxidative stress in cancer’ was proposed as the ROS-mediated mechanism of carcinogenesis. Persistent, rather than rapid and pronounced oxidative damage explains the characteristic biology of cancer cells (35).

An important issue that was addressed in this study was which cells at the squamocolumnar junction were the targets of oxidative damage in this model. In response to oxidative stress, cells overexpress certain genes as an adaptive or protective mechanism, such as HO1 and MT. HO1 is the highly inducible form of heme oxygenase, which catalyzes the initial and rate-limiting step in the oxidative degradation of heme to bilirubin (25). HO1 is rarely expressed in normal cells, except macrophages. When stimulated by inducers of oxidative stress, such as heme and metals, hydrogen peroxide, UV irradiation, inflammatory cytokines and lipid peroxide, many cells overexpress HO1 as a protective mechanism: it decreases the level of free cytosolic iron by inducing ferritin

![Fig. 4. Expression of oxidative stress-responsive genes, HO1 and MT, in rat esophagi after EDA and i.p. iron supplementation. HO1 was expressed in CLE (A), and EAC (B). MT was also expressed in CLE (C) and EAC (D). Positive staining is dark. Se, squamous epithelium of the esophagus.](image-url)
and increases the production of the antioxidative bile pigments (36). Knockout of the HO1 gene made the cells hypersensitive to oxidative stress induced by hemin, hydrogen peroxide, paraglutathione S-transferase, cadmium and endotoxin, according to a recent study (37). MT is a low-molecular-weight protein with a high content of cystein residues, which detoxifies heavy metal and scavenges ROS (25). MT synthesis is known to be induced by various stresses, including heavy metals, UV irradiation, X-irradiation, producers of ROS (tert-butyl hydroperoxide, paraquat, cisplatin, etc.), glutathione consumer (diethyl maleate) and cytokines (38). Overexpression of MT protects against the cytotoxic and DNA-damaging effects induced by nitric oxide (39). Antisense downregulation of MT induced growth arrest and apoptosis in human breast cancer cells (40). Knockout of the MT gene promotes the mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (41).

Normal esophagus does not express HO1 and MT. Some of the columnar epithelial cells and goblet cells of the small intestine occasionally express a low level of MT (42). In this study, we observed overexpression of both HO1 and MT in both CLE cells and EAC. This provided further evidence for the idea that the CLE cells at the squamocolumnar junction were targeted by oxidative stress. Consistent with our results, increased expression of HO1 and MT has been observed in premalignant and malignant cells of various kinds of human and animal cancers (43,44).

In conclusion, we have observed oxidative damage in the esophagus after EDA and iron supplementation. Iron overload occurred at the squamocolumnar junction not only by overexpression of IR in the premalignant CLE cells, but also by migration of macrophages carrying ‘free’ iron. CLE cells were the targets of oxidative stress since they were overloaded by transferrin-bound iron, surrounded by iron-overloaded macrophages, and overexpressed two oxidative stress-responsive genes, MT and HO1. We believe iron plays an important role in the formation of EAC by promoting oxidative damage in the premalignant CLE cells at squamocolumnar junction. A similar situation may occur in humans with gastroesophageal reflux and iron over-nutrition.

Acknowledgements

This study was supported by NIH grant CA75683 and facilities from the NIEHS Center Grant ES05022 and the Cancer Center Support Grant CA72720. C.S.Y. is a member of the Environmental and Occupational Health Sciences Institute and the Cancer Institute of New Jersey.

References

Oxidative damage in rat esophageal adenocarcinoma model


Received June 30, 1999; revised October 4, 1999; accepted October 14, 1999

263