Studies of iron deposits, inducible nitric oxide synthase and nitrotyrosine in a rat model for esophageal adenocarcinoma

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Introduction

Esophageal adenocarcinoma (EAC*) is a disease with a rapidly increasing incidence and an extremely poor prognosis (1–4).

*Abbreviations: ABC, avidin–biotin peroxidase complex; BCH, basal cell hyperplasia; BE, Barrett’s esophagus; DAB, diaminobenzidine; EAC, esophageal adenocarcinoma(s); EDA, esophagoduodenal anastomosis; H&E, hematoxylin and eosin; h.p.f., high power field; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PBS, phosphate buffered saline; SCC, squamous cell carcinoma; TUF, target unmasking fluid.

Intestinal-type Barrett’s esophagus (BE), a condition in which the normal stratified squamous epithelium is replaced by metaplastic columnar epithelium containing goblet cells, is a frequent precursor lesion of EAC (5,6). Patients with BE have a 30–125-fold increased risk over the general population of developing EAC (7–9). It is widely accepted that BE is an acquired condition caused by chronic gastroesophageal reflux (3,7,10).

Esophagoduodenal anastomosis (EDA, surgery to connect the esophagus to the duodenum, also known as duodenoesopha-gostomy) produces gastric and duodenal reflux into the esophagus, but produces EAC in rats at very low rates, if at all (11–13). Treatment of the operated animals with a carcinogen markedly enhances cancer development (45–80%). Most of the esophageal tumors had mixed properties of squamous cell carcinomas (SCC) and EAC, with cells producing keratin in one area and mucin in another. Only a small percentage of tumors were pure well-differentiated EAC.

We reproduced these results with EDA and treatment with a carcinogen, methyl-N-aryl nitrosamine or N’-nitrosornicotine (14). We also found that the operated animals were severely anemic several months after surgery and that this condition was responsive to iron treatment (14,15). In a second experiment, iron dextran (50 mg of Fe/kg, i.p., monthly) was used to prevent the development of anemia in the operated rats. This treatment, without a carcinogen, produced a high rate of intestinal BE (91%) and EAC (73%) in 31 weeks (14). The morphology of rat BE and EAC resembled that of human BE and EAC in many respects, and all of the EAC was pure well-differentiated mucinous EAC.

Chronic gastroesophageal reflux is believed to damage the normal stratified squamous epithelia and produces a compensatory hyperproliferative response in the human and rat esophagi (12,16,17). Induced cell proliferation has been reported to play a key role in carcinogenesis (18–20). Cell proliferation facilitates the conversion of DNA damage to mutations, leading to the activation of oncogenes or inactivation of tumor suppressor genes and a dysregulation of proliferation controls. Damage to the normal human or rat esophageal squamous epithelium by chronic gastroduodenal reflux also produces an inflammatory response in the distal esophagus (12,16,17). Chronic inflammation has long been recognized as a risk factor for a variety of human cancers (21,22). Reactive oxygen species and reactive nitrogen species produced by inflammatory phagocytes are important components of inflammation and have also been shown to cause significant tissue injury. It is possible that these reactive species contribute to all stages of carcinogenesis: initiation, promotion and progression (23,24).

Iron is known to be the most effective metal ion in catalyzing the production of free radicals (25,26). Epidemiological studies have shown that increased body stores of iron are associated with an increased risk of cancer (27–29). An increased production of tumors was also observed in animal models (30–32). We hypothesize that iron supplementation to the EDA rats
enhanced esophageal inflammation and the production of reactive oxygen (nitrogen) species. These species could damage epithelial cells and play a major role in adenocarcinogenesis in the rat. To test this hypothesis we characterized cell proliferation and inflammation, investigated iron deposition, and examined evidence of nitric oxide production in the esophagi of rats that had undergone EDA and received iron or no iron supplement.

Materials and methods

Materials

Iron dextran was purchased from Henry Schein (Port Washington, NY). The chemicals hematoxylin, eosin, potassium ferrocyanide and neutral red were purchased from Sigma (St Louis, MO). Target unmasking fluid (TUF) was purchased from Microm (Carl Zeiss, Inc., Oakville, ON). Peroxidase avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) kits were purchased from Vector Laboratories (Burlingame, CA). Anti-NOS2, a rabbit polyclonal antibody, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-nitrotyrosine, a rabbit polyclonal antibody, was purchased from Upstate Biotechnology (Lake Placid, NY).

Animals and treatment procedures

Six-week old male Sprague–Dawley rats from Taconic Farms (Germantown, NY) were housed at one 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 24 h prior to surgery. EDA was performed according to the procedure described previously (12,14). This procedure was approved by the Animal Care and Facilities Committee, Rutgers University (protocol no. 94-017). Postoperatively, the animals were given water after 2 h and rat chow the following day. The surgical animals were given iron dextran (50 mg Fe/kg, i.p.), starting 2 weeks after surgery (i.e. week 3) and continuing once a month for the duration of the experiment to prevent anemia. The animals were weighed weekly.

Tissue preparation

The rats were anesthetized with CO2, 2 ml of blood were taken by cardiac puncture, and then they were killed by CO2 asphyxiation. The stomach, the duodenum and the part of the duodenum that were removed, were opened longitudinally, photographed and examined for gross abnormalities. The tissue was fixed in 10% buffered formalin for 24 h and then transferred to 80% ethanol. The esophagus was cut 2–3 mm above the blue proline sutures to eliminate the duodenal mucosa that may have puckered during the healing process. A Swiss roll of the esophagus was made, processed and embedded in paraffin. Sections (5 µm) of tissue were mounted onto glass slides and used for hematoxylin and eosin (H&E) staining and immunohistochemistry.

Morphological analyses

Epithelial cell proliferation on the distal third of the esophagus was graded based on morphology, with a scale of 0–3, using the H&E stained tissue slides. The normal stratified squamous epithelium with 1–3 basal cell layers was graded as 0. Basal cell hyperplasia was graded as 1 for mild, 2 for moderate and 3 for severe when the number of proliferating basal cell layers occupied over one-third, one-third to two-thirds and over two-thirds of the total epithelial thickness, respectively. Inflammation was also graded based on morphology using the H&E stained slides, with the following criteria: 0, normal (no inflammatory cells); 1, mild (a few scattered inflammatory cells); 2, moderate (the density of lymphocytes and macrophages was an enlargement of capillaries in the stromal tissues); and 3, severe (high density of lymphocytes and macrophages with an enlargement of capillaries in the stromal tissues).

The data were evaluated statistically using a linear regression model.

Iron deposition in the esophagus

Iron histochemical staining was done using the Prussian blue test for ferric iron. This test involves the following procedure: the tissue sections were dewaxed with xylene and then hydrated with graded ethanol to water. The slides were then immersed in ferrocyanide solution (containing 2% potassium ferrocyanide and 2% aqueous hydrochloric acid), washed, and then counterstained with 1% neutral red. After washing, the tissue sections were dehydrated in graded ethanol, cleared in xylene, and mounted using permount. The iron stained bright blue; the rest of the tissue stained red.

Inducible nitric oxide synthase (iNOS) and nitrotyrosine immunohistochemistry

The basic immunohistochemistry procedure (33) was as follows: the tissue sections were first dewaxed in xylene, and hydrated in a gradient of ethanol to phosphate buffered saline (PBS). Target unmasking fluid was used to unmask the antigen. The endogenous peroxidase activity was quenched with hydrogen peroxide. Normal sera (10% normal goat serum) was applied to minimize non-specific binding. A 1:1 incubation with the first antibody (5 µg/ml for iNOS and 2 µg/ml for nitrotyrosine) was followed by the application of the secondary antibody conjugated to biotin. An avidin–biotin peroxidase complex (ABC) was then applied, and the staining was visualized with diaminobenzidine (DAB). The sections were counterstained with Mayer’s hematoxylin, washed, and then dehydrated using a gradient of ethanol to xylene. The slides were cover-slipped using permount. The percentage of cases that stained positive for iNOS at each time point was determined. For nitrotyrosine evaluation, one slide per rat was stained using the nitrotyrosine antibody. Four slides were chosen at random from each group. For each slide, the number of cells staining positive for nitrotyrosine/h.p.f. were counted manually for three fields at the lower esophagus and three fields at the middle esophagus. Thus, a total of 12 measurements each for the lower and middle esophagus were made per group. The data were evaluated statistically using a linear regression model.

Results

Cell proliferation and inflammation

Cell proliferation and inflammation in the distal esophagus were analyzed as a function of time. Basal cell hyperplasia (BCH) and inflammation were seen at all time points after surgery. There was a significant increasing linear trend for both cell proliferation (P = 0.0003) and inflammation (P = 0.0001) (Table I). Overall, BCH was graded as mild at week 3, mild to moderate at week 5, moderate at weeks 9 and 17, and moderate to severe at weeks 23 and 31. The hyperplastic epithelium covered the bottom half of the esophagus at week 3, and the bottom three-quarters at the later time points. Based on the density of inflammatory cells and the ingrowth of capillaries in the submucosal stroma tissues, inflammation was graded as mild to moderate at 3, 5, 9 and 17 weeks, moderate to severe at week 23, and severe at week 31. The most severe inflammation and epithelial damage were observed in the esophagoduodenal junction, characterized by epithelial erosion and chronic ulceration with numerous inflammatory cells (lymphocytes, eosinophils and macrophages), dilation of veins and proliferation of the capillaries.

Iron deposition in the esophagus

A significant increasing linear trend (P = 0.0001) was seen in iron deposition in the distal esophagus (Table I). Positive
Iron deposits, iNOS and nitrotyrosine in esophageal adenocarcinogenesis

Fig. 1. Micrographs showing iron accumulation, at week 31, in the stromal tissue of a rat that had undergone EDA and received monthly iron supplementation (50 mg Fe/kg, i.p.). Normal, upper one-third of the esophagus (×500); BCH, iron accumulation next to moderate BCH (×500); BE, iron accumulation next to BE (×500); EAC, iron accumulation next to EAC (×500).

Table II. Time-dependent expression of iNOS in the esophagus of rats that had undergone EDA and iron supplementation

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<thead>
<tr>
<th>Week</th>
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<th>iNOS expression (%)</th>
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<td>3</td>
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<td>23</td>
<td>12</td>
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<td>31</td>
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Iron dextran (50 mg of Fe/kg) was administered i.p. monthly starting 2 weeks after surgery.

Percent of animals showing positive immunochemical staining.

Iron staining was seen in the muscle layer as well as in the submucosa (or stromal tissue) adjacent to the epithelia in the distal one-third of the esophagus in all of the iron-injected animals (Figure 1). Positive iron staining was not uniformly distributed in the esophagus; it was associated with severe inflammation in areas with or without erosion of the epithelial layers. Positive iron staining was seen along the edges of the tumors and not in the center of the cancer nests. The operated animals that were not injected with iron and the non-operated animals did not have positive iron staining in their esophagi.

iNOS and nitrotyrosine immunohistochemistry

iNOS expression was not observed at weeks 3, 5 and 9, but there was an increase in iNOS expression at later time points. At week 17, 36% of the animals had iNOS expression in the distal esophagus. This percentage increased to 83% at week 23 and 100% at week 31 (Table II, Figure 2). All of the positive iNOS staining was found in the stromal macrophages directly beneath the epithelium in the distal one-third to two-thirds of the esophagus. No positive iNOS staining was seen in the upper one-third of the esophagi of the operated animals, or in the non-operated animals.

There was a significant increasing linear trend (P = 0.0001) in nitrotyrosine formation (number of positive cells/h.p.f.) in the lower esophagus, but not in the middle esophagus (Figures 3 and 4). Strong positive nitrotyrosine staining was found in the stromal macrophages directly beneath the epithelium in the lower one-third to two-thirds of the esophagi of all the operated animals; most of the positive staining was seen in the lower esophagus and a lesser amount was seen in the middle esophagus. More importantly, weaker positive staining was seen in the basal and parabasal cells in BCH and in Barrett’s epithelium at weeks 17, 23 and 31 (Figure 4). No positive nitrotyrosine staining was seen in the upper esophagi.
of the operated animals or in the esophagi of the non-operated animals.  

Comparison of iron-treated rats with non-iron treated rats

In order to study the effect of iron supplementation on EDA rats, rats with iron supplementation were compared with those without (Table III). The supplemented group (killed at week 31) had significantly higher (P < 0.05) levels of inflammation, cell proliferation, iNOS expression and nitrotyrosine, as well as more tumors than the EDA rats, which did not receive iron supplementation (killed 26 weeks after surgery). Iron supplemented rats killed at week 23 also had significantly higher (P < 0.05) levels of cell proliferation, iNOS and nitrotyrosine than rats in the latter group (data not shown).

**Discussion**

Iron supplementation to EDA rats produced a high incidence of BE (91%) and EAC (73%) in week 31 with the EDA model (14), whereas EDA without iron supplementation produced a low incidence (0–18%) of EAC (11–13). We propose that iron enhances inflammation-induced oxidative stress, which damages the esophageal epithelium and contributes to the development of BE and EAC. This hypothesis is supported by the comparison of the iron-treated EDA rats with EDA rats that received no iron supplementation. The iron-treated rats had significantly higher levels of iron deposition, inflammation, cell proliferation, iNOS expression and nitrotyrosine, as well as more tumors (Table III). We have characterized BCH and inflammation in the development of BE and EAC with the rat EDA model. BCH and inflammation increased from mild, to moderate, to severe over the course of the experiment. There was a significant increasing linear trend in both cell proliferation and inflammation. Iron deposition in the stromal tissue adjacent to the epithelium in the distal one-third of the esophagus, increased with time and was associated with areas of severe inflammation. Since the iron was administered i.p., we believe that the iron was picked up by the macrophages and then deposited in the esophageal stromal tissue. These iron deposits could enhance inflammation as well as the production of reactive oxygen radicals.

We saw positive iNOS immunohistochemical staining in the stromal macrophages in the distal one-third of the esophagus, starting at week 17 after surgery (36%) and with increased frequencies at week 23 (83%) and week 31 (100%). However, positive nitrotyrosine immunohistochemical staining was seen in the stromal macrophages in the distal one-third of the esophagus at all time points after surgery, starting at 3 weeks. A significant increasing linear trend was seen in nitrotyrosine expression (number of positive cells/ha, p.f.) in the stromal macrophages. We expected the expression of iNOS and nitrotyrosine to match, because nitric oxide is required for the synthesis of peroxynitrite, which reacts with tyrosine to form nitrotyrosine (34). One explanation for this disparity might be that the nitrotyrosine immunohistochemical staining was more sensitive than that for iNOS; the iNOS expression was not visualized until high protein levels were reached. Nitric oxide might have been produced continuously at low levels from weeks 3 to 9, which might have been at levels high enough to generate nitrotyrosine accumulation.

A significant finding of our present work was the detection of positive nitrotyrosine immunostaining in the epithelial cells, (both BE and BCH) in the distal one-third of the esophagus, starting at 17 weeks after surgery. Although the epithelial staining was weaker than that seen in the stromal macrophages, this result links nitric oxide production in the stromal macrophages with oxidative damage in the esophageal epithelial tissue, which may be important in carcinogenesis.

Although nitric oxide has been shown to have many normal physiological functions, excess nitric oxide production can have many potentially damaging effects on epithelial cells, including (i) interaction with mitochondrial iron–sulfur proteins and interfering with their functions; (ii) nitrosation of amines to produce nitrosamines, many of which are potent carcinogens; (iii) reaction with primary amine groups in DNA to result in deamination and mutation; (iv) inhibition of the repair of O²⁻-methylguanine in DNA by forming an S-NO adduct with O²⁻-methylguanine–DNA methyltransferase; and (v) reaction with superoxide anion to form the relatively long-lived strong oxidant, peroxynitrite, which can react with thiol groups and tyrosyl residues in proteins (21–23,35,36). Because tyrosine nitration is an irreversible reaction, 3-nitrotyrosine accumulation is considered to be a cumulative index of peroxynitrite production (35,37). Tyrosine nitration has been reported to interfere with phosphorylation by tyrosine kinases, which in turn may affect the ability of the protein to participate in important signaling pathways (23,35,37). Nitrotyrosine production has been associated with the induction of oxidative stress in human and animal models of diseases, including human rheumatoid arthritis (38) and atherosclerosis (34), human *Opisthochis viverrini* infection associated with cholangiocarcinomas (22), and animals chronically infected with hepatitis virus (21).

In previous work, we demonstrated that iron supplementation to rats given EDA enhanced the incidence of EAC, and this histopathogenic process is similar to that reported for human BE and EAC (14). In the present work, we observed that iron supplementation caused the deposition of iron and the induction of iNOS in the stromal macrophages of the distal esophagus. In human BE and EAC samples, similar patterns of iron deposition (G.-Y. Yang, N. Altork and C.S. Yang, unpublished results) and iNOS induction (39) have also been observed. Even though these events were detected only in inflammatory cells in the stroma, the reactive oxygen (nitrogen) species produced could elicit damage to micromolecules in epithelial cells and Barrett’s cells as indicated by nitrotyrosine formation in these cells. These could be important events in the formation
of BE as well as its progression to EAC. More quantitative data, however, are needed to further substantiate this point.

The present surgical model induces reflux of duodenal and gastric juice into the esophagus and produces BE and EAC. Although the pathogenic processes resemble those seen in humans, this is a rather drastic model, which produces features not shared by patients with BE. For example, the rats given EDA have a lower nutritional status in terms of iron, vitamin A and vitamin E because of malabsorption (X.X.Chen, G.-Y.Yang and C.S.Yang, unpublished results). A key feature of our model is iron supplementation. It is not known whether iron overload is a risk factor in human esophageal adenocarcinogenesis. The fact that the incidence of BE and EAC is more prevalent in North American and European males than females (a ratio of 8:1) (1) is consistent with the iron overload hypothesis. The consumption of red meat in these areas is high and males have a higher possibility of having iron overload because they do not have a normal route for iron elimination. The contribution of iron overload to human esophageal adenocarcinogenesis remains to be investigated.

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References