An esophagastroduodenal anastomosis model for esophageal adenocarcinogenesis in rats and enhancement by iron overload

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The aim of this study is to establish a good animal model for esophageal adenocarcinoma (EAC) and to test the hypothesis that iron over-nutrition enhances EAC formation. With rats, esophagastroduodenal anastomosis (EGDA) was accomplished by anastomosing the duodenum to the gastroesophageal junction. Iron supplementation was given by i.p. injection of iron dextran (4 mg Fe/kg/week). This model mimics the development of human EAC by introducing mixed reflux of gastric and duodenal contents. At 40 weeks after surgery, the body weight, food intake, hemoglobin, total serum iron, transferrin saturation, serum albumin, and plasma levels of α-tocopherol, γ-tocopherol and retinol of the EGDA rats were not significantly different from those of the non-operated controls. The animals generally had only mild esophagitis, except that the area surrounding the anastomosis opening had more severe esophagitis. Columnar-lined esophagus (CLE), CLE with dysplasia, and EAC were diagnosed in 53.5, 34.9 and 25.6%, respectively, of the 43 rats. All the tumors were well-differentiated mucinous adenocarcinomas at the squamocolumnar junction area, where most iron deposition was observed. EGDA avoids nutritional problems seen in other animal models for EAC. We believe that direct anastomosis of squamous epithelium to columnar epithelium and mixed reflux of gastric and duodenal contents lead to the formation of CLE and EAC. With this model, we demonstrated that iron supplementation significantly enhanced EAC formation, suggesting that iron over-nutrition could also be a risk factor for human EAC.

Introduction

Esophageal adenocarcinoma (EAC) is the most rapidly increasing cancer in this country. During the past three decades, the incidence rate of EAC and gastric cardia adenocarcinoma in the US increased by 5- to 6-fold, with a yearly increase of ~4–10% (1–3). The prognosis for EAC patients is extremely poor; the 5 year survival rate is ~10% (4). Therefore, it is very important to understand the disease process and to prevent this deadly disease at an early stage.

Gastroesophageal reflux, especially the combined reflux of both duodenal and gastric contents, is known to be a risk factor for EAC. In Western countries, reflux esophagitis is a commonly seen clinical identity, with >30% of the general population experiencing the symptoms at least once every month (5–7). An average of 10% reflux esophagitis patients will eventually develop columnar-lined esophagus, also known as Barrett’s esophagus (BE), which is characterized by replacement of the squamous epithelium of the esophagus by columnar epithelium (1,8). According to a large autopsy study, the incidence of CLE in the general US population was estimated to be 1 out of 80 individuals (9). The risk for EAC among CLE patients ranged from 1 in 46 to 1 in 441 patients/year, with a median of ~1 in 100 patients/year (10). The risk of CLE patients developing EAC is 30–125 times higher than the general population. CLE, especially the intestinal or ‘specialized’ type, is considered the precursor lesion of EAC (1).

Animal models can usually provide important information on human disease. Several surgical procedures have been used to produce EAC by inducing reflux of duodenal contents, with or without gastric contents, into the esophagus. These include the esophagoduodenal anastomosis (EDA) model, the duodenoforestomach reflux model, the pancreatico-esophageal reflux model, and the bilio-esophageal reflux model (11–15). In some models, esophageal adenocarcinogenesis was further potentiated with nitrosamines, such as 2,6-dimethylNitrosomorpholine, methyl-N-amylnitrosamine and N-nitrosornicotine (11,16). The resulting tumors showed both squamous cell carcinoma and EAC characteristics with nests of cells producing keratin in one area and mucin in another, and only a small percentage of tumors were pure well-differentiated EAC (12,17,18).

Recently, the EDA rat model was adapted in this laboratory (16,19). The animals (7%) developed EAC at 30 weeks after surgery. When iron was administered to prevent post-operative anemia, the incidence rate of EAC rose to 73% at 30 weeks after surgery. All of the tumors were well-differentiated mucinous adenocarcinomas. This could be a very interesting model for further studies. However, the EDA model and similar models have inherent problems. The malabsorption of certain nutrients due to partial loss of the function of the stomach resulted in compromised nutritional status after surgery. The EDA rats had much lower body weight, lower iron nutritional status, lower serum albumin level and lower levels of fat-soluble vitamins than the non-operated controls. Since the esophagus was used as the partial replacement of stomach function (i.e. temporary food storage), the animals usually had very severe inflammation across the whole esophagus, manifested by esophageal shortening, enlargement of the esophageal cavity (especially the lower and middle parts), hyperkeratinization and large-area epithelial sloughing and ulceration, which are not commonly seen in human patients (20; X.Chen and

Abbreviations: CLE, columnar-lined esophagus; DAB, diaminobenzidine; EAC, esophageal adenocarcinoma; EDA, esophagoduodenal anastomosis; EGDA, esophagastroduodenal anastomosis; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography.
C.S.Yang, manuscript in preparation). The lower nutritional status of iron and fat-soluble vitamins in these animals also limited the usefulness of this model in mechanistic and chemopreventive studies, since these nutritional deficiencies may affect carcinogenesis (21).

In order to avoid these problems and to better mimic the human situation, we developed a novel surgical model for EAC by making an anastomosis between the gastroesophageal junction and the duodenum. This procedure, named esophagogastrroduodenal anastomosis (EGDA), produced CLE and EAC without causing nutritional complications and severe large-area esophagitis. The effect of iron overload in this EGDA model was also investigated. This model enabled us to demonstrate that esophageal adenocarcinogenesis was enhanced by iron overload, which may have an important implication in the etiology of human EAC.

Methods and materials

Animals and treatment

Six-week-old male Sprague–Dawley rats from Taconic Farms (Germantown, NY) were housed two per cage, given an AIN93M diet (45 mg Fe/kg) (22) and water ad libitum, and maintained on a 12 h light–dark cycle. They were allowed to acclimatize for 2 weeks prior to surgery. Solid food was withdrawn 1 day before and for 1 day after the surgery. EGDA was performed under general anesthesia (80 mg ketamine and 12 mg xylazine per kg body wt, i.p.) through an upper midline incision. Two 1.5 cm incisions were made each on the gastroesophageal junction and the duodenum on the anti-mesenteric border, and then were anastomosed together with accurate mucosal to mucosal opposition. Care was taken not to reach the glandular stomach when the incision on the gastroesophageal junction was made (Figure 1). The original idea was to create a shortcut for mixed reflux of duodenal and gastric contents, and to damage the lower esophageal sphincter for direct reflux of gastric contents, with minimal alteration of gastrointestinal functions. This procedure was approved by the Animal Care and Facilities Committee, Rutgers University (protocol #94-017). The 90 surgically treated animals were allocated into two groups: group A rats received EGDA only; group B rats also received iron dextran (4 mg Fe/kg/week, i.p.) starting 4 weeks after surgery and continuing for the duration of the experiment. Two additional groups of animals (groups C and D) were the respective non-operated controls (Table I). Diet was prepared by Research Diets (New Brunswick, NJ) and stored at 4°C. The diet was C and D) were the respective non-operated controls (Table I). Diet was prepared by Research Diets (New Brunswick, NJ) and stored at 4°C. The animals were weighed weekly. Food intake was measured three times during the experiment (4, 16 and 30 weeks after surgery), each time for 3 consecutive days, and the average values of food intake (g diet/day) for the 3 days was calculated. All the animals were kept for 40 weeks after surgery.

Tissue preparation

Before killing, all the rats were anesthetized with CO₂, blood samples (4 ml) were taken by cardiac puncture or through the retro-orbital venous sinus and then they were killed by CO₂ asphyxiation. The esophagus was removed, opened longitudinally and examined for gross abnormalities. The perimeter of the anastomosis opening was measured for calculating the radius of the anastomosis opening. Tumor volume was determined by measuring the height, length and width of all visible tumors, and by using the average of the three measurements as the diameter. Tumor volume was calculated by: volume = \( \frac{4}{3} \pi r^3 \). Special care was taken to remove the esophagus from the duodenum and the stomach based on the suture line. The esophagus was cut longitudinally, fixed in 10% buffered formalin for 24 h and then transferred to 80% ethanol. The formalin-fixed esophagus was swiss-rolled, processed and embedded in paraffin. Serial sections (5 μm) were mounted onto glass slides and used for histopathological analysis.

Nutritional assessment

Fresh blood samples were used for determination of albumin, hemoglobin, total serum iron and transferrin saturation with kits from Sigma (St Louis, MO). Instructions from the manufacturer were followed with slight modifications. Retinol, α-tocopherol and γ-tocopherol were measured using a well-established high performance liquid chromatography (HPLC) method (23).

Histopathology, iron staining and immunohistochemistry

Histopathological analysis was performed on three hematoxylin and eosin (H&E)-stained slides (the 1st, 20th and 40th slides) for each rat. Reflux esophagitis was diagnosed with the observation of infiltration of inflammatory cells, basal cell hyperproliferation, dilation of venules, in-growth of the capillaries, and epithelial erosions and ulcers. Inflammation was also graded on morphology using the H&E-stained slides, as reported previously (19). CLE was characterized by the presence of intestinal columnar epithelium containing a villiform surface, mucus glands and intestinal-type goblet cells, above the blue prolene suture. Dysplastic lesions were diagnosed by the partial loss of cell polarity and maturation, nuclear atypia and an increase in mitotic figures. EAC was diagnosed when dysplastic columnar epithelial cells invaded through the basement membrane (20). Alcian blue staining was used to confirm the diagnosis of CLE and EAC (24). Ferric iron in the tissue sections was detected using the Prussian blue staining plus intensification with diaminobenzidine (DAB) (25).

Statistical analysis

The results on precancerous lesions and carcinogenesis were analyzed by the \( \chi^2 \) test. Other data were analyzed by the Student’s \( t \)-test using the computer software Statview 4.2.

Results

General health and nutritional status of the rats

Ninety-three percent of the animals (84/90) survived after surgery and six died before the termination of the experiment. Of these, four rats looked sick and inactive after surgery; they rapidly lost weight and died within 2 weeks. They might have died from anesthesia, excessive bleeding, breakage of the suture or esophageal stricture. The other two rats died after the second post-operative week without any obvious signs or reasons.

The body weights of the animals in the EGDA groups were only slightly lower than those of the non-operated control groups without significant difference (\( P > 0.05 \)) (Figure 2). EGDA and iron supplementation did not significantly change the food intake. The average dietary intake was ~45 g/day/kg body wt for rats in all the groups when measured at 4, 16 and 30 weeks after surgery (data not shown).

Iron nutrition was monitored by measuring hemoglobin, total serum iron and transferrin saturation at the time of killing (Table II). Hemoglobin was not statistically different among
the four groups \( (P > 0.05) \). Total serum iron and transferrin saturation were not significantly different among groups A, B and C \( (P > 0.05) \). The only statistical difference was observed for total serum iron and transferrin saturation of group D versus those of group A \( (P < 0.05) \). Serum albumin and plasma levels of α-tocopherol, γ-tocopherol and retinol were measured to monitor the status of protein and fat-soluble vitamin nutrition; significant difference among the four groups was not observed (Table II).

**Esophageal pathogenesis**

The gross appearance of the esophagi of the control animals was smooth and light pink. CLE and other lesions were not observed in the two non-operated control groups (Figure 3A). In the autopsy samples of the six animals that died before the experiment ended, healing of the anastomosis opening was seen within 1 week after surgery. Mild esophagitis began to develop in the lower end of esophagus, especially the area close to the anastomosis opening. At 40 weeks after EGDA, the esophagus appeared hyperemic in the lower part. Dilatation of superficial vein could be seen in some cases. However, severe esophagitic changes, such as ‘white cobblestone appearance’, ulcer, erosion and mucosal friability, which were observed after EDA (16), were not seen. The sizes of the anastomosis openings of the animals in groups A and B were \( 0.44 \pm 0.16 \) and \( 0.45 \pm 0.12 \) cm, respectively (all rats were measured), and there was no significant difference among these groups. Visible esophageal tumors were observed on the esophageal side of the anastomosis opening in seven and 19 rats in groups A and B, respectively. Only single tumor was seen in each of these animals. Tumor volume of group B was significantly higher than that of group A (Table I).

Under the microscope, the upper and middle parts of the esophagus were normal. Slight esophagitic changes, e.g. infiltration of inflammatory cells and basal cell hyperploration, were observed in the lower part of the esophagus. Signs of severe chronic esophagitis, such as hyperkeratinization, extensive epithelial sloughing, ulceration or intensive infiltration of plasma cells and lymphocytes, were only seen in the area close to the anastomosis opening (Figure 3B). Esophagitis near the anastomosis opening was graded on H&E slides. Iron-supplemented animals (group B) had significantly higher esophagitis grade than those without iron supplementation (group A) \( (P < 0.05) \). The middle and upper parts of the esophagi were normal in most EGDA rats. Slight esophagitis was only occasionally observed in the middle part of the esophagi of a few EGDA rats. Columnar epithelium (or CLE) was seen at the lower end of the esophagus extending upwards by \( <1 \) mm. There seemed to be no obvious difference in the length of CLE between group A and group B, although accurate measurement was difficult to make. The important thing was that, CLE was continuous from the columnar epithelium of the duodenum in all the cases (Figure 3C). An isolated island of columnar cells far removed from the anastomosis, which was observed in the EDA model as the sign of metaplasia (16), was not seen along the whole esophagus. This observation suggested upward creeping of the duodenal epithelium as the origin of CLE in the EGDA rats. In some cases, columnar cells moved upwards under the squamous epithelium of the esophagus, and then ‘broke through’ the above-lined squamous epithelium to appear on the luminal surface. Alcian blue staining confirmed the diagnosis of CLE by showing the mucin-secreting nature of the cells.

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**Table I. Histopathology of rat esophagi at 40 weeks after EGDA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Iron supplementation</th>
<th>n</th>
<th>Esophagitis grade</th>
<th>CLE</th>
<th>CLE with dysplasia</th>
<th>EAC</th>
<th>Tumor vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>EGDA</td>
<td>none</td>
<td>43</td>
<td>1.7 ± 0.35</td>
<td>23</td>
<td>15 (34.9%)</td>
<td>11</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>B</td>
<td>EGDA</td>
<td>i.p.</td>
<td>41</td>
<td>2.3 ± 0.20</td>
<td>32‡</td>
<td>22 (53.7%)</td>
<td>22</td>
<td>0.6 ± 0.23d</td>
</tr>
<tr>
<td>C</td>
<td>non-operated control</td>
<td>none</td>
<td>10</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>D</td>
<td>non-operated control</td>
<td>i.p.</td>
<td>10</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

*aAnimals were on the AIN93M diet (containing 45 mg Fe/kg diet). Groups A and C received no iron supplementation. Groups B and D received 4 mg Fe/kg/week i.p.*

*bEsophagitis was seen in all the EGDA rats of groups A and B. Esophagitis near the anastomosis opening was graded according to the method reported previously (19). The criteria were as follows: 0, normal (no inflammatory cells); 1, mild (few scattered inflammatory cells/high power field, ×400 in the stromal tissue); 2, moderate (moderate density of lymphocytes and macrophages); 3, severe (high density of lymphocytes and macrophages with an enlargement of capillaries in the stromal tissue). Significant difference was seen between groups A and B. No esophagitis was graded in groups C and D.

*cThe values are means ± SD of all visible tumors.*

*dSignificantly different from that of group A \( (P < 0.05) \).*

**Table II. Nutritional status of iron and fat-soluble vitamins in EGDA rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemoglobin (g/dl)</th>
<th>Serum iron (µg/dl)</th>
<th>Transferrin (µg/dl)</th>
<th>Serum albumin (g/dl)</th>
<th>α-Tocopherol (µg/dl)</th>
<th>γ-Tocopherol (µg/dl)</th>
<th>Retinol (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17.4 ± 6.1</td>
<td>144.3 ± 53.6</td>
<td>0.206 ± 0.071</td>
<td>4.3 ± 0.5</td>
<td>1495.5 ± 460.3</td>
<td>13.1 ± 9.2</td>
<td>47.0 ± 9.5</td>
</tr>
<tr>
<td>B</td>
<td>16.9 ± 2.7</td>
<td>173.6 ± 63.3</td>
<td>0.272 ± 0.095</td>
<td>4.0 ± 0.5</td>
<td>1409.5 ± 301.1</td>
<td>11.6 ± 6.0</td>
<td>45.3 ± 8.8</td>
</tr>
<tr>
<td>C</td>
<td>18.3 ± 3.7</td>
<td>171.8 ± 27.8</td>
<td>0.250 ± 0.038</td>
<td>4.1 ± 0.5</td>
<td>1908.3 ± 189.1</td>
<td>15.6 ± 0.6</td>
<td>57.8 ± 4.5</td>
</tr>
<tr>
<td>D</td>
<td>16.7 ± 1.2</td>
<td>215.3 ± 16.9</td>
<td>0.327 ± 0.026</td>
<td>4.0 ± 0.2</td>
<td>1840.3 ± 218.8</td>
<td>10.1 ± 0.9</td>
<td>52.1 ± 4.7</td>
</tr>
</tbody>
</table>

*bBlood samples were collected at 40 weeks after surgery. The values are means ± SD of samples from all rats shown in Table I.*

*bThere was no significant difference among groups A, B and C \( (P > 0.05) \). Group D was significantly higher than group A \( (P < 0.05) \), but not groups B and C.*
in CLE (Figure 3D). Intraperitoneal iron supplementation significantly increased the incidence of CLE from 53.5 to 78% ($P < 0.05$; Table I). Dysplastic cells were observed within the CLE in many samples. There were increased proliferation, partial loss of cell polarity and maturation, nuclear atypia, and an increase in mitotic figures (Figure 3E). Intraperitoneal iron supplementation significantly increased the incidence of dysplasia from 34.9 to 53.7% ($P < 0.05$; Table I). All of the EAC were pure, well-differentiated mucinous adenocarcinomas at the squamocolumnar junction with an adjacent area of CLE with dysplasia (Figure 3F). Other types of tumors of esophagus and other organs were not seen in this study. The incidence of EAC was 25.6% with EGDA alone (group A), and significantly increased to 53.7% ($P < 0.05$) by i.p. iron supplementation (group B) (Table I).

Iron deposition in the rat esophagus

Extensive staining was observed in the rats which received i.p. iron supplementation (Figure 4A), whereas EGDA alone produced only weak iron staining, which was identified with DAB intensification (Figure 4B). Iron staining was not observed in the non-operated control rats including those which received iron supplementation. Scattered iron staining was seen in areas with inflammation, the lower part of the esophagus. However,
Discussion

In this study, EGDA resulted in the development of well-differentiated mucinous adenocarcinomas in 25.6% of the rats at 40 weeks after surgery without the treatment with any known carcinogens and tumor promoters. The incidence rate was comparable with that induced by EDA (12). More importantly, the EGDA model overcomes the problems inherent to the EDA model, namely malnutrition and very severe inflammation. We did not see significant difference among the four groups in body weight, food intake, iron nutrition (hemoglobin, total serum iron and transferrin saturation), serum albumin and plasma fat-soluble vitamins (Figure 2; Table II). Severe inflammation was only limited to the area close to the anastomosis opening after EGDA. The upper and middle parts of most esophagi were free of any inflammation, even when combined with iron supplementation. Iron supplementation only promoted inflammation near the anastomosis opening (Table I).

EGDA mimics the two essential features for the development of human EAC: the duodeno-gastro-esophageal reflux and the presence of intestinal or ‘specialized’ columnar epithelium in the esophagus. Continuity of CLE from the duodenal epithelium in all the cases with CLE highly suggested that the specialized columnar cells most likely came from creeping substitution after EGDA. Since 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 gastric acid and bile acids than squamous epithelium (26), columnar epithelium may start to expand into the esophagus as an adaptation to the reflux-induced damage to the squamous epithelium. In human BE, at least two possible origins have been proposed for the specialized columnar epithelium: (i) columnar cells moving up to the esophagus from the gastric cardia; and (ii) differentiation of esophageal pluripotent stem/basal cells to columnar cells due to gastric reflex (20). The present model resembles the first possibility and, more importantly, it demonstrates that the columnar cells in the esophagus can progress to dysplasia and adenocarcinoma in the absence of any exogenous carcinogens or promoters. We believe this carcinogenesis process is driven by oxidative stress produced by reflux esophagitis.

Compared with the other existing animal models, EGDA has the following advantages: (i) it avoids the problems related to other procedures, such as loss of function of the stomach. Food passes along the normal alimentary tract, thus EGDA rats have normal nutritional status; (ii) there is substantial reflux of both gastric and duodenal contents into the esophagus. Recent studies employing ambulatory 24 h esophageal pH and bilirubin monitoring showed that a mixed reflux of gastric and duodenal juices is more harmful to the esophagus than gastric juice alone. There is synergism between duodenal content and gastric content in inducing BE and EAC, although either of the contents alone is also damaging (13,27,28); (iii) recirculation of bile through the stomach will raise the antral pH, thus resulting in gastrin release by the antral G cells. Gastrin is known to have a trophic effect on the gastrointestinal epithelium by encouraging the growth of esophageal carcinoma (29,30).

Iron is known to promote carcinogenesis in several animal models (31,32). Increased body stores of iron are associated with an increased risk of cancer (33,34). Intraperitoneal iron supplementation was found to enhance esophageal adenocarcinogenesis in our previous studies on the EDA model (16,19; X.Chen and C.S.Yang, manuscript in preparation). In these studies, we showed that oxidative damages to protein, DNA and lipid were present in the rat esophagus as a result of iron supplementation. Oxidative stress was believed to be a major factor in inducing EAC after EDA. However, because the EDA rats were iron-deficiency anemic, a second possibility is that anemic rats may not have enough metabolic energy for the tumor to develop, and iron supplementation provides the metabolic energy. In the present study, EGDA did not produce anemia, and low level of iron supplementation by i.p. injection significantly enhanced esophageal adenocarcinogenesis, in terms of both tumor incidence and tumor volume. Our present results clearly eliminated the second possibility and provide strong support for the hypothesis that iron enhanced esophageal carcinogenesis by increasing oxidative stress. The marked overexpression of oxidative stress-responsive genes, heme oxygenase 1 and metallothionein, in the columnar cells at the squamocolumnar junction and EAC is also consistent with this hypothesis (data not shown) (35).

Iron dextran is a complex of ferric hydroxide and low molecular mass dextran. After i.p. injection, most of the iron can be absorbed within 12–24 h (36). The body’s ability to excrete parenterally administered iron is limited, and the reticuloendothelial system sequesters excess iron. During inflammation, however, macrophages in the reticuloendothelial
system incorporate the free iron into siderophore, and then carry the iron to the site of inflammation (37). This may explain why the EGDA rats all had excess iron deposited in the esophagus after i.p. iron supplementation (Figure 4A). In the esophagi of group A which did not receive iron supplementation, light positive esophageal iron staining was also observed (Figure 4B). Iron administered i.p. further loaded the esophagi of EGDA rats with more iron, which greatly enhanced the development and increased the incidence of EAC. Iron deposition was also observed in liver and spleen, which are believed to be the sites for storage of excessive circulating iron. However, no tumor was observed in these organs.

Several previous epidemiological studies identified major risk factors for human EAC and most were related to gastroesophageal reflux (38–40). However, iron over-nutrition could also be a risk factor. Patients with hemochromatosis had a high risk for esophageal cancer (standardized incidence ratio, 42.9), according to an epidemiological study in Denmark (41). In the US, there was a substantial increase in dietary iron intake during the past two decades; the daily iron intake per capita was much higher than the requirement suggested by FDA (42). It is possible that iron over-nutrition in humans, especially due to the consumption of heme iron in red meat by males, may be an important risk factor for EAC. We also tested this possibility by supplementing EGDA rats with 2-fold dietary iron (90 mg/kg Fe instead of 45 mg/kg Fe inAIN93M). However, the incidence of EAC (29.7%, 11/37) and tumor volume were only slightly, but not significantly, higher than those of the non-supplemented EGDA rats (X.Chen and C.S.Yang, unpublished results). Probably, the sample size was not big enough to reveal any statistical difference. Further investigation is warranted to address this issue.

Acknowledgements

This work was supported by NIH grant CA75683 and facilities from the NIEHS Center Grant ES05022 and the NCI 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.

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*Received December 21, 1998; revised April 16, 1999; accepted May 5, 1999*