Chemoprevention of Carcinogenic Progression to Esophageal Adenocarcinoma by the Manganese Superoxide Dismutase Supplementation

Robert C.G. Martin, Qiaohong Liu, John M. Wo, Mukunda B. Ray, and Yan Li

Abstract

Purpose: Oxidative stress is related to the carcinogenic pathway of reflux esophagitis to Barrett’s metaplasia to esophageal adenocarcinoma (EAC). Recent studies have shown that a decreased manganese superoxide dismutase (MnSOD) level is associated with the increased incidences of Barrett’s esophagus (BE) and EAC. The aim of this study was to investigate MnSOD supplementation as a chemopreventive agent to prevent oxidative injury and subsequent BE and EAC formation.

Experimental Design: Our esophagoduodenal anastomotic (EDA) model was done on rats according to our established procedure and treated with Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP; 10 mg/kg, i.p. every 3 days). Histologic changes were determined after the EDA model at 1, 3, and 6 months. Lipid peroxidation and 8-hydroxy-deoxyguanosine for DNA oxidative damage were determined by thiobarbituric acid-reactive substance assay and immunohistochemical staining. Enzymatic activities of MnSOD and Cu/ZnSOD were evaluated, and the rate of proliferation was determined by proliferating cell nuclear antigen staining.

Results: Severe esophagitis was seen in 100% of the EDA rats, and morphologic transformation within the esophageal epithelium was observed with intestinal metaplasia (40% of animals) and cancer (40% of animals) identified after 3 months. Decreased oxidative damage, along with the decreased degree of esophagitis and incidence of BE (20%) and EAC (0%), was found in MnTBAP-treated EDA rats comparing with the saline-treated EDA control. Decreased proliferation (46%) and increased SOD enzymatic activities (25%) were also found in the EDA rats treated with MnTBAP.

Conclusion: MnTBAP protected rat esophageal epithelium from oxidative injury induced by EDA, and it could prevent the transformation of esophageal epithelial cell to BE to EAC by preservation of antioxidants.

Esophageal adenocarcinoma (EAC) has received considerable attention because of the dramatically increased incidence in the past 2 decades and the poor prognoses with a 5-year survival rate of 10% to 20% (1–5). Currently, it is widely accepted that a cause-effect relationship exists between gastroesophageal reflex disease and EAC. Oxidative stress has been proposed to be closely related to the carcinogenic progression of reflux esophagitis, Barrett’s metaplasia, and EAC. It has been shown that reactive oxygen species increase with the grade of reflux esophagitis and are highest in Barrett’s esophagus (BE) in clinical patients (6). Several studies have also shown that reactive oxygen species cause DNA injury, such as strand breakage, alterations in guanine and thymine bases, and DNA cross-linkage (7, 8). Therefore, oxidative DNA damage may contribute to the accumulation of genetic damage, and the accumulation of genetic and epigenetic aberrations produces one or more clones with metaplastic and/or malignant potential (9).

In our previous studies using an external esophageal perfusion rat model (10–13), we have shown significant increased levels of lipid peroxidation and 8-hydroxy-deoxyguanosine (8-OH-dG) and a significant decrease in the antioxidants manganese superoxide dismutase (MnSOD) and reduced glutathione (GSH) during external esophageal perfusion with both acid and bile. We have also shown that, in an esophagoduodenal anastomosis (EDA) rat model, the relationship between oxidative damage and the transition from chronic inflammation to adenocarcinoma remains consistent. Loss of MnSOD protective enzymatic activity was also found at a very early stage, and alteration of MnSOD protein level was closely related to the progression from esophagitis to metaplasia to EAC in EDA rats (13).

The use of SOD as a therapeutic agent to attenuate oxidative injury has been attempted (14–16); however, the drawback of SOD applications is that these natural products are large in size and therefore have limited penetration to cellular compartments. Several classes of low-molecular-weight SOD mimetics
have been developed to overcome some of these limitations. Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP), a non-peptidyl mimic of SOD, belongs to the metalloporphyrin class of catalytic antioxidants (17). MnTBAP has shown a broad-spectrum reactive species scavenger effect and prevents oxidative injury in a wide variety of cell lines and animals; that is, in cell culture models, MnTBAP has shown to be effective against injury produced by hydrogen peroxide (18) and excitotoxic agents N-methyl-d-aspartate (19) and to be a potent inhibitor of apoptosis (20). MnTBAP is also effective in animal models of injury, such as carcinogen-induced models (21).

SOD represents the first line of defenses against the harmful effects of superoxide radicals. The loss of SOD suggests that antioxidative defense system is compromised, which in turn exacerbates the gastroesophageal reflux disease condition, induces DNA injury, and allows for the accumulation of genetic alternation, which may lead to uncontrolled cell replication and increase malignant potential. We hypothesize that oxidative stress induces DNA damage, which drives the metaplastic and carcinogenic transformation in esophageal epithelium. MnTBAP supplement protects the esophagus from esophageal reflux injury through preservation of antioxidants and thereby prevention against BE and EDA. In this study, we proposed to investigate possible mechanisms by which MnTBAP is protective against oxidative injury in the esophageal epithelium of the EDA rat model during the transformation of Barrett’s to metaplasia and to EAC.

Materials and Methods

Animals and treatment. Eight-week-old Sprague-Dawley rats (Harlan) were housed three per cage, given commercial rat chow and tap water, and maintained on a 12-h light/dark cycle. They were allowed to acclimate for 2 weeks before surgery. Solid food was withdrawn 1 day before and 1 day after surgery. EDA was done on rats according to the operating procedure described previously. In brief, the animals were anesthetized with 60 mg/kg sodium pentobarbital, and the pedal reflex was used to monitor the depth of anesthesia. The gastroesophageal junction was ligated flush with stomach and the distal esophagus was transected proximal to the ligature. An enterotomy (4 mm) was made 1 cm distal to the pylorus on the antimesenteric border. The distal transected proximal to the ligature. An enterotomy (4 mm) was made 1 cm distal to the pylorus on the antimesenteric border. The distal esophagus was then anastomosed to the duodenal enterotomy with 8-OH-dG levels are defined by the percentages of computer program then quantified the threshold area represented by JPG data files (the resolutions were fixed as 200 pixels/inch). The procedure for the computer image analysis is done, and the acquired color images from the immunohistochemical staining were defined a standard threshold according to the software specification. The computer program then quantified the threshold area represented by color images. 8-OH-dG levels are defined by the percentages of threshold area in acquired color images.

Thiobarbituric acid–reactive substance assay. Lipid peroxidation is quantified by an OXitek TBARS Assay kit (ZepitoMetrix Corp.) measuring the malondialdehyde (MDA) concentrations as described in the provided instruction. Briefly, the tissue homogenate is processed for thiobarbituric acid reaction following the procedure described. The reaction mixture is covered and incubated at 95°C for 60 min and then cooled to room temperature in an ice bath for 10 min. The samples were centrifuged at 3,000 rpm for 15 min, and the absorbance is obtained with a microplate reader reading at 532 nm.

Western blot analysis of MnSOD expression. Western blot is done to determine the MnSOD protein expression in the esophageal mucosal layer and muscle layer. In brief, total protein is isolated from fresh tissue samples by homogenization in ice-cold buffer containing 20 mmol/L HEPES (pH 7.5), 1.5 mmol/L MgCl₂, 20 mmol/L DTT, 0.4 mol/L NaCl, 20% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L leupeptin at 4°C. Insoluble cellular material is removed by microcentrifugation at 16,000 × g for 5 min, and total protein is determined spectrophotometrically. The protein samples are separated via SDS-PAGE and subsequently transferred to the nitrocellulose membrane for Western blot described previously (11).

SOD enzymatic activity assay. SOD activity is determined by a SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.) according to the provided instruction. In brief, this kit allows highly sensitive SOD assay by using a highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium,
monosodium salt], which produces a water-soluble formazan dye on reduction with a superoxide anion. Samples are tested and a standard curve ranging from 0.156 to 20 units/mL is prepared. The colorimetric assay is done measuring formazan produced by the reaction between WST-1 and superoxide anion (O$_2^-$); the rate of the reduction with O$_2^-$ is linearly related to the xanthine oxidase activity and is inhibited by SOD. The absorbance was obtained with a microplate reader reading at 450 nm. MnSOD activity is determined by adding 1 mmol/L KCN to samples to block Cu/ZnSOD activity completely and then subtracting the Cu/ZnSOD activity from total SOD activity.

**Glutathione assay.** Glutathione is determined by a Cayman’s GSH assay kit (Cayman Chemical) using an enzymatic recycling method. In brief, using the sulfhydryl group of GSH reacts with 5,5'-dithio-bis-2-nitrobenzoic acid (Ellman’s reagent) and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB), which is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in the sample. Because of the use of glutathione reductase in this assay, both GSH and oxidized glutathione are measured and the assay reflects total glutathione.

**Catalase assay.** Catalase was determined by a Cayman’s GSH assay kit using the peroxidatic function of catalase for determination of enzyme activity. In brief, the method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H$_2$O$_2$. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which on oxidation changes from colorless to a purple color. Measurement of the absorbance at 540 nm provides an accurate estimation of catalase enzymatic activity in the sample.

**Statistical analysis.** Student’s $t$ tests assuming unequal variance were done. The results are expressed as mean values ± SD. Comparisons were made among the bile perfusion groups and saline control groups by ANOVA. A $P$ value of <0.05 was considered statistically significant.

### Results

**Esophageal pathogenesis.** In the 1-, 3-, and 6-month animals, the macroscopic evaluation of the esophagus showed a thickened and friable mucosal layer and surface with no evidence of these changes in the saline-treated and MnTBAP-treated animals. Microscopically, EDA rats with saline treatment, squamous epithelium showed hyperplasia and extensive defects with erosions or ulcersations in some regions, and 4 of

<table>
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<th>Time point</th>
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<th>No. animals (incidence %)</th>
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<tr>
<td>Month 3</td>
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<tr>
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<td>3.5 ± 0.21</td>
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<td>Month 6</td>
<td>EDA</td>
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<td>Month 6</td>
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**Table 1. Effects of cancer on histologic changes of rat esophagi after EDA**

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

Abbreviation: Non-OP, nonoperation.

**Fig. 1.** TUNEL-positive cells in nonoperation (A) and EDA esophageal epithelium from month 6 (B).
15 rats showed intestinal metaplasia at both months 3 and 6, with 2 of 15 animals with diagnoses of EAC at month 6. In the EDA rats with MnTBAP treatment, the treatment significantly affected the EDA-induced reflux esophagitis in all stages. Squamous hyperplasia, erosions, ulcerations, and extension of the lamina propria papillae within the esophageal mucosa were ameliorated in the MnTBAP-treated EDA rats from month 1 to month 6. The MnTBAP-treated group showed only one rat with intestinal metaplasia at month 6, with no evidence of tumor development. The histologic findings are summarized in the Table 1.

Proliferation and apoptosis of esophageal epithelial cells. The apoptotic index in all EDA rats was significantly increased in comparison with the nonoperation controls (P < 0.05). In the nonoperated esophageal epithelium, a few TUNEL-positive cells were found mainly in the keratinized layer; however, numerous TUNEL-positive cells were seen throughout the esophageal mucosal, especially in the enlarged papillae from the EDA esophageal epithelium (Fig. 1). The apoptotic indexes of EDA rats treated with MnTBAP for 1 and 2 months were significantly decreased in continuity compared with that in the EDA rats of saline controls. The apoptotic index in EDA rats with MnTBAP treatment after month 6 was nonsignificantly decreased when compared with saline treatment (Fig. 2A).

PCNA index was significantly increased in the EDA esophageal mucosa with both MnTBAP-treated groups and saline-treated groups compared with the nonoperation controls (P < 0.05). The proliferation indexes of EDA rats with MnTRAP treatment after month 3 were statistically decreased compared with that in the EDA rats of saline controls (P < 0.05; Fig. 2B).

Oxidative damage in the rat esophagus. MDA measurement for lipid peroxidation and 8-OH-dG measurement for oxidative damage to DNA resulted in significantly elevated MDA concentrations in the saline controls when compared with the MnTBAP-treated rats at both 1 and 3 months (Fig. 3A). MDA concentrations in 6-month MnTBAP-treated EDA rats were also decreased (P > 0.05) but no statistical significance when compared with EDA saline controls.

The levels of 8-OH-dG in the esophageal epithelium were significantly increased in the esophageal epithelium in the saline EDA rats compared with the nonoperated controls. Treatment with MnTBAP significantly decreased the level of 8-OH-dG in the esophageal epithelium of EDA rats after 1, 3, and 6 months (P < 0.05) when compared with saline controls. The level of 8-OH-dG in MnTBAP-treated EDA rats at month 6 was also decreased compared with that in the EDA saline controls; however, there were not statistically significant differences (P > 0.05; Fig. 3B).

Antioxidants in the rat esophagus. A time course study of MnSOD expression was also done in the esophageal tissue from the mucosal layer by Western blot analysis. Levels of MnSOD
protein expression showed a dramatic decrease in the rats after EDA 1 month; however, there were significantly increased levels of MnSOD in the rats at EDA 3 and 6 months compared with the nonoperated controls.

About SOD enzymatic activities, there was a significant loss of MnSOD activities ($P < 0.05$), which contributed to the loss of total SOD activity in esophageal epithelium of EDA rats with saline treatment for 1 month, compared with that of nonoperation animals ($P < 0.05$). This loss of SOD activity was from MnSOD loss only and not Cu/ZnSOD activity because these activities were increased in EDA rats. The loss of total SOD activity in esophageal epithelium of EDA rats was ameliorated in the MnTBAP-treated EDA rats. There was a significant difference between EDA with MnTBAP treatment and EDA only ($P < 0.05$), and both MnSOD activity and Cu/ZnSOD activity were increased after MnTBAP treatment in EDA rats. The SOD enzymatic activities are shown in Fig. 4.

The levels of GSH were significantly lowered in the esophageal epithelium of EDA rats in all stages compared with nonoperation controls, and the levels of GSH were also not augmented in EDA rats treated with MnTBAP ($P > 0.05$). The catalase enzymatic activities were also observed in the EDA rats; however, no significant difference was found among three study groups (nonoperation, EDA with saline treatment, and EDA with MnTBAP treatment; $P > 0.05$). The levels of GSH and catalase enzymatic activities are shown in Fig. 5.

**Discussion**

The EDA is the most commonly used surgical model to induce duodenogastroesophageal reflux and has successfully reproduced reflux esophagitis, Barrett’s metaplasia, and EAC (23–25). In this study, we used an EDA rat model to analyze the effect of antioxidative treatment by MnTBAP on the known carcinogenic sequence of esophagitis to Barrett’s metaplasia to EAC. MnTBAP inhibits EDA-induced lipid peroxidation and MDA oxidative damage. These results further confirm the known antioxidant function of MnTBAP, thus showing the role of oxidative stress in the transformation of esophageal epithelial cell to BE to EAC and providing evidence that MnTBAP protection against metaplasia and carcinogenesis may result from its antioxidant action. In this current study, reflux esophagitis, Barrett’s metaplasia, and carcinoma were all successfully reproduced in our EDA rat model, as we have shown in prior studies (26). The decreased SOD protein expression and enzymatic activity are found at 1 month after EDA, and MnSOD, but not Cu/ZnSOD, contributes to the loss of total SOD. It has been shown that the generation of the superoxide anion as the main free radical is involved in mucosal damage in reflux esophagitis (27–29), and superoxide anion is mainly scavenged by SOD. The increased levels of lipid peroxide and 8-OH-dG along with increase of apoptosis after EDA suggest that oxidative insult is involved in the initiation of reflux-induced esophageal cell death. On the other hand, one of the most important adaptive responses to cell loss in reflux esophagitis is an increase of the proliferation...
to maintain epithelial thickness and to increase the fold of the basal epithelium (papillae formation; refs. 30, 31), and superoxide anion might also act as endogenous carcinogens by damaging DNA and initiating cells to transformation if oxidative mutations are not correctly repaired under compromised condition of SOD.

A progressive increase of MnSOD is found after EDA 3 and 6 months, which is corresponding to our previous observation (13, 26). The increased MnSOD levels in the esophageal epithelium of EDA rat may reflect an adaptive process and the reasons could be as follows: (a) MnSOD is an inducible antioxidant and it can be induced to a high level by several stimuli, including oxidative stress and inflammatory (32), or (b) a compensatory mechanism against the decreased MnSOD enzymatic activity (14). In this regard, although there is a relative higher MnSOD level in the EDA esophageal epithelium, it is possible that the scavenging effect of SOD might be insufficient to decrease mucosal injury induced by continuous reflux extending over a much longer period. Therefore, supplementation of extraneous antioxidant may favor the compromised antioxidant defense system under the EDA condition.

Most catalytic SOD mimetics are designed with a redox-active metal center that catalyzes the dismutation reaction with superoxide anion in a manner similar to the active site metals of the mammalian (Cu/Zn or Mn) SODs. There are at least three classes of metal-containing SOD mimetics, including the salen, macrocyclic, and metalloporphyrins (33). We chose the MnTBAP for the following reasons: first, it is the most stable metal chelates belonging to metalloporphyrin class; second, it has high specificity for interaction with superoxide anion and with a high rate constant; third, it is nontoxic much like the native SODs; and finally, it has been shown to possess very potent effects in vivo against oxidative stress (17, 34, 35). We explore the role of MnTBAP against esophageal oxidative damage and in the prevention of Barrett’s metaplasia and adenocarcinoma arising in rat esophagus after EDA. An important issue addressed in this study is that administration of MnTBAP prevented esophageal mucosa damage from EDA-induced reflux esophagitis, and the decreased degree of reflux esophagitis is associated with the decreased risks of Barrett’s metaplasia and EAC. To our current knowledge, no available data have shown that MnTBAP has any directly inhibitive effect on the growth of cancer cells and its effects most likely underlying its ability to eliminate both O2•− and H2O2 to water to protect esophageal epithelium from EDA-induced oxidative mutation.

Although most observations linking lowered MnSOD activity to malignancy are that MnSOD opposes cancer by its antioxidative ability, MnSOD might also act as a new type of protectant (such as on Barrett’s metaplasia and EAC) antioxidative defense against oxidative damage. This concept is supported by the evidence that MnSOD expression is increased, rather than decreased, in gastric and colon cancer (36), as well as the effect of the MnSOD transgene in decreasing both acute and chronic ionizing irradiation induced damage to the esophagus (37, 38). In this study, we also observed the increases of index of apoptosis, MDA level, and 8-OH-dG level in the later EDA stage (6 months) and decreased GSH even with MnTBAP treatment. Therefore, it should be noted that some other mechanisms could be involved in these cellular events against the hyperproliferation in the EDA later stage, in which the esophageal epithelium is at high risk for metaplasia and EAC. It is also possible that, once Barrett’s metaplastic and/or carcinogenic transformation have been initiated at a very early stage after EDA, administration with MnTBAP may even worsen the transforming process. There is potential that MnSOD plays a dual role, one as a protectant to prevent initiation in the earlier stage and the second as promoter of carcinogenesis in the EDA at the later stage.

In conclusion, our results suggest that administration with MnTBAP is effective in preventing EDA-induced reflux esophagitis by its antioxidative ability, and this antioxidative effect is closely related to the prevention against Barrett’s metaplasia and EAC. The alternation of MnSOD is in response to the reflux injury after EDA, and the inhibition of oxidative stress is critical for prevention against intestinal metaplasia and cancer. We propose that oxidative damage is a causative factor for BE and EAC and believe that a chemoprevention against Barrett’s metaplasia and/or EAC by using antioxidant in reflux-induced esophagitis is reasonable. Further studies are needed to identify which naturally occurring antioxidants have the greatest potential for MnSOD supplementation and augmentation.

References

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