Association of Manganese Superoxide Dismutase Expression With Progression of Carcinogenesis in Barrett Esophagus

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Hypothesis: The down-regulation of manganese superoxide dismutase (MnSOD) expression plays a role in the progression of Barrett esophagus (BE).

Design: An evaluation of 92 esophageal samples, including 17 patients with normal esophagus, 22 with intestinal metaplasia, 22 with indefinite/low-grade dysplasia, 16 with high-grade dysplasia (HGD), and 15 with esophageal adenocarcinoma were evaluated for MnSOD expression. We evaluated MnSOD expression using immunohistochemistry and graded it separately on a 2-category ordinal scale in relation to the mucosa and submucosa that ranged from 0 (no staining) to 3 (strong staining). The total grading score of MnSOD immunoreactivity was the addition of mucosa and submucosa intensity, from 0 (no immunoreactivity in any of the anatomic sites) to a maximum score of 6 (strong staining reaction in both of the histoanatomic sites).

Setting: Study subjects were recruited from the Barrett’s Esophageal Registry at the University of Louisville, Louisville, Ky.

Main Outcome Measure: Manganese superoxide dismutase expression in established groups of progressive BE.

Results: Ninety-two samples were evaluated for MnSOD expression. The expression of MnSOD was found to be significantly reduced in samples with specialized intestinal metaplasia (mean score, 1.8), low-grade dysplasia (mean, 2.2), high-grade dysplasia (mean, 2.4), and esophageal adenocarcinoma (mean, 2.4) when compared with normal esophagus (mean, 3.9; \( P =.002 \)). Manganese superoxide dismutase expression was similar for esophageal adenocarcinoma and high-grade dysplasia.

Conclusions: Manganese superoxide dismutase expression is significantly reduced in patients with BE with high-grade dysplasia and esophageal adenocarcinoma. Manganese superoxide dismutase is related to the progression of BE and may represent one of the primary factors in oxidative stress protection. Further evaluation within genotypic expression and the role of antioxidants is needed in the effective screening and treatment of BE.

Arch Surg. 2005;140:1204-1209

Gastroesophageal reflux disease represents one of the most common diseases, affecting more than 20% of the adult American population. The repetitive reflux causes progressive mucosal damage, leading to reflux esophagitis. Approximately 10% to 20% patients with chronic reflux develop Barrett esophagus (BE), defined by the replacement of the normal esophageal squamous lining by a specialized intestinal metaplasia (SIM). Patients with BE have a 30 to 40 times greater risk of developing esophageal adenocarcinoma (EAC) compared with the general population, with EAC becoming one of the most rapidly increasing cancers in white men in the United States. Because, after a diagnosis of EAC, the probability of 5-year survival is approximately 15%, more effective screening is required to improve on this dismal prognosis.

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Because nearly all EAC arises from BE, a premalignant disease caused by gastroesophageal reflux disease, patients with BE should remain the true “high-risk” patient population. However, it remains unclear why some patients with BE develop EAC and some do not. Endoscopic surveillance remains the only current clinical strategy in evaluating the risk of BE. However, there is no clinically useful marker to stratify patients into low- and high-risk groups. A comprehensive understanding of the basic mechanisms in the progression of esophaga-
Manganese superoxide dismutase expression has been reported. There is increasing evidence that oxidative stress plays a key role in the progression of BE to dysplasia to EAC, with normal aerobic cellular metabolism producing several reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, and nonradicals like hydrogen peroxide. When normal cells are inflamed through chronic reflux, the production of ROS can be overwhelming, resulting in injury to the cells, which can induce gene mutations and lead to the progression from normal cells to cellular dysplasia and cancer.

Superoxide dismutase catalyzes the dismutation of 1 of the most common oxidative stress elements, superoxide oxygen radical to water and oxygen. Three distinct types of superoxide dismutases (SODs) have been identified in human cells: a homodimeric cytosolic copper and zinc superoxide dismutase, an extracellular homotetrameric glycosylated SOD, and a mitochondrial matrix homotetrameric manganese superoxide dismutase (MnSOD). Deficiency in the expression of mitochondrial MnSOD has been reported to promote carcinogenesis.

Numerous reports have demonstrated a relative deficiency of SOD catalytic activity, including mitochondrial MnSOD, in many types of solid tumors. Manganese superoxide dismutase expression has been evaluated in esophageal carcinoma, but little is known about the expression changes that occur in MnSOD from normal esophagus to BE to EAC. Thus, the aim of our study was to explore the relationship of MnSOD and its role in the initiation and progression of BE from normal esophagus. We hypothesized that MnSOD expression would be decreased in cells that had undergone changes from normal squamous esophageal cells to different stages of BE and on to EAC.

**STUDY SUBJECTS AND SPECIMEN COLLECTION**

Study subjects were recruited from the Barrett’s Esophageal Registry at the University of Louisville (Louisville, Ky) as previously described. Informed consent was obtained, and the study was conducted using endoscopic specimens from subjects with the established diagnosis of BE, the presence of SIM in histologic analysis. The length of columnar lined epithelial was defined as the distance from the proximal margin of the gastric fold to the squamous columnar junction. Short-segment BE was defined as a columnar lined epithelium length less than or equal to 2 cm, and long-segment BE was defined as a columnar lined epithelium length greater than 2 cm. The columnar lined epithelium was biopsied using jumbo biopsy forceps in a 4-quadrant fashion at 2-cm intervals starting at the distal margin and proceeding approximately to the squamous columnar junction. Specimens were separated for each level and placed in 10% formalin solution.

A control group consisted of subjects without BE. Subjects with erosive or ulcerative esophagitis as well subjects with a history of using bismuth compounds, prior radiotherapy, and chemotherapy were excluded from the study. The study was approved by the institutional review board for human study at the University of Louisville.

**PATHOLOGIC ANALYSIS**

The pathologic specimens were reviewed independently by 2 gastrointestinal pathologists. Pathologists were blinded to the subject’s clinical history, the endoscopic findings, and the results of the immunohistochemistry staining assay. Ablation blue staining was used to identify any presence of SIM. Hematoxylin-eosin staining was used to identify any presence of dysplasia. Dysplasia was classified into negative, indefinite/low-grade dysplasia, high-grade dysplasia, and EAC. Both pathologists had to concur on all specimens with high-grade dysplasia or EAC. Pathologic reading was determined for each biopsy slide with an overall pathologic diagnosis determined for each subject.

**IMMUNOHISTOCHEMICAL ASSAY**

The paraffin-embedded specimens were stained using the DAKO EnVision + System Kit (DAKO Corporation, Carpinteria, Calif). After the staining process was completed, the specimens were then deparaffinized and subsequently hydrated. A Tris buffer was used to wash the slides, and the specimens were then subjected to 5 minutes of peroxidase blocking. After the rewashing was complete, the polyclonal rabbit Ab-SOD (Upstate USA, Inc, Charlottesville, Va) was applied for a total of 30 minutes. The slides were rinsed after the allotted time, and the specimens were incubated with labeled polymer for 30 minutes at room temperature. After incubation, a visualization reagent, substrate-chromagen solution (diaminobenzidine), was added to the specimens. Finally, all slides were counterstained with methyl green. Each run was complete with the inclusion of a negative control.

**ASSESSMENT**

The specimens were graded separately on a 2-category ordinal scale. The scale took into account 2 factors, the staining in the mucosa and the staining in the submucosa. Each was assessed on the amount of immunostaining present and was graded on a scale from 0 (no staining) to 3 (strong staining). Specimens were evaluated by both a pathologist and our laboratory staff, and an average score was calculated from their evaluations. The total grading score of SOD immunoreactivity was the addition of mucosa and submucosa intensity, from 0 (no immunoreactivity in any of the anatomic sites) to a maximum score of 6 (strong-staining reaction in both of the histoanatomic sites). The total grading score was then assimilated according to the histologic type of the specimen, whether it was normal esophagus, metaplasia, dysplasia (high or low grade), or adenocarcinoma. Figure 1 demonstrates representative staining of MnSOD in the samples.

**STATISTICAL ANALYSIS**

A Spearman rank correlation coefficient (ρ) was used to analyze the correlation between MnSOD expressions with the various histologic stages of BE. Multiple analyses of variance were used to determine the differences of MnSOD expression, if any, between the different histologic stages of carcinogenesis. Differences between groups were regarded as statistically significant when the P values were less than .05.
A total of 92 samples, from 81 men and 11 women, were examined for measurement of MnSOD expression. There was an even distribution among all 5 groups in relation to age and degree of short- and long-segment columnar lined epithelium (Table 1). These samples were found to be representative of typical patient populations with BE and EAC.

The study population consisted of 17 patients with normal esophagus, 22 with SIM, 22 with low-grade dysplasia, 16 with high-grade dysplasia, and 15 with EAC. The control population was primarily made up of patients with reflux symptoms without endoscopic pathologic abnormalities.

Manganese superoxide dismutase expression was detectable in each histologic stage of BE and in the control patients in both the mucosa and the submucosa (Table 2). The combined mean±SD MnSOD expression in the control patients was 3.9±2.2, which was significantly more than in the patients with SIM (1.8±1.5, \( P = .002 \)) with a near 2-fold loss of combined MnSOD expression with intestinal metaplasia (Table 2). The combined MnSOD expression in low-grade dysplasia (2.2±1.7) was elevated when compared with SIM; however, it was significantly less than the controls (\( P = .02 \)). A similar expression was seen in the high-grade dysplasia (2.4±1.8) and the EAC (2.4±1.7) as compared with the low-grade dysplasia but remained significantly less than the control population (Figure 2).

The results from this study demonstrated a significant decrease in MnSOD expression in BE and EAC when compared with matched controls. This loss of MnSOD expression was most pronounced from normal to BE but remained...
consistent from dysplasia to adenocarcinoma. To our knowledge, this is the first study to examine the relationship between MnSOD and the progression of BE to EAC.

Several studies have demonstrated a relative deficiency of SOD catalytic activity, including mitochondrial MnSOD, in many types of solid tumors. Further interest in this relative deficiency of MnSOD activity has been greatly increased by observations that overexpression of MnSOD in tumor cells will suppress cell division in culture and tumor growth in vivo. In addition, recent reports have suggested a possible association between decreased MnSOD activity and malignant phenotype. While the precise reasons for this relationship between tumor cell growth rate and intracellular MnSOD activity are not known, these findings support the general idea that decreased expression of SOD may promote tumor growth. In fact, as a result of these and other observations, MnSOD is considered a tumor suppressor gene.

Further evaluation of MnSOD suggests that it is critically important in maintenance of mitochondrial function. Mice with deficiency of this enzyme exhibit progressive cardiomyopathy, neurodegeneration, and perinatal death. These studies went on to confirm that transgenic mice that express human MnSOD in the mitochondria are protected from environmental oxygen-induced lung injury and adriamycin-induced cardiac toxicity. In contrast, disruption of the other 2 SODs yielded viable mice that were normal in nonstressful conditions. Thus, the mitochondrial MnSOD represents a major cellular defense against oxidative stress.

Additional clinical studies have evaluated MnSOD expression with conflicting results. In contrast to our study, Janssen et al demonstrated that immunohistochemical expression of MnSOD was found to be enhanced in adenocarcinomas of the stomach and squamous cell carcinomas of the esophagus. This finding was contrasted by the loss of expression of copper and zinc superoxide dismutase in carcinomatous tissues when compared with normal tissues. The authors evaluated 81 gastric adenocarcinomas and found a significantly higher MnSOD expression within the tumor when compared with the normal mucosa. This higher expression did not correlate to a worse outcome; however, the ratio of MnSOD expression of tumor compared with the normal tissue was correlated of a worse overall survival rate. The significance of this up-regulation and worse outcome was felt to be related to chemoresistance, but this was not elucidated in this study. The difference in the Janssen et al study compared with the present study primarily remains the types of tissues that were examined, gastric (in the Janssen et al study) vs esophageal (in the present study), with similar histologic analysis of adenocarcinoma. The reason for a lack of significant up-regulation in the EAC samples of the present study cannot be defined from this data and remains an important future question.

The study by Malafa et al also reported MnSOD expression in gastric carcinoma, evaluating the difference in expression of MnSOD in metastatic vs nonmetastatic gastric cancers. Although the nonmetastatic gastric cancers showed no increase in expression vs normal gastric epithelial cells, 93% of primary tumor cells of metastatic gastric cancer cells showed an up-regulation of MnSOD expression.
MnSOD enzymatic activity by increased staining. These findings correlate with the theory that the MnSOD role changes during the transformation of a cell from normal to dysplasia to cancer. As also demonstrated in this present study, it appears that initially MnSOD acts as a tumor suppressor by inhibiting the ROS and preventing cellular damage; however, after the transformation to malignant cells takes place, the results of several studies infer that the MnSOD acts to protect the malignant cell from both chemotherapy\(^{20,31}\) and radiation therapy\(^{32,33}\) to allow for its progression and replication. This can be explained by the fact that the defenses against malignant cells, whether the body’s host defenses or exogenous treatments such as radiation or chemotherapy, often use ROS as a mechanism for cellular destruction. Malignant cells that have an increased expression in the MnSOD would therefore be more resistant to cellular destruction and therefore more likely to be resistant to treatment. This increase in MnSOD expression would lead to an increase in the probability of proliferation and metastasis.

The research of Izutani et al\(^{31}\) correlates the role of MnSOD after progression to cancer. This report demonstrated an up-regulation of MnSOD messenger RNA in gastric carcinoma and postulated that this served as a protective mechanism from tumor necrosis factor α cell toxicity from ROS. The later report from Izutani et al\(^{31}\) confirmed these findings by demonstrating an up-regulation of MnSOD messenger RNA in squamous esophageal carcinoma. This up-regulation of MnSOD messenger RNA in active malignant cells not only acts to protect from the body’s host defensive mechanisms, but a recent report by Hur et al\(^{31}\) demonstrated that the MnSOD up-regulation also gives gastric cancers protection against current chemotherapy agents. Further research by Izutani et al\(^{31}\) indicated that MnSOD expression inhibited the tumor sensitivity of Adriamycin in esophageal and gastric cancers, and furthermore, use of transforming growth factor β to inhibit MnSOD showed an increase in effectiveness of Adria- mycin as a suppressor of these tumors. Not only does this research solidify the theory that MnSOD protects the tumor cells after their transformation, but it leads us to believe that by inhibiting the MnSOD, chemotherapy may be made more effective.

In parallel with these reports, other reports have attempted to evaluate using MnSOD markers in both human serum and whole blood in an effort to find screening markers for early detection of certain cancers.\(^{35,36}\) These studies demonstrated promising results that may one day give us an accurate marker for earlier detection of some cancers.

The results of our study demonstrate that MnSOD expression is decreased in BE and EAC when compared with normal esophagus. These results further strengthen the idea that MnSOD can act as a tumor suppressing enzyme. These findings also suggest that different genotypes may exist depending on MnSOD messenger RNA expression, leading us to believe that certain genotypes may show an increased predilection to progress to dysplasia and adenocarcinoma. Determining these genotypes in patients with BE could be used in conjunction with other risk factors (tobacco or alcohol exposure, length of reflux symptoms, etc) to better determine which patients are at increased risk of progressing to esophageal cancer.

The limitation of this study is the range of MnSOD expression that was seen in formalin-fixed tissue; however, the sample size of each pathologic stage is enough to demonstrate an overall significance of expression.

In conclusion, MnSOD expression is significantly reduced in patients with BE with high-grade dysplasia and EAC. Manganese superoxide dismutase is related to the progression of BE and may represent one of the primary factors in oxidative stress protection. Further evaluation within genotypic expression and the role of antioxidants is needed in the effective screening and treatment of BE.

Accepted for Publication: March 25, 2005.

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REFERENCES


Invited Critique

Gastroesophageal reflux disease appears to cause cancer through an inflammatory pathway driven by oxygen free radicals. We have recently found extensive oxidative DNA damage in an experimental animal model of gastroesophageal reflux disease–induced esophageal cancer. It is reasonable to think that SOD could modulate the degree of inflammation and would be important in this type of carcinogenesis.

Superoxide dismutase has at least 2 important potential clinical roles in cancer. First, inadequate levels of SOD can accelerate oxidative inflammatory processes and carcinogenesis. Second, it can be expressed at high levels in tumors and make them nonresponders to radiotherapy.

This report by Hermann et al showed most importantly that MnSOD levels were lower in Barrett metaplasia but also in cancer. This suggests a role for SOD in promoting carcinogenesis but not in causing resistance to radiotherapeutic treatment.

The reader should be aware that this story is not completely convincing. Only 1 enzyme is assessed. There are 3 forms of SOD. It would be relatively easy for the authors to assess all 3 enzymes. It is important to note that MnSOD levels in this study were not increased in the cancer specimens. This runs counter to other published work. It makes it particularly important for the authors to look at the other forms of SOD to put this unusual finding in perspective. Furthermore, the group should assess SOD by both Northern and Western techniques.

We look forward to hearing more from this group in the years ahead and perhaps even finding a means to augment SOD levels in Barrett esophagus as a cancer prevention strategy.

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