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The Association of Manganese Superoxide Dismutase Expression in Barrett's Esophageal Progression With MnTBAP and Curcumin Oil Therapy

Suzanne C. Schiffman, M.D., Yan Li, Ph.D., and Robert C. G. Martin, M.D., Ph.D.[1]

Department of Surgical Oncology, Division of Surgical Oncology, University of Louisville, Louisville, Kentucky

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Background. **The aim of this study was to investigate the relationship between reflux induced bile insult and MnSOD expression, as well as to examine therapies to preserve MnSOD expression. Additionally, we sought to examine the relationship between MnSOD protein expression and MnSOD enzymatic activity.**

Methods. **MnSOD protein expression was determined by Western blot assay and enzymatic activity was determined by SOD assay. The enzymatic activity of the Het-1A and Bar-T cells were compared both before and after treatments.**

Results. **MnSOD expression in Het-1A cells was decreased after bile salt exposure. The cells that received MnTBAP or curcumin oil pretreatment showed increased MnSOD expression compared with control untreated cells. The Bar-T cells showed an increase in MnSOD expression after treatment with bile salts. The cells that were pretreated with MnTBAP displayed a larger increase in MnSOD expression compared with the cells that were not pretreated prior to bile salt exposure. The MnSOD activity was significantly different between the untreated cell lines ($P = 0.01$) and after treatment with bile salt ($P = 0.03$). Additionally, Bar-T cells had significantly less MnSOD activity than Het-1A cells after each of the pretreatments.**

Conclusions. **We demonstrated preservation of MnSOD expression in Het-1A cells that were pretreated with antioxidants including MnTBAP, curcumin oil, and certain berry extracts. Additionally, we demonstrated that Bar-T cells have significantly less MnSOD activity than Het-1A cells. These finding have important implications for future studies regarding chemoprevention and the treatment of esophageal cancer.**

Key Words: **manganese superoxide dismutase; curcumin oil; bile salt; esophageal cancer.**

INTRODUCTION

The superoxide dismutase (SOD) family represents the first line of defense against oxidative stress. SOD catalyzes the reaction of the superoxide radical into water and oxygen [1]. The three types of SOD are cytosolic copper/zinc-dependent SOD (CuZnSOD), iron-dependent extracellular SOD (EC-SOD), and manganese-dependent mitochondria SOD (MnSOD). The polypeptide form of MnSOD requires a mitochondrial location to acquire manganese and become enzymatically active [2]. MnSOD is considered a protective enzymatic protein for esophageal reflux-induced oxidative damage. Reactive oxygen species (ROS) play a role in the cell signaling network and cellular homeostasis and must be finely regulated [2].

Deficiency of MnSOD has been associated with carcinogenesis because MnSOD acts a tumor suppressor by inhibiting ROS and preventing cellular damage [3]. Evidence has demonstrated that SOD activity is decreased in reflux esophagitis patients and reflux animal models [4–11]. In our previous studies, we determined that SOD enzymatic activity was decreased in rat esophageal tissue after esophagoduodenal anastomosis (EDA) and external bile acid perfusion [7–9, 11]. Additionally, there was a significant decrease in MnSOD expression in patients with BE with high grade dysplasia and EAC compared with matched controls [5]. Supplementation with MnSOD mimetic Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) can protect rat esophageal epithelium from oxidative injury induced

[1] To whom correspondence and reprint requests should be addressed at Department of Surgical Oncology, Division of Surgical Oncology, 315 E. Broadway #312, Louisville, KY 40202. E-mail: rcmart03@louisville.edu.

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from increased bile acid exposure [12], thus demonstrating the need for therapeutic strategies to enhance a patient's endogenous ROS defense system.

We have recently confirmed the role of ROS and bile acids in esophageal carcinogenesis [13]. We found that MnSOD expression was significantly decreased in EAC normal esophageal epithelial cells and BE cells. This loss of MnSOD has interesting implications for chemoprevention and the molecular marker that could be evaluated for efficacy in patients with reflux esophagitis and Barrett's esophagus.

Chemoprevention is the administration of chemical entities, either as individual drugs or as dietary supplements, to prevent the initiation of premalignant lesions or their progression to cancer [14]. Chemoprevention can play an integral role in the overall strategy of reducing the incidence of cancer and is a potentially viable approach for reducing the risk of esophageal cancer in high risk individuals [15].

Our hypothesis for this study was that MnSOD protein and enzymatic expression could be increased in normal esophageal cells when pretreated with curcumin and berry extract in order to overcome the damage from oxidation stress. The aim of this study was to investigate the relationship between reflux induced bile insult and MnSOD expression, as well as to examine therapies to preserve MnSOD expression. Additionally, we sought to examine the relationship between MnSOD protein expression and MnSOD enzymatic activity.

METHODS

Cell Culture

Human esophageal cell line (Het-1A) was derived from esophageal autopsy tissue and purchased from ATCC (Manassas, VA). The cell line was maintained in bronchial epithelial cell medium (BEBM BulletKit; Clonetics Corporation, Walkersville, MD) and supplemented with 100 U/mL penicillin and streptomycin and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. The flasks and cell culture plates were pre-coated with 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I, and 0.01 mg/mL bovine serum albumin dissolved in culture medium.

A Human hTERT-immortalized non-neoplastic Barrett's cell line (BAR-T) was utilized as a generous gift from Rhonda Souza, M.D. and S.J. Spechler at the Department of Medicine, VA North Texas Health Care System and the University of Texas Southwestern Medical School, Dallas, Texas. The cell line was maintained in keratinocyte basal medium 2 (KBM-2; Clonetics Walkersville, MD) at 37°C in a humidified atmosphere of 5% CO_2.

The cells were maintained in Roswell Park Memorial Institute–1640 (RPMI–1640; ThermoScientific, South Logan, UT) and supplemented with 2 mM L-glutamine and 10% FBS and 100 U/mL penicillin and streptomycin and at 37°C in a humidified atmosphere of 5% CO_2.

Chemopreventative Pretreatments

Cells were seeded at 5×10^5/mL into 6-well plates per standardized protocol. Certain cells were pretreated prior to bile salt exposure. We added 200 uL of an MnSOD mimetic, Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), prior to addition of bile acids. Curcuma volatile oil was isolated from *Curcuma wenyujin. C. Rhizomes* are extracted at room temperature with light petroleum ether. The extract was concentrated at a low temperature to remove petroleum ether and the oily residue was collected. The curcuma volatile oil extract is typically found to be germacrone, curzerenone, β-elemene. Curcuma volatile oil is stored at 4°C until use [16]. The final concentrations of curcuma volatile oil were 50, 100, 200, and 500 μg/mL. This dosage was based on our animal experiments and is related to the clinical usage (100 mg/kg body weight) of curcuma volatile oil solved directly in the culture medium. We added curcuma volatile oil to each of the designated wells 1 h prior to bile salt treatment.

The black raspberry extracts were generously gifted from the Dr. Gary D. Stoner, Ohio State University. The extracts were prepared per standardized protocol [17]. Briefly, the black raspberries (*Rubus occidentalis*) were obtained from Stokes Fruit Farm in Wilmington, OH and subsequently freeze-dried and ground in a pulverizer to 40 mesh. Freeze-dried black raspberries (25 g) were placed is Soxhlet extraction thimble and extracted with either 400 mL of methylene chloride (berry extract C) or 400 mL of absolute ethanol (berry extract B) for 16 h. This procedure was repeated. The residue was removed and sonicated three times with 100 mL of water for 30 min each time. The extracts from each like-extraction were combined and the solvents removed by evaporation. A stock solution was prepared for berry extract B (0.025 g/2 mL PBS) and berry extract C (0.0248 g/2 mL PBS) and diluted to concentration 1:100 mM. We added 50 uL of extract (either berry B or berry C) to the designated wells 1 h prior to bile salt treatment. This dosage was based on our preliminary *in vitro* studies in which we determined the optimal dosages and these black raspberry extracts can solve directly in the culture medium.

Protein Isolation and Detection

Cells were seeded at 5×10^5/mL into 6-well plates per standardized protocol. One day after the cells were seeded, they were treated with 200 uL of a 1:1 mixture of bile salts reagents [choleate (CA), sodium deoxycholate (DCA), sodium glycocholate (GCA), and sodium taurocholate (TCA)] at 0.4 mM concentration for 4 h.

Cells were washed with ice-cold PBS and then scraped from plates using ice-cold lysis buffer [20 mmol.L HEPES (9 pH 7.4), 10 mmol/L NaCl, 1.5 mmol/L MgCl2, 20% glycerol, 0.1% Triton X-100, 1 mmol/L DTT, and protease inhibitor mixture]. The cells were centrifuged at 1500 RPM at 4°C for 15 min. BioRad (Newark, NJ, USA) protein assay stock solution was diluted with 1 part dye reagent concentrate with 4 parts distilled deionized water (DDH2O) and 1 mL was added to each clean, dry test tube. Four dilutions of bovine serum albumin standard (BSA 1.25 mg/mL) were prepared. Samples were prepared by adding 5 uL of sample to each tube. GeneQuant Pro spectrophotometer was utilized to measure the absorbance at 595 nm.

Western Blot Assay

The protein samples were separated by sodium-dodecyl sulfate electrophoresis and transferred to a nitrocellulose membrane for Western blotting. MnSOD expression and MnSOD enzymatic activity were evaluated using Western blot. The antibodies for MnSOD and glutathione peroxidase (GPX) were purchased from Millipore (Temecula, CA) and the antibodies for catalase (CAT) and ß-actin was purchased from Sigma Corp. (St Louis, MO). Thirty μg of protein was subjected to SDS/PAGE using a 10% gel, followed by transfer to a PVDF membrane. The membrane was blocked with 5% milk, incubated with the primary antibody at a dilution of 1:2000, 1 h at room temperature and detected with chemiluminescent detection using appropriate secondary antibodies (1:5000–10,000 dilution; depending on previous standardization). The specific protein bands were detected based on their molecular weight with reference to pre-labeled markers and quantitated using UnScanIt software (Silk Scientific Inc., Orem, UT). The average pixel of each protein band was divided by that of