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Original Research Communication

MnSOD Up-Regulates Maspin Tumor Suppressor Gene Expression in Human Breast and Prostate Cancer Cells

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ABSTRACT

Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme with tumor suppressor activity; however, the molecular mechanisms of MnSOD antitumor effects remain unclear. We hypothesized that MnSOD activity in cancer cells might cause downstream changes in the expression of other tumor suppressor genes. To determine whether maspin, a tumor suppressor gene that inhibits breast cancer cell invasion and metastasis, might be a target of MnSOD, we forced MnSOD expression in several human breast and prostate cancer cell lines by adenovirus-mediated gene transfer and measured maspin mRNA expression. Forced expression of MnSOD caused maspin mRNA to accumulate in a dose-dependent manner in both human breast and prostate cancer cells. Normal p53 was not necessary to mediate the effect of MnSOD because MnSOD up-regulated maspin in cells that harbor wild-type p53 and in cells that harbor mutant p53. Moreover, the effects of MnSOD on maspin were not due to demethylation of the maspin promoter. Analyses of maspin promoter activity, transcriptional run-on, and mRNA stability showed that maspin mRNA stability was the major mechanism for maspin up-regulation by MnSOD. Our findings identify a mechanism underlying MnSOD antitumor effects and provide evidence to support MnSOD as a genetic therapy in the treatment of human breast and prostate cancers. *Antioxid. Redox Signal.* 5, 677–688.

INTRODUCTION

CONVINCING EVIDENCE suggests that aberrant regulation of reactive oxygen species (ROS) production and removal is involved in the development of cancer (3, 23). Antioxidant enzymes scavenge ROS and inhibit the tumor cell malignant phenotype (24). Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme and a nuclear-encoded mitochondrial matrix protein. MnSOD converts superoxide, the first ROS generated from one-electron reduction of oxygen from cellular respiratory chain, to hydrogen peroxide (H_2O_2), which is further metabolized to water by catalase or glutathione peroxidase (24, 32). A variety of the tumor cells examined thus far have been found to exhibit a decrease or loss of MnSOD expression (3, 11, 23, 31, 39). Forced overexpression of MnSOD inhibits malignant transformation and suppresses tumor growth in a variety cancer cells both *in vitro* and *in vivo* (1, 15, 19, 24). This collection of evidence suggests that MnSOD is a new type of tumor suppressor gene (3, 12, 23–25, 29). However, the molecular mechanisms by which MnSOD suppresses the malignant phenotype are still unclear. Previous studies suggest that MnSOD may exert its tumor

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suppressor activity through modulation of redox-sensitive oncogenes and transcription factors (13). Nevertheless, direct evidence that relates MnSOD overexpression to other cell phenotype-related genes, especially tumor suppressor genes, is beginning to be addressed but still lacking (10). Li *et al.* previously reported that transfection of MnSOD cDNA into MCF-7 breast cancer cells reactivated mRNA expression of the tumor suppressor gene maspin (16), a bona fide tumor suppressor gene in human breast and prostate cancer cells (34, 36). More recently, Zou *et al.* reported a rapid and robust induction of maspin expression in prostate cancer cells (LNCaP, DU145, and PC3) and breast tumor cell MCF-7 following wild-type p53 expression from an adenovirus p53 expression vector (41). Based on these studies, we hypothesized that overexpression of MnSOD may up-regulate maspin expression not only in breast cancer cells, but also in prostate cancer cells, and functional p53 might be a factor that works between MnSOD and maspin. Our results indicate that forced MnSOD overexpression induces expression of maspin in a manner that appears independent of p53 status.

Maspin (mammary serine protease inhibitor) is a 42-kDa protein and a unique member of the serpin family (27, 40). Maspin is abundantly expressed in normal human mammary and prostate epithelial cells, but down-regulated in primary mammary and prostate cancer cells and lost in advanced cancer cell lines (27, 35, 40). Forced expression of maspin has been found to inhibit breast and prostate tumor formation, cell motility, invasion, and metastasis (28, 40). Recently, it was found that maspin works as an effective inhibitor of angiogenesis (37). Maspin has been classified as a class II tumor suppressor gene because no genetic mutations, deletions, or rearrangements have been found in maspin in association with its loss of expression. We previously found that silencing of maspin in breast cancer cells was closely associated with its promoter methylation status (6) and that this methylation pattern was comparable to that in normal cell types that were negative for maspin expression (8). We were interested to determine whether the effect of MnSOD on maspin expression in breast tumor cells was mediated through demethylation of the maspin promoter. Therefore, we measured the maspin promoter methylation status before and after Ad-MnSOD (recombinant adenovirus containing MnSOD cDNA) infection in human breast cancer MDA-MB-435 cells, which is a maspin-negative cell line and contains a highly methylated maspin promoter.

The balance between mRNA synthesis and degradation determines the steady-state level of an individual mRNA species. Differential maspin expression in normal and carcinoma-derived prostate and mammary epithelial cells has been shown to be due to differences in transcriptional control (34, 35). To explore the mechanism(s) involved in the MnSOD effect on maspin mRNA accumulation, we studied the effect of MnSOD on maspin transcription initiation rate *in vitro* by nuclear run-on analysis and maspin promoter activity by promoter-reporter analysis. Recently, the posttranscriptional regulation of mRNA stability has emerged as an important control mechanism of gene expression. Therefore, we also assessed the effect of MnSOD on maspin mRNA stability. Our results indicated that increasing maspin mRNA stability, but not transcriptional activity, contributes to up-regulation of maspin by MnSOD.

MATERIALS AND METHODS

Cell culture

Human breast cancer cell lines MDA-MB-435, MDA-MB-231, ZR-75–1, MCF-7, and normal immortalized human mammary epithelial cell MCF-10A were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). MDA-MB-435 and MDA-MB-231 cells were maintained in RPMI 1640 containing 10% fetal bovine serum supplemented with 50 μg/ml gentamycin. ZR-75–1 was cultured in RPMI 1640 with 10% fetal bovine serum supplemented with 100 units/ml penicillin and 50 μg/ml streptomycin. MCF-10A cells were grown in mammary epithelial growth medium (Clonetics, Walkersville, MD, U.S.A.) supplemented with 100 ng/ml cholera toxin (Calbiochem–Novabiochem Corp., La Jolla, CA, U.S.A.). MCF-7 cells were cultured in Eagle's minimum essential medium containing nonessential amino acids, 1 m*M* sodium pyruvate, and 10% fetalbovine serum. MCF-7 cell derived stable transfectants SOD15, Mn1, Mn40, and neomycin transfection control cell line Neo were maintained in the mediumsupplemented with 400 μg/ml geneticin (Life Technologies, Inc., Gaithersburg, MD, U.S.A.). Prostate cancer cell lines LNCaP, PC3, and DU145 were kindly provided by Dr. Michael Cohen at the University of Iowa. These three prostate cancer cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin and 50 μg/ml streptomycin. All the cells were cultured in a 5% CO_2/95% humidified air environment at 37°C. Cultures were fed with fresh medium two or three times per week.

Adenovirus infections

Replication-deficient recombinant type 5 adenovirus containing human MnSOD cDNA (Ad-MnSOD) or bacteria LacZ cDNA (Ad-LacZ) were from Gene Transfer Vector Core in the University of Iowa. A CMV promoter was used to drive expression of all the cDNAs. Cells were subcultured in 60-mm dishes (0.5–1 $\times$ 10^6 cells/dish) the day before infection in normal growth medium. On the day of the infections, cells were washed twice in prewarmed 1$\times$ phosphate-buffered saline (PBS) and infected with adenovirus in 2.5 ml of serum-free medium for 24 h. Cells were washed twice with 1$\times$ PBS and returned to full growth medium. After 48 h of recovery, infected cells were used for the analysis of MnSOD and maspin protein expression, maspin mRNA expression, or maspin promoter methylation status.

Western blot analyses

Cells were scrape-harvested and sonicated in 0.05 *M* potassium phosphate buffer (pH 7.8) with two bursts of 20 s each at maximum power using a Vibra Cell Sonicator equipped with an ice water-chilled cup horn tip. After centrifugation of the lysates, protein concentrations of the supernatants were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Ten micrograms (for MnSOD) or 20 μg (for maspin) of denatured protein samples was separated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to nitrocellulose membranes. For MnSOD immunoblotting, blots

were sequentially incubated with primary rabbit antiserum against human kidney MnSOD and secondary anti-rabbit IgG conjugated with horseradish peroxidase (HRP) as previously described (33). For maspin immunoblotting, blots were sequentially incubated with mouse monoclonal antibody against human maspin (dilution 1:500, Pharmingen, San Diego, CA, U.S.A.) and secondary anti-rabbit IgG conjugated with HRP. The HRP-conjugated antibodies were visualized by exposure of the blot to enhanced chemiluminescence staining (Amersham, Arlington, IL, U.S.A.) and bands were revealed by exposure of x-ray film.

Northern blot analysis for maspin mRNA

Total cellular RNA was isolated using GibcoBRL Total RNA Isolation Reagent (Life Technologies, Inc.). Twenty micrograms of total cellular RNA from each cell line was electrophoresed on an agarose gel containing formaldehyde and transferred to a nylon membrane. Nucleic acids were cross-linked to the membranes by UV irradiation and the membranes were incubated in prehybridization solution (50% formamide, 10× Denhardt's solution, 10% dextran sulfate, and 200 μg/ml salmon sperm DNA) for 12 h at 42°C. A KpnI-XhoI restriction fragment containing a ~1.1-kb partial human maspin cDNA was gel-isolated and labeled with [^{32}P]-dCTP using a random primed DNA labeling kit following the instructions provided by the supplier (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). The denatured cDNA probe was added to the prehybridization solution and incubated with the blots for 18 h at 42°C. The membranes were washed twice in 1× saline–sodium citrate buffer (SSC)/1% SDS at 65°C for 20 min. The labeled mRNA signal was detected and quantified by a Typhoon 8600 variable Mode Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Each blot was then stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GADPH) probe to confirmequal loading and transfer of RNA.

Isolation of genomic DNA and sodium bisulfite genomic sequencing of maspin promoter

Cells were treated with DNA lysis buffer (containing 100 m*M* NaCl, 10 m*M* Tris-HCl, 50 m*M* EDTA, 1% SDS) and harvested into a 1.5-ml centrifuge tube. Genomic DNA was then isolated and purified using routine phenol/chloroform/isoamyl procedure. Five micrograms of genomic DNA was modified with sodium bisulfite under conditions previously described (5). The maspin promoter was amplified from the bisulfite-modified DNA by nested PCR reactions using primers specific to the bisulfite-modified sequence of the maspin promoter. The first-round forward primer 5'-AAA AGA ATG GAG ATT AGA GTA TTT TTT GTG-3' and reverse primer 5'-CCT AAA ATC ACA ATT ATC CTA AAA AAT A-3' allowed the amplification of the fragment from −283 to +181 relative to the transcription start site. PCR amplifications were performed under the following conditions: 94°C for 4 min; 5 cycles of 94°C for 1 min, 56°C for 2 min, 72°C for 3 min; then 35 cycles of 94°C for 30 s, 56°C for 2 min, 72°C for 1.5 min; and a final extension of 72°C for 10 min. One microliter of first PCR product was further used as template in a nested PCR amplification. The nested forward and reverse primers 5'-GAA ATT TGT AGT GTT ATT ATT ATT ATA-3' and 5'-AAA AAC ACA AAA ACC TAA ATA TAA AAA-3' were used to generate the fragment from −237 to +133. PCR amplifications were performed under the following conditions: 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1.5 min; and a final extension of 72°C for 10 min. Fresh nested PCR products were gel-isolated and cloned into the TA Original vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). Twenty-five positive recombinants from MDA-MB-435 cells and 10 positive recombinants from adenovirus-infected cells were isolated, and plasmids were prepared using a Qiaprep Spin Plasmid Miniprep kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions and sequenced on an ABI automated DNA sequencer. The number of methylated CpG at each site was divided by the number of clones analyzed to yield the percentage of methylation.

MnSOD expression plasmids, maspin reporter gene construct, and transient transfections

pcDNA3-MnSOD expression vector, which contains the full-length human MnSOD cDNA driven by a CMV promoter, was constructed as described (33). The maspin promoter reporter construct (pGL3-MP) was made by fusing the maspin gene 5'-flanking region from −396 to +131 relative to the transcription start site to the pGL3/Basic vector (Promega, Madison, WI, U.S.A.). The identity and orientation of the insert were verified by DNA sequencing. Two micrograms of each pGL3-MP, pcDNA3-MnSOD was used for transient transfections. One microgram of pCMV-β-galactosidase vector was used as a control for normalizing variations in transfection efficiencies within and between experiments. The pcDNA3 or pGL3-Basic vectors wereadded to normalize the total amount of DNA transfected in each assay. Transfection was done in 5×10^5 MDA-MB-435 cells and DU145 cells by using 20 μl of SuperFect transfection reagent (Qiagen). After 4 h of incubation in 1 ml of full growth medium, transfection mixture was removed and cells were allowed to recover in full medium for an additional 48 h. Plasmid DNA was transferred into ZR-75-1 cells by electroporation. Ten micrograms of pGL3-MP or pGL3-Basic with 5 μg of pCMV-β-galactosidase was transferred into 5×10^7 cells by a single electric pulse at 260 V and 1,180 μF. ZR-75-1 cells were grown in 5 ml of growth medium in a 60-mm dish after transfection. Four hours later, culture medium was changed. Cells were grown in fresh medium for 48 h. All the transfected cells were harvested and lysed using 200 μl of passive lysis buffer (Promega). The resultant cell lysate was used for luciferase reporter gene assay and β-galactosidase activity assay.

Promoter activity assay

Luciferase activity of 20 μl of cell lysate was measured after addition of 100 μl of luciferase assay reagent following the manufacturer's protocol (Promega). Luciferase activity was normalized to β-galactosidase activity from the same amount of extract in each sample. The relative maspin promoter activity in each cell type was represented as a fold increase relative to that of pGL3-Basic vector. The mean and standard deviation from multiple plates in two independent transfection experiments were used as one datapoint.

Nuclear run-on assay for maspin mRNA in vitro *transcription*

Five million PC3 cells were infected with 100 MOI (multiplicity of infection) Ad-MnSOD or Ad-LacZ for 24 h and recovered in full growth medium for another 24 h. Cells were harvested by scraping into 1× PBS and centrifugation at 1,500 rpm for 2 min. The resulting cell pellet was resuspended in 1 ml of hypotonic buffer [10 m*M* KCl, 10 m*M* Tris-HCl (pH 7.5), 1.5 m*M* $MgCl_2$, 0.3 *M* sucrose, 0.25% NP-40, 1 m*M* dithiothreitol, and 0.5 m*M* phenylmethylsulfonyl fluoride] on ice for 15 min and gently disrupted in a Dounce homogenizer. Lysed cells were centrifuged at 1,500 rpm for 15 min, and nuclear pellet was immediately resuspended in 200 µl of transcription reaction buffer [50 m*M* Tris-HCl (pH 7.5), 0.1 m*M* ammonium sulfate, 1.8 m*M* dithiothreitol, 1.8 m*M* $MnCl_2$, 2 µl of RNasin, 300 µ*M* each of ATP, CTP, and GTP, and 100 µCi of [^{32}P]-UTP]. Transcription was carried out for 30 min at room temperature and terminated by incubation with 5 µl of RNase-free DNase and 2 µl of tRNA (20 mg/ml) for 15 min. Twenty microliters of 10% SDS and 2 µl of proteinase K (0.1 mg/ml) were added and the incubation continued for 45 min at room temperature. RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Equal amounts of radioactivity (~3 × 10^6 cpm/ml) were used to hybridize dot blots with denatured 2 µg of maspin and GAPDH cDNA in hybridization mixture [5.8× SSC, 58% formamide, 0.12% SDS, 1.2 m*M* EDTA, 12 m*M* Tris-HCl (pH 7.5), 4.7× Denhardt's, 0.4 mg/ml yeast tRNA, and 0.4 mg/ml sheared ssDNA] for 72 h. Blots were washed twice at 65 °C for 1 h. ^{32}P signals were visualized and quantitated by a PhosphorImager.

Maspin mRNA stability assay

To measure the maspin mRNA stability, PC3 cells were incubated with 10 µg/ml actinomycin D (Sigma Chemical Co., St. Louis, MO, U.S.A.) for the indicated times. To study the effect of MnSOD on maspin mRNA stability, cells were infected with 100 MOI Ad-MnSOD or Ad-LacZ for 24 h and recovered for 24 h before the treatment with the same dose of actinomycin D. Total cellular mRNA was harvested at each time point and subjected to northern blot analysis.

Statistical analysis

Maspin promoter activity data were expressed as the means of two independent experiments ± SD. Statistical significance among data groups was determined by one-way ANOVA and further two-sample *t* test at $p < 0.05$ level using S-PLUS 2000.

RESULTS

Ad-MnSOD increased MnSOD protein expression in human breast and prostate cancer cells

Adenovirus has proven to be a powerful gene delivery vehicle for the expression of transgenes. In this study, we used a type 5 recombinant adenovirus vector containing the full-length human MnSOD cDNA driven by a CMV promoter (Ad-MnSOD) to infect human breast and prostate cancer cells. Similarly, Ad-LacZ was used as the infection control. To verify the expression of MnSOD protein in the studied cells, we infected one breast cancer cell line MDA-MB-435 and one prostate cancer cell line DU145 with Ad-MnSOD and measured MnSOD protein expression by western blot analysis. The SDS-PAGE western blot results are shown in Fig. 1. As seen in Fig. 1, Ad-MnSOD infection led to MnSOD expression detectable as low as 10 MOI and abundantly at 100 MOI in both of the two cell types compared with controls. Ad-LacZ as high as 100 MOI did not induce MnSOD protein expression. We also observed a band with a higher molecular weight of MnSOD in DU145 cells. This is likely the MnSOD protein precursor, which has an extra 24 amino acids that are responsible for the transportation of the precursor to the mitochondria (30). Previous immunohistochemical studies have demonstrated the correct mitochondrial localization of this adenovirally expressed MnSOD (14).

MnSOD up-regulated maspin expression in human breast and prostate cancer cells

To determine whether overexpression of MnSOD could up-regulate maspin, we performed northern blot analyses to measure maspin mRNA expression in a variety of untreated, Ad-LacZ-infected, and Ad-MnSOD-infected human breast and prostate cancer cells. The northern blot results from human breast cancer cells MDA-MB-435 and MDA-MB-231 are shown in Fig. 2A and C. The results from human prostate cancer cells DU145, PC3, and LNCaP are shown in Fig. 3A, B, and C, respectively. As shown in Fig. 2, we observed a dose-dependent increase of maspin mRNA expression in MDA-MB-435 and MDA-MB-231 cells infected with 50–250 MOI Ad-MnSOD. Importantly, MnSOD expression also induced expression of

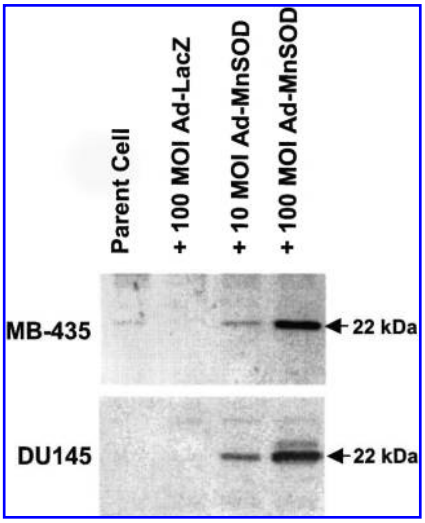

FIG. 1. Overexpression of MnSOD protein in Ad-MnSOD-infected human breast cancer cell MDA-MB-435 and human prostate cancer cell DU145. Cells were infected with Ad-LacZ or Ad-MnSOD for 24 h and recovered for 48 h. Ten micrograms of whole cell lysate was separated by SDS-PAGE and analyzed by immunoblotting with rabbit antiserum against human MnSOD.

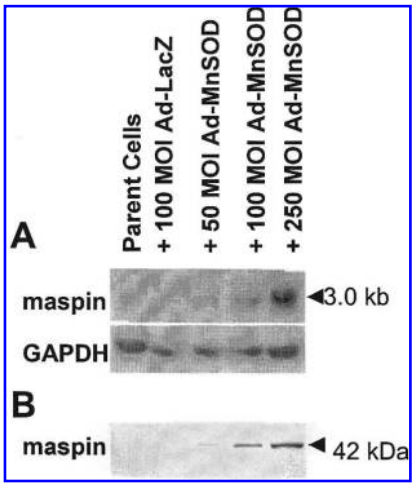

FIG. 2. Ad-MnSOD infection reactivates maspin mRNA (A) and protein (B) expression in human breast cancer cells MDA-MB-231. Ad-MnSOD infection also reactivates maspin mRNA expression in MDA-MB-435 human breast carcinoma cells (**C**). Cells were infected with Ad-LacZ or Ad-MnSOD for 24 h and recovered for 48 h. Twenty micrograms of total RNA was used for northern blot analysis of maspin mRNA expression. GAPDH mRNA expression was used as RNA loading and transfer control. Twenty micrograms of total cellular protein was used for western blot analysis of maspin protein expression.

the maspin protein in MDA-MB-231 cells as determined by western blot analysis shown in Fig. 2B. The induced maspin mRNA transcript was ~3.0 kb determined by its location relative to the 18S and 28S RNA. Infection with Ad-LacZ did not induce maspin expression. Interestingly, in Ad-MnSOD-infected human prostate cancer cells, we observed two maspin transcripts corresponding to 3.0 kb and 1.2 kb, respectively. Careful inspection of the 3'-untranslated region (3'-UTR) of maspin cDNA revealed two potential poly(A) signals located at nucleotides 1,239 to 1,244 and nucleotides 2,551 to 2,555 (Fig. 3C) relative to the transcription start site. These two poly(A) signals apparently lead to the generation of the shorter 1.2-kb and longer 3.0-kb maspin transcripts. Between the two transcripts, the smaller 1.2-kb transcript was much more inducible than the 3.0-kb transcripts as seen in the northern blots. The mechanisms of this change will be discussed later from the perspective of altered mRNA stability.

MnSOD up-regulated maspin mRNA expression in human breast and prostate cancer cells independent of the methylation status of the maspin promoter

Our previous study showed that aberrant CpG methylation of maspin promoter participated in the silencing of maspin in most of the studied breast carcinoma cell lines (6). To find out whether the effect of MnSOD on up-regulation of maspin mRNA in human breast cancer cells is through demethylating the maspin promoter, we examined the cytosine methylation status of the maspin 5' regulatory region from −237 to +133 in MDA-MB-435 cells before and after adenovirus infection.

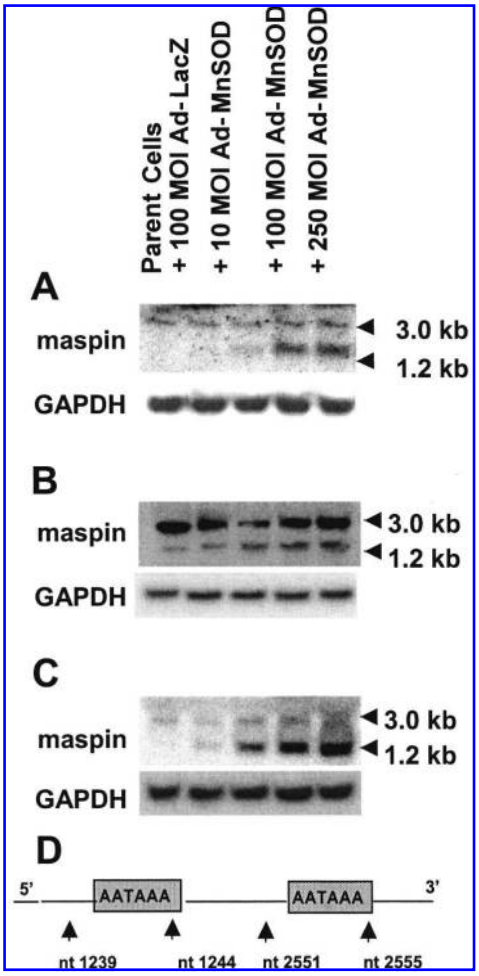

FIG. 3. Ad-MnSOD infection up-regulated maspin mRNA expression in human prostate cancer cells DU145 (A), PC3 (B), and LNCaP (C). Cells were infected with Ad-LacZ or Ad-MnSOD for 24 h and recovered for 48 h. Twenty micrograms of total RNA was put to northern blot analysis of maspin mRNA expression. GAPDH mRNA expression was used as RNA loading and transfer control. (**D**) Schematic representation of maspin 3'-UTR showing the two poly(A) signals.

To increase the amplification efficiency of bisulfite-modified DNA, we performed nested PCR reactions. The two pairs of primers in the maspin promoter region are shown above the arrows in Fig. 4A. This amplified region contains 19 CpG dinucleotides that are potential sites for cytosine methylation. The bisulfite genomic sequencing results of maspin promoter methylation status in the untreated MDA-MB-435 cell, Ad-LacZ-infected cell, and Ad-MnSOD-infected cell are shown in Fig. 4B–D. As seen in Fig. 4B, nearly all the 19 CpGs are hypermethylated in MDA-MB-435 cells. Neither Ad-LacZ nor Ad-MnSOD infection changed the maspin promoter methylation pattern under the same conditions that provided maximal maspin reactivation. Therefore, the effect of MnSOD on maspin expression is not due to the demethylation of maspin promoter in MDA-MB-435 cells.

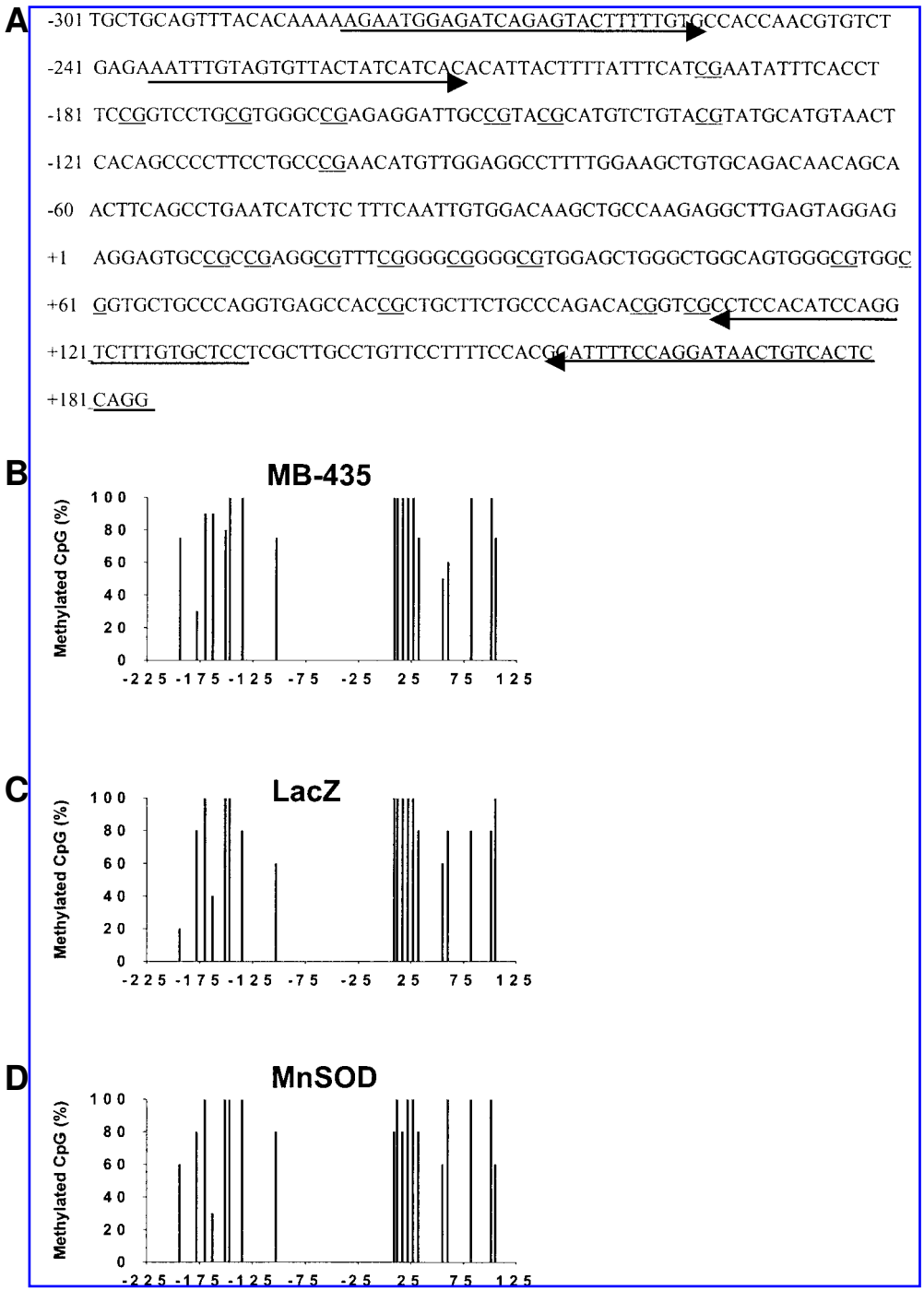

FIG. 4. Ad-MnSOD did not change the maspin promoter methylation status in human breast cancer MDA-MB-435 cells. (**A**) DNA sequence of maspin 5' regulatory region. Two pairs of arrows indicate the four primers for nest PCR. The 19 CpG potential sites for cytosine methylation are underlined. Cells were infected with 100 MOI Ad-LacZ or Ad-MnSOD for 24 h and recovered for 48 h. The cytosine methylation status of maspin in untreated MDA-MB-435 cells, Ad-LacZ-infected cells, and Ad-MnSOD-infected cells is shown in **B–D**.

MnSOD up-regulated maspin expression in human breast and prostate cancer cells independent of cellular p53 status

Zou *et al.* reported that p53 up-regulated maspin expression in both prostate and breast cancer cells, including all three prostate cancer cells we used here and one breast cancer cell MCF-7 (41). Thus, it is reasonable to hypothesize that MnSOD up-regulated maspin in a p53-dependent manner. To clarify whether this was the case, we examined the p53 status of the cells that were studied here and the MCF-7 cell, which was studied previously (16), based on the published literature. Results of this analysis are summarized in Table 1. Among the three breast cancer cell lines, MCF-7 harbors wild-type p53,

TABLE 1. OVERVIEW OF MASPIN PROMOTER METHYLATION AND P53 STATUS IN HUMAN BREAST AND PROSTATE CANCER CELL LINES

Cell types	*p53 status*	*Promoter methylation status (6)*
Breast cancer cells		
MDA-MB-435	mt-p53	+
MDA-MB-231	mt-p53	+
MCF-7	wt-p53	+
Prostate cancer cells		
PC3	p53(−/−)	−
DU145	mt-p53	−
LNCaP	wt-p53	−

mt, mutant; wt, wild type.

whereas MDA-MB-435 and MDA-MB-231 cells harbor mutant p53. Among the prostate cancer cell lines, LNCaP harbors wild-type p53 expression, whereas DU145 has a mutant p53 expression and PC3 is a p53 null cell line. Therefore, the up-regulation effect of MnSOD on maspin mRNA expression was independent of cellular p53 status.

MnSOD did not influence maspin promoter activity or mRNA transcription rate in PC3 cells

Several studies have shown that maspin promoter activity is abolished or down-regulated in human breast and prostate cancer cells compared with their normal counterpart (34, 35). To elucidate whether the effect of MnSOD is due to activation of the maspin promoter, we measured maspin promoter activity in transient and stable MnSOD overexpression models. The maspin promoter luciferase reporter construct pGL3-MP consists of the 5' regulatory region from −396 to +131 relative to the maspin transcription start (Fig. 5A). This region, which contains the binding sites of Ets, p53, and AP-1, has been characterized to be necessary and sufficient for the maspin transactivation (34). To test the function of this construct, we first transfected it into an immortalized but nontumorigenic human breast epithelial cell line MCF-10A that constitutively expresses a high level of maspin. As shown in Fig. 5B, maspin promoter activity in MCF-10A cells was higher than the activity of pGL3-Basic construct. Compared with the promoter activity in normal MCF-10A cells, the maspin promoter was less active in human breast cancer cells MDA-MB-435 and ZR-75-1. To determine whether MnSOD up-regulated the maspin promoter, we cotransfected pGL3-MP and an MnSOD expression vector pcDNA3-MnSOD into MDA-MB-435 and DU145 cells. As shown in Fig. 5C, MnSOD had no effect on maspin promoter activity in either of the two cell lines. We also measured maspin promoter activity in MnSOD stable transfectants, SOD15, Mn1, and Mn40, which were derived from MCF-7 human breast cancer cells (33). The MnSOD enzyme activities in the stable transfectants are 19, 62, and 230 U/mg of protein for SOD15, Mn1, and Mn40, respectively. However, no significant changes were observed in maspin promoter activity among the three MnSOD overexpressing cell lines (Fig. 5D).

To explore further whether MnSOD modulates maspin mRNA expression at the transcriptional level, we measured the maspin mRNA *in vitro* transcription rate. The nuclear run-on assay results in the Ad-MnSOD- and Ad-LacZ-infected PC3 cells are shown in Fig. 6. Again, we detected an almost equal amount of the newly transcribed maspin mRNA signal in the two groups. This result indicates that MnSOD has no effect on the rate of transcriptional initiation of maspin as determined by this *in vitro* transcription assay.

MnSOD increased maspin mRNA stability in PC3 cells

Much attention has been focused on the transcriptional regulation of maspin in normal and cancer cells. Thus far, an effect of maspin mRNA stability has not been explored. In this study, we examined maspin mRNA stability in PC3 cells, which have the most abundant endogenous maspin mRNA expression among the cancer cells we used here. We inhibited new maspin mRNA synthesis by treating the cells with 10 μg/ml actinomycin D and measured the residual levels of the 3.0-kb and 1.2-kb maspin transcripts at various time points. A representative northern blot result is shown in Fig. 7A. Quantitative analysis of the maspin half-life based on two independent experiments is shown in Fig. 7B. It shows that the half-life of the 3.0-kb transcript was ~20 h and the half-life of 1.2-kb transcript was much longer, ~30–36 h.

To determine whether the effect of MnSOD on the up-regulation of maspin mRNA was due to the modulation of maspin mRNA stability, we infected the PC3 cells with 100 MOI Ad-LacZ and Ad-MnSOD, then treated cells with actinomycin D, and assessed maspin mRNA levels at each time point. The northern blot results are shown in Fig. 8A. Quantitative analysis of the maspin mRNA remaining as a function of time is shown in Fig. 8B for the 3.0-kb transcript and Fig. 8C for the 1.2-kb transcript. These data suggest that MnSOD increased the stability of both transcripts compared with the Ad-LacZ infection control. For the 3.0-kb transcript, the residual maspin mRNA dropped to 30% within 36 h. In contrast, the 1.2-kb transcript consistently remained at nearly 80% from 12 to 36 hours. Therefore, the stabilizing effect of MnSOD expression on the 1.2-kb transcript was more significant than that on the 3.0-kb transcript. Taking into account that the 1.2-kb transcript has a longer half-life, it might explain why we observed a significant accumulation of 1.2-kb transcript, but not the 3.0-kb transcript, after MnSOD overexpression.

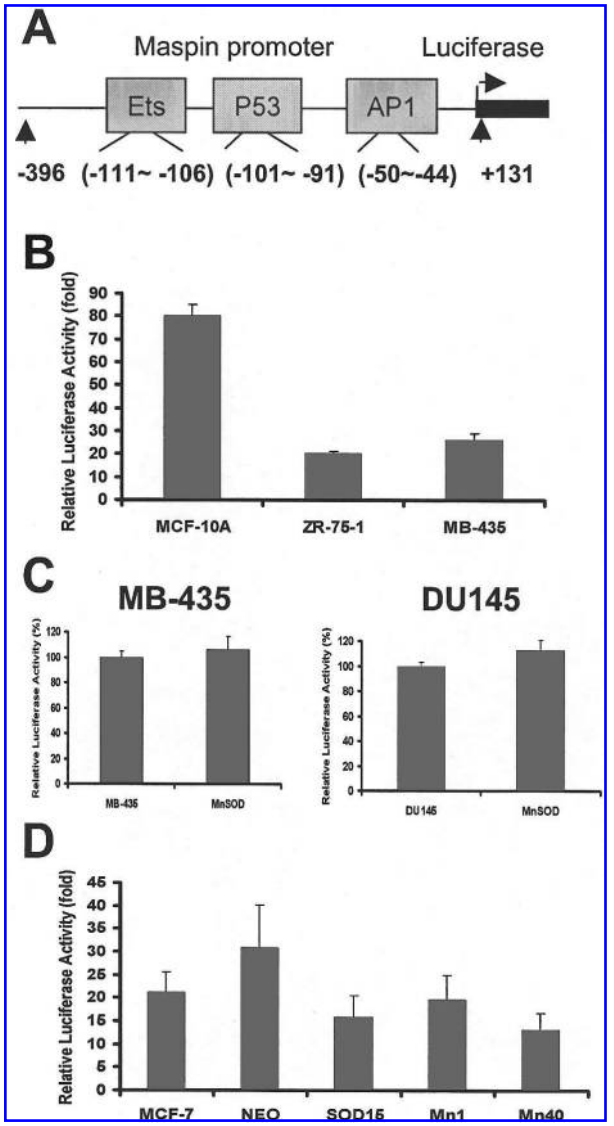

FIG. 5. Maspin promoter activity in human breast and prostate cancer cells. (**A**) Schematic representation of maspin promoter construct with the important cis-elements labeled in the shaded areas. (**B**) Maspin promoter activity was down-regulated in human breast cancer cells. MCF-10A and MDA-MB-435 cells were transiently transfected with 2 μg of maspin promoter construct pGL3-MP or pGL3-Basic together with 1 μg of pCMV-β-galactosidase as internal control. ZR-75–1 cells were transfected by electroporation. Ten micrograms of pGL3-MP or pGL3-basic was transfected into 5×10^7 cells with 5 μg of pCMV-β-galactosidase as the internal control. The relative luciferase activity normalized by β-galactosidase activity was represented as the fold increase compared with pGL3-Basic vector activity in each cell types. (**C**) MnSOD did not influence maspin promoter activity in human breast cancer MDA-MB-435 cells and human prostate cancer DU145 cells. Two micrograms of pcDNA3-MnSOD expression plasmid was cotransfected with 2 μg of pGL3-MP. The relative maspin promoter activity in the control cells was designated as 100%. (**D**) Maspin promoter activity in MnSOD transfected cells was represented as a percentage increase compared with control. Results shown here are the means and standard deviations from two independent transfection experiments.

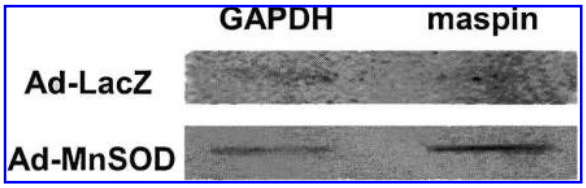

FIG. 6. Influence of MnSOD on the maspin mRNA *in vitro* transcription rate in PC3 cells. Cells were infected with 100 MOI Ad-LacZ or Ad-MnSOD for 24 h. After recovery for another 24 h, total RNA was isolated and put into *in vitro* transcription analysis. [^{32}P]-UTP-labeled mRNA samples were hybridized with slot-blotted 2 μg of maspin and GAPDH cDNA. ^{32}P signals were detected and quantified by PhosphorImager.

DISCUSSION

Tumor cells have often been found to display lower levels of antioxidant enzyme expression than their normal counterparts. For example, tumor cells are nearly always low in MnSOD and catalase activity and usually low in copper/zinc superoxide dismutase activity, whereas glutathione peroxidase activity is variable (3, 25, 33). MnSOD is a nuclear-encoded mitochondrial matrix protein and serves a protective function by

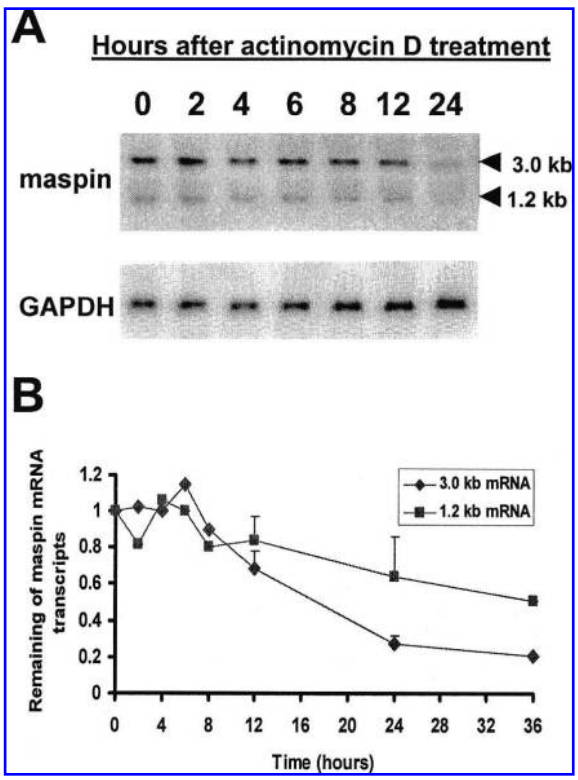

FIG. 7. Maspin mRNA stability in human prostate cancer PC3 cells. (**A**) Northern blot analysis for maspin mRNA stability assay. Cells were treated with 10 μg/ml actinomycin D. Total RNA was harvested at the times indicated and processed for northern blot analysis. The mRNA signals were quantified by PhosphorImager. (**B**) The remaining maspin mRNA transcripts normalized to GAPDH loading control were plotted versus time.

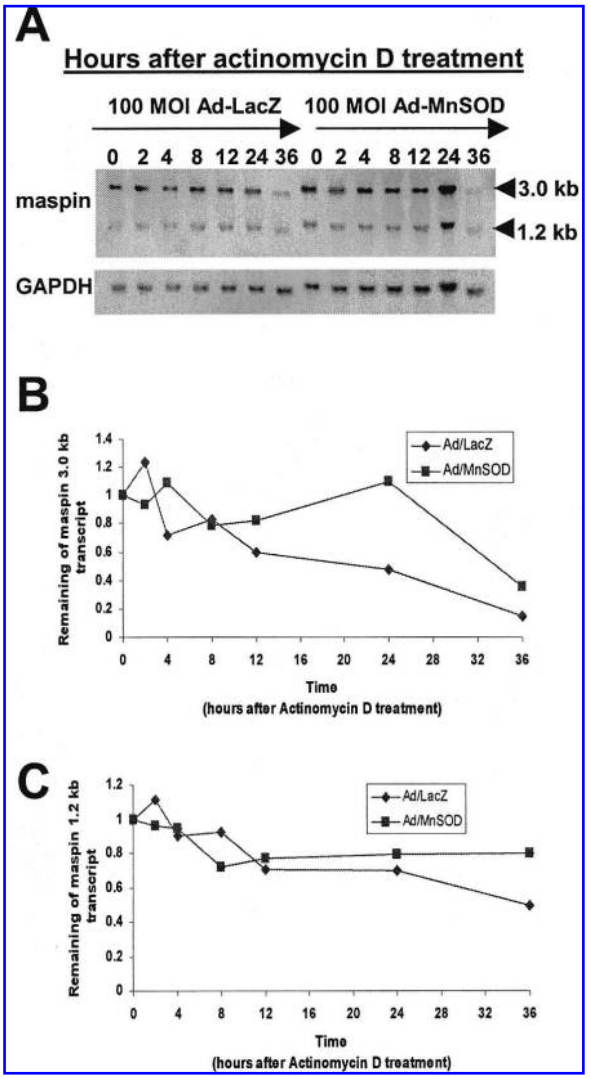

FIG. 8. MnSOD increased maspin mRNA stability in human prostate cancer PC3 cells. (**A**) Northern blot analysis showing the maspin mRNA stability. Cells were infected with 100 MOI Ad-LacZ or Ad-MnSOD for 24 h. After recovery for another 24 h, cells were further treated with 10 μg/ml actinomycin D. Total RNA was harvested at the times indicated after actinomycin D treatment and processed for northern blot analysis. The mRNA signals were quantified by PhosphorImager. (**B**) The remaining maspin mRNA 3.0-kb transcript normalized to GAPDH was plotted versus time. (**C**) The remaining maspin mRNA 1.2-kb transcript normalized by GAPDH loading control was plotted versus time.

detoxifying superoxide. MnSOD is a particularly important primary antioxidant enzyme in part because of its strategic location in mitochondria. The mitochondria consume >90% of the oxygen metabolized by aerobic cells, and ~2% of the oxygen consumed results in the formation of superoxide as a result of electron leakage from the respiratory chain. Superoxide is not only the first ROS derived from one-electron reduction from cellular respiratory chain in mitochondria, it can also initiate a cascade generation of other ROS, for example, H_2O_2, hydroxyl radical ($^{\cdot}OH$), peroxynitrite ($ONOO^-$), and alkoxyl radical ($RO^{\cdot}$) (25). Thus, decreased expression of MnSOD might contribute to the carcinogenic process by allowing aberrant accumulation of ROS in the cell. Moreover, the aberrant accumulation of diffusible, more stable ROS such as H_2O_2 may lead to changes in gene expression in the cell (10).

Substantial evidence has demonstrated that forced overexpression of MnSOD is sufficient to suppress the malignant phenotype in a great variety of human tumors, including melanoma (4), human glioma (38), prostate cancer (19), and breast cancer (18). The parameters of these suppressed malignant phenotypes included not only *in vitro* growth in soft agar, but also more importantly the *in vivo* growth in nude mice. However, the mechanisms by which MnSOD accomplished this suppression are poorly understood. It appears that the effects of MnSOD overexpression on cancer cells are due to a noncytotoxic tumor suppressive effect (22). Previous studies suggested that MnSOD may exert its tumor suppressor activity through modulation of the function of redox-sensitive oncogenes and transcription factors (13). MnSOD overexpression is known to affect c-Jun expression (13) and AP-1 and nuclear factor-κB activities (17). This may occur through redox activation of these molecules by diffusible ROS as has been previously suggested. These factors may in turn regulate the expression of downstream genes related to tumor initiation, progression, and metastasis. But the direct evidence relating MnSOD to the expression of other tumor suppressor genes is still lacking. In this study, we demonstrated that overexpression of MnSOD could up-regulate maspin tumor suppressor gene expression in human breast and prostate cancer cells. Moreover, the effect of MnSOD overexpression was independent of maspin promoter methylation status and tumor cell p53 status. Thus, these results not only confirm the previous study by Li *et al.* that looked at only a single MnSOD-expressing clone of MCF-7 cells (16), but also extend those findings to two other human breast carcinoma cell lines as well as three human prostate cancer cell lines where MnSOD expression was forced in heterogeneous populations of cells by an adenoviral vector.

Aberrant cytosine methylation within CpG dinucleotides in the 5' regulatory regions is an important epigenetic mechanism to silence important tumor suppressor genes (7). Our previous work showed that aberrant cytosine methylation and chromatin condensation of the maspin promoter participated in the silencing of maspin expression during neoplastic progression of breast cancer cells (6). In addition, 5-aza-deoxycytidine treatment reactivated maspin mRNA expression (6). In this study, however, we found that MnSOD did not change the maspin promoter methylation pattern even though it reactivated maspin expression. In addition, MnSOD up-regulated maspin expression not only in maspin promoter hypermethylated breast cancer cells but also in maspin promoter largely unmethylated prostate cancer cells (unpublished observations). Taking these finding together, we concluded that the effect of MnSOD on maspin was independent of maspin promoter methylation status. We know that aberrant promoter methylation participates in the silencing of many important tumor suppressor genes including RB, VHL, BRCA1/2, WT-1, p16/Ink4b, p15/ink4b, p27/kip, hMLH1, and E-cadherin (2). Our study regarding the reactivation of epigenetically silenced maspin by MnSOD suggests that other epigenetically silenced tumor suppressor genes potentially could be reactivated or up-regulated by MnSOD.

Indeed, recent results of microarray expression studies in MnSOD-overexpressing cells would support this hypothesis (10). These effects may explain in part the mechanisms of MnSOD's extensive antitumor effect in tumor cells.

Zou *et al.* reported that p53 regulated expression of maspin in both breast and prostate cancer cells (41). They found that p53 activated the maspin promoter by binding directly to a p53 consensus site present in the maspin promoter. DNA-damaging agents and cytotoxic drugs induced endogenous maspin expression in cells containing the wild-type p53. Maspin expression was refractory to the DNA-damaging agents in cells containing mutant p53. Thus, it seems that wild-type p53 is a critical upstream regulator of maspin. However, our study showed that MnSOD up-regulated maspin expression not only in p53 wild-type cells LNCaP, but also in p53 mutant cells (MDA-MB-435, MDA-MB-231, PC3, and DU145). Taken together, these studies strongly suggest that even though maspin is a downstream effecter of MnSOD, functional p53 is not a necessary mediator between them.

The level of specific mRNA species depends on either their new transcription or posttranscriptional degradation or both. Our study in PC3 human prostate carcinoma cells showed that the effect of MnSOD on the up-regulation of maspin expression was through increasing maspin mRNA stability, but not increasing its transcription. We did not measure the effect of MnSOD on the maspin mRNA stability and *in vitro* transcription in human breast cancer cells due to the undetectable endogenous maspin mRNA expression. But we predicted that increasing maspin mRNA stability might also be the major mechanism for its accumulation after MnSOD treatment in human breast cancer cells. As shown in Fig. 5, maspin promoter activity is down-regulated in human breast cancer cells MDA-MB-435 and ZR-75-1. Consistent with our study, Zhang *et al.* showed that the maspin promoter transactivation activity was decreased in primary tumor cells and was abolished in MDA-MB-231 cells (34). The aberrantly hypermethylated maspin promoter is maintained in a closed chromatin structure and likely inaccessible to transcriptional factors that may otherwise bind to their binding sites in the promoter region. Epigenetic silencing of the maspin promoter is one of the major mechanisms for the loss or down-regulation of the maspin expression in human breast cancer cells. MnSOD could not demethylate the maspin promoter in MDA-MB-435 human breast cancer cells, so it is unlikely that MnSOD can render the maspin promoter more accessible and lead to a stronger transactivation. The possibility still exists, however, that MnSOD expression could lead to other epigenetic changes at the maspin promoter that render it more accessible to transcriptional transactivation, as has recently been shown for p53-mediated activation of maspin expression (26). Nevertheless, our promoter reporter assay in MDA-MB-435 cells showing that MnSOD did not increase maspin promoter activity, as well as the results from our nuclear run-on studies, all support the conclusion that it is unlikely that MnSOD increases maspin transcription.

Recently the posttranscriptional regulation of mRNA stability has been identified as an important control mechanism of gene expression. Our study here demonstrated that MnSOD increased maspin mRNA accumulation through increasing maspin mRNA stability and therefore identified a possible mechanism that contributes to the regulation of other tumor suppressor genes by MnSOD. However, increasing mRNA stability is not the only effect of MnSOD on other genes. Melendez and Davies reported several years ago that MnSOD and hypoxia modulated interleukin-1α levels in HT-1080 fibrosarcoma cells by destabilizing interleukin-1α mRNA (21). Although the mechanisms that alter mRNA stability of different genes have unique features, in general, sequences located in the 3'-UTR and their interactions with specific proteins regulate mRNA stability. These motifs could mediate the stabilization or destabilization of individual mRNA depending on the specific protein factors binding to them. The binding of some protein factors to the 3'-UTR are redox-sensitive (9, 20), and the activities of these modifiers of posttranscriptionalmRNA stability could be affected either directly or indirectly by diffusible ROS such as H_2O_2, the product of MnSOD. Defining the redox-sensitive cis-element in the 3'-UTR and protein factors binding to maspin mRNA should be an interesting area to explore in the elucidation of redox regulation of maspin by MnSOD.

Perspectives

In summary, MnSOD overexpression up-regulates maspin tumor suppressor gene expression in human breast and prostate cancer cells. The effect of MnSOD on the up-regulation of maspin expression is mainly due to the posttranscriptional effect of increasing maspin mRNA stability. Moreover, the effect of MnSOD on maspin expression is independent of maspin promoter methylation status and tumor cell p53 status. As the loss of p53 function and epigenetic silencing of tumor suppressor genes are two critical determinants of gene expression and response to tumor therapy, our findings provide a rationale for the use of MnSOD as a gene therapy target to treat human breast and prostate tumors.

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ABBREVIATIONS

Ad-LacZ, recombinant adenovirus containing β-galactosidase cDNA; Ad-MnSOD, recombinant adenovirus containing MnSOD cDNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MnSOD, manganese superoxide dismutase; MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SSC, saline–sodium citrate buffer; 3'-UTR, 3'-untranslated region.

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