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MANGANESE SUPEROXIDE DISMUTASE EXPRESSION IN HUMAN CANCER CELLS: A POSSIBLE ROLE OF mRNA PROCESSING

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The level of manganese superoxide dismutase (MnSOD) as determined by an immunoreactive assay was reduced in human cancer cells. The reduced amount of immunoreactive MnSOD in these cells was observed regardless of the growth state of the cells. The decrease in the enzyme protein was associated with a decrease in the mature form of MnSOD transcript as determined by Northern Analysis. The decreased amount of the mature form of MnSOD transcript was accompanied by an increase in the amount of the partially processed form of MnSOD transcript. These results suggest that RNA processing or translation of MnSOD mRNA may be responsible for the decreased amount of MnSOD activity in human tumor cells.

KEY WORDS: MnSOD, human cancer, mRNA processing, Western blot, Northern blot.

INTRODUCTION

Superoxide dismutases (SODs) are metalloproteins which catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide.[1] This enzyme is thought to play an important role in protection of aerobic cells against oxygen toxicity.[2] Two forms of SODs are found in eukaryotic cells: a copper and zinc containing superoxide dismutase (CuZnSOD) found in the cytosol, and a manganese containing superoxide dismutase (MnSOD) found primarily in mitochondrial matrix.[3] It has been shown that tumor cells have lower MnSOD activity when compared to their normal cell counterparts.[4-10] The decrease in enzyme activity is attributable to a decreased amount of enzyme protein[9,10] and a decreased level of translatable MnSOD mRNA.[10]

To further investigate the molecular basis for the loss of MnSOD activity in human tumor cells, we performed Western and Northern analyses of MnSOD in SV40 transformed human fibroblasts (SV40-WI38) and their corresponding normal cell counterparts (WI38). The results suggest that defects in the processing of MnSOD transcripts or translation of MnSOD mRNA may be the cause for the reduced MnSOD protein observed in human cancer cell.

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MATERIALS AND METHODS

Cell Growth and Maintenance

Normal human lung fibroblasts (WI38), SV40 transformed human lung fibroblasts (WI38 VA 13 subline 2 RA) and human colon carcinoma (HT29) cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Eagle's basal medium containing 200 μmole/l glutamine, 10% FCS, 2.2 g/l sodium bicarbonate, 100 units/ml sodium penicillin and 100 μg/ml streptomycin sulfate. Cells were grown in monolayer culture in 175 cm^2 flasks (Falcon) and maintained at 37° C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 3 days. Cells were harvested in both the exponential and confluent growth phases. Confluency was defined as that time when cell contact was established throughout the flasks but before any significant piling up had occurred in the SV40 transformed cells. When samples were required under "fed" conditions, medium was changed 24 hours prior to harvest. "Unfed" cells were obtained by not changing medium during the 4 days prior to harvest.

Antibody to MnSOD

Two polyclonal antibodies were used in this study. The first antibody used was produced against mouse heart MnSOD as previously described.[11] The second antibody used was produced against human kidney MnSOD. The human kidney MnSOD was purified according to the method of Weisiger and Fridovich.[12] For this antibody, whole serum was used to perform the Western analysis.

Immunological detection of MnSOD

The MnSOD immunoreactive protein content in all cell lines was determined by a modification of a previously described Western blotting procedure.[11] Electrophoresis of cell homogenates was performed in a 12.5% polyacrylamide gel following pretreatment with sodium dodecylsulfate (SDS) and β-mercaptoethanol at 100° C for 4 minutes. The proteins from the finished gel were electrotransferred to nitrocellulose filters (BA85) or Gene Screen. The filters were washed, and then treated with polyclonal antibody against mouse heart MnSOD or human kidney MnSOD for one hour at room temperature or 24 hours at 4° C. After removal of the MnSOD antibody, the immobilized antigen-antibody complex was reacted with horseradish-peroxidase-conjugated anti-rabbit IgG for 30 minutes at room temperature. After washing, immunological active MnSOD was visualized by incubating the blot with 4-chloro-1-napthal in phosphate buffered saline (PBS) and H_2O_2.

For quantification, the density of each MnSOD band on the blots was analyzed with a BioRad Video Densitometer using a standard curve generated from serial dilution of purified MnSOD.

DNA Probes

The MnSOD probe used in this study is a full length cDNA coding for human MnSOD. The cDNA was prepared from λgt 11 human colon carcinoma (HT29) cDNA library by screening with antibody as described by Hyunh *et a.*[13] Primary

screening of 700,000 recombinants yielded 4 positive clones. An Eco RI fragment of an approximately 1000 bp insert from one of these clones was further subcloned into an M13 mp 18 vector and sequenced by the chain termination technique,[14] using Sequenase™ and the protocol provided by the supplier.[15] Analysis of the cDNA sequence confirmed that the insert encoded human MnSOD because it was virtually identical to the previously published sequence for human MnSOD from various normal human sources.[16-18]

The β-actin probe was obtained from Oncor, Inc. (Gaithersburg, MD).

RNA Isolation and analysis

Total cellular RNA was isolated by the method described by Chrigwin *et al.*[19] Cells were placed in a 4 M solution of guanidine isothiocyanate containing 0.5% sarcosine, 25 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% antifoam A and homogenized in a Dounce homogenizer. This solution (8 ml) was layered over 3 ml of 5.7 M CsCl and 1 ml of 2.4 M CsCl in a 12 ml polyallomer centrifuge tube, and the material was centrifuged at 34,000 rpm for 20 hrs at 20° C in an SW41 rotor. The pelleted RNA was resuspended in sterile water (DEPC treated to inactivate ribonuclease). The RNA was extracted with an equal volume of (4:1) chloroform: butanol and then precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol.

Northern Analysis

Poly A^+ RNA was isolated from total RNA by oligo dT cellulose chromatography as described by Aviv and Leder.[20] Samples of isolated mRNA were electrophoresed in a 1.1% formaldehyde agarose gel and transferred to a nitrocellulose filter (BA85). The RNA on the filter was then hybridized to a ^{32}P cDNA probe ($5 \times 10^5 - 1 \times 10^6$ cpm/ml) at 42° C for 24–48 hr in a solution containing 5X SSC, 50% formamide, 50 mM sodium phosphate pH 7.0, 0.2% SDS, 5 X Denhardt's solution, and 0.1 mg/ml denatured salmon sperm DNA. The probe was labelled with $\alpha^{32}P$ dCTP by the random hexamer method.[21] The filters were washed twice with 5X SSC, 0.1% SDS

TABLE I

Comparison of immunoreactive MnSOD in normal and tumor cells. Data from WI38 cells were arbitrarily given a value of 1.00 and the tumor cells were given a value relative to it. All samples were obtained when cells were in the confluent fed condition. In the confluent state, tumor cells were harvested at a time when cells contact had been established, before piling up occurred. The ratio of immunoreactive MnSOD was determined by Western blotting as previously described[9]

Cells	Immunoreactive MnSOD Ratio	
	Mouse Heart[1]	Human Kidney
WI38	1.00	1.00
SV40-WI38	.21	.29
HT29	ND[2]	.07

[1]Taken from reference
[2]Not determined

and 0.05% sodium pyrophosphate at room temperature and then twice with 0.1 X SSC, 0.1% SDS and 0.05% sodium pyrophosphate at 68°C. The filters were then exposed to Kodak XAR film at −70°C using an intensifying screen.

RESULTS

The amount of MnSOD protein in human cells as determined by an immunoreactive assay using antibodies to MnSOD is shown in Table I. Two antibody preparations were used in this study. The first antibody was raised in rabbits against purified mouse heart MnSOD as described previously.[9] The second antibody was raised in rabbits against purified human kidney MnSOD using standard procedures. Both antibodies predicted similar ratios of immunoreactive MnSOD in the SV40 transformed WI38 cells to the normal WI38 cells. Since the MnSOD cDNA used in this study was isolated from HT29 cells, we also assayed for the level of MnSOD protein in these cells. The results (Table I) show that HT29 cells have a very low level of immunoreactive MnSOD. This corresponds to the low abundance of MnSOD mRNA represented in the HT29 cDNA libraries. The difference between the antibody against mouse heart MnSOD and human kidney MnSOD is that the antibody to mouse heart MnSOD also recognized an extra band that migrates above the MnSOD band at about 45 kDaltons. As illustrated in Figure 1, this extra band serves as an internal control for the amount of protein loaded and transferred to nitrocellulose filter. Figure 1 shows that the SV40 transformed WI38 cells have less MnSOD protein compared to WI38 cells regardless of the conditions under which the cells are grown.

Northern analysis of total cellular RNA using the human tumor MnSOD cDNA probe showed that both normal and transformed WI38 cells have two classes of

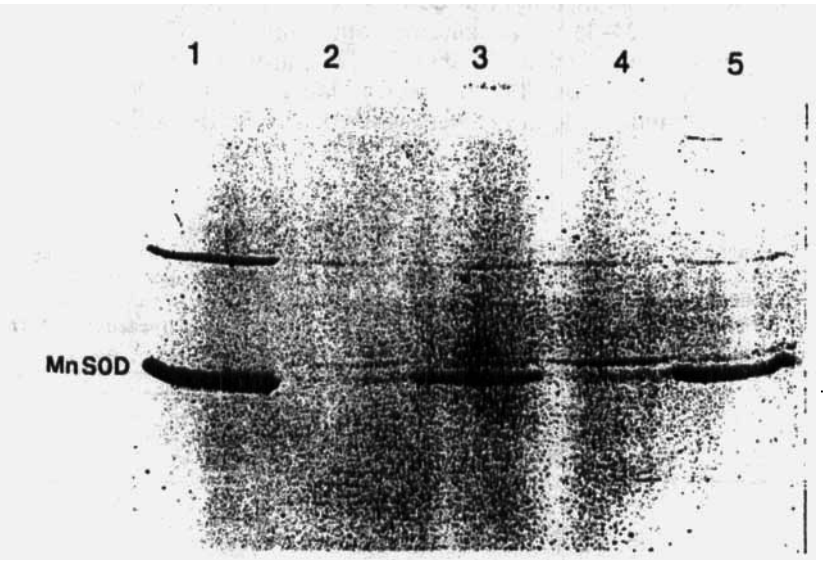

FIGURE 1 Detection of immunoreactive MnSOD. The western blot was performed using antibody to mouse heart MnSOD. Lane 1 contained 10 μg of chicken liver MnSOD. Lane 2 and 3 contained 250 μg of fed, exponentially growing SV40-WI38 and WI38 cells and lane 4 and 5 contained 250 μg of unfed, confluent SV40-WI38 and WI38 cells, respectively.

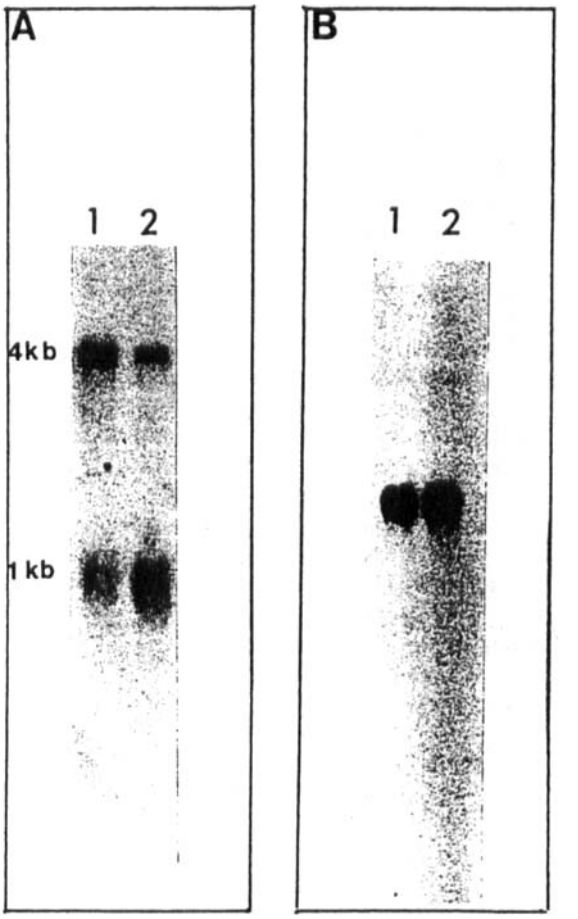

FIGURE 2A Northen analysis of human MnSOD transcripts in confluent cells. Lane 1 contained SV40 transformed WI38 cells. Lane 2 contained WI38 cells. Poly A^{P} RNA (5 μg each) was fractionated on a 1.1% formaldehyde agarose gel, transferred to nitrocellulose paper and hybridized with ^{32}P MnSOD cDNA as described in Materials and Methods.

FIGURE 2B The same filter was washed and rehybridized to a β-actin probe.

MnSOD transcripts, 1000 nt and 4000 nt (Figure 2A), that correspond to the previously reported MnSOD transcripts in human cells.[21,22] The larger transcript represents a partially processed transcript because it also hybridized to the fifth intron of the MnSOD gene.[22] The amount of the fully processed transcript is reduced in confluent SV40 transformed WI38 cells (Figure 2A, Lane 1) compared to confluent normal WI38 cells (Figure 2A, Lane 2). The decrease in the amount of the fully processed 1000 nt transcript in the SV40 transformed cells was accompanied by an increase in the amount of the partially processed 4000 nt transcript. Specific reduction of the 1000 nt transcript in the SV40 transformed WI38 cells was further verified by reprobing the RNA blot with a β-actin probe (Figure 2B) which showed similar amounts of the transcripts in both cells lines. Variability in the ratio of the fully processed to the partially processed transcripts was observed in exponentially growing cells (not shown). The cause for this variability is currently under investigation.

DISCUSSION

The results reported in this study clearly demonstrate that SV40 transformed WI38 cells have less MnSOD protein compared to their "normal" cell counterparts regardless of the growth stage of the cells. It can be argued that the MnSOD protein in the transformed cells have different determinants from those found in normal cells. Therefore, the protein could react less with the antibody. However, this is not likely because 1) the migration of the protein in the transformed cells that is recognized by MnSOD antibodies was the same as for their normal counterparts, 2) antibodies against MnSOD from two different sources gave the same result: the transformed cells have less immunoreactive MnSOD protein compared to their normal cell counterparts and 3) the amino acid sequence of the human tumor MnSOD is virtually identical to that of normal human MnSOD as predicted from the nucleotide sequence of cDNAs that encoded MnSOD from both tumor and normal sources.

The level of MnSOD protein in these cells is associated with the amount of the fully processed MnSOD (1 kb) transcripts. Thus, the reduced level of MnSOD in human tumor cells is probably due to the reduced amount of the mature mRNA for MnSOD. Since the decrease in the amount of the fully processed transcript in the transformed cells was accompanied by an increase in the amount of the partially processed transcript, it appears that the expression of the fully processed transcript is related to the expression of the partially processed transcript. The significance of 4 kb transcript remains unknown, a major question is whether this transcript is translated into protein. Several investigations, including our previous work, have demonstrated that the loss of MnSOD activity in tumor cells is due to the reduced amount of MnSOD protein. Marlhens *et al.* have demonstrated that the reduced amount of MnSOD protein in SV40 transformed human skin fibroblasts is due to a reduced amount of translatable MnSOD mRNA using *in vitro* translation technique.[10] Our data indicated that the level of MnSOD in cells is associated with the amount of mature MnSOD transcript. Taken together, all the results obtained thus far support the hypothesis that the loss of MnSOD activity in human tumor cells is due to a reduced amount of translatable MnSOD mRNA and that RNA processing or translation of MnSOD mRNA plays a role in the apparent loss of MnSOD activity in human tumor cells. However, other possibilities exist to explain the loss of MnSOD activity in tumor cells. The mRNA for MnSOD might be less stable in tumor cells than normal cells. A second possibility is that transportation of transcripts from the nuclease to the cytoplasm might be defective in tumor cells. A third possibility is that the two mRNA species observed in human cells might be transcripts from two different MnSOD genes. The levels of mRNA process from one of these genes might be lower in tumor cells. These possibilities will be the subject of future investigations.

An increase in MnSOD activity in human tumor cells has been shown to be associated with the differentiation of Friend erythroleukemia cells.[23] In addition, treatment of undifferentiated Friend erythroleukemia cells with liposome entrapped SOD caused these cells to become differentiated.[23] In addition, induction of MnSOD in tumor cells has also been assoicated with the development of resistance to killing by tumor necrosis factor (TNF) or interleukin-1 (IL-1). TNF and IL-1 have been shown to cause the induction of both MnSOD transcripts and MnSOD protein in tumor cells.[24,25] Since treatment of human tumor cells with low levels of TNF or IL-1 conferred resistance to killing by subsequent treatment of cells with TNF and cycloheximide, suggesting that TNF and IL-1 induced a protective protein, Wong and

Goeddel have suggested that MnSOD is one of the proteins involved in protecting cells from the cytotoxic effect of TNF.[24] Thus resistance to TNF may be due in part to induction of MnSOD. Asoh et al. have shown that MnSOD is induced to a much greater extent in the human breast cancer MCF-7 cell line than its TNF resistant variant.[26] Wong *et al.*[27] have demonstrated that overexpression of MnSOD caused by transfection of the MnSOD gene confers increased resistance to TNF plus cycloheximide of the 293 embryonic kidney cell and the ME-180 cervical carcinoma cell lines. Conversely, expression of antisense MnSOD renders these cells sensitive to TNF. Our finding that differential expression of the two MnSOD transcripts is associated with the level of MnSOD protein in cells may provide a clue to the development of specific measures to modulate cellular MnSOD activity. This approach may be useful in preventing the emergence of resistance to cancer treatment or in promoting development of differentiation in cancer cells.

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