Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells

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ABSTRACT Introduction of a normal human chromosome 6 into human melanoma cell lines results in suppression of tumorigenicity. This suggests that a gene(s) on chromosome 6 controls the malignant phenotype of human melanoma. Because antioxidants can suppress the tumor-promotion-phase of carcinogenesis, and because the antioxidant enzyme manganese superoxide dismutase (MnSOD) has been localized to a region of chromosome 6 frequently lost in melanomas, we have examined the effect of introducing sense and antisense human MnSOD cDNAs into melanoma cell lines. Cell lines expressing abundant (+)-sense MnSOD-5 cDNAs significantly altered their phenotype in culture and lost their ability to form colonies in soft agar and tumors in nude mice. In contrast, the introduction of antisense MnSOD cDNA or +psvneo had no effect on melanoma tumorigenicity. These findings indicate that stable transfection of MnSOD cDNA into melanoma cell lines exerts a biological effect that mimics that observed after introduction of an entire human chromosome 6.

Evidence that tumor cells fail to express genes negatively regulating malignancy continues to accumulate, with several candidate tumor suppressor genes identified (1–8). Direct support for the role of tumor suppressor genes in malignancy has come from studies introducing single human chromosomes by microcell hybridization (8). In human melanoma cell lines, introduction of a human chromosome 6 by microcell hybridization resulted in suppression of the transformed phenotype (9), suggesting that a gene(s) on chromosome 6 has a role in the tumorigenic expression of melanoma.

Reactive oxygen species may play a role in tumor promotion and in regulation of cell division (10–12). Reactive oxygen species-generating compounds promote skin tumors in mice, whereas antioxidants, which prevent or act as terminators of reactive oxygen species chain reactions, antagonize this process (13). Included in the family of antioxidant enzymes are superoxide dismutase enzymes (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) that catalyze the conversion of O2− to H2O2 plus O2 (14). One of the antioxidant SOD enzymes commonly decreased and not generally inducible in tumor cells is manganese SOD (MnSOD) (15–18). MnSOD is found predominantly in the mitochondria of eukaryotes (14). Like most mitochondrial proteins, MnSOD is encoded by a nuclear gene, synthesized in the cytosol as a larger precursor with an N-terminal transit peptide, and subsequently imported into the mitochondria with proteolytic processing to the mature form (19). The human MnSOD gene has recently been localized to chromosome 6q25 (20), a region of chromosome 6 frequently lost in malignant melanoma (21). The human MnSOD gene product is alternatively spliced and polyadenylated to give rise to three small (1 kb) and one large (4.2 kb) MnSOD mRNAs (22). MnSOD is induced by mediators of oxidant stress, including tumor necrosis factor, interleukin 1, and lipopolysaccharide (23). MnSOD is also induced during normal cellular differentiation (24, 25).

To determine whether MnSOD can modulate the tumorigenicity of melanoma, we examined its expression in parental and human chromosome 6-containing (+6) microcell hybrids of the human UACC-903 melanoma cell line (9). MnSOD expression in the parental UACC-903 cell line was modulated by using expression vectors containing MnSOD cDNAs. Our results demonstrate that increased MnSOD gene expression modifies the transformed phenotype of melanoma cells, suggesting that MnSOD may be important in melanoma tumorigenesis.

MATERIALS AND METHODS

Reagents. EcoRI-digested plasmid SFFV-neo Bluescript (26) was provided by Dennis Loh (Washington University). The simian virus 40 promoter-driven neomycin gene expression vector (psvneo) was the gift of Nadia Rosenthal (Boston University). The MnSOD cDNA-5 is designated arbitrarily to correspond to the original reference (22). Following the stop codon and 18 nt of shared 3' untranslated sequence, MnSOD-5 is alternatively spliced and polyadenylated at the terminal MnSOD AATAAA polyadenylation signal. The MnSOD and copper/zinc SOD (Cu/ZnSOD) antibodies have been characterized (27). Cu/ZnSOD cDNA was obtained from the American Type Culture Collection. Mitochondrial malate dehydrogenase (MMDH) cDNA and rabbit antihuman MMDH polyclonal antibodies were provided by Arnie Strauss (Washington University). Detailed characterization of parental UACC-903 and +6 microcell hybrids has been reported (9). Newly transfected UACC-903 melanoma cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, l-glutamine, penicillin, streptomycin (9), and 500 μg of geneticin (Sigma). MnSOD expression studies were performed with exponentially growing cells (data not shown) and as cells reached confluence (Fig. 1).

Creation of MnSOD-Expressing Melanoma Cell Lines. Full-length MnSOD-5 cDNA was ligated into EcoRI-digested SFFV-neo Bluescript in the sense and antisense orientations. The psvneo or MnSOD cDNA expression vectors were introduced into parental UACC-903 cells by calcium phosphate coprecipitation (28), and stable cell lines were selected in G418-containing medium.

Abbreviations: SOD, superoxide dismutase; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc SOD; MMDH, mitochondrial malate dehydrogenase; PCNA, proliferating cell nuclear antigen.

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Fig. 1. MnSOD and Cu/ZnSOD gene expression in near-confluent +6 microcell hybrids and stable transfectants of the human melanoma cell line UACC-903. (A) RNA blot analysis of MnSOD and Cu/ZnSOD. Total cellular RNAs (15 μg) were hybridized with a MnSOD cDNA probe (i), a MnSOD complementary RNA probe (ii), and a human Cu/ZnSOD cDNA probe (iii). Solid arrowheads indicate endogenous MnSOD RNAs. Open arrowheads indicate transfected MnSOD RNAs. Positions of 28S and 18S rRNAs are indicated. (B) Western blot analysis. Whole cell extracts (20 μg) of near-confluent cells subjected to SDS/PAGE, transferred to nitrocellulose, and allowed to react with anti-MnSOD antiserum (27). Antigen–antibody complexes were detected with goat anti-rabbit IgG antibody conjugated with alkaline phosphatase. Lanes: 1, parental UACC-903; 2, MCH-361CL; 3, +psv-neo; 4, (+)-sense MnSOD-5-1; 5, (+)-sense MnSOD-5-3; 6, (+)-sense MnSOD-5-5; 7, (+)-antisense MnSOD-5-2.

MnSOD Expression in Stable Transfectants and in +6 Microcell Hybrids. Detection of MnSOD sequences, SFFV-neo Bluescript-specific sequences, and Cu/ZnSOD sequences were accomplished with specific 32P-labeled probes as described (24). Genomic Southern blots (29) were performed to estimate copy number of MnSOD expression vectors. Autoradiograms of the SFFV-neo Bluescript versus the Cu/ZnSOD hybridization signals in 10 μg of genomic DNA on Southern blots were compared following densitometric scanning. The relative plasmid copy number was confirmed by quantitative PCR (30). The 5' exon 4 (5'-GGCTGCACTCTTTGGTGTC-3') and 3' exon 5 (5'-CTCCCCAGTTGATACATT-3') PCR oligonucleotide primers spanned intron 4 of the human MnSOD gene (S.L.C. and J.W.G., unpublished data). PCRs using human genomic DNA isolated from placenta, UACC-903, +psv-neo, and UACC-903 +6 microcell hybrids or a genomic subclone containing exons 4 and 5 as templates yielded a 2.4-kb MnSOD genomic fragment. PCRs using human MnSOD cDNA as a template yielded a 224-nt product. PCRs using RNase-treated genomic DNA from (+)-sense or (+)-antisense MnSOD UACC-903 human melanoma cell lines yielded fragments of both 2.4 kb and 224 nt. MnSOD copy number was estimated for each transfected cell line using the ratio of the 224-nt MnSOD cDNA signal and the 2.4-kb genomic signal (which corresponds to two copies of the MnSOD gene in genomic DNA). MnSOD copy number estimates by Southern blots and PCR were similar (Table 1).

Table 1. Properties of melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Copy no. of MnSOD cDNAs</th>
<th>SOD activity</th>
<th>Transformed phenotype</th>
<th>HMB expression</th>
<th>% anti-PCNA</th>
<th>Soft agar cloning efficiency, %</th>
<th>No. of tumors/ injection sites (day 10)</th>
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<tr>
<td>Parental</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>UACC-903</td>
<td>0</td>
<td>80</td>
<td>15</td>
<td>65</td>
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<td>+ + +</td>
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<td>+6 microcell</td>
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<tr>
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<td>14</td>
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SOD activity is expressed as units per mg of protein. Homogenates of UACC-903 cell lines as they reached confluence were assayed by the xanthine oxidase nitroblue tetrazolium assay for SOD activity (31). Enzyme activities are an average of three (+)-sense MnSOD-5-5 or two (+)-sense MnSOD-5-2 separate samples. Reproducibility of the SOD enzyme assay is 10–20% on a single sample (L.W.O., unpublished data). Soft agar cloning efficiency is reported as percentage ± SD. pop, Population; ND, not done.
ined both by phase-contrast microscopy and by light microscopy following methanol fixation (24) and staining with Harris' hematoxylin.

Soft agar cloning efficiency was calculated as described (33) by suspending 1 x 10^4 cells in 0.33% soft agar in RPMI 1640 medium and counting colonies of >50 μm at 21 days.

The monoclonal antibody HMB-45, which is specific for melanocytic tumors (33), was used to evaluate the immunocytochemistry of melanoma cell lines. Also, the proliferating cell nuclear antigen (PCNA) antibody (DAKO, Carpinteria, CA) diluted 1:40 or anti-PCNA (PC10; DAKO) diluted 1:30 and then incubated with biotinyl horse anti-mouse IgG (Vector Laboratories). Interpretation of immunocytochemical staining of all antibodies was performed by a single blinded observer. Reactivity to HMB-45 was defined as positive >30% of cells were unequivocally stained (+ + +), intermediate [5-30% of cells were unequivocally stained (+ +)], and negative [0-4% of tumor cells were positive (− −)]. The percentage of melanoma cell nuclei staining positively with anti-PCNA was determined by analysis of at least 500 nuclei.

After injection of parental UACC-903 melanoma cells into athymic (nu/nu) mice, palpable tumors formed in 100% of animals within 5-7 days. Growth of MnSOD-expressing cell lines in athymic (nu/nu) mice was evaluated for up to 120 days after injection of tumor cells (5 x 10^6 or 1 x 10^6) (Table 1).

Confocal Microscopy. Human melanoma cell lines were prepared for staining as described (36). Primary rabbit antihuman MnSOD or MMDH antibody diluted 1:200 was used with fluorescein-conjugated goat anti-rabbit IgG (Sigma) at 1:100 dilution. Cells were mounted in 50% (vol/vol) glycerol/0.1% (wt/vol) n-propyl gallate (Sigma) and photographed using a Lasersharp MRC500 scanning confocal microscopy system (Bio-Rad).

RESULTS

Description of Cell Lines Examined. We established multiple clonal cell lines to determine whether differences in growth characteristics might depend on differences in MnSOD expression in the transfected lines. All experiments compared our transfected variants of the UACC-903 cell line to the parental line (which expressed the melanoma phenotype) and to a +psv2neo UACC-903 microcell hybrid (which expressed the melanoma phenotype) (9). In addition, a control UACC-903 cell line was established expressing psv2neo to detect any effect of growth in G418-containing medium on MnSOD gene expression. After selection, 14 independent clones and two populations of G418-resistant cell lines were isolated and analyzed for functional MnSOD expression (by RNA blot and SOD activity analyses) and growth characteristics. Two cell lines were clearly different morphologically, containing abundant steady-state MnSOD RNAs (Fig. 1) and the highest MnSOD enzyme activities (Table 1). Two cell lines expressing abundant antisense MnSOD mRNA, 2 cell lines expressing intermediate steady-state MnSOD mRNAs and MnSOD enzyme activity, and 10 cell lines expressing low steady-state MnSOD mRNAs and MnSOD enzyme activities were indistinguishable phenotypically from the psv2neo or UACC-903 parental controls (Table 1). Complete analyses were performed on clonally derived cell lines with different copy numbers of the (+)-sense MnSOD-5 cDNA (sense MnSOD-5-1, sense MnSOD-5-3, and sense MnSOD-5-5). These were compared to a clonal cell line expressing high levels of antisense MnSOD-5 mRNA, the parental UACC-903, +6 microcell hybrids, and the psv2neo cell line.

MnSOD Expression in Parental UACC-903 Human Melanoma Cells, +6 Microcell Hybrids, and in Stable MnSOD Transfectants. RNA blot analyses using a double-stranded cDNA probe (Fig. 1A, i) or a single-stranded complementary RNA probe (Fig. 1A, ii) demonstrate that new sense MnSOD mRNAs are expressed in sense MnSOD-5-1, -5-3, and -5-5 cell lines and new antisense MnSOD mRNA is expressed in the antisense MnSOD-5-2 cell line. The new MnSOD mRNA products of the MnSOD expression vectors are all larger than the 1-kb MnSOD mRNAs, possibly by addition of the 900 nt of the simian virus 40 polyadenylation signal present in the expression vector. Increased expression of the transfected sense MnSOD mRNAs correlates with increased expression of the endogenous MnSOD gene (Fig. 1, solid arrowheads). The abundance of the transfected sense MnSOD-5 RNAs correlates approximately to the transfected plasmid copy number (Table 1). Increases in steady-state MnSOD mRNAs correspond to increased MnSOD enzyme expression and MnSOD protein abundance by Western blot analyses (Fig. 1). Neither Cu/ZnSOD (Fig. 1A, iii) nor MDMH (data not shown) gene expression is altered in any melanoma cell line. Cu/ZnSOD activity measured in the sense MnSOD-expressing melanoma cell lines is generally decreased and is increased in the antisense MnSOD-expressing cell line (Table 1). Immunoreactive Cu/ZnSOD protein content in selected cell lines (data not shown) is unchanged.

Comparison of MnSOD Expression and Growth Characteristics of Human Melanoma Cell Lines. RNA blot analyses demonstrate that confluent parental, +psv2neo, and antisense MnSOD cell lines have little steady-state MnSOD expression of any sense MnSOD RNA species. These cell lines also have low levels of MnSOD enzyme activity (Table 1) or protein detected by Western blot analyses (Fig. 1B). The +psv2neo and antisense MnSOD-5-expressing cells phenotypically resembled parental melanoma cells (9). All of these cell lines grew quickly to confluence and formed rounded grape-like cell clusters even at low plating density (Fig. 2A). Immunohistochemical staining with HMB-45 (34) labeled most of the parental, +psv2neo, and antisense MnSOD-5 cells uniformly positive. Anti-PCNA (35, 36) staining of the

![Fig. 2. Micrographs of hematoxylin-stained monolayer cultures of the +psv2neo (A) and (+)-sense MnSOD-5-3 (B) stable transfectants of the human melanoma UACC-903 cell lines. (A) The +psv2neo cells were small and rounded and formed clusters of cells even at low plating density. (x40.) (B) In contrast, the (+)-sense MnSOD-5-3 cells displayed distinct nuclei and nucleoli, a more differentiated appearance with a flattened stellate morphology, and a higher cytoplasmic/nuclear ratio, and they failed to form foci even at high plating density. (x20.)](image-url)
+psv-neo and antisense MnSOD-5 cell lines were similar to the parental UACC-903 melanoma cells. The +psv-neo and antisense MnSOD-5 cell lines were able to form colonies in soft agar and palpable tumors in 100% of injected animals within 5-7 days (Table 1).

In contrast, RNA blot analyses demonstrate that +6, (+)-sense MnSOD-5-1, and (+)-sense MnSOD-5-3 cell lines express increased amounts of steady-state MnSOD mRNAs (Fig. 1A). These cell lines also have increased MnSOD enzyme activity and MnSOD protein content (Fig. 1B), which is coincident with a dramatic change in cellular morphology (Fig. 2B). These cell lines also had distinct nuclei and nucleoli, appeared more differentiated (with a higher cytoplasmic to nuclear ratio), and failed to form cell foci even at high cell density or to form colonies in agar. HMB-45 staining was also markedly reduced. A significant decrease in staining with anti-PCNA was found in the (+)-sense MnSOD-5-1 and -5-3 cell lines compared to the +psv-neo, or (+)-antisense MnSOD-5 cell lines (40-55% compared to 70%, respectively). Finally, animals injected with the sense MnSOD-5-1 or -5-3 cell lines failed to develop tumors, even 120 days after injection (Table 1).

The sense MnSOD-5-5 cell line had a much smaller increase in steady-state MnSOD mRNAs, protein abundance (Fig. 1), and MnSOD enzyme activity (Table 1). This low (+)-sense MnSOD-5-5-expressing cell line had moderately reduced HMB-45 and anti-PCNA staining and decreased soft agar cloning efficiency (Table 1). In contrast to the high MnSOD-expressing lines, 100% of the animals injected with (+)-sense MnSOD-5-5 developed palpable tumors within 5-7 days (Table 1).

Taken together, the altered cell morphology, decreased melanoma-associated antigen expression, decreased anti-PCNA staining, and reduced agar clonogenicity suggest that increased expression of MnSOD-5 leads to suppression of the in vitro transformed phenotype and to in vivo suppression of tumorigenicity in these human melanoma cells.

Confocal Microscopy. Because mitochondria in tumor cells can be different from mitochondria in normal cells (37-39), we examined the effect of altered MnSOD gene expression on the fluorescence localization of MnSOD and MMDH in melanoma cell lines. All cells had a punctate distribution of MnSOD staining, suggesting that the expressed MnSOD protein is localized to an organelle. Immunofluorescence staining patterns of MnSOD and MMDH antibodies in the parental, +psv-neo, and (+)-antisense MnSOD-5 cell lines were similar. In the cell lines that express little MnSOD, MnSOD immunostaining was poor and most MnSOD and MMDH staining was perinuclear (Fig. 3 a and b). Immunofluorescence staining patterns of MnSOD and MMDH were similar in the (+)-sense MnSOD-5-1, (+)-sense MnSOD-5-3, and +6 cell lines (Fig. 3 c-e), suggesting that MnSOD staining is coincident with the distribution of mitochondria. The +6, (+)-sense MnSOD-5-1 and (+)-sense MnSOD-5-3 cell lines, which increase MnSOD expression, had intense punctate MnSOD or MMDH staining that extended into the cytoplasm (Fig. 3 c and e).

This cytoplasmic extension and increased staining by two mitochondria-located antibodies in the +6, (+)-sense MnSOD-5-1 and (+)-sense MnSOD-5-3 cell lines suggest that increased MnSOD expression may alter mitochondrial intracellular location or number. Electron microscopy will be required to confirm either of these possibilities.

DISCUSSION

Our experiments demonstrate clear differences in cellular morphology, growth, and tumorigenicity that correlate with increased MnSOD gene expression in UACC-903 human melanoma cells. Sense MnSOD-5-1 and -5-3-expressing cell lines closely resemble +6 microcell hybrids in their low level of clonogenicity in soft agar, lack of staining with HMB-45, inability to form tumors in nude mice, and mitochondrial staining patterns.

Our results suggest further that MnSOD gene expression alters either mitochondrial intracellular location or mitochondrial number. Many studies have suggested a role for altered mitochondrial number, size, shape, distribution (37), membrane potential, and function (38–40) in neoplastic transformation. The protooncogene bcl2, which interferes with programmed cell death, is a mitochondrial membrane protein (36). A recently described fos transformation effector gene (fte-1), which cooperates with fos to produce a transformed phenotype, is highly homologous to a yeast protein involved in mitochondrial protein importation (41). The results presented in this study suggest that MnSOD gene expression may have effects on both the growth of melanoma cells and their mitochondria.

Oberley et al. (17) postulated that decreased SOD activity plays a causal role in malignant transformation and that experimental increases in SOD activity should induce differentiation in tumor cells. Differentiation of a mutant nondifferentiating strain of Physarum (42) and of human Friend erythroleukemia cells (43) by the experimental addition of exogenous liposomal Cu/ZnSOD has supported this hypothesis. Our results suggest that an increase in MnSOD gene expression can induce reversion of the transformed phenotype of human melanoma cells.
The sense MnSOD-5-5 melanoma cell line, which has only a small increase in MnSOD gene expression, MnSOD enzyme activity (Fig. 1), and is only moderately phenotypically differentiated (Table 1) suggests that there may be a minimal required dose of MnSOD-5 to suppress the malignant melanoma phenotype. A remarkable effect of overexpression of MnSOD-5 is increased expression of the endogenous MnSOD gene, which may contribute to the increment in MnSOD activity. Our data support the notion that quantitatively similar increases in MnSOD gene expression, MnSOD enzyme activity, and changes in melanoma growth characteristics requiring multiple copies of sense MnSOD-5 are achieved by addition of a single extra copy of chromosome 6. These data clearly do not exclude the possibility of additional tumor suppressor genes on chromosome 6 playing a direct role in reversion of melanoma tumorigenesis. It is striking that introduction of MnSOD resulted in an effect that mimicked the introduction of an entire chromosome 6.

Clarification of the regulatory mechanisms perturbed by MnSOD-5 overexpression will be important in elucidating the mechanism of observed effects of MnSOD-5 on the transformed phenotype. In this regard, the impact of MnSOD on superoxide metabolism is critical. Of interest, Schreck et al. (44) have recently proposed that superoxide may function as a second messenger capable of modifying the activity of transcription factors such as NF-κB. Alternatively, regulatory signals in the MnSOD mRNAs may modulate gene expression controlling the melanoma phenotype. These mechanisms may not be mutually exclusive and additional experiments will be necessary to discriminate between these alternatives.

Finally, our previous studies introducing chromosome 6 into melanoma cells have suggested that this chromosome may contain one or more genes with tumor suppressor function. Studies are needed to determine whether alterations in the coding or regulatory sequences of MnSOD occur in biopsy specimens or established cell lines from human malignant melanoma. Although the present results will require corroboration, they nevertheless suggest that the role of MnSOD in melanoma tumorigenesis deserves further evaluation.

Note Added in Proof. Nine additional cell lines expressing low steady-state MnSOD mRNAs and MnSOD enzyme activities (8–23 units per mg of protein) were also indistinguishable phenotypically from the UACC-903 parental controls.

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