ANTIOXIDANTS & REDOX SIGNALING
Volume 3, Number 3, 2001
Mary Ann Liebert, Inc.

Forum Original Research Communications

Overexpression of MnSOD Protects Murine Fibrosarcoma Cells (FSa-II) from Apoptosis and Promotes a Differentiation Program upon Treatment with 5-Azacytidine: Involvement of MAPK and NFκB Pathways

YUNFENG ZHAO, KELLEY K. KININGHAM, SHU-MEI LIN, and DARET K. ST. CLAIR

ABSTRACT

Stable transfection of neomycin and human manganese superoxide dismutase (MnSOD2) expression plasmids into a murine fibrosarcoma cell line (FSa-II) was previously done in our laboratory. Treatment with 10 μM 5-azacytidine induced apoptosis in the control cell line (NEO), whereas the MnSOD-overexpressing cell line (SOD-H) demonstrated differentiated-appearing morphology. The levels of the myogenic transcription factor, MyoD, and the muscle-specific marker, α-actin, were increased over time with 5-azacytidine treatment in the SOD-H cell line. Nuclear transcription factor NFκB was activated in the SOD-H cell line, whereas inhibition of NFκB activation reduced the levels of MyoD and α-actin. Members of mitogen-activated protein kinase pathway and the Raf1/MEK/ERK cascade were shown to play a positive role in this event. Overexpression of MnSOD not only can protect cells from the toxic effects of 5-azacytidine, but can also promote the fibrosarcoma cells to enter a differentiation program. Antioxid. Redox Signal. 3, 375–386.

INTRODUCTION

MANGANESE SUPEROXIDE DISMUTASE (MnSOD) is an essential antioxidant enzyme that protects mitochondria from oxidative injury resulting from aerobic respiration. It has been suggested that MnSOD may be a candidate tumor suppressor gene because abnormal MnSOD activity has been found in numerous cancer cell lines *in vitro* and *in vivo* (26, 35, 39). We have previously reported that mutations in the human MnSOD promotor region may cause the reduced expression of MnSOD in some tumors (46). Our laboratory has established a human MnSOD cDNA transfected murine fibrosarcoma cell line, FSa-II, which itself exhibits low endogenous levels of MnSOD. A clone transfected with the pSV2-neomycin (NEO) plasmid was selected as control. Overexpression of MnSOD increased the fibronectin levels and reduced tumorigenicity and metastatic capability in the FSa-II cells expressing a high level of the human MnSOD gene (SOD-H) (32, 37). We have also investigated the levels of several redox-sensitive oncogenes, and found that overexpression of MnSOD selectively reduced the binding activity of Jun-associated transcription factors (activator protein-1 and cyclic AMP-response element binding protein) when compared with the NEO cell

Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536.

line (18). These results suggest that MnSOD may alter tumorigenicity by modulating the activity of selected oncoproteins.

DNA methylation in mammals plays a crucial role in development, epigenetic silencing, aging, carcinogenesis, and certain human genetic diseases (2, 14, 20). The methylation of CpG dinucleotides can negatively regulate the transcription of the adjacent genes and can, for an example, silence a number of tumor suppressor genes (12, 27). 5-Azacytidine (5-AzaC) and 5-aza-2′-deoxycytidine, as methylase inhibitors, exert antineoplastic activity and have been used in the clinic (13, 24). 5-AzaC is also known to promote differentiation. Davis *et al*. (5) found that 5-AzaC treatment of mouse C3H10T1/2 embryonic fibroblasts could convert them to myoblasts, and they subsequently identified MyoD, the first myogenic transcription factor to be cloned, as the myogenic regulator. In studies on antineoplastic activity of 5-AzaC, both apoptotic and differentiation effects were found in several systems, but the mechanisms underlying these events have not been determined (24, 40). Previously, we found that transfection of MnSOD can promote cellular differentiation of the mouse embryonic fibroblast, C3H10T1/2 cell line, following 5-AzaC treatment (36). This result suggests that elevation of the MnSOD level in cancer cells may promote the possibility of differentiation therapy with 5-AzaC treatment. When we treated the FSa-II NEO and SOD-H cells with 5-AzaC, we found that overexpression of MnSOD showed both antiapoptotic and differentiation-promotion effects. The possible signaling events involved in apoptosis and myogenic differentiation were investigated.

MATERIALS AND METHODS

Cell culture

A murine fibrosarcoma cell line (FSa-II) was stably transfected with a pSV2-NEO plasmid or a human MnSOD cDNA plasmid (SOD-H) as previously described (32, 42). On the basis of the plating efficiencies, 3×10^5 cells for the SOD-H cell line and 5×10^5 cells for the NEO cell line were seeded in 100-mm dishes or 75-cm^2 flasks in McCoy's 5A medium. After 72 h, 10 μM 5-AzaC (Sigma, St. Louis, MO, U.S.A.) was added with or without MG-132 (Sigma), a proteasome inhibitor dissolved in phosphate-buffered saline (PBS). To verify the role of extracellular signal-regulated protein kinase (ERK) kinase, the ERK kinase inhibitor, PD98059 (Calbiochem, La Jolla, CA, U.S.A.), was dissolved in dimethyl sulfoxide to a concentration of 20 mM and added to the culture medium to a final concentration of either 10 or 50 μM.

Colorimetric MTT assay

Five thousand NEO cells or 3,000 SOD-H cells were seeded onto a 96-well plate in 100 μl of medium. After a 48-h incubation, 20 μM 5-AzaC was added with fresh medium. Cells were incubated for another 24 h, then washed twice with PBS. MTT dye [10 μl; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] dissolved in PBS was added to the cells and incubated for 6 h. Isopropyl mix (100 μl; 0.1 M HCl in 2-propanol) was added, and the plate was shaken at room temperature for 30 min. Colorimetry was performed on a Spectra Max Plus ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 615 nm. The absorbance of the untreated cells was set up as 100% of survival standard.

Detection of apoptosis with flow cytometry

After treatment with 5-AzaC for 12 or 24 h, the medium in the cells was aspirated, and the attached cells were removed with cell dissociation solution (Sigma) and then passed through a nylon screen cloth (Small Parts Inc., Miami Lakes, FL, U.S.A.) to obtain a single cell suspension. Combined Hoechst 33342 (Molecular Probes, Eugene, OR, U.S.A.) and merocyanine 540 (MC 540; Molecular Probes) staining was used to detect apoptosis (28).

DNA fragmentation

Cells were plated in 100-mm dishes and treated with 0–10 μM 5-AzaC for 24 h. DNA was extracted following the protocol as previously described (19).

Caspase-3 activity

5-AzaC-treated cells were collected and washed twice with PBS. The number of cells was counted using a hemacytometer. Each sample (2×10^6 cells) was analyzed for caspase-3 protease activity by the ApoAlert Caspase-3 Assay Kit (Clontech, Palo Alto, CA, U.S.A.).

Preparation of cytoplasmic and nuclear fractions and total cell lysate

FSa-II NEO and SOD-H cells were plated in 100-mm dishes and, following treatment, were collected and washed with PBS twice. The pellets were resuspended in 400 μl of Buffer A [10 m*M* HEPES-KOH with 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 μ*M* dithiothreitol (DTT), and protease inhibitors, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], kept on ice for 15 min, and then 12.5 μl of 10% NP-40 was added and vortex-mixed vigorously for 15 s. Lysate was centrifuged at 12,000 rpm (17,500 *g*) for 30 s. The supernatant was removed as the cytoplasmic fraction, and the pellet was dissolved in 100 μl of buffer B [20 m*M* HEPES-KOH with 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 35% glycerol, 0.2 m*M* PMSF, 5 μ*M* DTT, 0.2 m*M* EDTA (pH 8.0) containing the protease inhibitors, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin]. The sample was kept on ice for 20 min, centrifuged at 12,000 rpm (17,500 *g*) for 2 min, and the supernatant frozen at −80°C. For total cell lysis, cell pellets were suspended in homogenization buffer (20 m*M* HEPES, pH 7.4 with 5 m*M* EGTA, 10 m*M* BME, 1 m*M* PMSF, and protease inhibitors, pepstatin, leupeptin, and aprotinin) and homogenized with 10 up-and-down strokes using a 10-ml pestle. The unlysed cells and nuclei were separated by centrifugation at 500 rpm (50 *g*) for 3 min, and the supernatant (total cell lysate) was kept at −80°C. Protein concentration was measured by a colorimetric assay using bovine serum albumin as the standard (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Western-blot analysis

Nuclear extracts (30 μg) were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel at 65 V for 14 h, and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). Proteins transferred onto the membrane were verified by Ponceau staining. The membrane was washed with distilled water to remove the excess stain and blocked with Blotto [5% milk, 10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.05% Tween 20] for 2 h at room temperature. Membranes were probed with anti-MyoD (M-318, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a 1:200 dilution. To detect the protein level of α-actin, a monoclonal anti-α-actin (Sigma, A-4700) antibody (1:200 dilution) was used. For examining phosphorylated ERK (p-ERK), 20 μg of the cytoplasmic fraction was loaded and a monoclonal anti-p-ERK antibody (E-4, Santa Cruz) was used at a 1:200 dilution. After visualization of protein bands, the membrane was stripped and reprobed with an anti-ERK antibody (K-23, Santa Cruz) at a 1:1,000 dilution. To detect the protein level of Raf1, 50 μg of the cytoplasmic fraction was loaded, and goat polyclonal IgG against Raf1 (C-12, Santa Cruz) was used at 1:200 dilution. Before addition of the secondary antibody, the membranes were washed twice with TBST (10 m*M* Tris-HCl with 150 m*M* NaCl and 0.05% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) at a 1:4,000 dilution. The final washing steps included three rinses (5 min each) of TBST, followed by two rinses (5 min each) of TBS (10 m*M* Tris-HCl with 150 m*M* NaCl). The antibody bands were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Electrophoretic mobility shift assays (EMSAs)

Nuclear factor-κB (NFκB) DNA binding activity was analyzed in nuclear extracts. The consensus NFκB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was purchased from Promega (Madison, WI, U.S.A.). After labeling with γ-^{32}P, the probe was purified by separating the labeled double-strand and single-strand DNA on a 20% polyacrylamide native gel. A 20-μl reaction solution containing 5 μg of nuclear extract, 4 μl of 5× binding buffer

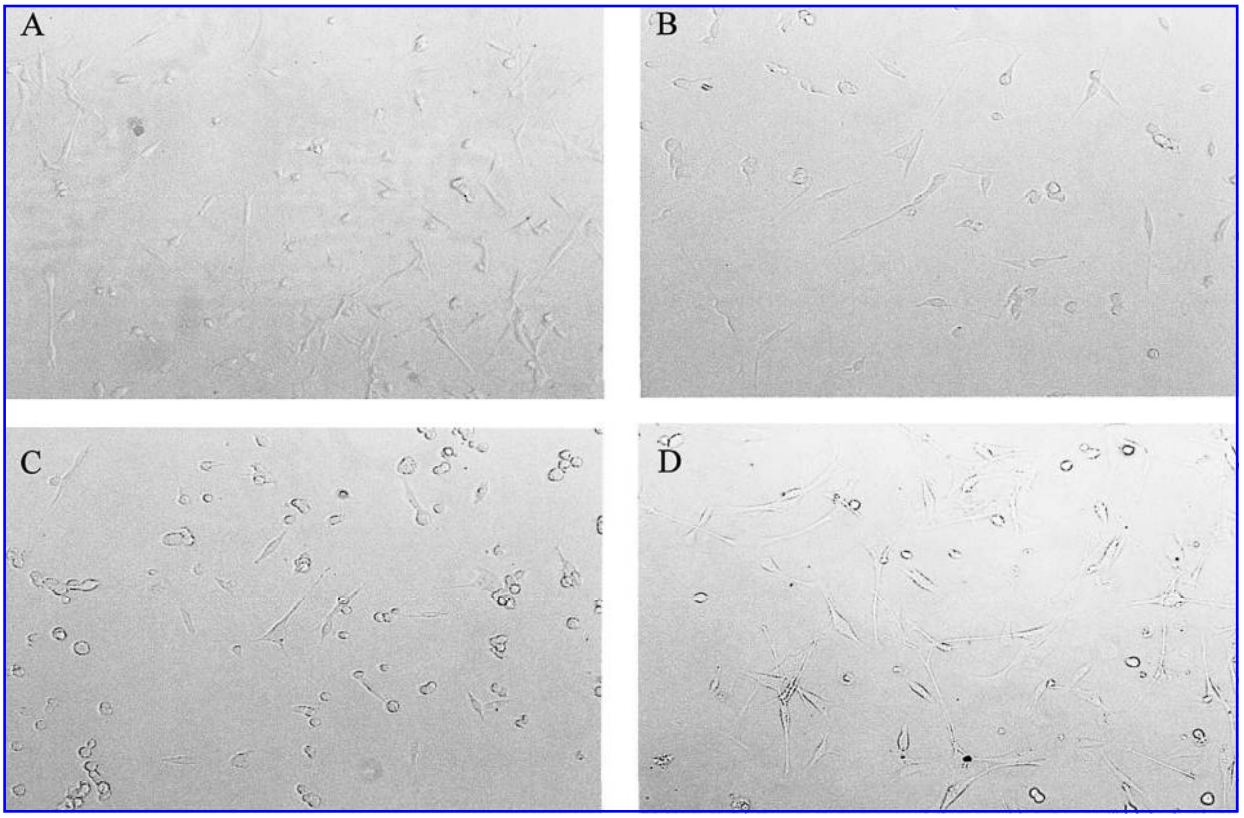

FIG. 1. Morphology of FSa-II NEO and SOD cells treated with 10 μM 5-AzaC for 24 h. (**A**) NEO control cells, (**B**) SOD-H control cells, (**C**) NEO treated cells, (**D**) SOD-H treated cells. Magnification: 10×.

[50 m*M* Tris-HCl, pH 7.4 with 250 m*M* NaCl, 20% glycerol, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 5 m*M* DTT, and 0.25 mg/ml poly(dI-dC)], and 50,000 cpm of labeled probe was incubated for 20 min at room temperature. After incubation, 2.5 μl of 10× loading buffer was added, and samples were separated on a 6% native gel for 3–4 h. DNA–protein complexes were visualized by exposing the gels to Kodak film. For supershift, 2.5 μg of polyclonal rabbit anti-NFκB p65 (sc-372x, Santa Cruz) or anti-NFκB p50 (sc-1190x, Santa Cruz) was mixed with nuclear extracts for 1 h at room temperature before addition of labeled probe.

RESULTS

5-AzaC treatment induced apoptosis in the FSa-II NEO cell line; the FSa-II SOD-H cell line was resistant to the apoptotic effect and exhibited a differentiated-like morphology

FSa-II NEO and SOD-H cells were incubated with 10 μM 5-AzaC. After 12 h, many of the NEO cells had detached, in contrast to only a small number of the SOD-H cells, and some of the attached SOD-H cells exhibited an elongated morphology. After 24 h, a large number

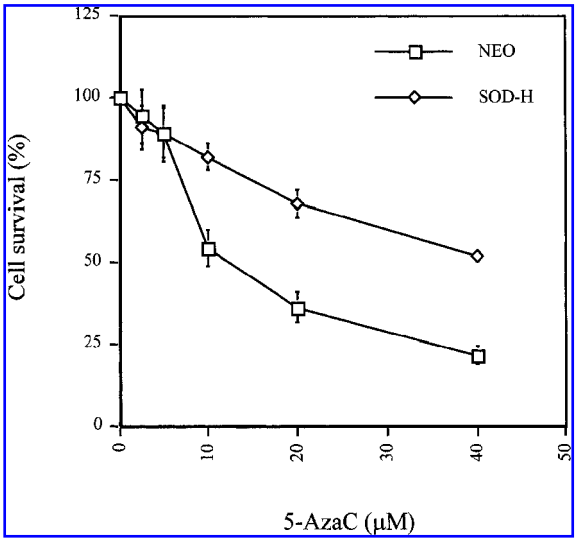

FIG. 2. 5-AzaC-induced cytotoxicity determined by MTT staining of FSa-II NEO and SOD cells after 20 μM 5-AzaC treatment.

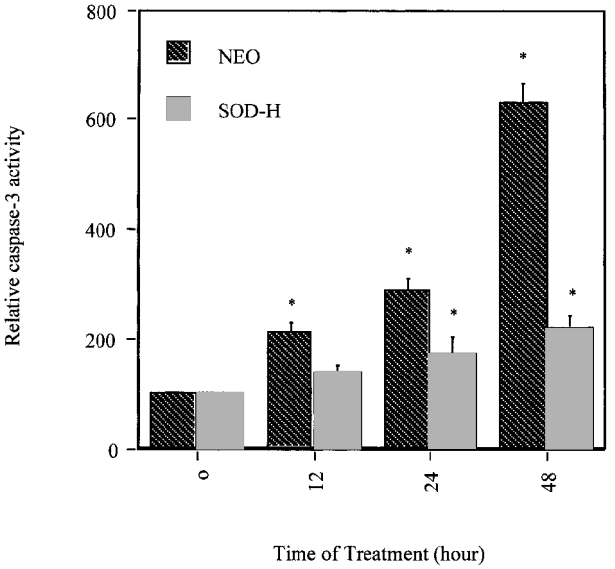

FIG. 3. Caspase-3 activity in 5-AzaC-treated FSa-II NEO and SOD cells. *Significant difference from the vehicle-treated control group ($p < 0.01$) by using Student's *t*-test.

of the NEO cells detached (Fig. 1C), whereas the SOD-H cells developed further differentiated-appearing morphology. The cells stretched out, and some of them had two or three nuclei in a single cell (Fig. 1D). These results suggested that overexpression of MnSOD not only could protect the fibrosarcoma FSa-II cells from toxicity by 5-AzaC, but may also promote the differentiation effect of 5-AzaC on fibrosarcoma cells.

As MnSOD is localized within mitochondria, the mitochondria damage marker, MTT, was used to determine cytotoxicity after 5-AzaC treatment in a dose-dependent manner. 5-AzaC induced cytotoxicity in both NEO and SOD-H cells (Fig. 2); however, from three experiments, for 50% survival of the treated NEO cells, the concentration of 5-AzaC was between 9 and 11 μM, and for SOD-H cells, it was >40 μM. At 20 μM 5-AzaC, ~70% of the NEO cells were injured, whereas only 30% of the SOD-H cells were affected.

Caspase-3 plays a direct role in proteolytic degradation of cellular proteins responsible for progression to apoptosis. The results in Fig. 3 show that a trend of increased caspase-3 activity with time was observed in both cell lines. However, it was significantly increased only in the NEO cell line at all time points after 5-AzaC treatment ($p < 0.01$).

A DNA fragmentation assay was used to detect apoptosis after 5-AzaC treatment. As shown in Fig. 4, 5 μM AzaC treatment resulted in DNA fragmentation in the NEO cells (lanes 3 and 4), but not in the SOD-H cells (lanes 6 and 7). To further identify apoptotic cell death, dual staining of FSa-II cells with Hoechst 33342 and MC 540 was used. Hoechst 33342 is a DNA-specific dye, whereas MC 540 detects membrane phospholipid domain changes. In Fig. 5, where the *x* axis stands for fluorescence and the *y*-axis stands for events or counts (cell number), a shift to strong fluorescence indicated significant apoptotic progression in the 24-h 5-AzaC-treated FSa-II NEO cells (Fig. 5C), compared with no clear shift of treated SOD-H cells (Fig. 5D).

Two differentiation markers, MyoD and α-actin, were increased in the SOD-H cells after 5-AzaC treatment

5-AzaC-treated FSa-II SOD-H cells showed some features of differentiated-appearing morphology (Fig. 1D). It is well known that 5-AzaC can convert some nonmuscle cell lines to myogenic lineage by inducing muscle-specific transcription factors like MyoD (5). Therefore, we investigated MyoD expression after 5-AzaC treatment. Western analysis showed that the levels of MyoD protein were increased with time after 5-AzaC treatment in the SOD-H cells, but not in the NEO cells (Fig. 6A). The levels of α-actin, one of the muscle-specific markers, and also a MyoD target gene, were also increased in the SOD-H cells after incubation with 5-AzaC (Fig. 6B, lanes 4 and 6). There was a slight decrease in

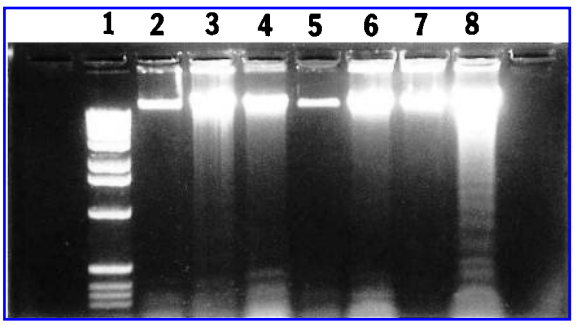

FIG. 4. DNA fragmentation. Cells were treated with 0–10 μM 5-AzaC for 24 h. Lane 1, 1 kb DNA ladder; lane 2, NEO, vehicle treatment control; lane 3, NEO, 5 μM 5-AzaC; lane 4, NEO, 10 μM 5-AzaC; lane 5, SOD-H, vehicle treatment control; lane 6, SOD-H, 5 μM 5-AzaC; lane 7, SOD-H, 10 μM 5-AzaC; lane 8, NEO, positive control [treated with 1 μM A23187 (calcium ionophore)].

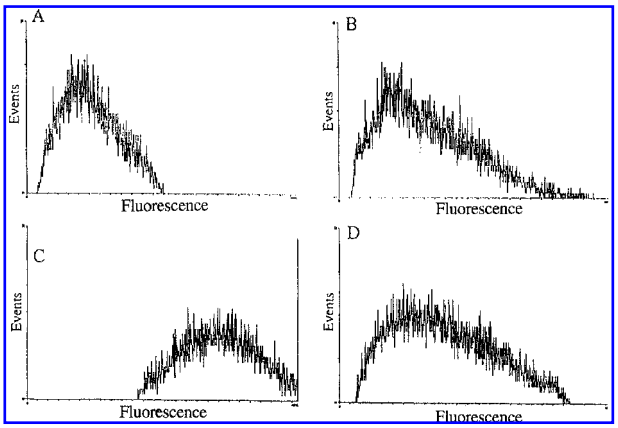

FIG. 5. Flow cytometry analysis of FSa-II NEO and SOD-H cells staining with Hoechst 33342 and MC 540. (**A**) NEO control. (**B**) SOD-H control. (**C**) NEO, 20 μM 5-AzaC, 24-h incubation. (**D**) SOD-H, 20 μM 5-AzaC, 24-h incubation.

the α-actin levels in the NEO cells at both time points (Fig. 6B, lanes 3 and 5).

NFκB plays an important role in the FSa-II cells after 5-AzaC treatment

There is increasing evidence to show the antiapoptotic effect of NFκB activation. We examined the NFκB binding activity in our 5-AzaC-treated FSa-II cells (Fig. 7A). EMSA results show that in the SOD-H cells, NFκB binding activity was significantly increased with 5-AzaC treatment (Fig. 7A, lanes 4, 6, 8, 10, and 12). The NFκB binding activity was only slightly increased in the NEO cells at 15 min after 5-AzaC treatment (Fig. 7A, lane 3), and the increase was not detectable at any of the later time points (Fig. 7A, lanes 7, 9, and 11). To verify further the role of NFκB in 5-AzaC activity, we used a proteasome inhibitor, MG-132, to inhibit IκB degradation. After treatment of the SOD-H cells for 15 h with 10 μM MG-132, the activation of NFκB was reduced (Fig. 7B); the increase in the MyoD and α-actin levels was also reduced (Fig. 7C and 7D).

Raf1/MEK/MAPK cascade was involved in signaling differentiation

To investigate further the upstream regulators in this differentiation event, we examined the mitogen-activated protein kinase (MAPK) pathway. ERK, a member of MAPK, is sug-

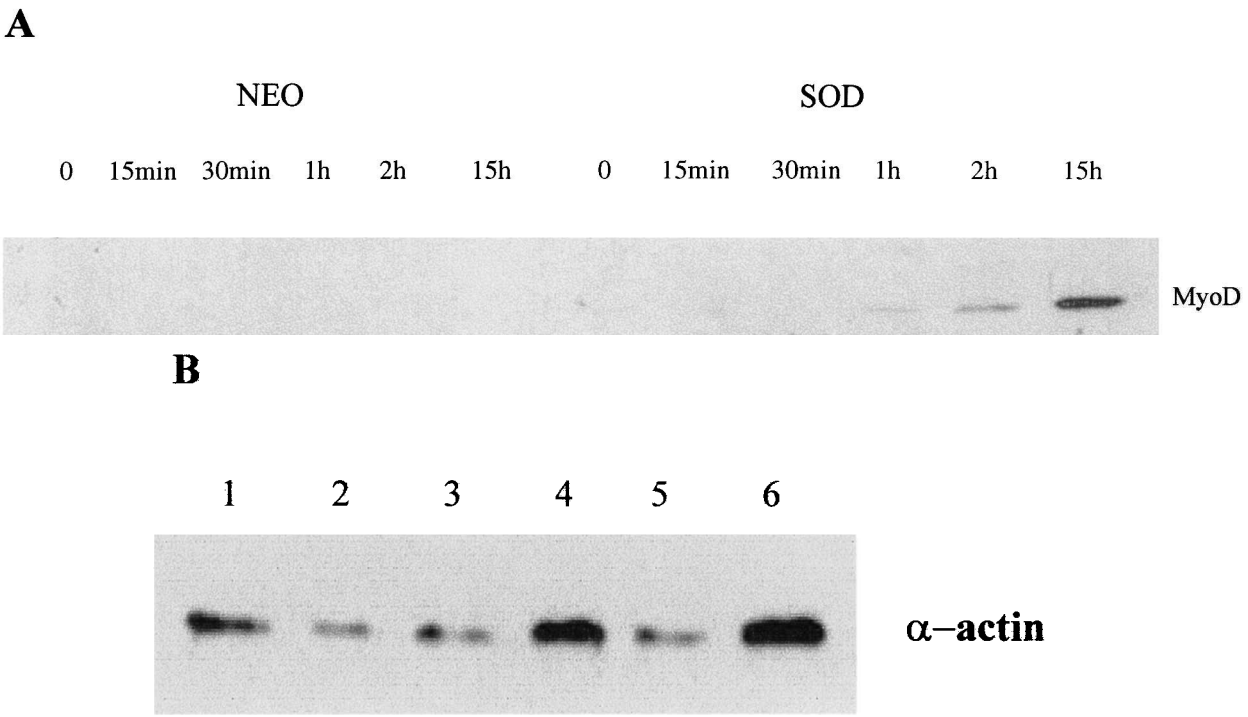

FIG 6. The levels of differentiation markers in NEO and SOD-H cells. The concentration of 5-AzaC was 10 μM. (**A**) Western blot showing MyoD levels in both cell lines, (**B**) Western analysis of α-actin levels in the two cell lines. Lane 1, NEO control; lane 2, SOD-H control; lane 3, NEO, 5-AzaC, 12-h incubation; lane 4, SOD-H, 5-AzaC, 12-h incubation; lane 5, NEO, 5-AzaC, 24-h incubation; lane 6, SOD-H, 5-AzaC, 24-h incubation.

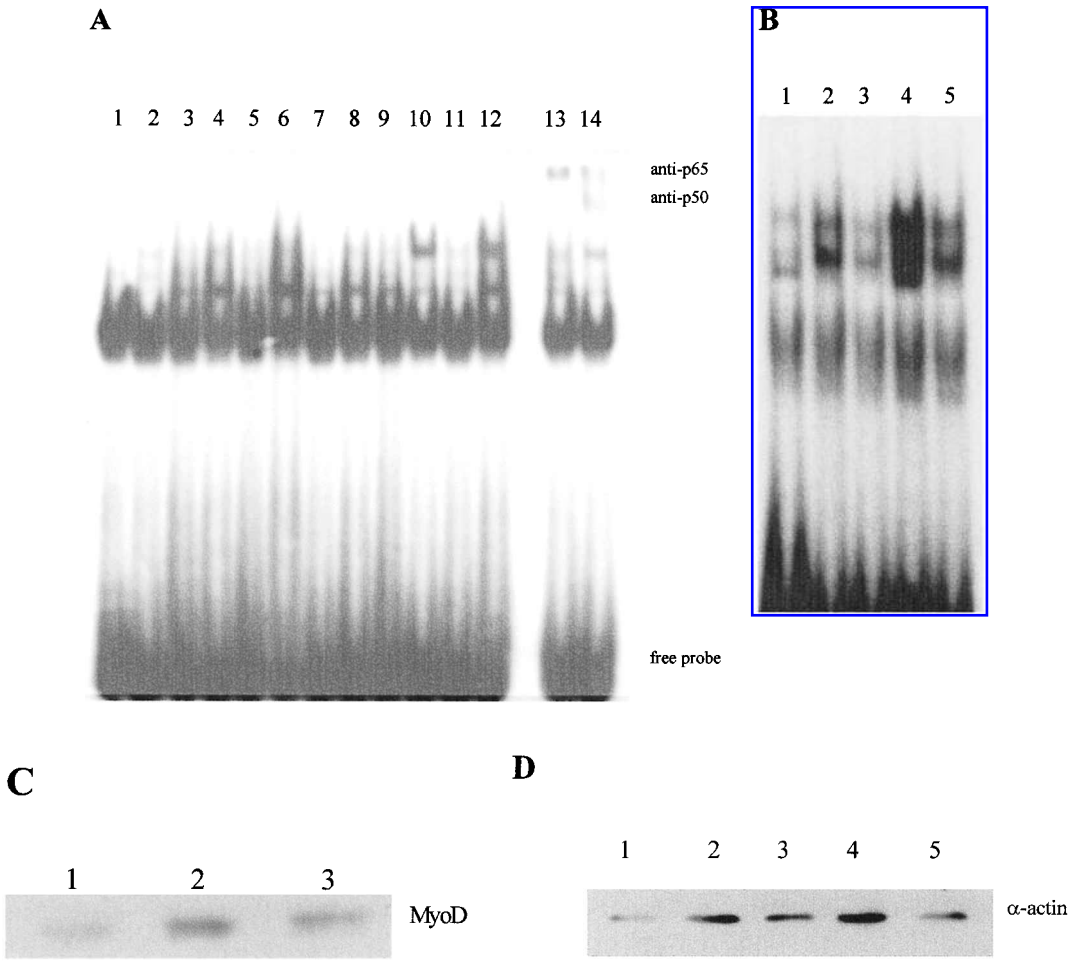

FIG. 7. Involvement of NFκB in the molecular mechanism of 5-AzaC action. The concentration of 5-AzaC was 10 μM. (**A**) EMSA of NFκB binding activity as a function of time after 5-AzaC treatment. Lanes 1, 3, 5, 7, 9, and 11; NEO cells; lanes 2, 4, 6, 8, 10, and 12, SOD-H cells. Lanes 1 and 2, control; lanes 3 and 4, 15 min; lanes 5 and 6, 30 min; lanes 7 and 8, 1 h; lanes 9 and 10, 2 h; lanes 11 and 12, 15 h, SOD-H (2 h); lanes 13 and 14, supershift with anti-p65 or anti-p50 antibody. (**B**) Detection of NFκB in SOD-H cells after addition of proteasome inhibitor MG-132. Lane 1, controls; lane 2, 5-AzaC for 12 h; lane 3, 5-AzaC + MG-132 for 12 h; lane 4, 5-AzaC for 24 h; lane 5, 5-AzaC + MG-132 for 24 h. (**C**) Western blot of MyoD in the SOD-H cells after addition of MG-132. Lane 1, SOD-H control; lane 2, 5-AzaC-treated for 15 h; lane 3, 5-AzaC combined with 10 μM MG-132. (**D**) Western blot of α-actin in the SOD-H cells after cotreatment of MG-132 with 5-AzaC for 0–24 h. Lanes 1, SOD control; lane 2, 5-AzaC only for 12 h; lane 3, 5-AzaC + 10 μM MG-132 for 12 h; lane 4, 5-AzaC for 24 h; lane 5, 5-AzaC + 10 μM MG-132 for 24 h.

gested to be involved in cell proliferation and differentiation (34). There is also some evidence to suggest that activation of MyoD can be induced by ERK. The activation of ERK is an inherent property of muscle cells, and not of the parental fibroblast cells (8). We used a phosphorylated-specific ERK antibody to detect the active forms of ERK, p42 and p44. As shown in Fig. 8A, the activated form of ERK (p-ERK) was higher in the nontreated NEO cell, and then was gradually reduced with increasing time after 5-AzaC treatment. In the SOD-H cells, the level was increased shortly after 5-AzaC treatment (within 30 min), decreased after 2 h, and then increased again after 15-h treatment. The nonphosphorylated forms of ERK did not change in either cell line during the treatment (Fig. 8B). Therefore, the increase in p-ERK was not due to an increase in the total level of ERK proteins.

To probe the role of p-ERK in the early differentiation program, we used a noncompetitive inhibitor of MAPK kinase, PD98059. PD98059 can block the increase of MyoD protein (Fig. 8C) and the activation of NFκB (Fig. 8D), but it had no influence on the α-actin level (data not shown).

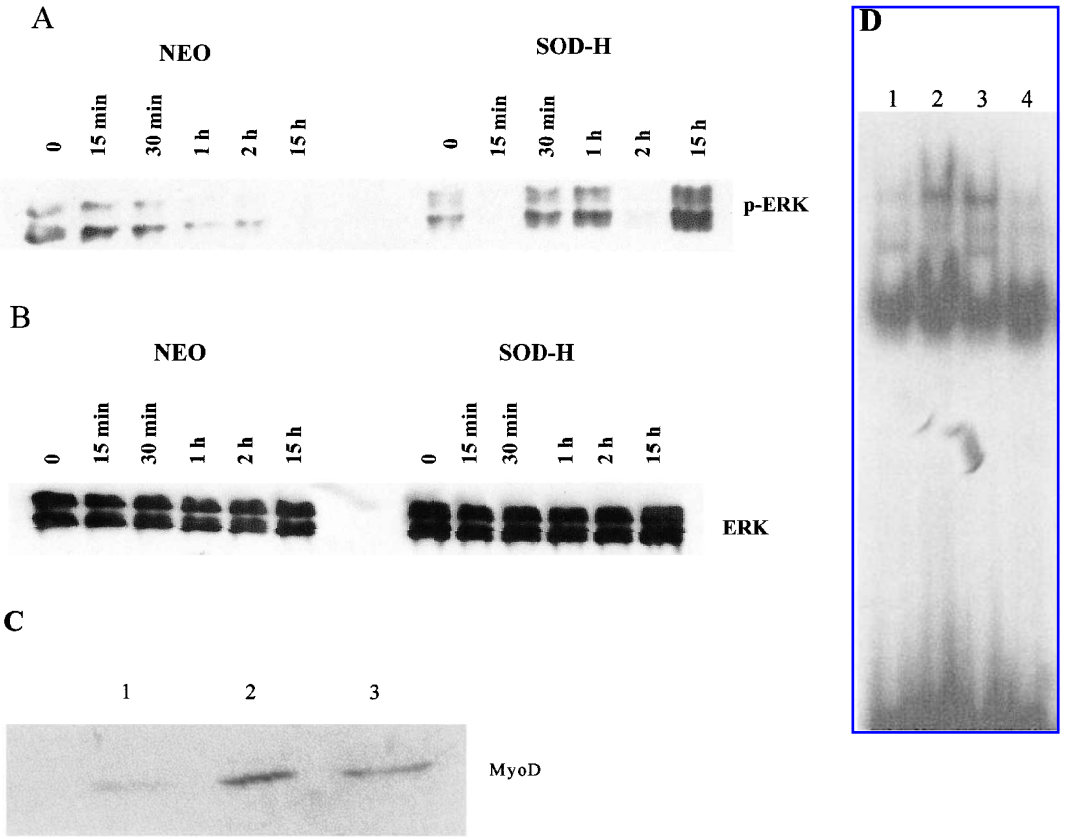

FIG. 8. ERK kinase in an early differentiation event. FSa-II NEO and SOD-H cells were incubated with 10 μM 5-AzaC for the indicated times. (**A**) Western analysis of phosphorylated forms of ERK. (**B**) Nonphosphorylated forms of ERK. (**C**) Western blot showing MyoD levels after 15 h of 5-AzaC treatment in the presence or absence of PD98059. Lane 1, SOD-H control; lane 2, 5-AzaC 15 h; lane 3, 5-AzaC plus 10 μM PD98059. (**D**) EMSA showing that PD98059 blocked NFκB activation in SOD-H cells. Lane 1, control; lane 2, 5-AzaC for 15 h; lane 3, 5-AzaC + 10 μM PD98059; lane 4, 5-AzaC + 50 μM PD98059.

Raf1 is a candidate that may activate MAPK. To identify the potential upstream effector of ERK, we performed western analysis of Raf1 in both NEO and SOD-H cells. Figure 9 shows that in the SOD-H cells, Raf1 was increased after 2 h, and this effect was sustained at 15 h after 5-AzaC treatment. There were no detectable levels of Raf1 in the NEO cells at all time points examined.

DISCUSSION

MnSOD is a nuclear encoded mitochondrial matrix enzyme that scavenges toxic superoxide radicals. The parental murine fibrosarcoma cell line (FSa-II), originally developed as a spontaneous tumor in C3Hf/Sed mice, exhibits low endogenous levels of MnSOD. We have previ-

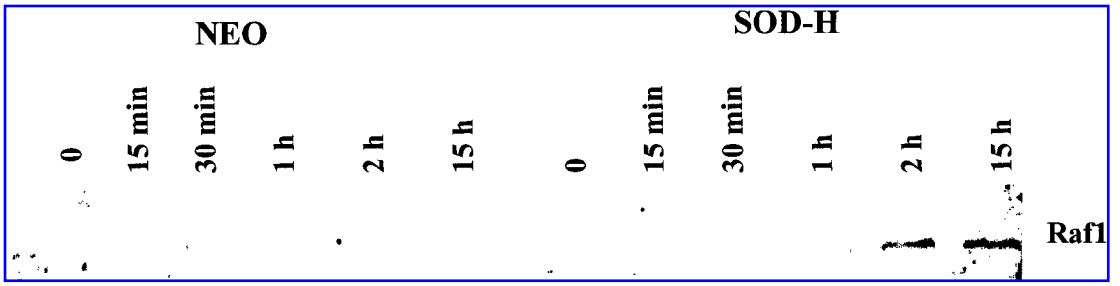

FIG. 9. Western analysis of Raf1. FSa-II NEO and SOD cells were incubated with 10 μM 5-AzaC for 15 min to 15 h.

ously demonstrated that transfection of the human MnSOD cDNA into FSa-II cells, leading to an elevated expression of MnSOD, can reduce tumorigenicity and suppress tumor metastasis *in vivo* (32, 37). In this study, we found that 5-AzaC treatment induced toxicity in the FSa-II NEO cells, whereas in the SOD-H cells, not only was the toxicity attenuated, but the cells also showed differentiated-appearing morphology. Two features shown in the NEO cells suggest that 5-AzaC induced apoptosis in these cells. First, the activity of caspase-3, an apoptotic execution protease, increased with time after 5-AzaC treatment. Second, DNA fragmentation and changes in membrane phospholipid structure were observed after 5-AzaC treatment. Although 5-AzaC-induced programmed cell death in cultured cells has been reported, the mechanism of apoptosis induced by 5-AzaC is unknown. Our findings that increased expression of MnSOD prevents apoptosis in the SOD-H cells suggest that an oxidative activation of programmed cell death may be involved. However, further experiments are required to elucidate fully this point.

The antineoplastic activity of 5-AzaC has been recognized and used for cancer therapy in the clinic, and demethylation of tumor suppresser genes has been postulated as a possible mechanism (13, 24). In several 5-AzaC-treated cancer cell lines, differentiated-appearing morphology has been described (24, 44, 47), but the possible mechanism has not been elucidated. Here we detected the increase of an early differentiation marker, MyoD, in SOD-transfected FSa-II cells, and also found an increased level of α-actin, a MyoD target gene. MyoD was the first member of the myogenic transcription factor family to be cloned (5). MyoD and Myf-5 are the primary myogenic regulatory factors. Either MyoD or Myf-5 is first induced independently of one another during development. Activation of either of them allows entry into the myogenic program (30, 31). α-Actin is one of the muscle-specific proteins and a MyoD target gene. The promoter region of the α-actin gene contains an E box that is directly activated by MyoD or myogenin (3, 23). Thus, 5-AzaC in the presence of MnSOD converted fibrosarcoma cells into an early, but not fully differentiated, myoblast lineage.

We have also examined the possible upstream regulators of MyoD. ERK, a classical member of the MAPK cascade, is known to exert a pleiotropic role in cell proliferation and differentiation. Our results showed that p-ERK was increased within 30 min of 5-AzaC treatment in the FSa-II SOD-H cells, whereas it gradually decreased in the NEO cells, suggesting that ERK may participate in the early differentiation program. In the NEO cells, the decrease of p-ERK may partly be responsible for the toxicity of 5-AzaC. In the SOD-H cells, the activation of ERK activity may be linked to the entry of the myogenic program by activation of MyoD.

Signaling transduction cascade of Raf1/MEK/MAPK is suggested to be involved in proliferation and differentiation. In the SOD-H cell line, the levels of Raf1 were increased by 2 h, with no significant change in the NEO cell line, suggesting a positive role in this cascade. When a noncompetitive inhibitor of MAPK kinase (PD98059), which can block its activation by Raf1 (1), was used in 5-AzaC-treated SOD-H cells, the increase of MyoD was inhibited, whereas the level of α-actin was not changed. These results are consistent with those reported by Gredinger *et al*. (8), who demonstrated that PD98059 could block induction of MyoD in the differentiation medium, whereas none of the examined muscle-specific markers such as myosin light chain and muscle creatine kinase were inhibited. Taken together, these results suggested that PD98059 may have a limited effect on differentiation or that additional pathways are involved.

Another interesting result is the gradual activation of NFκB in the SOD-H cells after 5-AzaC treatment. Activation of NFκB has been suggested to be a pivotal regulator of inflammation, cell differentiation, apoptosis, and proliferation (6). There is some evidence to suggest that activation of NFκB can trigger early events during myogenesis. Kaliman *et al*. (15) reported that insulin-like growth factor-II induces NFκB activity, which is one of the critical events for myogenesis. Similarly, Wang and Passaniti (43) demonstrated that extracellular matrix inhibits apoptosis and enhances endothelial cell differentiation by a NFκB-dependent mechanism. In our study, when we treated the SOD-H cells

with the proteasome inhibitor, MG-132, the cells lost their differentiated-appearing morphology (data not shown). In addition, we also found that MG-132 inhibited the increase of MyoD after 5-AzaC treatment, suggesting a link between NFκB activation and MyoD expression. However, a recent study by Guttridge *et al*. (10) demonstrated that tumor necrosis factor-induced NFκB activity suppressed MyoD mRNA at the posttranscription level in differentiating C2C12 myocytes, but not in differentiated myotubes. Thus, it is possible that the effect of NFκB on MyoD levels may be dependent on inducing agents, cell types, and stages of differentiation. It will be interesting to elucidate further if NFκB plays a distinct role in the differentiation of nontransformed versus transformed cells.

It has been reported that Raf1 activates NFκB via an autocrine feedback loop (25), and the ERK inhibitor PD98059 can block MAPK activation by Raf1. Our study demonstrated that adding PD98059 to SOD-H cells blocked the NFκB activation effect of 5-AzaC, suggesting a link between MAPK pathways and NFκB activation.

Evidence has accumulated that supports a direct role for mitochondria in cell death and differentiation. Previous studies from this and other laboratories suggest that mitochondria dysfunction by superoxide accumulation may play pivotal roles in normal tissue injury induced by diverse insults *in vitro* and *in vivo* (7, 9, 17, 22, 48). Overproduction of MnSOD has been shown to prevent mitochondria from injury by cytokine, chemical hypoxia, nitric oxide, alkalosis, and radiation (7, 9, 19, 22, 45). The data presented here will add to the role of MnSOD in preventing apoptosis induced by an agent known to induce differentiation. Rochard *et al*. (29) showed that mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. Li *et al*. (21) reported mitochondria mediated tumor necrosis factor-α/NFκB signaling in skeletal muscle myotubes. Biswas *et al*. (4) reported that the NFκB level declined after mitochondrial DNA contents were reduced by mitochondrial metabolic inhibitors. Our previous studies demonstrated that overexpression of MnSOD promotes myogenic differentiation of embryonic fibroblast cells (36). The data reported here suggest that expression of MnSOD can enhance the differentiation program of cancer cells.

Reactive oxygen species have been shown to act as signal mediators that affect both cell transformation and differentiation (11, 16, 33, 38). Our finding that overexpression of MnSOD in the mitochondria modulates the activation of MAPK and NFκB pathways further signifies the role of mitochondrial antioxidants in the regulation of cell death and differentiation. These results indicate that MnSOD could play an important role in cancer therapy.

ACKNOWLEDGMENTS

The authors wish to thank Jennifer Strange and Greg Bauman for their technical contributions to the manuscript. This work was supported by NIH grants CA 80152, CA 49797, CA 73599, and HL 03544.

ABBREVIATIONS

5-AzaC, 5-azacytidine; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MC 540, merocyanine 540; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEO, neomycin; NFκB, nuclear factor-κB; PBS, phosphate-buffered saline; p-ERK, phosphorylated ERK; PMSF, phenylmethylsulfonyl fluoride; SOD-H, human MnSOD gene.

REFERENCES

1. Alessi DR, Chuenda A, Cohen P, Dudley DT, and Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* 270: 27489–27494, 1995.
2. Baylin SB. Tying it all together: epigenetics, genetics, cell cycle, and cancer. *Science* 277: 1948–1949, 1997.
3. Biesiada E, Hamamori Y, Kedes L, and Sartorelli V. Myogenic basic helix–loop–helix proteins and Sp1 interact as components of a multiprotein transcriptional

complex required for activity of the human cardiac alpha-actin promoter. *Mol Cell Biol* 19: 2577–2584, 1999.
4. Biswas G, Adebanjo OA, Freedman BD, Anandatheerthavarada HK, Vijayasarathy C, Zaidi M, Kotlikoff M, and Avadhani NG. Retrograde Ca^{2+} signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk. *EMBO J* 18: 522–533, 1999.
5. Davis RL, Weintraub H, and Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51: 987–1000, 1987.
6. Ghosh S, May MJ, and Kopp EB. NF-kappa B and Rel proteins: evolutionary conserved mediators of immune responses. *Annu Rev Immunol* 16: 225–260, 1998.
7. Gonzalez-Zulueta M, Ensz LM, Mukhina G, Lebovitz RM, Zwacka RM, Engelhardt JF, Oberley LW, Dawson VL, and Dawson TM. Manganese superoxide dismutase protects nNOS neurons from NMDA and nitric oxide-mediated neurotoxicity. *J Neurosci* 18: 2040–2055, 1998.
8. Gredinger E, Gerber AN, Tamir Y, Tapscott SJ, and Bengal E. Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. *J Biol Chem* 273: 10436–10444, 1998.
9. Guidot DM, McCord JM, Wright RM, and Repine JE. Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase-deficient *Saccharomyces cerevisiae* in hypoxia. Evidence for electron transport as a major source of superoxide generation in vivo. *J Biol Chem* 268: 26699–26703, 1993.
10. Guttridge DC, Mayo MW, Madrid LV, Wang CY, and Baldwin AS Jr. NF-κB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science* 289: 2363–2366, 2000.
11. Halliwel B and Gutteridge JMC. *Free Radicals in Biology and Medicine,* 2nd ed, Oxford, U.K.: Clarendon Press, 1989, pp. 86–123.
12. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, and Baylin SB. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 95: 6870–6875, 1998.
13. Hossain MM, Nakayama H, Takashima A, Goto N, and Doi K. 5-Azacytidine (5Az) induces apoptosis in PC12 cells: a model for 5Az-induced apoptosis in developing neuronal cells. *Histol Histopathol* 12: 439–445, 1997.
14. Jaenisch R. DNA methylation and imprinting: why bother? *Trends Genet* 13: 323–329, 1997.
15. Kaliman P, Canicio J, Testar X, Palacin M, and Zorzano A. Insulin-like growth factor-II, phosphatidylinositol 3-kinase, nuclear factor-kappaB and inducible nitric-oxide synthases define a common myogenic signaling pathway. *J Biol Chem* 274: 17437–17444, 1999.
16. Kamata H and Hirata H. Redox regulation of cellular signaling. *Cell Signal* 11: 1–14, 1999.
17. Keller JN, Kindy MS, Holtsberg FW, St. Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, and Mattson MP. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci* 18: 687–697, 1998.
18. Kiningham KK and St. Clair DK. Overexpression of manganese superoxide dismutase selectively modulates the activity of Jun-associated transcription factors in fibrosarcoma cells. *Cancer Res* 57: 5265–5271, 1997.
19. Kiningham KK, Oberley TD, Lin S, Mattingly CA, and St. Clair DK. Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. *FASEB J* 13: 1601–1610, 1999.
20. Lewin B. The mystique of epigenetics. *Cell* 93: 301–303, 1998.
21. Li YP, Schwartz RJ, Waddell ID, Holloway BR, and Reid MB. Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-kappaB activation in response to tumor necrosis factor alpha. *FASEB J* 12: 871–880, 1998.
22. Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen HC, Szweda LI, and St. Clair DK. Prevention of mitochondria injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J Biol Chem* 273: 8217–8224, 1998.
23. Marsh DR, Carson JA, Stewart LN, and Booth FW. Activation of the skeletal alpha-actin promoter during muscle regeneration. *J Muscle Res Cell Motil* 19: 897–907, 1998.
24. Momparler RL, Cote S, and Eliopoulos N. Pharmacological approach for optimization of the dose schedule of 5-aza-2′-deoxycytidine (Decitabine) for the therapy of leukemia. *Leukemia II* (Suppl 1): S1–S6, 1997.
25. Norris JL and Baldwin AS. Oncogenic Ras enhances NFκB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *J Biol Chem* 274: 13841–13846, 1999.
26. Oberley LW and Buettner GR. Role of superoxide dismutase in cancer: a review. *Cancer Res* 39: 1141–1149, 1979.
27. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, and Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 54: 2552–2555, 1994.
28. Reid S, Cross R, and Snow EC. Combined Hoechst 33342 and merocyanine 540 staining to examine murine B cell cycle stage, viability and apoptosis. *J Immunol Methods* 192: 43–54, 1996.
29. Rochard P, Rodier A, Casas F, Cassar-Malek I, Mar-

chal-Victrion S, Daury L, Wrutniak C, and Cabello G. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. *J Biol Chem* 275: 2733–2744, 2000.
30. Rudnicki MA, Braun T, Hinuma S, and Jaenisch R. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* 71: 383–390, 1992.
31. Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, and Jaenisch R. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75: 1351–1359, 1993.
32. Safford SE, Oberley TD, Urano M, and St. Clair DK. Suppression of fibrosarcoma metastasis by elevated expression of manganese superoxide dismutase. *Cancer Res* 54: 4261–4265, 1994.
33. Sauer H, Rahimi G, Hescheler J, and Wartenberg M. Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cell. *FEBS Lett* 476: 218–223, 2000.
34. Schaeffer HJ and Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 19: 2435–2444, 1999.
35. St. Clair DK. Manganese superoxide dismutase: roles in cancer therapy. In: *2nd International Symposium Book,* edited by Manuchair E. Scottsdale, AZ: Prominent Press, 2000, pp. 1–24.
36. St. Clair DK, Oberley TD, Muse KE, and St. Clair WH. Expression of manganese superoxide dismutase promotes cellular differentiation. *Free Radic Biol Med* 16: 275–282, 1994.
37. St. Clair DK, Wan XS, Kuroda M, Vichitbandha S, Tsuchida E, and Urano M. Suppression of tumor metastasis by manganese superoxide dismutase is associated with reduced tumorigenicity and elevated fibronectin. *Oncol Rep* 4: 753–757, 1997.
38. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, and Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401: 79–82, 1999.
39. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med* 8: 583–599, 1990.
40. Taylor SM. 5-Aza-2′-deoxycytidine: cell differentiation and DNA methylation. *Leukemia* 7(Suppl 1): 3–8, 1993.
41. Urano M and Kahn J. Some practical questions in the tumor regrowth assay. In: *Rodent Tumor Models in Experimental Cancer Therapy,* edited by Kallman RF. Elmsford, NY: Pergamon Press, 1987, pp. 122–127.
42. Urano M, Kuroda M, Reynolds R, Oberley TD, and St. Clair DK. Expression of manganese superoxide dismutase reduces tumor control radiation dose: gene-radiotherapy. *Cancer Res* 55: 2490–2493, 1995.
43. Wang W and Passaniti A. Extracellular matrix inhibits apoptosis and enhances endothelial cell differentiation by a NFkappaB-dependent mechanism. *J Cell Biochem* 73: 321–331, 1999.
44. Wang XM, Wang X, Li J, and Evers BM. Effects of 5-azacytidine and butyrate on differentiation and apoptosis of hepatic cancer cell lines. *Ann Surg* 227: 922–931, 1998.
45. Wong GHW, Elwell JH, Oberley LW, and Goeddel DV. Manganese superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. *Cell* 58: 923–931, 1989.
46. Xu Y, Krishnan A, Wan XS, Majima H, Yeh CC, Ludewig G, Kasarskis EJ, and St. Clair DK. Mutations in the promoter reveal a cause for the reduced expression of the human manganese superoxide dismutase gene in cancer cells. *Oncogene* 18: 93–102, 1999.
47. Yamada T, Ohwada S, Saitoh F, Adachi M, Morishita Y, and Hozumi M. Induction of Ley antigen by 5-aza-2′-deoxycytidine in association with differentiation and apoptosis in human pancreatic cancer cells. *Anticancer Res* 16: 735–740, 1996.
48. Yen HC, Oberley TD, Vichitbandha S, Ho YS, and St. Clair DK. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J Clin Invest* 98: 1253–1260, 1996.

Address reprint requests to:
Dr. Daret K. St. Clair
Graduate Center for Toxicology
University of Kentucky
Lexington, KY 40536, U.S.A.

E-mail: dstcl00@pop.uky.edu

Received for publication November 22, 2000; accepted January 25, 2001.

This article has been cited by:

1. Aaron K. Holley, Sanjit Kumar Dhar, Daret K. St. Clair. 2012. Curbing cancer's sweet tooth: Is there a role for MnSOD in regulation of the Warburg effect?. *Mitochondrion* . [CrossRef]
2. Mohammed Emamussalehin Choudhury, Kana Sugimoto, Madoka Kubo, Hirotaka Iwaki, Tomoaki Tsujii, Win Thiri Kyaw, Noriko Nishikawa, Masahiro Nagai, Junya Tanaka, Masahiro Nomoto. 2012. Zonisamide up-regulated the mRNAs encoding astrocytic anti-oxidative and neurotrophic factors. *European Journal of Pharmacology* . [CrossRef]
3. Vani Ramesh, Prabakaran Ravichandran, Clinton L. Copeland, Ramya Gopikrishnan, Santhoshkumar Biradar, Virupaxi Goornavar, Govindarajan T. Ramesh, Joseph C. Hall. 2011. Magnetite induces oxidative stress and apoptosis in lung epithelial cells. *Molecular and Cellular Biochemistry* . [CrossRef]
4. Sudhakar Baluchamy, Prabakaran Ravichandran, Vani Ramesh, Zhenhua He, Ye Zhang, Joseph C. Hall, Olufisayo Jejelowo, Daila S. Gridley, Honglu Wu, Govindarajan T. Ramesh. 2011. Reactive oxygen species mediated tissue damage in high energy proton irradiated mouse brain. *Molecular and Cellular Biochemistry* . [CrossRef]
5. Yan Chen, Liwen Chang, Wenbin Li, Zhihui Rong, Wei Liu, Ruiyan Shan, Rui Pan. 2010. Thioredoxin protects fetal type II epithelial cells from hyperoxia-induced injury. *Pediatric Pulmonology* **45**:12, 1192-1200. [CrossRef]
6. Sumihiro Kawajiri, Yutaka Machida, Shinji Saiki, Shigeto Sato, Nobutaka Hattori. 2010. Zonisamide reduces cell death in SH-SY5Y cells via an anti-apoptotic effect and by upregulating MnSOD. *Neuroscience Letters* **481**:2, 88-91. [CrossRef]
7. Aaron K. Holley, Sanjit Kumar Dhar, Yong Xu, Daret K. St. Clair. 2010. Manganese superoxide dismutase: beyond life and death. *Amino Acids* . [CrossRef]
8. Isabel Quirós, Rosa M. Sáinz, David Hevia, Olivia García-Suárez, Aurora Astudillo, Manuel Rivas, Juan C. Mayo. 2009. Upregulation of manganese superoxide dismutase (SOD2) is a common pathway for neuroendocrine differentiation in prostate cancer cells. *International Journal of Cancer* **125**:7, 1497-1504. [CrossRef]
9. Kinsley K. Kiningham, Zina-Ann Cardozo, Carla Cook, Marsha P. Cole, John C. Stewart, Mariela Tassone, Mitchell C. Coleman, Douglas R. Spitz. 2008. All-trans-retinoic acid induces manganese superoxide dismutase in human neuroblastoma through NF-#B. *Free Radical Biology and Medicine* **44**:8, 1610-1616. [CrossRef]
10. Liang-Yo Yang, Wei-Li Chen, Jia-Wei Lin, Shu-Fen Lee, Chin-Cheng Lee, Tsung I Hung, Yau-Huei Wei, Chwen-Ming Shih. 2005. Differential expression of antioxidant enzymes in various hepatocellular carcinoma cell lines. *Journal of Cellular Biochemistry* **96**:3, 622-631. [CrossRef]
11. Archana Jaiswal McEligot, Sun Yang, Frank L. Meyskens, Jr.. 2005. REDOX REGULATION BY INTRINSIC SPECIES AND EXTRINSIC NUTRIENTS IN NORMAL AND CANCER CELLS. *Annual Review of Nutrition* **25**:1, 261-295. [CrossRef]
12. D STCLAIR, Y ZHAO, L CHAISWING, T OBERLEY. 2005. Modulation of skin tumorigenesis by SOD. *Biomedecine & Pharmacotherapy* **59**:4, 209-214. [CrossRef]
13. Mojgan Djavaheri-Mergny, Delphine Javelaud, Juana Wietzerbin, Françoise Besançon. 2004. NF-#B activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNF#-treated Ewing sarcoma cells. *FEBS Letters* **578**:1-2, 111-115. [CrossRef]
14. Terry D. Oberley , Yi Xue , Yunfeng Zhao , Kelley Kiningham , Luke I. Szweda , Daret K. St. Clair . 2004. In Situ Reduction of Oxidative Damage, Increased Cell Turnover, and Delay of Mitochondrial Injury by Overexpression of Manganese Superoxide Dismutase in a Multistage Skin Carcinogenesis Model. *Antioxidants & Redox Signaling* **6**:3, 537-548. [Abstract] [Full Text PDF] [Full Text PDF with Links]
15. Vuokko L Kinnula, James D Crapo. 2004. Superoxide dismutases in malignant cells and human tumors. *Free Radical Biology and Medicine* **36**:6, 718-744. [CrossRef]

16. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]