Enhancing the Antitumor Activity of Adriamycin and Ionizing Radiation

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Abstract

Overexpression of manganese superoxide dismutase (MnSOD), when combined with certain chemicals that inhibit peroxide removal, increases cancer cell cytotoxicity. Elevating MnSOD levels in cells enhances the conversion of superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$), combined with inhibiting the removal of H$_2$O$_2$, further increases H$_2$O$_2$ levels, leading to increased cytotoxicity. We hypothesized that increasing endogenous O$_2^-$ production in cells that were pretreated with adenoviral MnSOD (AdMnSOD) plus 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) would lead to an increased level of intracellular H$_2$O$_2$ accumulation and increased cell killing. The cytotoxic effects of Adriamycin or radiation, agents known to produce O$_2^-$, were determined in MDA-MB-231 breast cancer cells pretreated with AdMnSOD plus BCNU both in vitro and in vivo. In vitro, AdMnSOD plus BCNU sensitized cells to the cytotoxicity of Adriamycin or radiation. In vivo, AdMnSOD, BCNU, and Adriamycin or ionizing radiation inhibited tumor growth and prolonged survival. The results suggest that agents that produce O$_2^-$ in combination with AdMnSOD plus BCNU may represent a powerful new antitumor regimen against breast cancer. [Cancer Res 2009;69(10):4294–300]

Introduction

Within the antioxidant system, manganese superoxide dismutase (MnSOD) is found in the mitochondrial matrix, from which ~75% of cellular superoxide (O$_2^-$) is generated. Cancer cells almost always express low levels of MnSOD, and if the activity of MnSOD is increased, the phenotype of cancer cells should be at least partially reversed as hypothesized by Oberley and Buettner (1). MnSOD catalyzes the dismutation of O$_2^-$ to hydrogen peroxide (H$_2$O$_2$), thus changing the balance between O$_2^-$ and H$_2$O$_2$ and affecting signal transduction pathways that modulate cell proliferation (2, 3). The tumor-suppressive effect of MnSOD is supported by many studies demonstrating that overexpression of MnSOD in transformed cell lines leads to the reversion of the malignant phenotype (4–7) and MnSOD overexpression alone has a largely nontoxic tumor suppressive effect in many cancer cell types (8). H$_2$O$_2$ may be the effector species involved in the tumor suppressive effect of MnSOD due to the fact that addition of pyruvate, a scavenger of H$_2$O$_2$, can enhance the proliferation of MnSOD-overexpressing cells (9). Also, coexpression of MnSOD and either catalase (10) or glutathione peroxidase (11) can reverse the inhibition of cell growth induced by MnSOD overexpression. These findings implicate H$_2$O$_2$ as an important mediator for the inhibition of cell growth induced by MnSOD overexpression. On the other hand, without adequate peroxidase or catalase, SOD-overexpressing cells will be exposed to an increased steady-state concentration of H$_2$O$_2$. In the mitochondrial microenvironment, there are many electron transport enzymes containing iron, and the reaction between H$_2$O$_2$ and Fe$^{2+}$ can lead to either the production of HO$^*$ through the metal-catalyzed Haber-Weiss reaction or the production of ferryl or perferryl species (12). This is consistent with studies demonstrating that overexpression of SOD can sensitize cells to oxidant stress (13). Moreover, buthionine sulfoximine, an inhibitor of glutathione synthesis that results in inhibition of peroxide detoxification, caused dramatic cell killing in glioma cells that were stably transfected with MnSOD cDNA and had little effect on the parental cells (14).

1,3-Bis-chloroethyl-1-nitrosourea (BCNU) is a chemotherapy drug that decomposes in aqueous buffer at physiologic pH to form an alkylation moiety and a carbamoylating moiety. The alkylation moiety reacts in the cell to alkylate purines or pyrimidines, resulting in DNA and RNA cross-linking. The carbamoylating moiety acts on nucleophilic alkyl side chain groups of amino acids inactivating proteins, including glutathione reductase (GR; refs. 15, 16). After exposure to BCNU, cells increased the synthesis of new glutathione (GSH; ref. 17) and also increased the percentage of glutathione disulfide (GSSG; ref. 18), most likely due to the inactivation of GR, which converts GSSG to GSH. If GR is inhibited, cells cannot remove H$_2$O$_2$ as well. In a previous study from our laboratory, Weydert and colleagues (8) showed that MnSOD-overexpressing human oral squamous carcinoma cell lines were more sensitive to BCNU. Cells treated with adenovirus containing MnSOD (AdMnSOD) alone showed >90% survival, whereas cells treated AdMnSOD plus BCNU had the greatest cytotoxicity with <20% survival. In vivo studies showed a 4- to 20-fold inhibition of tumor growth and prolonged animal survival by the combined treatment of AdMnSOD plus BCNU.

The purpose of the present study was to determine if a superoxide radical generator, such as Adriamycin or ionizing radiation, could increase the antitumor effect of AdMnSOD plus BCNU both in vitro and in vivo. The hypothesis was that if we would increase the levels of the substrate for MnSOD (O$_2^-$), we would generate more product (H$_2$O$_2$) and thus obtain more cytotoxicity.
Materials and Methods

Cell culture. The human breast carcinoma cell line MDA-MB-231 (MB231), purchased from American Type Culture Collection, was cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were incubated under a humidified atmosphere of 95% air/5% CO2 at 37°C. Cells were passed weekly by treatment with 0.25% trypsin/0.02% EDTA. Mycoplasma was tested at 3-mo intervals so only Mycoplasma-free cells were used.

Adenovirus infection. AdMnSOD was manufactured at the University of Iowa’s Vector Core Facility and is prepared by inserting the MnSOD gene into the estrone (E1) region of an Ad5 E1/partial E3-deleted replication-deficient adenoviral vector and has previously been described (8). The cDNA is under the control of a cytomegalovirus (CMV) promoter. AdEmpty (adenovirus with empty shuttle vector and a CMV promoter) was used as a vector control.

Immunoblotting and activity gels. The primary polyclonal antibodies against human MnSOD and copper zinc SOD were developed in our laboratory (19). Glutathione peroxidase (GPx1) and GR primary antibodies were obtained from Lab Frontier. Western blots were performed according to the method described by Laemmli (20) using the same technique that has been previously described in our laboratory (8). The SOD activity gel assay is based on the inhibition of the reduction of nitroblue tetrazolium by SOD (21). The catalase activity gel assay was carried out according to the methods described by Sun and colleagues (22). For the GR activity gel assay, equal amounts of protein from different samples were subjected to electrophoresis in 8% native polyacrylamide gels in nondenaturing running buffer (pH 8.3). For GR band visualization, after electrophoresis, the gel was stained with 250 mmol/L Tris (pH 8.0) containing 3/4 mmol/L GSSG, 0.36 mmol/L NADPH, 0.052 mmol/L dichlorophenol-indophenol, and 1.1 mmol/L 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide until blue precipitate GR bands began to form.

Glutathione measurement. The intracellular levels of GSH and GSSG were measured according to the methods described by Anderson (23). After reduced GSH was removed by mixing samples with 2-vinylpyridine, the cellular GSSG level was measured by the same DTNB assay (24). Reduced GSH was determined by subtracting the GSSG content from the total GSH content. The concentration of samples was calculated by comparing the rates of samples to the rates obtained from the corresponding standard curve. The concentrations were then normalized to the protein content.

Plating efficiency. Cell survival was measured by clonogenic ability of cells as previously described (25). After different treatments, cells (250–3,000) were seeded in 6-well plates in RPMI 1640 with 10% FBS and

![Figure 1.](image-url) BCNU altered GR activity and glutathione in AdMnSOD-infected MB231 cells in a dose-dependent manner. Cells were infected with either 100 MOI AdEmpty or AdMnSOD and then treated with different concentrations of BCNU for 2 h. A, the activities of major antioxidant proteins were measured by activity gel assay. AdMnSOD infection increased MnSOD activity in MB231 cells, and increasing concentrations of BCNU inhibited GR activities in both parental and AdMnSOD-infected cells in a dose-dependent manner. Activities of other antioxidant proteins [copper zinc SOD (CuZnSOD) and catalase] were not changed. B, cells were collected and the GSH and GSSG concentrations were measured. AdEmpty infection, AdMnSOD infection, and BCNU alone had no effect on %GSSG, whereas AdMnSOD (100 MOI) infection + BCNU (50 μmol/L) treatment resulted in a 2.1-fold increase in %GSSG (*, P < 0.01 versus AdEmpty; n = 3).

![Figure 2.](image-url) Pyruvate reversed the increase in BCNU cytotoxicity induced by MnSOD overexpression. A, clonogenic survival after BCNU treatment without pyruvate. There was a dose-dependent decrease in cell survival after 5 to 10 μmol/L BCNU treatment. AdEmpty infection did not decrease cell survival. AdMnSOD alone without BCNU slightly decreased cell survival to 85%. AdMnSOD infection sensitized cells to BCNU treatment, at a dose of BCNU (5 and 10 μmol/L, points, means; bars, SE; *, P < 0.05. AdMnSOD plus BCNU versus BCNU only, or AdEmpty plus BCNU; n = 3). B, clonogenic survival after BCNU treatment in the presence of 10 mmol/L pyruvate. The presence of pyruvate reversed the decrease in clonogenic survival seen with the AdMnSOD plus BCNU treatment when compared with BCNU or AdEmpty plus BCNU (points, mean; bars, SE; n = 3).
allowed to grow for 14 d to form colonies. After staining with Coomassie Blue solution containing 0.1% crystal violet and 2% citric acid in H2O, colonies (>50 cells) were counted under a dissecting microscope. Plating efficiency was calculated as follows:

\[
\text{Plating efficiency} = \frac{\text{Colonies formed}}{\text{Cells seeded}} \times 100%.
\]

**In vivo tumor xenografts.** Female athymic nude mice at 4- to 7-wk-old, weighing 20 to 23 grams (Harlan Sprague-Dawley, Inc.), were injected s.c. with \(4 \times 10^5\) MB231 cells suspended in PBS into the right flank region with a 1 cc tuberculin syringe equipped with a 25-gauge needle. Tumors were allowed to grow and the tumor size was monitored using a vernier caliper. The tumor volume was calculated according to the following formula:

\[
V = \frac{L \times W^2 \pi}{6},
\]

where \(V\) is tumor volume, \(L\) is length, and \(W\) is width (5). After the tumors reached ~70 mm\(^3\), \(1 \times 10^6\) plaque-forming unit (pfu) of Ad\textit{MnSOD} suspended in a 3% sucrose PBS solution to a final volume of 100 \(\mu\)L were delivered directly into the tumor using a 25-gauge needle attached to a 1-cc tuberculin syringe. PBS with 3% sucrose (100 \(\mu\)L) was administrated as control and Ad\textit{Empty} alone was also administered as a vector control. Two days later, upon maximal protein expression, 5 mg/kg dose of BCNU (prepared as mentioned above, ~50 \(\mu\)L) was injected directly into the tumor mass. Controls were injected with the same amount of ethanol in PBS. Four hours after BCNU injection, mice were treated with either Adriamycin (3 mg/kg, diluted into injectable NaCl with a final concentration of 50 \(\mu\)L) delivered intratumorally or 30 Gy radiation given at a dose rate of 1.27 Gy/min. Before radiation, nude mice were anesthetized with 80 to 100 mg/kg ketamine/10 mg/kg xylazine i.p. and shielded with a lead block with only the tumor-bearing right hind flank exposed to radiation. BCNU and Adriamycin were obtained from the clinical pharmacy at the University of Iowa Hospitals and Clinics. Tumor volumes were measured every 3 d using a vernier caliper until the animals showed obvious signs of illness. Animals were sacrificed by CO\(_2\) asphyxiation at the end of the experiment at the University of Iowa Hospitals and Clinics. Previous work in our laboratory has shown that Ad\textit{MnSOD} alone was also administered as a vector control. Two days later, upon maximal protein expression, 5 mg/kg dose of BCNU (prepared as mentioned above, ~50 \(\mu\)L) was injected directly into the tumor mass. Controls were injected with the same amount of ethanol in PBS. Four hours after BCNU injection, mice were treated with either Adriamycin (3 mg/kg, diluted into injectable NaCl with a final concentration of 50 \(\mu\)L) delivered intratumorally or 30 Gy radiation given at a dose rate of 1.27 Gy/min. Before radiation, nude mice were anesthetized with 80 to 100 mg/kg ketamine/10 mg/kg xylazine i.p. and shielded with a lead block with only the tumor-bearing right hind flank exposed to radiation. BCNU and Adriamycin were obtained from the clinical pharmacy at the University of Iowa Hospitals and Clinics. Tumor volumes were measured every 3 d using a vernier caliper until the animals showed obvious signs of illness. Animals were sacrificed by CO\(_2\) asphyxiation at the end of the experiment at the University of Iowa Hospitals and Clinics.

**Results**

Previous work in our laboratory has shown that Ad\textit{MnSOD} infection increased MnSOD in cells at the RNA, protein, and enzymatic activity levels (27). The increase was both dose and time dependent. Once again Ad\textit{MnSOD} [100 multiplicity of infection (MOI)] infection increased MnSOD immunoreactive protein and activity in MB231 cells 24 hours postinfection, peaked at 48 hours after infection, and persisted for >72 hours (data not shown).

Our previous work showed that BCNU inhibited GR in a both dose- and time-dependent manner. To determine the effect of BCNU on GR in MB231 cells, Ad\textit{Empty}- or Ad\textit{MnSOD}-infected cells were treated with different concentrations of BCNU for 2 hours. Treatment with 5 to 50 \(\mu\)mol/L BCNU caused a dose-dependent decrease of GR activity (Fig. 1A). BCNU (25 mmol/L or above) resulted in undetectable levels of GR activity. Inhibition of GR activity after BCNU treatment was independent of the MnSOD levels in the cells as both Ad\textit{Empty} and Ad\textit{MnSOD}-infected cells...
showed a similar inhibition. Also, the activities of other major antioxidant enzymes (catalase and copper zinc SOD) were not affected by either AdMnSOD infection or BCNU treatment (Fig. 1A).

MB231 cells infected with 100 MOI AdMnSOD or AdEmpty were treated with different concentrations of BCNU (Fig. 1B). Cells were collected and GSH and GSSG were measured. BCNU (50 μmol/L) resulted in a 1.35-fold increase in the percentage of GSSG when compared with controls, whereas AdMnSOD plus BCNU (5 μmol/L) caused a 1.62-fold increase in the percentage of GSSG (P < 0.05 versus controls; means ± SE, n = 3). AdMnSOD plus BCNU (50 μmol/L) resulted in a further significant 2.1-fold increase in the percentage of GSSG in cells (P < 0.05 versus controls; means ± SE, n = 3). Thus, there was a significant increase in oxidative stress at BCNU concentrations that only partially inhibit GR as shown in Fig. 1A.

To determine if increased H$_2$O$_2$ played a role in the sensitization of MnSOD-overexpressing cells to BCNU, pyruvate, a H$_2$O$_2$ scavenger (28), was used. After AdMnSOD infection, MB231 cells were treated by BCNU with or without pyruvate. MnSOD overexpression sensitized MB231 cells to BCNU (5 μmol/L). BCNU alone or BCNU plus the AdEmpty vector control resulted in 72% clonogenic survival, whereas MnSOD-overexpressing cells in the presence of 5 μmol/L BCNU decreased clonogenic survival to 41% (Fig. 2A). Pyruvate reversed the MnSOD + BCNU–induced cytotoxicity. In the presence of pyruvate (10 mmol/L), MnSOD-overexpressing cells treated with BCNU had a similar clonogenic survival as control or vector-infected cells also treated with BCNU (Fig. 2B). These results suggest that increased cell killing by AdMnSOD plus BCNU is largely due to the presence of increased levels of H$_2$O$_2$.

Adriamycin is a quinone containing antitumor antibiotic (29) that can be reduced to the Adriamycin-free radical semiquinone (30), which then redox cycles with O$_2$ to produce O$_2^-$(31). AdMnSOD plus BCNU-sensitized cells to the cytotoxicity of Adriamycin as determined by clonogenic survival (Fig. 3A). In the clonogenic assay, Adriamycin (0.05 μmol/L) alone resulted in 30% clonogenic survival in MB231 cells, whereas the combination of AdMnSOD 100 MOI, BCNU 5 μmol/L, and Adriamycin 0.05 μmol/L resulted in 0.3% clonogenic survival (P < 0.05; means ± SE, n = 3).

Radiation produces various ROS including O$_2^-$, H$_2$O$_2$, and HO$^*$. MB231 cells were pretreated with AdMnSOD or AdEmpty for 48 hours and BCNU (5 μmol/L) for 2 hours, and then exposed to 1, 2, and 3 Gy delivered by $^{137}$C and clonogenic survival determined (Fig. 3B). Three Gy irradiation decreased clonogenic survival to 17%, whereas BCNU decreased clonogenic survival to 9%. The combination of MnSOD, BCNU, and 3 Gy-irradiation resulted in a survival fraction to 2% (P < 0.05; means ± SE, n = 3).

To test if intratumoral delivery of AdMnSOD or BCNU could alter the protein and activity levels of MnSOD and GR, respectively, MB231 cells were injected into the flank of mice and allowed to grow to ~70 mm$^3$. AdMnSOD or BCNU were injected directly into the tumor. Both Western blots and activity gels showed increased MnSOD protein and activity in tumor tissue 48 hours after AdMnSOD infection (Fig. 4A and B), whereas the activity of copper zinc SOD was not affected (data not shown). BCNU treatment did not change GR protein levels in the tumors, but it did inhibit GR activity (Fig. 4C).

In all of the in vivo experiments, the statistical analyses focused on the effects of different treatments on cancer progression. The primary outcomes of interest are time to death and tumor growth over time. To test the in vivo effects of increasing O$_2^-$ levels with Adriamycin on AdMnSOD plus BCNU, tumor xenografts were treated with nothing (controls), Adriamycin alone, AdMnSOD alone, BCNU alone, AdMnSOD + Adriamycin, AdMnSOD plus BCNU, BCNU + Adriamycin, AdEmpty plus BCNU plus Adriamycin, or the combination of AdMnSOD, BCNU, and Adriamycin.
Figure 5A provides tumor growth curves of the observed tumor volumes for all mice in the experiment. Supplementary Table S1A summarizes the mean tumor sizes in the nine groups. The sample sizes given in the table are the total number of measurements available within each group. The $P$ value was < 0.0001 for the global test of equality between the growth curves across treatment groups. Pairwise group comparisons were carried out to identify where the group differences occurred. The pairs of groups for which the $P$ values were < 0.05 are presented in Supplementary Table S2A. The combination of $\text{AdMnSOD} + \text{BCNU} + \text{Adriamycin}$ had the greatest effect in decreasing tumor volumes (Fig. 5A) and prolonging survival (Fig. 5B). As shown in Fig. 5A, at day 51, the combination of $\text{AdMnSOD}$, BCNU, and Adriamycin decreased tumor volume to an average of 64 mm$^3$ compared with 1,100 mm$^3$ in controls (Means, $P < 0.05$ versus controls; $n = 6–8$). The Log-rank test comparing the survival times across the 9 groups had a $P$ value of < 0.0001. Median survival estimates are provided in Supplementary Table S1C. Kaplan-Meier plots for the nine treatment groups are presented in Fig. 5B.

Figure 5. $\text{AdMnSOD}$ infection plus BCNU sensitized the antitumor effect of Adriamycin in vivo and increased animal survival. A, in vivo tumor growth. The combination of $\text{AdMnSOD}$ plus BCNU sensitized xenografts to the antitumor effect of Adriamycin. At day 26, the average tumor volume for the untreated control group was 950 mm$^3$; the average tumor volume for groups treated by the combination of $\text{AdMnSOD}$ plus BCNU was 30 mm$^3$. All the other treatment combinations prolonged the tumor volume growth but were not as significant as the combination of $\text{AdMnSOD}$ plus BCNU plus Adriamycin. B, Kaplan-Meier plots of estimated survival. The combination of $\text{AdMnSOD}$ plus BCNU plus Adriamycin significantly increased tumor-free survival time in nude mice. At day 54, 5 of 8 control mice were sacrificed due to the large size of the tumors. At day 26, the survival of mice treated with $\text{AdMnSOD}$ plus BCNU plus radiation was 100%. At day 80, the survival for the group treated by the combination of three reagents was 85%.

Figure 6. $\text{AdMnSOD}$ infection plus BCNU sensitized the antitumor effect of ionizing radiation in vivo and increased animal survival. A, in vivo tumor growth. The combination of $\text{AdMnSOD}$ plus BCNU plus radiation decreased MB231 tumor growth in nude mice when studied up to 34 d postradiation. At day 34, the average tumor volume for the control group was 942 mm$^3$, whereas the average tumor volume for groups treated by the combination of $\text{AdMnSOD} + \text{BCNU} + \text{radiation}$ was 47 mm$^3$. Other treatment groups including radiation alone, BCNU plus radiation, $\text{AdMnSOD}$ plus BCNU, and the combination of $\text{AdEmpty}$ plus BCNU with radiation also inhibited tumor growth but were not as effective as the combination of $\text{AdMnSOD}$ plus BCNU plus radiation. B, Kaplan-Meier plots of estimated survival. The combination of $\text{AdMnSOD} + \text{BCNU} + \text{radiation}$ significantly increased tumor-free survival time in nude mice. At day 28, 7 of 8 mice in the control group were sacrificed due to the large size of the tumors. At day 28, the survival of mice treated with $\text{AdMnSOD}$ plus BCNU plus radiation was 100%. At day 80, the survival for the group treated by the combination of three reagents was 85%.

The Log-rank test comparing the survival times across the 9 groups had a $P$ value of < 0.0001. Median survival estimates are provided in Supplementary Table S1C. Kaplan-Meier plots for the nine treatment groups are presented in Fig. 5B.
Table S1D summarizes the \( P \) values for pairwise comparison among treatment groups. Of note, the tumor-free animal survival was 62.5\% in the AdMnSOD + BCNU + Adriamycin group compared with no survivors with all other treatment protocols at day 327 \((P < 0.05\) versus other treatment combination groups; Fig. 5B).

To confirm the in vivo effects of increasing \( \text{O}_2^- \) levels with Adriamycin on AdMnSOD plus BCNU, tumor xenografts were treated with nothing (controls), Adriamycin alone, AdMnSOD + Adriamycin, BCNU + Adriamycin, or the combination of AdMnSOD, BCNU, and Adriamycin. Tumor volume in mice treated with the combination of AdMnSOD, BCNU, and Adriamycin once again was decreased significantly compared with all other treatment groups (Supplementary Fig. S1A). In addition, survival was greatest in mice with tumors treated with the combination of AdMnSOD, BCNU, and Adriamycin (Supplementary Fig. S1B).

To test the in vivo effects of increasing \( \text{O}_2^- \) levels with ionizing radiation on AdMnSOD plus BCNU, tumor xenografts were treated with nothing (controls), with radiation alone, BCNU + radiation, AdMnSOD + radiation, AdMnSOD + BCNU, AdEmpty + BCNU + radiation, and AdMnSOD + BCNU + radiation (Fig. 6A and B). The same experiment was repeated (Supplementary Fig. S2A and B), and in both sets of experiments, tumor volume was compared among the groups. The group of mice that had the combination of AdMnSOD + BCNU + radiation had the greatest inhibition of tumor growth. Supplementary Table S3A summarizes the mean tumor sizes in seven groups. Figure 6A shows the estimated growth curves from the fitted mixed linear regression model. The sample sizes given in the table are the total number of measurements available within each group. The \( P \) value was < 0.0001 for the global test of equality between the growth curves across treatment groups. Pairwise group comparisons were carried out to identify where the group differences occurred. The pairs of groups for which the \( P \) values were < 0.05 are presented in Supplementary Table S3B.

The groups of mice that received AdMnSOD + BCNU + radiation had the greatest tumor-free survival than other groups as shown in Fig. 6B and Supplementary Table S3C. Supplementary Table S3C presents the mean survival times in the seven groups. The Log-rank test comparing the survival times across the 7 groups had a \( P \) value of < 0.0001. Further pairwise comparisons identified where the group differences occur. The results are presented in Supplementary Table S3D. In this set of experiments, only 1 out of 8 controls were alive at day 28 (Fig. 6B). At day 80, AdMnSOD + BCNU + radiation increased survival and resulted in complete eradication of tumors in 6 of 7 mice resulting in an 87.5\% survival that was significantly greater than any other group of mice.

To confirm the in vivo effects of increasing \( \text{O}_2^- \) levels with ionizing radiation on AdMnSOD plus BCNU, the same experiment was repeated (Supplementary Fig. S2). Once again, tumor volume in mice treated with the combination of AdMnSOD, BCNU, and ionizing radiation was decreased significantly compared with all other treatment groups (Supplementary Fig. S2A). The \( P \) value was 0.0006 for the global test of equality between the growth curves across treatment groups. In addition, survival was greatest in mice with tumors treated with the combination of AdMnSOD, BCNU, and ionizing radiation (Supplementary Fig. S2B). Log-rank test for comparing the survival across the seven groups had a \( P \) value of 0.006. In the groups of mice that received AdMnSOD + BCNU + radiation, tumor-free survival was 80\% on day 126, whereas survival was 37\% in the group of animals treated with AdEmpty + BCNU + radiation. 28\% in the group animals receiving BCNU + radiation, and 12\% in the group of animals treated either with radiation alone or AdMnSOD + radiation. At day 126 there were no survivors in the control group (Supplementary Fig. S2B).

Discussion

Previous work in our laboratory has shown that MnSOD combined with BCNU increases cancer cell killing in contrast to the largely nontoxic tumor suppressive effect for MnSOD alone (8). Elevating MnSOD in cells enhances the conversion of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and inhibiting peroxide removal through the GPx system causes accumulation of \( \text{H}_2\text{O}_2 \) in cells, contributing to cell killing. The purpose of our current study was to further extend the cytotoxic effect of MnSOD plus BCNU by increasing endogenous \( \text{O}_2^- \). We had hypothesized that malignancies that can induce the production of \( \text{O}_2^- \) are added to MnSOD and BCNU-pretreated cells, higher concentration of \( \text{H}_2\text{O}_2 \) would be produced leading to an increase in cell death.

Our current study shows that BCNU effectively inhibited GR activity both in tissue culture cells and in tumor xenografts. The inhibition of GR by BCNU was independent of the MnSOD levels in the cells. Also, of the major antioxidant enzymes surveyed, only GR was inhibited by BCNU; other enzymes, such as MnSOD, copper zinc SOD, and CAT were not affected. Our study also showed that enforced expression of MnSOD and inhibition of GR decreased tumor cell clonogenicity in vitro, and decreased tumor xenograft growth in vivo. Most importantly, the addition of exogenous superoxide, either by Adriamycin or ionizing radiation, can enhance the antitumor effect of AdMnSOD plus BCNU.

A reasonable explanation of these results is that \( \text{H}_2\text{O}_2 \) is the main mediator in the cell killing induced by MnSOD plus BCNU. First, elevating the MnSOD level in cells enhances the conversion of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \). Second, inhibition of the GR activity in cells by BCNU resulted in inhibition of peroxide removal through the GPx pathway. Zhong and colleagues (32) showed a significant correlation between the sensitivity of glioma cells to BCNU and catalase levels suggesting that inhibition of the glutathione system results in catalase protecting against peroxide toxicity. To further support the effect of \( \text{H}_2\text{O}_2 \), our current study showed that pyruvate could reverse the sensitization to BCNU induced by MnSOD overexpression.

Adriamycin is a quinone containing antitumor antibiotic (29). It is electron affinic and the acceptance of one electron causes Adriamycin to be reduced to the Adriamycin free radical semiquinone (30). This semiquinone free radical cannot only induce DNA damage by itself but also redox cycles with \( \text{O}_2 \) to produce \( \text{O}_2^- \) (31). However, the potential of Adriamycin as a widely used anticancer drug is compromised by the development of life-threatening cardiac toxicity. It has been reported that MnSOD overexpression can alleviate the Adriamycin-induced mitochondrial damage in the heart of transgenic mice (33).

Together with surgery and chemotherapy, radiation therapy is one of the major modalities for breast cancer treatment. After ionizing radiation, MnSOD protein increased in a biphasic manner with the first peak due to a preformed MnSOD protein or MnSOD mRNA and the second peak due to an increase in new protein synthesis (34). Chronic exposure to ionizing radiation induces an adaptive response that decreases the cytotoxicity of radiation. This adaptive response is caused by the alteration of nuclear factor-\( \kappa \)-B, a stress-responsive transcription factor that regulates MnSOD
expression, which in turn enhances the expression of genes that participate in radiation-induced adaptive responses (35). Overexpression of MnSOD reduces the levels of irradiation-induced inflammatory cytokines (36), and reverses radiation-induced bone marrow inhibition, cystitis, gastroenteritis, and esophagitis (37–40). It has been reported that the murine hematopoietic progenitor cell line that overexpresses MnSOD results in significant radioprotection compared with the parental cell line (41). However, MnSOD overexpression did not protect hypoxic cells either morphologically or in a clonogenic survival study (42). Thus, overexpression of MnSOD has been shown to protect against both Adriamycin- and radiation-induced damage and cytotoxicity. Our current study shows that MnSOD overexpression can have the opposite effect resulting in increased cell damage, if peroxide removal is inhibited. Increased superoxide radical production with either ionizing radiation or Adriamycin can increase the cell killing effect of AdMnSOD plus BCNU. Our work suggests that superoxide radical when given with AdMnSOD plus BCNU could be an effective anticancer combination.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

References
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