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Forum Review

Anticancer Therapy by Overexpression of Superoxide Dismutase

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ABSTRACT

Cancer cells are in general low in the enzymatic activities of both manganese-containing (MnSOD) and copper- and zinc-containing superoxide dismutase. We have hypothesized that part of the tumor cell phenotype is due to this loss of enzymatic activity. To test this hypothesis, we have overexpressed MnSOD via plasmid and adenovirus transfection in various cancer cell types and have shown tumor suppression. This tumor suppression is via a noncytotoxic mechanism and probably occurs due to cell-cycle perturbations. We have also shown that MnSOD overexpression causes the anticancer drug 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) to have increased cytotoxicity. Our hypothesis for the mechanism of action of this combination is that overexpression of MnSOD leads to increased peroxide levels and that BCNU inhibits peroxide removal. We currently are investigating the use of adenovirus *MnSOD* plus BCNU in the treatment of cancer. Results thus far are consistent with the idea that we can use the alterations in antioxidant enzymes observed in cancer cells to therapeutic advantage. Antioxid. Redox Signal. 3, 461–472.

INTRODUCTION

OVER THE LAST THREE DECADES, a great deal of evidence has accumulated linking reactive oxygen species (ROS) and cancer. ROS have been shown to be involved in cancer formation, basic cancer cell biology, and cancer treatment. This review will focus primarily on the role of ROS in cancer biology and treatment. ROS are molecules that contain oxygen and have higher reactivity than ground state molecular oxygen. These species include not only oxygen radicals like superoxide, hydroxyl, and peroxyl radicals, but also nonradical molecules like singlet oxygen and hydrogen peroxide. ROS are generated during normal aerobic metabolism, and increased amounts of these species are produced during various forms of oxidative stress. ROS are known to react with various intracellular targets, including lipids, proteins, and DNA. ROS-induced damage can result in cell death, mutations, or carcinogenesis (8). The net intracellular concentration of ROS is the result of the production of ROS and the ability of substances to remove them.

In recent years, much evidence has accumulated suggesting that ROS at high concentrations are cytotoxic, whereas ROS at low concentrations are involved in the regulation of several key physiological processes. These processes include cell differentiation (2), apoptosis (13), and cell proliferation (41) and are thought to be regulated by redox-sensitive signal transduction pathways. As key examples, many kinases [such as Ste20-like kinase (38), c-Jun NH_2-

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terminal kinase (25), and big mitogen-activated protein kinase1 (1)], phosphatases (14), and transcription factors [including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and p53 (44)] have been shown to be redox-regulated by ROS. Also, many genes have been shown to be turned on by ROS (11, 41). Thus, it appears that ROS act as regulatory molecules in an analogous fashion to what has been observed with phosphorylation. Our work as described below suggests a key role for ROS and antioxidants in controlling cell growth in cancer cells.

ANTIOXIDANTS

Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS. These antioxidants include small molecular weight compounds, such as vitamins E and C, as well as the larger molecular weight antioxidant enzymes. There are three major types of primary intracellular antioxidant enzymes in mammalian cells: superoxide dismutase (SOD), catalase (CAT), and peroxidase, of which glutathione peroxidase (GPx) is the most prominent. The SODs convert superoxide radical into hydrogen peroxide, whereas the CATs and peroxidases convert hydrogen peroxide into water. In this way, two toxic species—superoxide radical and hydrogen peroxide—are converted to the harmless product water. These antioxidant enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells (27). SOD and CAT need no co-factors to function, but GPx requires several co-factors and secondary enzymes. Two such proteins [glutathione reductase (GR) and glucose-6-phosphate dehydrogenase] are considered secondary antioxidant enzymes because they do not act on ROS, but they enable GPx to function. The antioxidant enzyme scheme is shown in Fig. 1.

An important feature of these enzymes is that they are highly compartmentalized. In general, manganese-containing SOD (MnSOD) is localized in the mitochondria, copper- and zinc-containing SOD (CuZnSOD) in the cytoplasm and nucleus, CAT in peroxisomes and cytoplasm, and GPx in many subcellular compartments. Each of these enzymes is also found in several isoforms. One reason for the existence of many forms of each of these enzymes is to reduce oxidative stress in the various parts of the cell; different proteins are needed for different cellular and subcellular locations.

ANTIOXIDANT ENZYMES AND CANCER

It has been over 25 years since the first report was published demonstrating that the activity of MnSOD was diminished in transformed cells when compared with the normal cells from which they were derived (47). Since that time, numerous articles have been published showing altered levels of antioxidant enzymes in cancer cells; this subject matter has been reviewed several times (31–36). Cancer cells are nearly always low in MnSOD and CAT activity, and usually low in CuZnSOD activity (31–36). GPx activity is variable. Recently, it has been shown that in some cancer cells, reduced expression of MnSOD is due to mutations in the promoter of the gene (46).

Even though there is a large body of literature linking free radicals and antioxidant enzymes to cancer, most of the evidence is correlative and does not demonstrate a causal relationship. There are several lines of evidence that do imply a causal relationship. Powerful evidence for a causal relationship is that in various model systems, ROS cause cancer; moreover, antioxidants in general, and SOD and SOD-mimetics in particular, inhibit malignant transformation (8, 33, 35). Molecular biological techniques have been also used to demonstrate an important role for SOD in transformation; overexpression of MnSOD by cDNA transfection led to inhibition of radiation-induced transformation in a mouse fibroblast cell line (43).

EFFECT OF INCREASING SOD ON THE CANCER PHENOTYPE

If antioxidant enzymes are important in cancer, then normalization of the levels of these enzymes should result in reversal of at least part of the cancer cell phenotype. This hypothesis

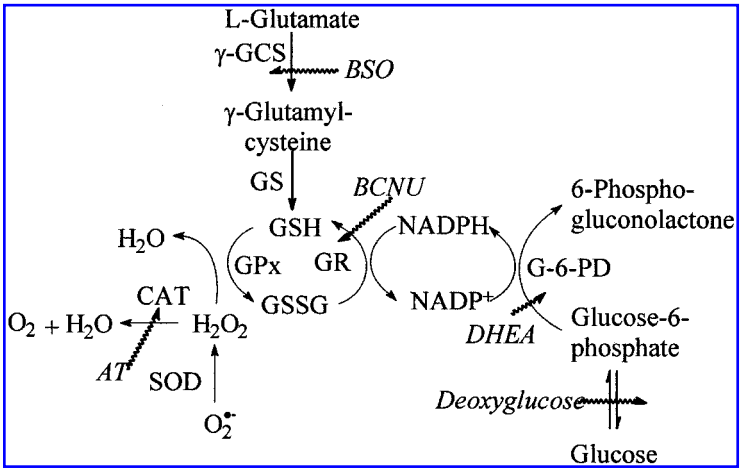

FIG. 1. Antioxidant enzyme scheme. γ-GCS, γ-glutamylcysteine synthetase; G-6-PD, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; $O_2^{\cdot -}$, superoxide. The inhibitors of the pathway are also shown: AT, 3-amino-1,2,4-triazole; BCNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; deoxyglucose, 2-deoxy-D-glucose; DHEA, dehydroepiandrosterone.

was first suggested by Oberley and Buettner in 1979 (32) and has been tested with regards to SOD in three different ways: (a) elevation of SOD by exposure to a superoxide generator and subsequent isolation of resistant cells; (b) addition of liposomal CuZnSOD protein; and (c) elevation of SOD, particularly MnSOD, by sense cDNA transfection. Each of these techniques is discussed below.

Increased SOD by exposure to superoxide

Using drug resistance experiments, Fernandez-Pol *et al.* (10) have shown a relationship between SOD and the malignant phenotype. They examined the effect of paraquat, a known superoxide producer, on Kirsten virus-transformed NRK (normal rat kidney) cells. Kirsten virus-transformed cells had much lower SOD activities than normal NRK cells. Virus-transformed cells were largely killed by paraquat, but a small fraction of these cells became resistant to paraquat. These resistant cells had much higher SOD activities than the parental cells. Moreover, the resistant (revertant) cells had an apparently normal cell phenotype: they appeared normal morphologically, did not grow in soft agar, and had a normal saturation density and serum requirement. The authors concluded: "The overall mechanism underlying the reversion of the clone RE8G3 remains to be determined rigorously; however, it may be solely due to the increases in SOD levels, which may be sufficient to result in reversion of all the transformed properties examined." One weakness of this study is that the authors did not determine which form of SOD was involved; they only measured total SOD activity. Thus, these experiments need to be duplicated and all forms of SOD quantified.

Increasing SOD by using liposomes

A second technique to elevate SOD in cancer cells is the use of liposomal SOD protein. Native SOD does not penetrate well into cells so liposomes can be used to deliver the protein. Beckman *et al.* (5) have shown that Friend erythroleukemia cells differentiate and stop proliferating in the presence of liposomal SOD. Liposomes without SOD caused no differentiation. It should be emphasized that this study used CuZnSOD. No one has yet attempted an analogous experiment with MnSOD protein. It is possible that both forms of SOD protein suppress the malignant phenotype.

Increasing SOD by cDNA transfection

A third way to deliver SOD to cancer cells is the use of cDNA transfection. The first article using this technique with MnSOD was pub-

lished in 1993 (9). In collaboration with Drs. Sue Church and James Grant at Washington University, we demonstrated that the transfection of *MnSOD* cDNA into cultured human melanoma cells resulted in the loss of the malignant phenotype. The malignant phenotype was tested both *in vitro* by assays such as growth in soft agar and, more importantly, *in vivo* by growth in nude mice. All of the tests showed a loss of the malignant phenotype in clones that overexpressed MnSOD by at least fivefold. In culture, cells overexpressing MnSOD had a slower mitotic rate as demonstrated by proliferating cell nuclear antigen staining. In the nude mouse assay, 18 of 18 sites injected with the parental melanoma cell line developed tumors, whereas 0 of 16 sites injected with melanoma cells expressing high levels of MnSOD developed tumors.

A second article on *MnSOD* cDNA transfection demonstrated a suppression of the metastatic phenotype in a mouse fibrosarcoma cell line (40). Clones overexpressing MnSOD exhibited a decreased metastatic rate, with the highest overexpressor showing no metastases. This research group also has shown that in the fibrosarcoma cell line, clones overexpressing MnSOD demonstrate reduced tumorigenicity as evidenced by a large increase in the number of tumor cells required to cause tumor formation in one-half the mice (TD_{50}) in a syngeneic model system (43).

We have recently published articles on five other cancer cell types and one virally transformed cell line showing that overexpression of MnSOD in each of these cell lines led to suppression of cell growth both *in vitro* and *in vivo*. Growth suppression was observed in human breast carcinoma MCF-7 cells (17), virally transformed WI-38 human lung fibroblasts (48), A172R rat glioma (51), U118 human glioma (52), human oral squamous carcinoma SCC-25 (24), and human prostatic carcinoma DU145 cells (19; work done in collaboration with Dr. Terry Oberley at the University of Wisconsin). The results from the oral squamous carcinoma studies (24) were especially informative. In these experiments, MnSOD-overexpressing clones were isolated and shown to have two- to fivefold increased MnSOD activity compared with wild type or control vector-transfected cell clones. The growth of these cells in nude mice was inversely proportional to the MnSOD activity, *i.e.*, the higher the MnSOD activity, the slower the growth. When the tumor volume of individual clones was plotted against the MnSOD activity, a strong negative correlation was found, with a correlation coefficient of -0.920 ($p = 0.001$). A >90% inhibition of tumor growth was found in the highest MnSOD-overexpressing cell line. This result is strong evidence that increased MnSOD activity inhibits human oral cancer growth.

In addition to these published studies, Dr. Grace Wong while at Genentech demonstrated a suppression of the malignant phenotype by MnSOD overexpression in four other cancer cell lines (personal communication). Moreover, Dr. Andres Melendez at the Albany Medical College has completed work showing that elevation of MnSOD suppresses the malignant phenotype in human fibrosarcoma cells (28). Therefore, in 13 of 13 tumor types examined, overexpression of MnSOD led to suppression of at least part of the tumor cell phenotype. This work has been done at six different institutions (The University of Iowa, Washington University, University of Kentucky, University of Wisconsin, Genentech, and Albany Medical College). Thus, the evidence appears substantial that MnSOD elevation by cDNA transfection can suppress the malignant phenotype in a great variety of tumors. This work showing growth suppression and the fact that loss of heterozygosity for *MnSOD* has been found in human melanoma (29) and glioma (22) is the basis for the hypothesis proposed by us and others that *MnSOD* is a new type of tumor suppressor gene (7).

EFFECT OF MnSOD POLYMORPHISM ON TUMOR SUPPRESSIVE EFFECT OF MnSOD

One of our major findings is that a polymorphism in MnSOD can affect its tumor suppressive effect. It has been previously demonstrated that the MnSOD protein has two naturally occurring variants at amino acid 58; either isoleucine (Ile) or threonine (Thr) can be at this position in the protein (6). The isolated

Ile^{58} protein was found to possess twice the enzymatic activity of the Thr^{58} form and to be more stable against heat. We sequenced the cDNA we had been transfecting and found it contained the lesser activity Thr^{58} form. We used site-directed mutagenesis to make the Ile^{58} form. We then transfected both forms into wild-type MCF-7 cells and isolated overexpressing clones (49). Four clones overxpressing Thr^{58} MnSOD and eight clones overexpressing Ile^{58} MnSOD were isolated and characterized. The Ile^{58} clones had three times the specific activity of the Thr^{58} form. Both forms of the MnSOD had tumor suppressive activity that was in general proportional to the MnSOD activity. The Ile^{58} clones had a higher tumor suppressive effect apparently because they had higher MnSOD activity. These data suggest that the activity of the protein is the cause of the tumor suppressive effect and not a nonenzymatic property of the protein, or some effect of the RNA. We are currently working on making inactive mutant forms of the protein to examine further this question.

We believe our results have far-reaching implications. An article has recently appeared demonstrating that another polymorphism for MnSOD may cause increased risk for breast cancer (3). A valine or alanine can be at the -9 position in the MnSOD presequence. Premenopausal women who were homozygous for the alanine allele had a fourfold increase in breast cancer risk compared with those with one or two valine alleles. This suggests that MnSOD polymorphisms may be important in cancer susceptibility. It is very logical that individuals who have a MnSOD protein that is less active should be more susceptible to oxidative stress. Hence, we would predict that individuals who express the Thr^{58} form of MnSOD may be much more susceptible to cancer than those who express the Ile^{58} form.

BIOLOGICAL MECHANISM OF MnSOD AS A TUMOR SUPPRESSOR

We are currently investigating the mechanism of the tumor suppression by MnSOD overexpression. Our studies to date have eliminated necrosis, apoptosis, and inflammatory events. In other words, we see no evidence of cell death as a mechanism. We feel that the effects of MnSOD overexpression on cancer cells are due to a noncytotoxic tumor suppressive effect. We have demonstrated changes in cell-cycle parameters following MnSOD overexpression using flow cytometry (20). Our working hypothesis at present is that MnSOD overexpression leads to changes in the superoxide/hydrogen peroxide balance and this causes changes in the redox state that affects signal transduction pathways modulating cell proliferation. This is a reasonable hypothesis because in the last several years, there has been an explosion of literature demonstrating that kinases, phosphatases, and transcription factors are all redox-modulated by ROS (1, 14, 25, 38, 44). We recently published an article showing that MnSOD overexpression leads to an inhibition of the AP-1 transcription factor and an activation of the NF-κB transcription factor in human breast cancer cells (18). Our hypothesis is supported by the recent work in Gisela Storz's laboratory at the NIH. They have shown that the OxyR transcription factor in *Escherichia coli* is activated through the formation of a disulfide bond and is deactivated by enzymatic reduction with glutaredoxin (50). The OxyR transcription factor is sensitive to oxidation and activates the expression of genes in response to hydrogen peroxide. The redox potential of OxyR was determined to be -185 mV; as the redox potential of *E. coli* cytosol is -280 mV, OxyR is reduced in the absence of stress. These results are an example of redox signaling through disulfide bond formation and reduction. A similar example in *E. coli* is the Sox R transcription factor, which activates genes in response to superoxide radicals; Sox R is an iron-sulfur protein whose activation is redox-modulated through superoxide radical. Thus, in *E. coli* the redox potential of the cell is modulated by superoxide and hydrogen peroxide; the redox potential in turn governs the activity of these two transcription factors that regulate the expression of a number of genes. We believe similar regulation occurs in mammalian cells, although the regulation is far more complex.

In collaboration with Dr. Bharat Aggarwal at M.D. Anderson Cancer Center, we have shown

that MnSOD overexpression suppresses tumor necrosis factor (TNF)-induced apoptosis and activation of NF-κB and AP-1 transcription factors (26). Thus, overexpression of MnSOD in MCF-7 cells completely abolished NF-κB activation, IκBα degradation, p65 nuclear translocation, and NF-κB reporter gene expression. Besides TNF, phorbol ester-, okadaic acid-, ceramide-, and lipopolysaccharide-induced activation of NF-κB was blocked by MnSOD, indicating a common pathway of activation. In addition, MnSOD blocked the TNF-mediated activation of AP-1, stress-activated c-Jun protein kinase, and mitogen-activated protein kinase kinase. These results demonstrate that MnSOD overexpression can have a dramatic effect on signal transduction pathways.

MOLECULAR SPECIES RESPONSIBLE FOR TUMOR SUPPRESSIVE EFFECT OF MnSOD

So far, three species have been suggested as effectors for the MnSOD tumor suppressive effect: superoxide radical, hydrogen peroxide, and nitric oxide. Superoxide radical and hydrogen peroxide are logical because they are the substrate and product of SOD. Our work has focused on testing hydrogen peroxide as an effector, because there are enzymes that specifically remove this species. As will be discussed in more detail later, we have already transfected GPx1 into MnSOD-overexpressing cells and demonstrated an inhibition of the tumor suppressive effect. As GPx1 can also remove lipid hydroperoxides, it is unclear if its effect is due to hydrogen peroxide or other hydroperoxides. For this reason, we plan in the future to transfect both peroxisomal and mitochondrial CAT into MnSOD-overexpressing cells to determine the effect. CAT does not effectively act on lipid hydroperoxides, but does remove hydrogen peroxide effectively.

Andres Melendez has suggested that nitric oxide is involved in the suppression of cancer cell proliferation by MnSOD (28). He found that MnSOD overexpression enhanced the cytostatic action of three nitric oxide donor compounds. Thus, nitric oxide enhanced the inhibition of cell proliferation caused by MnSOD overexpression. This is in contrast to our earlier work showing that MnSOD overexpression greatly protected against nitric oxide toxicity (12). The two studies are not contradictory because they use different cell types and doses of nitric oxide and have different end points. The Melendez study uses nonkilling doses of nitric oxide and studied cell proliferation as an end point. In our study, we used lethal doses of nitric oxide and studied cell survival as an end point. Indeed, in the Melendez study, the effect of MnSOD overexpression is lost at high concentrations of nitric oxide donors. Hence, nitric oxide concentrations may determine the results.

EFFECT OF GPx ON THE TUMOR SUPPRESSIVE EFFECT OF MnSOD

The hypothesis driving our research has been that increasing MnSOD leads to an increase in hydrogen peroxide levels. However, others have claimed that increasing SOD will lead to a decline in superoxide levels, but no change or a decline in hydrogen peroxide levels. For this reason, Omar and McCord have suggested that increasing SOD will lead to an increase in levels of lipid hydroperoxides, but not in hydrogen peroxide (37). As a first attempt to study this question, we have transfected cytosolic GPx (GPx1) into cells already overexpressing MnSOD (21). We have found that the growth suppression caused by MnSOD overexpression is eliminated by also increasing GPx. That is, cancer cells overexpressing both MnSOD and GPx no longer show a growth inhibition. This result suggests that the tumor growth inhibition caused by MnSOD overexpression is due to hydroperoxides. It does not tell us what type of hydroperoxide because GPx is fairly promiscuous; it uses as substrate a wide variety of hydroperoxides, including lipid hydroperoxides, as well as hydrogen peroxide. In the future, we plan to transfect CAT into MnSOD-overexpressing cells. CAT acts on hydrogen peroxide, but only poorly on lipid hydroperoxides. In this way, we can show what is the molecular effector of the MnSOD tumor suppression. In these experiments, a mitochondrial presequence like that of MnSOD

must be ligated to the *CAT* gene; otherwise the CAT protein will not be targeted to the mitochondria. This was no problem with the GPx because we have shown using immunogold electron microscopy that the overexpressed GPx is found in mitochondria. In preparation for this work, we have obtained expression plasmids for both peroxisomal CAT and mitochondrial CAT from Dr. Andres Melendez of the Albany Medical College. He has already shown that both plasmids express CAT in the proper subcellular location (4). Very recently, his group has shown that transfection of either cytosolic or mitochondrial CAT into fibrosarcoma cells reversed the proliferative and clonogenic inhibition associated with MnSOD overexpression (39). As overexpression of GPx or CAT inhibits the tumor suppressive effect of MnSOD, inhibition of GPx or CAT should have the opposite effect, *i.e.*, greater growth suppression.

EFFECT OF CuZnSOD OVEREXPRESSION

Most of the SOD transfection work published so far has involved MnSOD. Some work has also been done with CuZnSOD. Recently, the importance of CuZnSOD has been examined by transfecting antisense *CuZnSOD* cDNA and demonstrating increased malignancy. Muramatsu *et al.* (30) examined two human oral squamous carcinoma-derived clones, SAS-H1 with high invasiveness and SAS-L1 with low invasiveness. Clone SAS-H1 exhibited significantly greater motility than SAS-L1, but had significantly lower levels of CuZnSOD activity than SAS-L1 cells. *CuZnSOD* antisense cDNA was transfected into SAS-L1 cells. Antisense cDNA transfected clones had lower CuZnSOD activity than vector control clones, and this was associated with increased motility. Invasiveness of the parental and antisense clones was enhanced by superoxide treatment, whereas the invasiveness of SAS-L1 was unaffected. The authors concluded that CuZnSOD was "involved in cell motility by virtue of its action in scavenging superoxide in the cells." This same group has found similar results in murine fibrosarcoma Meth A cells (45); expression of antisense *CuZnSOD* cDNA led to inhibition of CuZnSOD activity and increased metastases. In these cells, the inhibition of CuZnSOD did not cause a change in cell growth *in vitro* or *in vivo* in nude mice.

INCREASED ANTICANCER CYTOTOXICITY WITH BCNU IN CELL CULTURE

We have found that MnSOD in combination with certain chemicals can have an anticancer effect that causes cell killing in contrast to the noncytotoxic tumor suppressive effect described above for MnSOD alone. The enzymatic effect of MnSOD protein is to dismute superoxide radical into hydrogen peroxide. If we inhibit hydrogen peroxide removal, then we should kill cancer cells because of direct toxicity or hydrogen peroxide-mediated damage. We have tested this idea in tissue culture using stable plasmid transfected rat glioma cells and found very positive results (51). The higher the MnSOD levels, the higher the killing we observed in cells treated with 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) or buthionine sulfoximine (BSO). BCNU is a clinically used anticancer drug that causes alkylation and also inhibits GR. If GR is inhibited, cells cannot remove hydrogen peroxide (51). BSO is a competitive inhibitor of a glutathione synthesis protein. If BSO is given to cells, then glutathione is depleted and again hydrogen peroxide cannot be removed by the GPx system. As an example of the effectiveness of this combination, with wild-type and vector control cells, ~10% of the cells were killed by 50 μM BSO, whereas 100% of the high MnSOD-overexpressing cells were killed by the same dose of BSO (51).

ADENOVIRUS TRANSDUCTION OF *MnSOD* cDNA INTO HAMSTER ORAL CANCER *IN VITRO*

Plasmid transfection is notoriously inefficient with only a small percentage of the cells expressing the transfected gene. For this reason, plasmid transfer can be difficult to use effectively *in vivo.* To increase transfection effi-

cacy, we have shifted our studies to a much more efficient vector—adenovirus. We have demonstrated that adenovirus can be used *in vitro* to deliver MnSOD to HCPC-1 hamster oral squamous carcinoma cells (15). Nearly 100% of the transduced cells overexpressed MnSOD. A six- to sevenfold increase in MnSOD activity was found in cells transduced with 100 MOI Ad*MnSOD*, showing that the expressed protein had enzymatic activity. Overexpression of MnSOD activity via adenovirus transduction led to a 50% reduction in growth rate *in vitro* and a two-thirds reduction in plating efficiency and growth in soft agar. Thus, adenovirus transduction could be used effectively to deliver the *MnSOD* cDNA and inhibit hamster oral cancer growth.

ADENOVIRUS TRANSDUCTION OF MnSOD cDNA IN HAMSTER ORAL CANCER *IN VIVO*

To determine whether adenovirus infection could work *in vivo*, we did two preliminary short-term experiments. Two million hamster oral carcinoma cells (HCPC-1) were delivered subcutaneously into the flank region of nude mice. The tumors were allowed to grow until they reached $\sim$50 mm^3. At this time, $\sim 1 \times 10^9$ plaque forming units (PFU) of Ad*MnSOD* were delivered directly into the tumor. Subsequently, 5×10^8 PFU of Ad*MnSOD* were delivered on days 5 and 10 in one ("short term") experiment and on days 5, 10, 15, and 21 in the other ("long-term") experiment. Control animals received no treatment, serum-free media, or Ad*LacZ* in similar volumes and PFUs at the same time points. The same total volume of serum-free media or adenovirus constructs was delivered to four or more sites in the tumor, depending on tumor size at the time of injection.

The results of these two experiments suggested that Ad*MnSOD* alone caused growth inhibition (16). Most importantly, the tumors removed from the killed mice still showed an elevation of immunoreactive MnSOD protein as evidenced by western blotting. These results suggested that further investigation of Ad*MnSOD* was warranted and resulted in the work discussed below.

COMBINATION OF Ad*MnSOD* WITH CYTOTOXIC ANTICANCER AGENTS *IN VITRO*

Even though we were able to demonstrate a significant growth inhibitory effect of Ad*MnSOD* by itself, we did not think that this treatment would be an effective clinical antitumor therapy. This lack of effect is because when injection of the adenovirus is terminated, the cancer cells may start proliferating again. The resumption of proliferation is likely because, unlike the plasmid transfections, the adenovirus does not integrate into the genome, but replicates episomally. Thus, with time in a dividing cell population, the transgene is diluted. In humans, no more than a few injections of the adenovirus can be given, because an immune response is mounted against the virus. This presumed lack of a persistent growth regulatory effect prompted us to try a different approach to circumvent this shortcoming; we decided to try a combination of adenovirus with cytotoxic anticancer agents. In this approach, the short life of the adenovirus is not a shortcoming, but a benefit. The adenovirus is given, causes cell killing in conjunction with the cytotoxic agent, and then disappears. Thus, any negative effect of the adenovirus will not linger.

As discussed earlier, the rationale for the use of Ad*MnSOD* with cytotoxic agents is the following. The enzymatic effect of MnSOD protein is to dismute superoxide radical into hydrogen peroxide. If we inhibit hydrogen peroxide removal, then we should kill the cancer because of direct toxicity or hydrogen peroxide-mediated damage. It should be emphasized that our results are also consistent with increased lipid hydroperoxides produced by SOD overexpression as postulated by Omar and McCord (37).

To determine whether overexpression of MnSOD via an adenovirus construct will cause increased cytotoxicity when given with BCNU, we have studied the cytotoxicity exerted by the adenoviral vectors in combination with BCNU on human oral squamous carcinoma SCC-25 cells in culture. The annexin/propidium iodide survival assay and flow cytometry were used to measure apoptosis, necrosis, and total cell

killing. The control and all of the cells treated with adenoviruses alone showed >90% survival. Cells treated with 250 μM BCNU alone showed ~60% survival. Cells treated with Ad*MnSOD* or Ad*CuZnSOD* plus BCNU demonstrated ~20% survival. Ad*CAT* prevented the increased killing caused by SOD overexpression. We have also demonstrated in these cells that the adenovirus constructs perform as expected, leading to increases in antioxidant enzyme immunoreactive protein and enzymatic activity. These results show convincingly that SOD overexpression *in vitro* in the presence of BCNU can lead to a greater increase in the killing of human oral cancer cells.

ADENOVIRUS PLUS BCNU *IN VIVO*

Because of our success *in vitro* and in preliminary experiments with Ad*MnSOD* alone *in vivo*, we decided to do complete *in vivo* experiments with Ad*MnSOD* plus BCNU. Four million human oral carcinoma cells (SCC-25) were delivered subcutaneously into the flank region of nude mice. The tumors were allowed to grow until they reached ~50 mm^3. At this time, $\sim 1 \times 10^9$ PFU of Ad*MnSOD* were delivered directly into the tumor. Two days later, the mice were injected with BCNU intratumorally at 15 or 30 mg/kg. Control animals received no treatment, Ad*LacZ* in similar volumes and PFUs, 100% ethanol, 3% sucrose, BCNU alone at 30 mg/kg, Ad*MnSOD* alone, and Ad*MnSOD* plus Ad*CAT* at the same time points. None of the controls, including Ad*LacZ,* showed significant anticancer effects. Ad*MnSOD* plus BCNU was the most effective agent in inhibiting tumor growth. It should be noted that BCNU alone was given at 30 mg/kg, whereas in the combination BCNU was only given at 15 mg/kg. Thus, Ad*MnSOD* allows us to get a greater anticancer effect with BCNU at half the dose than obtained with BCNU alone at twice the dose. At day 604, animal survival was 50% in the Ad*MnSOD* plus BCNU group, whereas it was 0% in all the control groups including BCNU alone. Moreover, animals treated with BCNU alone were only 50% cancer-free on day 466, whereas those treated with Ad*MnSOD* plus BCNU were 100% cancer-free. Thus, these data showed a very significant anticancer effect of Ad*MnSOD* plus BCNU in human oral cancer.

Moreover, it should be emphasized that we have seen no evidence of any normal cell toxicity when the adenovirus is given intratumorally; almost no animals treated with our antitumor combination died, and the few that have died appeared to have been contaminated by microorganisms. We have recently done a toxicity experiment in which we used the highest virus concentration that the Gene Transfer Vector Facility at Iowa can make. Thus, to study the normal cell toxicity of Ad*MnSOD*, normal ND4 mice (five per group) were injected intraperitoneally with 1 ml of Ad*MnSOD* with final particle concentrations of 10^7, 10^8, 10^9, 10^{10}, and 2×10^{11} PFU per mouse. The highest concentration of adenovirus we could obtain was 200 times that normally given in our *in vivo* experiments. The mice were monitored for 2 weeks, and no toxic effects were observed. Thus, we feel that this anticancer combination shows great promise as a new type of anticancer therapy. It should be emphasized that all our experiments use intratumor injections because adenovirus given systemically has been shown to cause some normal cell toxicity.

CONCLUSIONS

We have shown that overexpression of MnSOD can inhibit tumor cell growth both *in vitro* and *in vivo* by a noncytotoxic mechanism. At the same time, overexpression of MnSOD in combination with BCNU inhibits tumor cell growth by a cytotoxic mechanism. To express MnSOD *in vivo*, we have used an adenovirus construct. As adenovirus given systemically shows normal cell toxicity, we feel the best way to put this into humans is to use some method of intratumor delivery. We are currently seeking ways to obtain clinical grade adenovirus made so that we can put this promising antitumor combination into a human clinical trial. Other methods to raise SOD in cells may also prove advantageous in the treatment of cancer. Thus, SOD mimics may prove useful. Also, the type and location of SOD may prove important and need to be investigated in the future. Thus,

CuZnSOD and extracellular SOD should also be studied, as well as SODs expressed in new locations. Therapies of cancer based on SOD activity show real promise for the future.

ABBREVIATIONS

Ad*CAT*, adenovirus *CAT*; Ad*CuZnSOD*, adenovirus *CuZnSOD*; Ad*MnSOD*, adenovirus *MnSOD*; AP-1, activator protein-1; BCNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; CAT, catalase protein; *CAT*, catalase gene; CuZnSOD, copper- and zinc-containing superoxide dismutase protein; *CuZnSOD*, copper- and zinc-containing superoxide dismutase gene; GPx, glutathione peroxidase protein; GR, glutathione reductase; Ile, isoleucine; MnSOD, manganese-containing superoxide dismutase protein; *MnSOD*, manganese-containing superoxide dismutase gene; NF-κB, nuclear factor-κB; PFU, plaque forming units; ROS, reactive oxygen species; SOD, superoxide dismutase; Thr, threonine; TNF, tumor necrosis factor.

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