INHIBITION OF TUMOR CELL GROWTH BY OVEREXPRESSION OF MANGANESE-CONTAINING SUPEROXIDE DISMUTASE

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It has been almost twenty years now since the first report appeared demonstrating that the activity of the antioxidant enzyme manganese-containing superoxide dismutase (MnSOD) was diminished in transformed cells when compared to an appropriate normal cell control (1). Since that time, numerous papers have been published showing altered levels of antioxidant enzymes in cancer cells; this subject matter has been reviewed many times (2-9). Cancer cells are nearly always low in MnSOD and catalase (CAT) activity, and usually low in CuZnSOD activity (2-9). Glutathione peroxidase (GPX) activity is variable.

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 such a causal relationship is that in various model systems, reactive oxygen species (ROS) cause cancer and antioxidant in general, and SOD and SOD-mimetics in particular, inhibit malignant transformation (4, 7, 10). Recently, molecular biological techniques have been utilized to demonstrate an important role for SOD in transformation; overexpression of MnSOD by cDNA transfection led to inhibition of radiation-induced malignant transformation in a mouse fibroblast cell line (11).

If antioxidant enzymes are important in cancer, then normalization of these enzymes should result in reversal of at least part of the cancer cell phenotype. This hypothesis was first suggested by Oberley and Buettner in 1979 (2). This hypothesis has been examined in three different ways with regard to SOD: 1) elevation of SOD by exposure to a superoxide generator and subsequent isolation of resistant cells; 2) addition of liposomal CuZnSOD; and 3) elevation of MnSOD by sense cDNA transfection. Each of these techniques will be discussed below.

Fernandez-Pol *et al.* (12) have shown using drug resistance experiments 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 very high SOD activities. 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. One weakness of this study is that the authors did not determine which form of SOD was involved; they just measured total SOD activity. Thus, these experiments need to be duplicated and all forms of SOD quantitated.

A second technique to elevate SOD in tumor cells is the use of liposomal SOD. Native SOD does not penetrate well into cells and so liposomes can be used to deliver the protein. Beckman *et al.* (13) 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. It is possible that both forms of SOD protein suppress the malignant phenotype.

A third way to deliver SOD to tumor cells is the use of cDNA transfection. The first paper using this technique appeared in 1993. Drs. Sue Church and James Grant at Washington University in collaboration with our group at Iowa showed transfection of MnSOD cDNA into cultured human melanoma cells resulted in the loss of the malignant phenotype (14). 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 these tests showed a loss of the malignant phenotype in clones that overexpressed MnSOD by at least five-fold. The most important observation was that from the nude mouse assay; 18 out of 18 sites injected with the parental melanoma cell line developed tumors, while 0 out of 16 sites injected with melanoma cells containing high levels of MnSOD developed tumors. Interestingly, clones that had only a three-fold elevation of MnSOD formed tumors in nude mice, suggesting a threshold level of the enzyme was necessary to suppress malignancy. A paper has recently appeared which claims that MnSOD does not suppress tumorigenicity or metastasis in another human melanoma cell line (15). I would like to emphasize that I feel that this paper is incorrect in its conclusions. This is because even though transfections were performed, the clones that were isolated had increased MnSOD mRNA, but not increased MnSOD

protein or MnSOD activity. Thus, the authors did show that increased MnSOD mRNA did not suppress the malignancy of this cell line, but they did not test the hypothesis that increased MnSOD protein or activity can suppress the malignancy of this cell line. Our work as discussed below showed a strong correlation between MnSOD enzymatic activity and tumor suppression.

We have published papers on four other malignant cell lines and one partially transformed line that demonstrate that transfection of MnSOD cDNA leads to suppression of at least part of the cancer cell phenotype. By order of publication, we have shown that overexpression of MnSOD inhibited transformed cell growth in human breast cancer MCF-7 cells (16), SV-40 transformed WI-38 human lung fibroblasts (17), A172R rat glioma cells (18), U118 human glioma cells (19), and human oral squamous carcinoma SCC-25 cells (20). This last paper was especially informative because we isolated several clones with different levels of MnSOD activity. As in the other papers, cells with high MnSOD activity grew slower when injected in nude mice. Importantly, in these experiments, tumor volume was inversely proportional to MnSOD activity (correlation coefficient of -0.929 and $p=0.001$). Since there was such a high inverse correlation between MnSOD activity and tumor growth, the data suggest, but do not prove, that the enzymatic activity of the MnSOD protein is what caused the tumor suppressive effect. Moreover, it suggests that the higher the MnSOD activity, the more the growth of the tumors is suppressed.

Thus, all three methods to increase SOD in tumor cells (selection of superoxide-resistant cells, addition of liposomal SOD, and transfection of MnSOD cDNA) have led to the same conclusion: elevation of SOD leads to suppression of at least part of the tumor cell phenotype. Thus, it appears that MnSOD functions as a tumor suppressor in a wide variety of cancers. Recently, the importance of SOD has been examined in a different way — by transfecting antisense CuZnSOD cDNA and demonstrating increased malignancy. Muramatsu *et al.* (21) examined two human squamous carcinoma-derived clones, SAS-HI with high invasiveness and SAS-LI with low invasiveness. Clone SAS-HI exhibited significantly greater motility than SAS-LI, but had significantly lower levels of CuZnSOD activity than SAS-LI cells. CuZnSOD antisense cDNA was transfected into SAS-LI 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 were enhanced by superoxide treatment, while the invasiveness of SAS-LI was unaffected. The authors concluded that CuZnSOD was “involved in cell motility by virtue of its action in scavenging superoxide in the cells.”

A central question that remains is why does elevation of SOD have any effect? In other words, what is the mechanism whereby SOD exerts its tumor suppressive action? Mechanism can be examined in several different ways. The first mechanistic question that can be asked

is what the molecule that is responsible for the effect? There are three likely candidates: superoxide radical, hydrogen peroxide, and nitric oxide radical. With regard to this question, the importance of hydrogen peroxide can and is being studied directly by GPX transfection into MnSOD-overexpressing cell lines; if hydrogen peroxide is the causative molecule, the GPX overexpression should have a dramatic effect on tumor cell growth. We have produced some evidence that nitric oxide is not likely the major causative molecule (22). A second mechanistic question is what is the target of the causative molecule? This question can be asked with regards to molecules like proteins, lipids, DNA, etc. as well as with subcellular organelles such as mitochondria, nucleus, etc. Since active MnSOD is only known to be found in mitochondria, this organelle is a likely target. A third question is whether the effects of MnSOD overexpression are due to cell protection, cell damage, or physiological regulation? This question will be the major focus of our future research. In general, SOD could have an effect because its presence protects important molecules, or damages certain molecules, or because its substrates or products affect key metabolic pathways such as those that control cell division.

One exciting hypothesis for mechanism is that MnSOD overexpression leads to changes in physiological pathways. This is a logical proposal because superoxide radical and hydrogen peroxide have been shown to modulate many cellular activities. Moreover, in recent years, it has been demonstrated convincingly that many transcription factors are redox-modulated (23). Both DNA binding and actual transcriptional activity (transactivation) have been shown to be modulated by redox-active factors. For this reason, we have examined two of the prominent redox-active transcription factors (AP-1 and NF- κ B) in MnSOD-overexpressing cells (24). The hypothesis behind this work was that MnSOD overexpression would lower the levels of superoxide radical and increase hydrogen peroxide levels and one or both of these species could affect transcription factor activity. Using both reporter construct and DNA-binding assays, we have found modulation of both AP-1 and NF- κ B by MnSOD overexpression. This data suggests that one way that increased MnSOD levels inhibits tumor cell growth is through modulation of transcription factor activity. This exciting work will be continued in the future to determine the true mechanism of tumor suppression by MnSOD.

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