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Invited Review

Antioxidant enzyme levels in cancer

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Summary. Normal cells are protected by antioxidant enzymes from the toxic effects of high concentrations of reactive oxygen species generated during cellular metabolism. Even though cancer cells generate reactive oxygen species, it has been demonstrated biochemically that antioxidant enzyme levels are low in most animal and human cancers. However, a few cancer types have been found to have elevated levels of antioxidant enzymes, particularly manganese superoxide dismutase. Morphologic studies of animal and human cancer have confirmed that although the majority of tumor cell types from several organ systems have low antioxidant enzymes, adenocarcinomas may have elevated manganese superoxide dismutase and catalase levels. However, all cancers examined to date have some imbalance in antioxidant enzyme levels compared with the cell of origin. Antioxidant enzyme importance in cancer genesis has been difficult to evaluate in early cancerous lesions using biochemical techniques because such lesions are small and therefore below the level of detection. Using immunohistochemical techniques, early lesions of human and animal cancers were demonstrated to have low antioxidant enzymes, thus suggesting a role for these enzymes both in the genesis of cancer and the malignant phenotype. All but one human cancer cell type (the granular cell variant of human renal adenocarcinoma) examined showed both low catalase and glutathione peroxidase levels, suggesting that most cancer cell types cannot detoxify hydrogen peroxide. Our results to date are used to propose new cancer therapies based on modulation of cellular redox state.

Key words: Antioxidant enzymes, Reactive oxygen species, Superoxide dismutase, Carcinogenesis, Malignant phenotype

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Introduction

Reactive oxygen species are molecules that contain oxygen and have higher reactivity than ground state molecular oxygen. These species include not only the oxygen radicals (superoxide, hydroxyl, and peroxyl radicals), but also non-radical molecules like hydrogen peroxide and singlet oxygen. Reactive oxygen species are produced during normal aerobic metabolism, and increased levels of reactive oxygen species are generated when cells are exposed to certain forms of stress. High levels of reactive oxygen species are detrimental to cells through reactions with many intracellular targets, including proteins, lipids, and DNA. However, numerous recent studies have indicated that reactive oxygen species at low concentrations have a variety of physiologic functions, including regulation of gene transcription (Schreck et al., 1991), signal transduction pathways (Chen et al., 1995; Lander et al., 1995; Russo et al., 1995; Sundaresan et al., 1995), mitosis (Shibanuma et al., 1988), apoptosis (Hockenbery et al., 1993), and senescence (de Haan et al., 1996).

The intracellular concentration of reactive oxygen species is a consequence of both the production of these species and their removal by various antioxidants. Cells contain a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species. These include a number of small molecular weight antioxidants such as glutathione and vitamins E, C, and A. The antioxidants also include three types of larger molecular weight primary antioxidant proteins - the enzymes superoxide dismutase (McCord and Fridovich, 1969), catalase (Percy, 1984), and glutathione peroxidase (Mills, 1957). Superoxide dismutase converts superoxide radical into hydrogen peroxide, while catalase and glutathione peroxidase convert hydrogen peroxide into water. In this way, two toxic species - superoxide radical and hydrogen peroxide - are converted into the harmless product, water. These enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells (McCord et al., 1971). In addition to the protective effects of antioxidant

enzymes, the fact that low levels of reactive oxygen species can affect physiologic processes suggests the possibility that antioxidant enzymes can also have a regulatory role in key cell functions.

An important feature of the primary antioxidant enzymes is that each one exists in several forms. It is thought that there are several forms so that each compartment in the cell is protected against reactive oxygen species. For example, there are two main forms of superoxide dismutase inside cells: a copper- and zinc-containing superoxide dismutase found predominantly in the cytoplasm (McCord and Fridovich, 1969), and a manganese-containing superoxide dismutase found primarily in the mitochondrial matrix (Weisiger and Fridovich, 1973). Another copper- and zinc-containing superoxide dismutase is found in extracellular fluids (Marklund, 1982). There are at least two forms of catalase found in mammalian cells; catalase is found both in the cytoplasm and the peroxisomes (Peeters-Juris et al., 1975). Four forms of glutathione peroxidase have been demonstrated to date; these glutathione peroxidase proteins are found in many subcellular locations, including nucleus, cytoplasm, and mitochondria (Muse et al., 1994).

II. Biochemical analysis of antioxidant enzyme levels in normal compared with cancer tissues

Biochemical studies of normal tissues or organs have shown a large variability in the levels of antioxidant enzymes (Marklund et al., 1982). Presumably, these levels reflect the unique metabolism of each organ. A major problem with most biochemical studies is that tissue or organ homogenates are used as the starting material; tissues and organs contain several cell types, and it is not possible by measuring activities of tissue homogenates to determine the contribution of each cell type to the total enzyme activity. Even in a relatively homogeneous organ such as the heart, which consists primarily of myocytes, other cell types are present, including endothelial cells, smooth muscle cells, fibroblasts, etc. Therefore, measurement of antioxidant enzyme activities in whole organs or tissues reflects the levels in the predominant cell type. As described below, additional studies are necessary in order to characterize antioxidant enzyme activities in individual cell types.

In numerous biochemical studies, it has been established that cancer tissue is nearly always low in manganese superoxide dismutase and catalase activities and usually low in copper, zinc superoxide dismutase activity. Glutathione peroxidase activity in tumor tissue is variable if measured with biochemical methods using tissue homogenates. The studies that support these conclusions have been reviewed numerous times (Sun, 1990; Oberley and Oberley, 1993, 1994). Some investigators, however, have challenged the concept that antioxidant enzymes are low in tumors, since at least one antioxidant enzyme, manganese superoxide dismutase, has very high activity in certain human tumors, including mesothelioma (Westman and Marklund, 1981) and renal cell carcinoma (Yang et al., 1987). In addition, several tumors show elevated levels of immunoreactive protein for manganese superoxide dismutase, including ovarian cancer (Nakata et al., 1992), neuroblastoma (Kawamura et al., 1992), and lung cancer (Iizuka et al., 1984). It is not known whether enzyme activities are correspondingly elevated in these latter tumors since enzyme activity studies were not performed. Because of these findings that challenge the concept that antioxidant enzyme levels are diminished in human tumors, our laboratories have begun an in-depth analysis of human tumors. The results, to be summarized below, suggest abnormal levels and abnormal regulation of antioxidant enzymes in all tumor cell types. The possible consequences of such abnormalities for cancer therapy will also be discussed below.

Our laboratories have concentrated on analysis of manganese superoxide dismutase in malignant versus normal tissue. This biochemical analysis has resulted in several important conclusions. Reduced cancer cell manganese superoxide dismutase activity have been observed in all species examined and does not depend on the nature of the transforming agent. Thus, lowered manganese superoxide dismutase activity has been observed in human and various rodent (rat, mouse, hamster) cancer cells, whether spontaneously transformed (Sun et al., 1993b) or transformed by viruses, chemicals, ionizing radiation, or hormones (McCormick et al., 1991). At least part of the reason that cancer cells are low in manganese superoxide dismutase activity is because the amount of translatable mRNA is low and thus less protein is synthesized (Sun et al., 1993a). In the one tumor examined so far, human colon adenocarcinoma, the coding region of the manganese superoxide dismutase gene was normal; this suggests that the reason for the lowered mRNA is changes in the regulatory regions of the manganese superoxide dismutase gene or changes in regulatory protein(s) that bind to these regulatory regions (St. Clair and Holland, 1991).

Manganese superoxide dismutase is an enzyme that is inducible by its substrate, superoxide anion. Inducible enzyme levels may be diminished in cells because their substrate levels are low, and thus biosynthesis of the inducible enzyme does not occur. However, it has been demonstrated that cancer cells have the capacity to produce superoxide radicals, the substrate for superoxide dismutase (Bize et al., 1980). It has also been hypothesized that tumor cells have diminished manganese superoxide dismutase activities because tumors often have a hypoxic core (Petkau et al., 1977). However, this cannot be the cause for the general lowering of cancer cell manganese superoxide dismutase since diminished levels of manganese superoxide dismutase have consistently been observed in cancer cells in culture compared with normal cells (Oberley et al., 1994a). In these experiments, the levels of oxygen are equivalent in the normal and the cancer cells.

Not only are the constitutive levels of manganese superoxide dismutase low in cancer cells, but cancer cells have lost much of the ability to undergo gene induction upon exposure to agents generating superoxide radicals (Oberley et al., 1987). It is now known that manganese superoxide dismutase may be induced by factors other than its substrate; these factors include tumor necrosis factor, interleukins, and phorbol ester (Masuda et al., 1988; Wong and Goeddel, 1988; Fujii and Taniguchi, 1991). The reason why oxidative stress fails to induce manganese superoxide dismutase in cancer cells while cytokines are successful in some cancer types is still unknown.

Our laboratories have studied one tumor, the human renal adenocarcinoma, in some depth; this tumor has extremely variable levels of manganese superoxide dismutase. The cell of origin of this tumor is thought to be the proximal tubule; biochemical measurements of isolated human proximal tubules showed manganese superoxide dismutase activity to be 126 units per milligram protein (Oberley et al., 1994b). Human renal adenocarcinomas, on the other hand, showed activity measurements that varied from 7 to 1,235 units per milligram protein, i.e., from approximately ten times less than normal to ten times greater than normal (Oberley et al., 1994b). Thus, even in our laboratories, not all human tumors have lowered manganese superoxide dismutase activity. However, morphologic studies to be discussed below do suggest that all tumors have abnormal (elevated or depressed) antioxidant enzymes levels compared to the cell of origin, including manganese superoxide dismutase. As discussed below, these alterations may have profound implications for cancer cell behavior and hence cancer cell therapy.

The relationship(s) between antioxidant enzymes and cancer is made even more complex by the fact that low levels of reactive oxygen stimulate cell proliferation in many cell types (Burdon, 1995), and it has been demonstrated in certain cell culture situations that antioxidant enzymes can suppress cell proliferation (Oberley et al., 1991a). There is also direct evidence linking manganese superoxide dismutase to the process of cell differentiation. St. Clair et al. (1993) demonstrated that elevation of MnSOD via transfection led to greatly increased levels of differentiation in C3H 10T1/2 cells exposed to the demethylating agent 5-azacytidine.

III. Biochemical analysis of the role of antioxidant enzymes in experimental skin carcinogenesis studies

Several experimental systems have suggested a role for reactive oxygen species and antioxidant enzymes in carcinogenesis. Experimental models of skin cancer in mice are one example. Papillomas can be induced in SENCAR mice by topical application of a single nontumorigenic dose of carcinogen (initiation), followed by repetitive treatments with the noncarcinogenic tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). With time, some of the papillomas arising from this protocol progress to squamous cell carcinomas, irrespective of continuing TPA treatment. Several lines of evidence suggest a role for reactive oxygen species in this model of chemical carcinogenesis. The rate of progression and the percentage of papillomas that progress to squamous cell carcinomas can be enhanced by treating the papillomas with oxidants (O'Connell et al., 1986). Long-term treatment of initiated skin with 5 or 10% solutions of hydrogen peroxide weakly promotes papilloma development (Klein-Szanto and Slaga, 1982). Topical application of the superoxide dismutase biomimetic CuII $[3,5\text{-diisopropylsalicylate}]_2$ inhibits TPA-dependent promotion (Egner and Kensler, 1985). Topical or intraperitoneal administration of several agents that possess antioxidant activity or are capable of enhancing the cellular antioxidant system also inhibit TPA-dependent promotion (Perchelett et al., 1985, 1987a-c). Reactive oxygen species levels are elevated in epidermal cells prepared from TPA-treated mice (Robertson et al., 1990). Superoxide dismutase and catalase activities are reduced in TPA-treated skin, while xanthine oxidase activities are increased (Reiners et al., 1991). Similarly, superoxide dismutase and catalase activities are decreased in papillomas and squamous cell carcinomas, while xanthine oxidase activity is increased. Reduced levels of antioxidant enzymes may result in oxidative stress. These combined results strongly suggest a role for reactive oxygen species in experimental animal models of chemically induced skin carcinogenesis. Recent preliminary studies in collaboration with Dr. Daret St. Clair at the University of Kentucky have demonstrated that transgenic mice overexpressing manganese superoxide dismutase are resistant to chemically induced skin carcinogenesis in the above described mouse model (unpublished observations).

IV. Biochemical analysis of reactive oxygen species and antioxidant enzymes in estrogen-induced kidney cancer in the Syrian hamster

A second experimental model in which there is considerable evidence for a role for reactive oxygen species in the development of cancer is estrogen-induced kidney cancer in the Syrian hamster. Natural (steroid) and synthetic (stilbene) estrogens have been known to be potent carcinogens in the Syrian golden hamster. Malignant renal neoplasms develop after long-term hormonal treatment (Kirkman, 1959). The mechanism by which estrogens cause cancer in this model is the subject of intense investigation. It seems most likely that both the hormonal properties of estrogens and their intracellular metabolites are involved. The renal cortex of the hamster is a bona fide estrogen-sensitive target, as has been ascertained by the presence of a specific estrogen receptor, its increase after prolonged estrogen stimulation, and a 13-fold increase in progesterone receptors after estrogen treatment (Li et al., 1974; Li and

Li, 1978). While it has been suggested that the hormonal property of estrogens is essential in causing *in vivo* neoplastic transformation of the renal cortex, other studies suggest an important role for estrogen metabolites in estrogen-induced carcinogenesis (Li and Li, 1987).

Diethylstilbestrol (DES), a synthetic estrogen that is often used to induce tumorigenesis in the hamster model both because of its potency and because of its role in human disease (Herbst et al., 1984), is metabolically oxidized to DES-4',4" quinone (DES Q) by cytochrome P450 (Liehr et al., 1985) and is extremely reactive chemically. It binds to small peptides, cellular proteins, and DNA. Roy and Liehr (1989b) found DES Q in tissues of hamsters given DES for a prolonged period. Because DES Q was found in organs other than kidney (in which DES does not induce cancer), these authors conclude that oxidation of DES to DES Q and the genotoxocity of DES Q may be necessary, but not sufficient, for tumor development. They hypothesized that hormone-dependent growth of initiated cells may also be necessary for the development of cancer (Roy and Liehr, 1989b). It has now been demonstrated that redox cycling occurs between DES and DES Q, catalyzed by microsomal drug-metabolizing enzymes (Liehr et al., 1986b). Such redox cycling generates oxygen free radicals (Roy and Liehr, 1988) that have been postulated to damage DNA or other cellular macromolecules. It has been directly demonstrated that peroxidative metabolism of DES induces formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative DNA damage (Rosier and Van Peteghem, 1989).

Antioxidant enzyme activities have been determined in Syrian hamster kidney tumors and kidney cortex from immature and adult estrogenized and untreated male hamsters (McCormick et al., 1991). Superoxide dismutase (both copper, zinc and manganese forms) and catalase activities were significantly lower in primary tumors than in renal cortex from untreated and estrogen-treated male hamsters. Primary tumors were serially passaged in the ascites form in estrogen-treated castrated hamsters; these tumors, if passaged long enough, become autonomous (hormone-independent). Catalase, copper, zinc superoxide dismutase, and manganese superoxide dismutase activities were lower in passaged autonomous renal tumors than in primary tumors. Quantitative Western blot analysis of immunoreactive proteins confirmed that catalase, copper, zinc superoxide dismutase, and manganese superoxide dismutase were lower in both primary tumors and passaged autonomous tumors than in normal adult renal cortex. Similar levels of antioxidant enzyme activities were found in tumor and newborn kidney, and these values were much lower than those in normal adult kidney cortex or isolated proximal tubules. These lowered antioxidant activities could cause oxidative stress in tumor cells; the possible effects of oxidative stress on tumor cells is discussed below.

In fact, it has been demonstrated that oxidative stress results from administration of estrogens to hamsters; Roy and Liehr (1989a) have demonstrated increases in glutathione, activity of glutathione peroxidase, and products of lipid peroxidation in kidneys of hamsters given prolonged estrogen treatment. Hamsters exposed to estrogens show oxidative damage to proteins (Winter and Liehr, 1991). Hamsters treated with estrogens have been reported to demonstrate DNA alterations. These include estrogen-DNA adducts (Liehr et al., 1986a), as well as oxidative damage to DNA, with the formation of the DNA oxidative product 8-hydroxydeoxyguanosine detected in kidneys of estrogen-treated hamsters (Roy et al., 1991).

V. Morphological studies of antioxidant enzymes in normal tissues

Measurement of antioxidant enzyme biochemical activities in whole organs or tissues does not provide an estimate of enzyme activities of individual cell types within these organs or tissues. We have used immunoperoxidase analysis of hamster tissues to demonstrate that each cell type within an organ has a unique antioxidant enzyme profile. The results appear to be organ-rather than species-specific; for example, we have found a similar distribution of antioxidant enzymes in kidneys of hamster (Muse et al., 1994), rat (Oberley et al., 1995a), and human (Oberley et al., 1996) and lungs of rat (Coursin et al., 1992) and human (Coursin et al., 1996).

The distribution of antioxidant enzymes was examined in normal hamster kidney is some detail (Muse et al., 1994). Immunoperoxidase and immunogold techniques were used to localize antioxidant enzymes in the adult hamster kidney. Each cell type in the kidney showed specific patterns of labeling of these enzymes. For example, proximal and distal tubular and transitional epithelial cells showed significant staining for all of these enzymes, while glomerular cells and cells of the thin loop of Henle did not show significant staining at the light microscopic level. In addition, high levels of glutathione peroxidase were found in smooth muscle cells of renal arteries. At the ultrastructural level, each enzyme was found in a specific subcellular location. Manganese superoxide dismutase was found in mitochondria, catalase was localized in peroxisomes, while copper, zinc superoxide dismutase was found in both the nucleus and cytoplasm. Glutathione peroxidase was found to have a broad intracellular distribution, with localization in mitochondria, peroxisomes, nucleus, and cytoplasm. Microvilli of tubular cells were labeled by antibodies to catalase, copper, zinc superoxide dismutase, and glutathione peroxidase. These observations demonstrate that there are large variations in the levels of antioxidant enzymes in different cell types, and that even within a distinct cell type, the levels of these enzymes vary in different subcellular locations. Presumably, these differences reflect the unique metabolism in various subcellular compartments of each

cell type.

These general conclusions have been confirmed in a study of rat lung, where type II pneumocytes and ciliated respiratory epithelium were shown to have higher levels of antioxidant enzymes than other lung cell types (Coursin et al., 1992). Each cell type had the same intracellular distribution of antioxidant enzymes as observed in the kidney; in addition, microvilli of type II pneumocytes and cilia of respiratory epithelium exhibited labeling for catalase, copper, zinc superoxide dismutase, and glutathione peroxidase. It is not presently known whether staining of microvilli and cilia represents true localization of antioxidant enzymes in these organelles or represents staining of extracellular fluid containing antioxidant enzymes adherent to these organelles.

Studies of cell renewal systems in adult hamster indicated greater staining for antioxidant enzymes in differentiated cells compared with precursor cells; for instance, villus cells of the small intestine showed much greater labeling than crypt cells (Oberley et al., 1990). The concept has thus been proposed that antioxidant enzymes are markers of cell differentiation (Allen and Balin, 1989), with lowered antioxidant enzymes being suggested to be a general feature of precursor cells. Immunoperoxidase studies of hamster kidney development have demonstrated that antioxidant enzymes are not detectable until later stages of kidney development, after extracellular basement membrane proteins have been deposited (Oberley et al., 1995b). These latter results are consistent with the hypothesis that antioxidant enzymes are markers of cell differentiation in at least some cell systems.

VI. Morphologic analysis of antioxidant enzymes in estrogen-induced hamster renal cancer

We have performed an extensive morphologic analysis of the development of cancer in the estrogen-induced hamster kidney cancer model. Syrian hamsters were treated with DES, and at monthly intervals, their kidneys were studied using light, immunoperoxidase, and ultrastructural techniques. At 4.5 months, DES-treated hamsters showed small interstitial lesions (between tubules) composed of clusters of small round cells with high nuclear-cytoplasmic ratio; normally, the renal interstitium is populated by a sparse number of elongated fibroblast-like (spindle) cells. Immunoperoxidase and ultrastructural studies showed these early lesions to be identical to fully formed tumors, which are large lesions present after 9 months of treatment. While biochemical studies had demonstrated that fully formed tumors had low activities for manganese and copper, zinc superoxide dismutase and catalase (McCormick et al., 1991), such studies could not be performed on early lesions since they were a minority population of cells in the whole kidney. However, immunoperoxidase techniques demonstrated that the earliest neoplastic lesions observed (4.5 months) had no detectable immunostaining with antibodies to copper, zinc superoxide dismutase, manganese superoxide dismutase, or catalase (Oberley et al., 1991b). These studies suggest that early lesions do not have significant levels of antioxidant enzymes.

VII. Morphologic analysis of antioxidant enzymes in human cancer

Immunogold analysis of human renal adenocarcinomas was first performed by Oberley et al. (1994b); this tumor was of considerable interest because of its documented extreme variability in manganese superoxide dismutase activities. Renal cell carcinomas were subclassified on the basis of light microscopy and ultrastructural analysis into clear cell, granular cell, or mixed clear and granular cell variants. In all three types of tumor, immunogold studies showed little staining using antibodies to copper, zinc superoxide dismutase or glutathione peroxidase. However, intensity of labeling for manganese superoxide dismutase and catalase depended on the cell type(s) in the tumor. Clear cell variants demonstrated trace staining for manganese superoxide dismutase and catalase, while granular cell variants exhibited heavy staining for both of these enzymes. Mixed types of tumors showed clear cells with trace staining for all antioxidant enzymes examined, while granular cells again showed intense labeling for manganese superoxide dismutase and catalase. With normal human kidney proximal tubule as a comparison, immunogold ultrastructural analysis using antibody to manganese superoxide dismutase demonstrated infrequent small lightly labeled mitochondria in clear cell variants, while granular cell variants exhibited numerous medium-sized heavily labeled mitochondria. These data suggest that: (a) the variability in activity values for manganese superoxide dismutase may be due to heterogeneity in cell types in these tumors; and (b) manganese superoxide dismutase immunoreactive protein was elevated in granular cells both because of an increase in the number of mitochondria and because the labeling density in mitochondria was increased compared with mitochondria in clear cell types or in normal proximal tubular cells.

After this analysis was performed, we studied antioxidant enzymes in normal human kidney and common renal cancers (renal adenocarcinoma, papillary carcinoma, transitional cell carcinoma of the renal pelvis, and Wilm's tumor) using immunogold techniques with polyclonal antibodies to copper, zinc and manganese superoxide dismutases, catalase, and glutathione peroxidase (Oberley et al., 1996). Normal tissue adjacent to human renal tumors had the same antioxidant enzyme immunoreactive protein profile as normal human kidney, thus establishing that the presence of tumor does not alter the levels of antioxidant enzyme immunoreactive proteins in adjacent kidney tissue; this fact allowed direct comparison of levels of antioxidant enzyme immunoreactive protein in tumor and adjacent

tissue. The results for renal adenocarcinoma were the same as that described above, with low levels of antioxidant enzymes in clear cell variants, but high levels of manganese superoxide dismutase and catalase immunoreactive protein in granular cell variants. However, the other three types of renal tumors showed low levels of staining for all antioxidant enzymes. Studies of transitional carcinoma of the renal pelvis was especially informative since it was possible to compare levels of antioxidant enzyme immunoreactive protein in tumors with that in adjacent normal transitional epithelium; all antioxidant enzyme antibodies demonstrated lower levels of immunoreactive protein in transitional cell carcinoma than in adjacent normal transitional epithelium.

To further analyze immunoreactive protein in human cancers, human lung tumors were analyzed using immunoperoxidase techniques (Coursin et al., 1996). Samples of normal lung and six major types of human lung carcinomas (adenocarcinoma, squamous cell carcinoma, bronchioloalveolar cell carcinoma, adenosquamous carcinoma, undifferentiated large cell carcinoma, and small cell carcinoma) were studied. Staining for antioxidant enzymes was generally low in tumor cells compared with the high level of staining noted in respiratory epithelium. A notable exception was heterogeneity in immunostaining for manganese superoxide dismutase in lung adenocarcinomas, which showed strongly positive and negative cells in the same tumor. Tumor stromal cells (fibroblast-appearing cells) often showed strong staining for manganese superoxide dismutase, while stromal cells were negative for other antioxidant enzymes. Small arteries within tumors showed strong staining for glutathione peroxidase in endothelial and smooth muscle cells. None of the carcinomas studied had significant levels of catalase or glutathione peroxidase; this finding has potential therapeutic relevance since it indicates that these tumors have a decreased capacity to detoxify hydrogen peroxide.

Recently, we have used polyclonal antibodies to manganese and copper, zinc superoxide dismutase and catalase to analyze antioxidant enzymes in human prostate carcinoma (manuscript in preparation). Compared with benign prostate epithelium, manganese superoxide dismutase and catalase showed statistically significant less immunolabeling in prostatic intraepithelial neoplasia (early cancer) and frank cancer. These results confirm that early human prostate cancer lesions have lowered levels of two antioxidant enzymes. Labeling for glutathione peroxidase was barely detectable in both benign and malignant prostate epithelium, while staining for copper, zinc superoxide dismutase was not significantly different in benign versus malignant epithelium. Interestingly, we were able to document that advanced infiltrating cancer actually had increased levels of manganese superoxide dismutase in some cases. Thus, adenocarcinomas in all three organs examined (kidney, lung, and prostate) showed some adenocarcinoma cells with low and some with high levels of manganese superoxide dismutase. It is not known whether these two adenocarcinoma cell types represent different degrees of differentiation or increased expression of the manganese superoxide dismutase gene; we speculate that cytokines could be responsible for this increase in manganese superoxide dismutase immunoreactive protein. Ultrastructural immunogold analysis of human prostate cancer using polyclonal antibody to manganese superoxide dismutase demonstrated small mitochondria that were lightly labelled compared with the larger more heavily labeled mitochondria in benign epithelium (unpublished observation). In agreement with light microscopy studies, infiltrating tumor epithelium in some cases showed small mitochondria that were heavily labeled; these results demonstrate that the same cancer can have abnormal mitochondria that either under- or overexpress manganese superoxide dismutase.

One of the major findings in these studies of human cancers is that antioxidant enzymes do not show expression in abnormal subcellular locations. For instance, manganese superoxide dismutase remains in the mitochondria and does not appear in the cytoplasm or nucleus of tumor cells. Copper, zinc superoxide dismutase, which is present in both cytoplasm and nucleus of normal cells, remains in these same subcellular locations in malignant cells. Thus, coding sequences in these genes for organelle localization and subcellular transport mechanisms must remain relatively intact in cancer cells.

VIII. Tissue culture studies of antioxidant enzyme regulation in normal and malignant tissues

Regulation of antioxidant enzymes activities was studied in cultured human renal adenocarcinoma cells (Yang et al., 1987). The activities of antioxidant enzymes were monitored in isolated renal adenocarcinoma tissue and in cultured renal adenocarcinoma cells. The results were compared with the activities of these enzymes in the proposed cell of origin - isolated human proximal tubular tissues and cultured human proximal tubular epithelial cells. In the tumor studied, manganese superoxide dismutase activity was greater in the tumor tissue than in isolated proximal tubules, while copper, zinc superoxide dismutase activity was equivalent in the two tissues. Catalase and glutathione peroxidase activities were less in tumor tissue than in isolated proximal tubules. When either proximal tubules or isolated tumor cells were grown in serum, all antioxidant enzymes showed significant decrease in activities. In contrast, when proximal tubules or isolated tumor cells were grown in a serum-free chemically defined medium, antioxidant enzymes activities were greater in tubular cells compared with cells grown in serum, whereas tumor cell antioxidant enzyme activities were low and were not stimulated by serum-free conditions. These studies demonstrate a significant difference in regulation of antioxidant enzyme activities

in normal versus malignant human renal epithelium.

A second culture system studied was the estrogen-induced hamster kidney cancer (Oberley et al., 1994a). Antioxidant enzyme activities were studied in normal hamster kidney proximal tubules and in estrogen-induced hamster kidney cancer. *In vivo*, hamster kidney tumor had lower activities of manganese superoxide dismutase, copper, zinc superoxide dismutase, catalase, and glutathione peroxidase than hamster kidney proximal tubules. Differences in antioxidant enzyme activities were, in general, maintained in tissue culture, with antioxidant enzyme activities remaining low in tumor cells compared with normal cells. Normal proximal tubular cells showed significant induction of manganese superoxide dismutase activity as a function of time in culture or following exposure to DES, while manganese superoxide dismutase activity remained low in tumor cells under these conditions. These results suggest than antioxidant enzymes, particularly manganese superoxide dismutase are regulated differently in estrogen-induced hamster kidney cells than in normal hamster kidney proximal tubular cells.

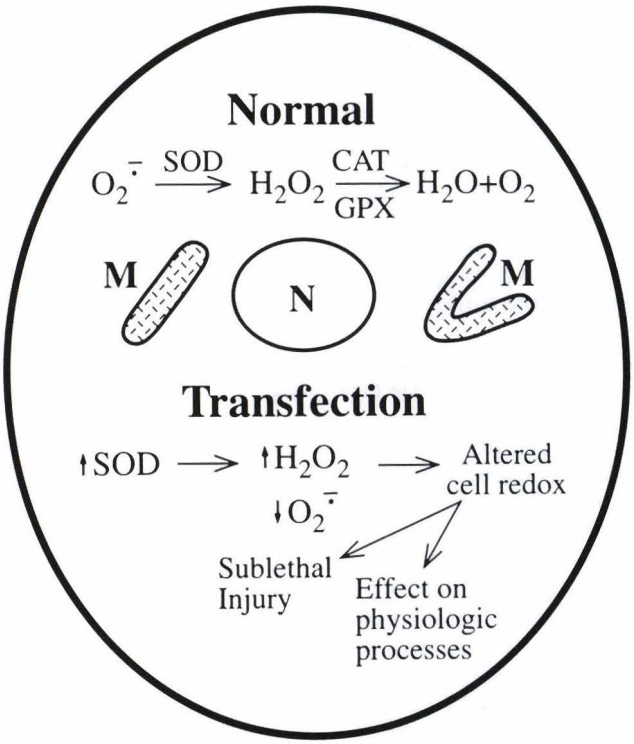

Fig. 1. Possible effects of transfection of cDNA for manganese superoxide dismutase on cell behavior. Normally superoxide dismutase (copper-zinc superoxide dismutase is found in the cell cytoplasm, manganese superoxide dismutase in mitochondria) converts superoxide anion into hydrogen peroxide, which is then detoxified by either glutathione peroxidase or catalase into water. Increasing levels of superoxide dismutase will result in decreased levels of superoxide anion and increased levels of hydrogen peroxide, thus altering the prooxidant/antioxidant balance (cell redox state) of cells. This may result in sublethal injury or may affect physiologic processes such as gene transcription, signal transduction, mitosis, apoptosis, and senescence. The lower level of superoxide dismutase in cancer cells compared with normal cells should allow the development of cytotoxic or normalization therapies for cancer treatment. N: nucleus; M: mitochondria; $O_2^{\cdot-}$: superoxide anion; H_2O_2: hydrogen peroxide; SOD: superoxide dismutase; GPX: glutathione peroxidase; CAT: catalase.

IX. Suppression of the malignant phenotype by manganese superoxide dismutase

Using cell culture systems, it has been possible to demonstrate that overexpression of manganese superoxide dismutase suppresses the malignant phenotype. Transfection of the cDNA for manganese superoxide dismutase resulting in an increase in enzymatic activity suppressed cell growth *in vitro* and *in vivo* of human melanoma (Church et al., 1993), human breast carcinoma (Li et al., 1995), human glioma (Zhong et al., 1996b), human squamous oral carcinoma (unpublished observations), and human prostate carcinoma (unpublished observations) cell lines. The mechanism by which overexpression of maganese superoxide dismutase suppresses cell growth is not certain, but cytotoxicity studies have demonstrated that cell death is not a contributing factor (Li et al., 1995; Zhong et al., 1996b). Increased manganese superoxide dismutase expression could result in decreased superoxide anion levels, a substrate for the enzyme, and an increase in hydrogen peroxide levels, a product of the enzyme; these changes would affect cell redox (Fig. 1). It is not certain whether these changes in cell redox would primarily cause sublethal injury or change key physiologic processes to cause a decrease in tumor cell growth; perhaps both processes occur depending on each cell type's unique biochemistry and on the level of enzyme activity expressed, with higher levels tending to result in more cell injury. One recent study has demonstrated that overexpression of copper, zinc superoxide dismutase results in overproduction of hydrogen peroxide (de Haan et al., 1996). Future studies are being designed to determine the mechanism by which overexpression of superoxide dismutase activity inhibits tumor cell growth.

X. Morphologic evidence for oxidative stress in early lesions and frank cancer

Recent studies have demonstrated biochemical evidence for oxidative stress in a wide variety of human cancers, by measuring levels of 8-hydroxy-2'-deoxyguanosine, which indicates oxidative DNA damage (Okamoto et al., 1994; Toyokuni et al., 1995). Using an antibody to 4-hydroxy-2-nonenal, we have recently demonstrated labeling of both prostate carcinoma and renal carcinoma (unpublished observations), demonstrating lipid peroxidation in both of these human tumors. Ultrastructural immunogold analysis with this antibody demonstrated a 2-fold increase in mitochondrial labeling in prostate adenocarcinoma tumor mitochondria compared with benign prostate epithelial cell mitochondria (unpublished observations),

suggesting the primary oxidative stress to be in tumor mitochondria. In keeping with this finding, tumors with large numbers of mitochondria (granular cell variant of renal adenocarcinoma) had greater labeling than other tumors studied (unpublished observations).

XI. Conclusions concerning the role of antioxidant enzymes in cancer

Our first major conclusion is that changes in manganese superoxide dismutase reflect the fact that cancer is at least in part an abnormality in cell differentiation (Tomlinson and Bodmer, 1995), and it seems likely that most cancers arise from precursor cells (Fig. 2). Manganese superoxide dismutase is a nuclear-encoded mitochondrial enzyme. There is strong evidence that mitochondria undergo specialization as cells differentiate (Ostronoff et al., 1996). Indeed, morphologic evidence from our laboratories show that in kidney development, mitochondria increase dramatically in size and number (Gonzalez et al., 1989) as animals mature. Both human renal cancers (Oberley et al., 1994b) and the estrogen-induced renal cancer (Oberley et al., 1991b) show small mitochondria, and we have demonstrated that human renal adenocarcinomas show abnormal mitochondrial levels of manganese superoxide dismutase (Oberley et al., 1994b). It thus seems likely that one of the major reasons for abnormalities in antioxidant enzymes in cancer is the lack of regulation of differentiation observed in cancer cells.

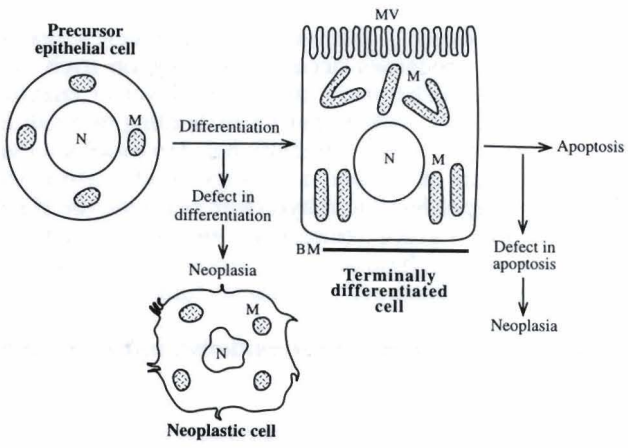

Fig. 2. Lowered manganese superoxide dismutase as a defect in differentiation. The majority of cancers arise in epithelial cells and most likely involve defects in differentiation. However, some epithelial cancers, such as prostate cancer, also have defects in apoptosis. Because the majority of cancers arise before terminal differentiation and because mitochondrial number and size increase with cell differentiation, epithelial cancers almost always have a decrease in the number and size of mitochondria. Because manganese superoxide dismutase is a mitochondrial enzyme, its level is almost always decreased in cancer. Exceptions are some adenocarcinomas in which mitochondria may be numerous but small; these mitochondria may have elevated manganese superoxide dismutase. Thus, in all cancers examined to date, both mitochondria and regulation of manganese superoxide dismutase are abnormal. N: nucleus; M: mitochondria; MV: microvilli; BM: basement membrane.

A second major conclusion is that there is an imbalance of antioxidant enzyme levels in most cancer cells. The majority of renal, lung, and prostate cancers lacked both catalase and glutathione peroxidase, which should result in an accumulation of hydrogen peroxide. This accumulation of hydrogen peroxide may have an important effect on cancer cell behaviour, because of the known effect of prooxidant-antioxidant (cell redox state) on cell physiology.

A third conclusion is that one subtype of cancer cell (adenocarcinoma) is heterogeneous with regard to manganese superoxide dismutase levels, with tumor cells showing either low or high levels of enzyme. This fact has practical implications for both tumor cell behavior and response to therapy. A recent paper has documented the prognosis of patients with cervical carcinoma correlates with manganese superoxide dismutase levels. The authors of this paper suggest that response to radiation therapy may correlate with manganese superoxide dismutase levels, since tumors with high enzyme activity levels would be resistant to the reactive oxygen species generated by ionizing radiation (Nakano et al., 1996).

A final conclusion is that cancer cells have abnormal regulation of antioxidant enzymes, particularly manganese superoxide dismutase. This difference in regulation between normal and malignant cells may allow the development of therapeutic modalities that exploit these differences (see below).

XII. Conclusions concerning the role of reactive oxygen species in carcinogenesis

Biochemical and immunohistochemical studies to date have documented the presence of oxidative stress in both human cancer and experimental animal models of cancer. However, the relation of the increase in reactive oxygen species to changes in antioxidant enzymes is not well understood. Do decreases in antioxidant enzymes cause increases in reactive oxygen species, or do increased reactive oxygen species alter the regulation of antioxidant enzymes? This is an important question for understanding the etiology of cancer. One of the major consequences of an increase in reactive oxygen species in cancer cells would be an increase in mutations (Moraes et al., 1991), which could affect the rate of tumor progression.

XIII. Implications of altered antioxidant enzymes for cancer therapy

Studies to date have demonstrated that over-expression of manganese superoxide dismutase suppresses the malignant phenotype in a large number of cancer cell lines both *in vitro* and *in vivo*. The mechanism by which suppression occurs is not known, though we (unpublished observations) and others (de Haan et al., 1996) have demonstrated that over-expression of superoxide dismutase results in over-

production of hydrogen peroxide, an enzymatic product of the enzyme's action. Several studies have demonstrated that transfection of the cDNA for manganese superoxide dismutase with resultant increase in enzyme activity does not result in loss of cell viability (Li et al., 1995; Zhong et al., 1996b). However, it is possible that overexpression of manganese superoxide dismutase could result in sublethal injury. We have found that overexpression of manganese superoxide dismutase (5-fold increase) in human prostate carcinoma DU145 cells resulted in suppression of cell growth both *in vivo* and *in vitro* (unpublished observations). Flow cytometry analysis using 2',7'-dichlorofluorescein-diacetate (DCFH-DA; a oxidation-sensitive fluorochrome) showed accumulation of peroxides within cells. Electron microscopic examination of these cells showed mitochondrial injury, with loss of mitochondrial cristae observed. In addition, these cells were sensitized to agents that generated reactive oxygen species, including menadione, mitomycin C, and hydrogen peroxide. Cells overexpressing manganese superoxide dismutase treated with hydrogen peroxide and examined ultrastructurally showed dramatic injury to mitochondria, manifested by mitochondrial swelling and loss of cristae. Rat glioma cells overexpressing manganese superoxide dismutase also show sensitivity to oxidative stress (Zhong et al., 1996a). These results suggest a basis for cytotoxic therapy for cancer cells.

Other cell lines that we have transfected did not show mitochondrial injury (Li et al., 1995; Zhong et al., 1996b), yet they did show inhibition of cell growth. It seems more likely that in these instances, changes in cell redox are affecting cell physiologic processes, including gene transcription or an effect on signal transduction pathways (Fig. 1).

It seems likely that altering cell redox offers tremendous potential for cancer therapy. While transfection of cDNA for antioxidant enzymes is one way to change the prooxidant-antioxidant balance of cells, low molecular weight compounds that possess antioxidant enzyme activities and liposomes containing antioxidant enzymes or antioxidant compounds are alternative possibilities. Further analysis of redox changes within cells is necessary before this type of therapy becomes a reality. While measuring antioxidant enzymes is illuminating, what is really needed is an in-depth knowledge of the prooxidant-antioxidant balance within cells and specific sites within cells. Then therapies could be developed for specific tumor cells. We should also be able to exploit the differences in antioxidant enzyme regulation between normal and malignant cells, and understanding the difference in this regulation may allow specific redox therapies to be developed.

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