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Invited Paper

Role of antioxidant enzymes in cell immortalization and transformation

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Summary

The role of antioxidant enzymes, particularly superoxide dismutase (SOD), in immortalization and malignant transformation is discussed. SOD (generally MnSOD) has been found to be lowered in a wide variety of tumor types when compared to an appropriate normal cell control. Levels of immunoreactive MnSOD protein and mRNA for MnSOD also appear to be lowered in tumor cells. Tumor cells have the capacity to produce superoxide radical, the substrate for SOD. This suggests that superoxide production coupled with diminished amounts of MnSOD may be a general characteristic of tumor cells. The levels of MnSOD in certain cells correlates with their degree of differentiation; non-differentiating cells, whether normal or malignant, appear to have lost the ability to undergo MnSOD induction. These observations are used to elucidate a two-step model of cancer. This model involves not only the antioxidant enzymes, but also organelle (particularly mitochondria and peroxisomes) function as a dominant theme in carcinogenesis.

Introduction

The hypothesis that free radicals are important in carcinogenesis is not a new one. Enthusiasm for this idea has waxed and waned over the years. Definitive evidence on this hypothesis has been difficult to obtain because of the elusive nature of free radicals. Recent advances in techniques and understanding have resulted in a clearer picture of the role of free radicals in cancer. It is the purpose of this paper to critically review the current state of our knowledge as well as suggest new avenues of study.

Antioxidant enzymes in tumor cells

Because of the difficulty in accurately measuring free radicals in living cells, investigators of the relationship between free radicals and cancer have focussed on measurements of antioxidant enzyme activities of normal and tumor cells. These enzyme activities will give an indication of how well a cell is protected against active oxygen species. Moreover, in normal cells, since the antioxidant enzymes are inducible, the levels of the antioxidant enzymes reflect the levels of their substrates – the active oxygen species. As we will see later, the latter assumption may not be true in tumor cells.

The data on the levels of antioxidant enzymes in tumor cells has been reviewed a number of times [1, 2, 3]. The general picture that has emerged is that tumor cells have abnormal activities of the antioxidant enzymes when compared to an appropriate normal cell control. In this paper, antioxidant enzymes will mean the superoxide dismutases (SOD), catalases (CAT), and peroxidases, of which

glutathione peroxidase (GPX) appears to be the most important in mammalian cells. Each of these enzymes is found in several forms and the enzymes are highly compartmentalized in the cell. For instance, a copper and zinc-containing SOD (Cu-ZnSOD) is found predominantly in the cytoplasm, whereas a manganese-containing SOD (MnSOD) is found primarily in the mitochondria. It has been found that tumor cells are nearly always low in MnSOD and CAT activity, and usually low in Cu-ZnSOD activity. GPX activity is variable. Since our work has focussed on SOD, we will summarize these studies below:

1. MnSOD activity is low in tumor cells only when compared to a proper cell type – the normal differentiated cell of the cell type from which the tumor arose. Thus, we found that MnSOD activity in dimethylhydrazine-induced large intestinal tumors was not significantly lower than that in the whole intestine, an organ comprised of many cell types [4]. However, the tumor MnSOD activity was greatly lowered when compared to the surface epithelial cell of the large intestine – the mature differentiated cell of this tissue compartment. Tumor cells most resembled crypt cells in their SOD activity; the crypt cell is the likely intestinal epithelial stem cell. A crucial unresolved question on this subject is whether the stem or the differentiated cell is the cell of origin for cancer.

MnSOD activity in tumor tissue is not necessarily low in absolute terms; MnSOD activity is low in the tumor when compared to an appropriate control. *In vivo*, the MnSOD activity in a tumor may actually be higher than that seen in other normal tissues. Mesothelioma tumors have higher MnSOD activity than most normal tissues [5]. However, to prove that mesotheliomas are an exception to the general rule that MnSOD activity is lowered in tumors would require a direct comparison to normal mesothelium; this test has not been done.

2. The reduction in MnSOD activity in tumor tissue is seen in several species and does not depend on the transforming agent [1, 2]. Thus, lowered MnSOD activity has been observed in human and rodent cells, whether spontaneously transformed or transformed by viruses, chemicals, or ionizing radiation.

3. We have recently shown that in both rodent and human cells, levels of immunoreactive MnSOD are lowered in tumor cells when compared to an appropriate normal cell control. Depending on *in vitro* growth conditions, levels of immunoreactive MnSOD were 2.3 to 14.9-fold lower in SV40-transformed human WI38 cells than in normal WI38 cells (submitted for publication). Marlhens *et al.* found that confluent *in vitro* normal human skin fibroblasts had 7.6 times the MnSOD protein than did their SV40 transformed counterparts [6]. Moreover, this same group found lowered levels of translatable messenger RNAs for MnSOD in human fibroblasts transformed by SV40. Much more work needs to be done in this area, but it appears that at least part of the reason MnSOD activity is low in tumor cells is because the translatable mRNA is low and thus less MnSOD protein is made.

4. In the one system studied, loss of MnSOD activity was found to be characteristic of tumor tissue and not of normal tissue, either quiescent or rapidly proliferating. Thus, regenerating mouse liver had elevated MnSOD activity when compared to normal mouse liver, while hepatoma tissue had the characteristic low activity [7]. Obviously, more tissues must be studied with regards to cell proliferation and SOD activity.

5. Inducible enzyme levels may be low in cells because their substrate levels are low and unable to induce biosynthesis of that enzyme. However, several studies have shown that neoplastic cells have the capability to produce superoxide radicals ($O_2^{\cdot -}$), the substrate for SOD [1, 2, 8]. Thus, it appears that SOD, an inducible enzyme, is not low in tumor cells because metabolically produced $O_2^{\cdot -}$ is low. This suggests that superoxide production coupled with diminished amounts of MnSOD may be a general characteristic of tumor cells. It has also been suggested that low SOD activity in tumors is a result of lowered oxygen concentrations [9]. Since many *in vivo* tumors are poorly vascularized, they may be anoxic at their centers. However, there is good evidence that the lack of oxygen or oxygen radicals is not the reason for the low MnSOD of neoplastic cells. First, it has been shown that increased oxygen concentrations causes increased SOD activity in normal cells, but not in HeLa or fibrosarcoma cells in

culture [10]. Second, superoxide (or a species derived from it) is thought to be the factor which causes MnSOD induction [11]. Paraquat, a known superoxide producer, caused SOD induction in normal rat kidney cells, but not in virus-transformed cells [12]. Last, we have shown that X-irradiation, which also produces oxygen radicals, caused MnSOD induction in mouse heart, but not in Ehrlich ascites tumor cells [13]. Thus, although oxygen might modulate the MnSOD activity of tumor cells to some extent, hypoxia is not the major cause of the low MnSOD levels in tumors. The fact is that even a high oxygen or superoxide concentration does not cause any appreciable induction of MnSOD in tumor cells. Thus, tumor cells are low in MnSOD activity because they have lost most of the capacity to undergo induction of the enzyme.

6. The loss of MnSOD appears to be associated with immortalization of cell lines. Normal mortal fibroblasts were found to possess MnSOD activity, whereas supposedly normal fibroblasts that are immortal possessed very little MnSOD activity [14]. Much more work needs to be done in the future to ascertain the generality of this phenomenon.

7. In both normal and tumor cells, activities of SOD, particularly MnSOD, correlate with the degree of differentiation of the cells. With regards to malignant cells, only one study has been reported. Both Cu-Zn and MnSOD activities were low in all the Morris hepatomas examined when compared to normal rat liver [15]. The SOD activities were correlated with the degree of differentiation of the tumors; the more differentiated the tumor, the higher the SOD activity.

The relationship between normal cell differentiation and the antioxidant enzymes has recently been reviewed [3]. As an example, *in vitro* human monocytes showed an increase in SOD activity, but a decline in CAT and GPX activity during differentiation [16]. Usually, MnSOD is induced during differentiation. However, one exception has been reported [17]. Human polymorphonuclear leukocytes (PMN) have low levels of both Cu-Zn and MnSOD activity. Low MnSOD activity is consistent with the loss of mitochondria seen during PMN differentiation. Moreover, the malignant HL-60 cell line, when caused to differentiate to PMNs by dimethylformamide, also underwent a loss of MnSOD activity [17]. Obviously, whether a cell loses or gains mitochondria during differentiation will in part determine whether MnSOD is induced or lost.

Two examples of differentiation are especially informative. Allen *et al.* [18] have studied differentiation of the slime mold *Physarum polycephalum*. Differentiation was induced by transfer of *Physarum* from growth medium to salts-only starvation medium. As differentiation proceeded, MnSOD activity increased 20-fold. The increase in MnSOD activity preceded morphological differentiation. Moreover, the W (white) strain, which did not differentiate, also did not show MnSOD induction. The H strain differentiated at a slower rate and showed less MnSOD induction. The H strain is a heterokaryon formed by fusion of a differentiating and non-differentiating strain. Thus, every strain of *Physarum* studied showed a correlation between differentiation competence and MnSOD induction.

We have recently performed studies on SOD in the X-REF-23 cell line [19]. This cell line was isolated after X-irradiation of rat embryo fibroblasts. X-REF-23 is an immortal, non-malignant, contact-inhibited cell line which has a normal rat chromosome number in all passages studied. Low passage X-REF-23 cells will spontaneously differentiate into adipose and muscle cells, if allowed to become confluent; high passage X-REF-23 cells demonstrate little or no spontaneous differentiation. In the laboratory of Dr. Duane Guernsey, Department of Physiology, subclones from the X-REF-23 cell population which differentiate to either muscle or adipose cells have been isolated. We studied the SOD activity of one muscle and one adipose clone (LW Oberley, L Ridnour, E Sierra-Rivera, TD Oberley, D Guernsey, submitted for publication). Cu-ZnSOD activity was induced in the adipose clone and the parental X-REF-23 cells with time in culture, but not in the muscle clone. In contrast, MnSOD activity was induced in both the muscle and adipose clones at the time when differentiation occurred, but not in the non-differentiating X-REF-23 cell line. A small amount of MnSOD induction did occur at a much later time in the X-REF-23 cell line. Since MnSOD is a protective enzyme, we examined with electron microscopy what if any structural damage occurred

in these cells. Since this enzyme is found primarily in the mitochondria, we were especially interested in examining these organelles. In the differentiating clones the mitochondria appeared normal. In the non-differentiating X-REF-23 cell line, dramatic increases (approximately 20-fold) in mitochondrial number occurred with time in culture. Moreover, most of the mitochondria seen were abnormal in three ways: they are small, have few cristae, and many have damaged membranes. Thus, in the differentiating subclones of the X-REF-23 cell line, MnSOD induction seems to be associated with differentiation; conversely, the late passage X-REF-23 line, which does not differentiate, does not undergo MnSOD induction at the proper time. MnSOD is only increased in the differentiation incompetent line at a time when mitochondrial numbers are greatly increased; this implies that in this cell line the amount of MnSOD per mitochondria decreases greatly with time in culture. It appears that in the non-differentiating cell line, MnSOD only increases because the number of mitochondria increases so drastically.

Our current hypothesis is that in most cells, MnSOD induction is necessary, but not sufficient, for differentiation. In other words, cells cannot differentiate unless MnSOD is induced, but MnSOD induction alone will not cause differentiation, as other pathways must also be induced or repressed. With modern molecular biological techniques, this hypothesis is testable. One could place an expression vector for MnSOD into both differentiation competent and incompetent lines to determine the effect on differentiation. Moreover, one could prevent MnSOD induction in a differentiating cell using antisense mRNA for MnSOD and determine if this prevents cell differentiation.

Two-step model of cancer

We have proposed a two-step model of carcinogenesis [20, 21, 3]. Our hypothesis is that the first step in carcinogenesis is the attainment of immortality and the second step is the loss of control of cell division. How do we integrate this hypothesis with the experimental data on SOD presented earlier? The data is consistent with the concept that the attainment of immortality is associated with the loss of MnSOD seen in tumor cells. There is some evidence that antioxidant enzymes are also involved in the second step, but that evidence is not firm at the present time [3].

As an example, the X-REF-23 cell line data discussed earlier can easily fit into the two-step hypothesis. As stated earlier X-REF-23 is an immortal, non-malignant cell line apparently immortalized by X-irradiation. X-REF-23 can undergo full neoplastic transformation after treatment with methylcholanthrene, X-rays plus 12-0-tetradecanoylphorbol-13-acetate (TPA), or a second dose of X-irradiation [19]. Thus, two distinct steps are present. The multistep nature of malignant transformation has been observed in a wide variety of systems besides the X-REF-23 cell line [3].

Immortalization and differentiation

The model that we have presented earlier [3] implies that immortalization of a population of cells occurs because of lack of differentiation. This idea was first proposed by Bell *et al.* in 1978 [22]. Immortalization is not caused by a particular cell living forever, but by a population always retaining the ability to divide. Conversely, mortality in a population of cells is caused by differentiation and the subsequent loss of ability of differentiated cells to undergo cell division. Thus, in a given population of cells, if the dividing (stem?) cells stay above a certain critical number, the population will be immortal; if the number of clonogenic cells falls below a critical number, the population will lose the ability to keep dividing.

It should always be remembered that immortality occurs in a population of cells. Because of the presence of a large number of cells, the fate of the population will be determined in large part by competition. In the simplest example, one could consider a population of dividing stem cells and differentiated, non-dividing cells. There will be competition between these two cells for available growing space. One would suppose that the dividing stem cell would win out in this competition since it can replicate.

However, the fate of the population depends not only on the competition, but also on the growth kinetics of each cell. The number of each cell type, if in isolation, depends on the birth rate and the death rate of each cell. A population of cells could be immortal because the stem cell population grows at a rate so as to insure its survival. Conversely, immortalization could occur because the normally long lived differentiated cell has become short-lived; i.e., its death rate has increased thereby allowing the normally slow growing stem cells to take over the population. Which of these two possibilities is true in general remains to be determined.

These concepts can be illustrated by the X-REF-23 cell line. X-REF-23 is an immortal cell line. At what stage in its growth does it become immortal? Certainly, the X-REF-23 cell line changes during passaging, since early passage cells are difficult to transform, whereas late passage cells are easily transformed [19]. Moreover, early passage cells spontaneously differentiate, whereas late passage cells show little or no differentiation. Thus, it is obvious that X-REF-23 changes during passaging. We hypothesize that the early passage cells are long lived (since they live longer than the rat embryo fibroblasts from which they were derived), but not immortal. We hypothesize that this cell line becomes truly immortal when it loses its ability to differentiate. This appears to be what is observed.

Role of organelles

In both the slime mold and X-REF-23 work discussed earlier, a similar phenomenon was observed: cells that could differentiate underwent MnSOD induction and cells that did not differentiate did not undergo MnSOD induction at the proper time. Since MnSOD is a mitochondrial enzyme, we wondered what effect the lack of MnSOD induction had on mitochondrial structure. For this reason, we performed transmission electron microscopy studies on the X-REF-23 cell line and two differentiated subclones. As described earlier, no change in mitochondrial morphology occurred in the two differentiating subclones, but in the parental non-differentiating cell line, confluency caused the appearance of numerous small abnormal mitochondria.

We have organized those observations into a model of transformation in the X-REF-23 cell line. Confluency (contact inhibition) is hypothesized to cause an oxidative burst in the cell and in particular in the mitochondria. In the differentiating clones, this results in induction of MnSOD. In the non-differentiating cell line, MnSOD induction does not occur. Thus, the cell is left without protection of the mitochondria against superoxide radicals. Oxidative damage to the mitochondria results in the production of the small abnormal mitochondria. The formation of these abnormal mitochondria could prevent normal differentiation or might simply be an effect of the lack of differentiaton.

The structure of the abnormal mitochondria seen in the X-REF-23 cell line is similar to that seen in yeast upon treatment with ethidium bromide [23]. Ethidium bromide destroys mitochondrial DNA. The proteins encoded by mitochondrial DNA cannot be made and thus abnormal small (petite) mitochondria result. Yeast with petite mitochondria survive because they can derive their energy from glycolysis. In contrast, ethidium bromide causes the formation of petite mitochondria in mammalian cells, but the cells cannot survive because they require respiration to produce enough energy.

Petite mitochondria have the following characteristis: 1) they are small; 2) they exhibit few cristae; 3) they have abnormal or damaged membranes; 4) they have damaged or missing mitochondrial DNA. The abnormal mitochondria seen in the non-differentiating X-REF-23 cell line has the first three characteristics of petite mitochondria. We are presently examining whether the fourth characteristic, damaged mitochondrial DNA, is present in the X-REF-23 cell line. Mitochondrial damage in this cell line seems certain, but whether this damage is due to oxidative attack of mitochondrial DNA, or some other target such as the membrane, remains to be seen.

Obviously, in the X-REF-23 cell line, attainment of confluency (contact inhibition) leads to abnormal mitochondria. One way to avoid this is for the cell to escape contact inhibition. Tumor cells do not exhibit contact inhibition of growth. Thus, we hypothesize that in the second step of malignant

transformation, a cell is made which does not exhibit contact inhibition. This enables the transformed cell to avoid the abnormal mitochondria seen in the immortalized cell ine and thus produce a more competitive cell.

The increase in mitochondria seen in the X-REF-23 cell line is reminescent to the increase in peroxisome number seen after giving peroxisome proliferators (e.g., plasticizers and hypolipidemic agents). Peroxisomes are ubiquitous cytoplasmic organelles. They contain a variety of enzymes including those capable of producing and destroying H_2O_2 (CAT). Induction of maximal peroxisome proliferation in liver cells is associated with an approximate two-fold increase in the activity and biosynthesis of CAT and up to a 30-fold increase in the activity of the H_2O_2-generating peroxisomal fatty acid β-oxidative system [24]. The effects of peroxisome proliferators is largely confined to the liver.

One of the effects of these agents is to cause the formation of liver tumors. Tumor formation occurs after peroxisome proliferation. Reddy has proposed that sustained proliferation of peroxisomes in liver leads to increased generation of hydrogen peroxide and oxidative stress [24]. This oxidative stress can then act as either an initiator or promotor (or both) of hepatocarcinogenesis. Reddy has listed the evidence for this hypothesis: 1) consistent association between the induction of peroxisome proliferation and hepatocellular carcinomas; 2) sustained and specific induction of H_2O_2-generating peroxisomal β-oxidative system; 3) increased intracellular levels of H_2O_2 in livers with peroxisome proliferation; 4) increased accumulation of lipofuscin following chronic induction of peroxisome proliferation; 5) increased lipid peroxidation and generation of highly reactive oxygen-free radicals; 6) DNA damaging capability of hypolipidemic drug-induced liver peroxisomes; 7) marked inhibition of peroxisome proliferation-induced hypatocarcinogenesis by antioxidants.

Thus, both peroxisome proliferation and mitochondrial proliferation seem to be associated with carcinogenesis. Perhaps lack of communication between organelle and nucleus is important in carcinogenesis. In both instances, there is increase in the production of active oxygen species but one of the antioxidant enzymes does not increase to an equivalent amount. This imbalance undoubtedly leads to oxidative damage.

Conclusions

Obviously, the model we have presented is based on a limited number of observations. Many other cell systems need to be studied to ascertain the general picture. Nevertheless, the idea that organelle disequilibrium plays a dominant role in transformation remains an attractive hypothesis.

Central to our model is the concept that MnSOD is not induced in immortalized cells in response to an oxidative insult. Why this induction does not occur is still a mystery, although several hypotheses have been presented [3]. Thus, it would seem essential to study why this induction does not occur.

Finally, even though our research has concentrated on one enzyme, MnSOD, it is important to study the total antioxidant picture in order to fully understand carcinogenesis. In particular, the other antioxidant enzymes may also play a large role in malignancy. Certainly, CAT appears to be a key enzyme in peroxisome proliferator-induced liver carcinogenesis. Moreover, different pictures may arise in different cells. Hopefully, the facts will become clearer in the near future.

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