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CELL DIVISION IN NORMAL AND TRANSFORMED CELLS: THE POSSIBLE ROLE OF SUPEROXIDE AND HYDROGEN PEROXIDE.

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ABSTRACT

A unified theory of cell division is presented. This theory explains the division of both tumor cells and normal cells and involves superoxide radicals, hydrogen peroxide, glucose, cAMP, and cGMP.

Key Words: cell division, superoxide, hydrogen peroxide, glucose, cyclic nucleotides

INTRODUCTION

We have recently presented a unified theory of cell differentiation, aging and cancer (1). This theory proposes that both aging and the cancer phenotype are caused by oxygen-derived radicals. Missing from this theory are the fine points of the controlling mechanism for normal and tumor cell division. In this paper, we present a hypothesis to explai the regulation of both normal and tumor cell division. This hypothesis is based on a number of cellular systems - procaryotic and eukaryotic, dividing and nondividing. We believe that an underlying biochemical unity is present in all these systems so that observations made in one system may be applicable to another.

MODELS FOR CELL DIVISION

Our model for normal cell division is shown in Figure 1. In this model, we propose that a signal to start cell division occurs at the plasma membrane. This causes H_2O_2 to be formed, allows glucose to enter the cell, and affects the ratio of the various cyclic nucleotides. These events cause the loss of catalase and peroxidase, a circumstance which allows intracellular hydrogen peroxide to accumulate. We hypothesize that H_2O_2 is the ultimate cause of normal cell division.

EVIDENCE FOR MODEL OF NORMAL CELL DIVISION

Our model for normal cell division is based on observations in experiments in several systems with special emphasis on the effect of

MODEL FOR REGULATION OF NORMAL CELL DIVISION - START SIGNAL

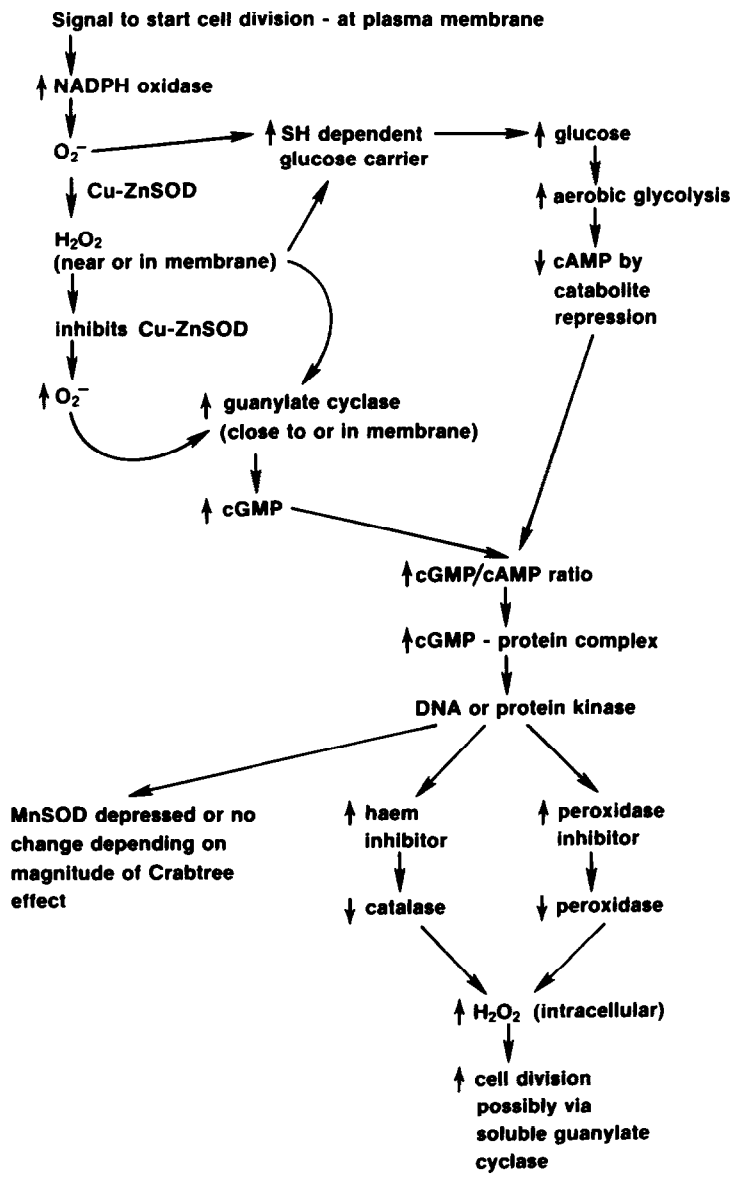

Figure 1. Model for regulation of normal cell division: Signal to start division.

insulin in rat adipocytes. We believe that similar effects are present in most mammalian cells. Insulin is an appropriate model because it can stimulate cell division in-vitro (2). We hypothesize that the signal to start cell division usually works in a manner similar to that of the action of insulin - i.e., the production of H_2O_2. Thus, insulin binds to the cell membrane and this stimulates intracellular H_2O_2 production. May and Haen have shown that insulin stimulates hydrogen peroxide production in rat epididymal fat cells (3). These authors suggested that H_2O_2 may act as a second messenger for the observed effects of insulin. H_2O_2 and insulin have both been shown to stimulate glucose transport in rat adipocytes (4). May and Haen have also shown that lipid synthesis from glucose is enhanced over a narrow range of H_2O_2 concentrations (0.15 to 0.5 mM) added to the incubation medium (5). As with insulin, H_2O_2 was found to stimulate greater glucose incorporation into glyceride-fatty acid than incorporation into glyceride-glycerol. H_2O_2, like insulin, also increased the amount of pyruvate dehydrogenase in the active form without increasing the total amount of pyruvate dehydrogenase. While medium glucose per se was found to activate the enzyme, it is unlikely that the effect of H_2O_2 was mediated by the known enhancement of glucose transport since the effects on the enzyme were maximal in the absence of glucose in the incubation medium. These findings add to the growing list of insulin effects that are reproduced by H_2O_2 and strengthen the hypothesis that assigns H_2O_2 the role of second messenger of insulin.

Other compounds have also been recently shown to affect H_2O_2 production (6). Oxytocin (10^{-8}M) and [Arg^8] vasopressin (10^{-6}M), both hormones with known insulin-like activities in fat cells, stimulated H_2O_2 production 123% and 119% respectively. Nerve growth factor β-subunit (10^{-6}M), an amino acid sequence homolog of insulin, stimulated H_2O_2 production to 112%, while relaxin (10^{-6}M), another homolog, had no effect. Glucagon inhibited H_2O_2 production with the same dose dependence as it stimulated lipolysis, depressing it maximally to 29%. Glucagon also inhibited insulin - and oxytocin - stimulated H_2O_2 production. Similar inhibition was obtained with β-adrenergic agonists and corticotropin. Dibutryl cyclic AMP (5×10^{-3}M) lowered H_2O_2 production to 52%, while nicotinic acid (10^{-6}M), an inhibitor of adenylate cyclase, stimulated H_2O_2 production to 182%. The authors conclude that inhibition of H_2O_2 production is the result of elevated 3',5'-cyclic adenylic acid (cAMP) levels. This fact is part of the basis for our model of stopping cell division which we will discuss later.

With this background, the question arises where does the H_2O_2 come from? One of the most obvious possibilities is from superoxide (O_2^-). It has repeatedly been shown that in phagocytic cells superoxide is produced (7,8,9). Although these are mostly nondividing cells, we believe similar biochemical pathways are probably found in dividing cells. The source of O_2^- in the neutrophil has been identified as a NADPH oxidase located in the plasma membrane (10). We hypothesize that a similar mechanism is present in most mammalian cells although the function of O_2^- and H_2O_2 produced in nonphagocytic cells is obviously

different from that in phagocytosis. The O_2^- once formed can nonenzymatically dismute or dismute enzymatically through the copper and zinc containing superoxide dismutase (Cu-Zn SOD) to form H_2O_2. Thus, H_2O_2, as well as being the second messenger of insulin and the bactericidal agent of phagocytic cells, may also be the second messenger for cell division. It is not essential in our model that H_2O_2 be formed from O_2^-; it is essential only that H_2O_2 be formed. It is possible that H_2O_2 is formed by a mechanism not involving superoxide.

In our scheme, two oxygen species have been formed - O_2^- and H_2O_2. We hypothesize that these species have at least three effects. One of these, the inhibition of Cu-Zn SOD, will be discussed later. The second effect is activation of guanylate cyclase near the plasma membrane. Plasma membrane guanylate cyclase may not be involved because only soluble and not particulate guanylate cyclase has been reported to be activated by H_2O_2 (11). This does not mean that the membrane guanylate cyclase could not be activated by other species such as O_2^- or O_2^- plus H_2O_2. Membrane guanylate cyclase has not been tested in this manner, as Mittal and Murad looked only at soluble guanylate cyclase (12,13). However, the identity of the guanylate cyclase is not essential to our theory; only the fact that H_2O_2 produced from the membrane activates a form of guanylate cyclase which releases 3',5'-cyclic guanylic acid (cGMP) into the cytoplasm is essential. H_2O_2, ·OH formed from H_2O_2, and O_2^- itself have been hypothesized to cause activation of guanylate cyclase (11,12,13,14). Mittal and Murad claim that both O_2^- and H_2O_2 are needed for activation (12,13). Activation of guanylate cyclase will cause an increase in cGMP, a compound which has been postulated to be responsible for the control of cell proliferation. Evidence for the role of cGMP in cell division has been provided by lymphocytes; in this system, levels of cGMP rise immediately after stimulation with mitotic agents (15). However, intracellular guanylate cyclase can only be activated by H_2O_2 or ·OH only if catalase and peroxidase are low in activity. This brings us to the third effect of the activated oxygen species. We postulate that the third effect of these activated oxygen species is to activate the glucose carrier (3,4,5). Most likely it is H_2O_2 alone that does this, although no one has tested O_2^-. The glucose carrier is a sulfhydryl dependent protein (16). Both O_2^- and H_2O_2 readily react with sulfhydryl compounds (17). Activation of the membrane glucose carrier causes an increase in glucose in the cell and this activates glycolysis. This depresses the level of cAMP in *E. coli* by the mechanism known as catabolite repression (18). We propose that glucose also depresses cAMP by a similar mechanism in mammalian systems.

Thus, we have two phenomena - rise of cGMP and decline of cAMP. We believe it is this ratio of cGMP to cAMP that is important in cell division. This is not a new idea, but has been championed by Rudland (19). Thus, it is not necessary that cAMP always decrease and cGMP always increase, but that the ratio $\frac{cGMP}{cAMP}$ increases. Thus, in some systems, cAMP may not change, but cGMP could increase, causing an increase in the ratio. This theory explains most of the observations on cAMP and cGMP. In most cases, cAMP appears to stimulate cells to proceed toward G_0 and towards fuller expression of their differentiated functions (20).

In a few cell types, cAMP may not be involved or may act in the opposing direction (20). In particular, regenerating liver has been reported to contain elevated levels of cAMP and no changes in cGMP (21,22). Hepatomas in-vivo also have been reported to have elevated levels of cAMP (23). These hepatomas also contained elevated levels of cGMP (24). However, there is conflicting data in this area because recent work in several rat Morris hepatomas showed no changes in cAMP but greatly increased levels of cGMP (25). This latter observation is consistent with our theory.

We believe the ratio of $\frac{cGMP}{cAMP}$ is important in dividing, mammalian cell systems for the same reason it is true in E. coli. In this system, cAMP binds to the cAMP-receptor protein (CRP). The binary complex of CRP with cAMP represents an effective catabolite gene activator complex to promote transcription of β-galactosidase and galactokinase mRNA. cGMP complexes as well as cAMP with CRP; it can compete with cAMP for binding; and it forms a complex with this receptor protein that does not permit transcription of the gal or lac operon (26). That the inhibition of transcription derives from an interaction of cGMP with CRP is indicated by the observation that in mutant strains of E. coli devoid of the binding protein, the induction of the nonconstitutive enzymes is not suppressed by cGMP (27).

We postulate a similar cAMP binding protein (CRP) exists in mammalian cells and is important in cell division. In mammalian cells, many proteins bind cAMP - especially the regulatory subunit of protein kinases. There is evidence for a CRP-like protein in mammalian cells. In calf thymus cells, a fraction (about 1%) of cyclic AMP-binding protein was found to have an affinity for DNA (28). Binding protein-cyclic AMP complex preferentially bound to the native forms of calf thymus DNA compared to denatured DNA. This protein may be similar to CRP protein in E. coli.

Thus, we feel that an increase in $\frac{cGMP}{cAMP}$ ratio will lead to an increase in the cGMP-protein complex and a decrease in the cAMP-protein complex. This complex will then bind to DNA and prevent transcription of some operons while activating others. Alternatively, cAMP or cGMP may bind to a regulatory unit of a protein kinase and activate or deactivate this protein. This may lead to activation or depression of nuclear genes via histone phosphorylation.

In any case, we hypothesize that the elevated glucose causes repressior of the synthesis of both catalase and peroxidase. The evidence that this occurs has been provided by Hassan and Fridovich in experiments with E. coli (29). They have shown that glucose suppresses the synthesis of catalase and of peroxidase in E. coli. Exhaustion of the glucose present in a complex medium, or abrupt transfer of cells from a glucose-containing to a glucose-free medium, resulted in a sharp increase in both catalase and peroxidase. The glucose effect was diminished by cAMP, thus identifying it as catabolite repression. Aerobic growth of E. coli compared to an anaerobic growth caused a large increase in catalase

and peroxidase. This was not dependent on either O_2^- or H_2O_2, since a similar induction was caused by the addition of nitrate under anaerobic conditions. It was concluded that catalase and peroxidase are co-induced with the components of the respiratory chain.

Hassan and Fridovich have shown that glucose also suppresses levels of superoxide dismutase (SOD) (presumably manganese containing superoxide dismutase (Mn SOD), although they do not say)(30,31). Thus, growth of E. coli using glucose is associated with a low intracellular level of superoxide dismutase. Exhaustion of glucose, or depression of the pH due to the accumulation of organic acids, caused these organisms to then obtain energy from the oxidative degradation of other substances in a rich medium. This shift in metabolism was associated with a marked increase in the rate of synthesis of superoxide dismutase. Depression of the synthesis of SOD by glucose was shown to be not due to catabolite repression since it was not eliminated by cAMP and since dimethyl glucoside did not mimic the effect of glucose. Moreover, glucose itself did not depress SOD synthesis when the pH had fallen low enough to cause a shift to non-fermentative metabolism. These authors conclude that SOD synthesis is controlled directly or indirectly by the intracellular level of O_2^- and that glucose depressed the level of this enzyme because glucose metabolism is not associated with as rapid production of O_2^- as is the metabolism of many other substances. In accord with this view is their observation that paraquat, which can increase the rate of production of O_2^- by redox cycling, caused a rapid and marked increase in SOD. Because evolution conserves key biochemical pathways, we believe higher organisms exhibit similar responses to glucose.

Thus, in our model of cell division, one might also expect Mn SOD to be depressed in normal cell division since glucose is elevated. In accord with this view is Yamanaka's observation that trypsin, which has been shown to stimulate cell division in many cell types, causes the loss of Mn SOD in WI-38 human lung fibroblasts (32). We feel trypsin could act by altering the cell surface so that more glucose is allowed to enter the cell. This will then cause suppression of Mn SOD because of inhibition of respiration, lack of O_2^- production and, thus, lack of need for Mn SOD activity.

However, this model is hard to reconcile with our observation in regenerating mouse liver (33). In this proliferating normal cell system, we did see inhibition of Cu-Zn SOD at 4 days (the time of maximum mitotic activity) as predicted by our model. However, Mn SOD did not decline, showing that something is different here than in E. coli and in the lung fibroblasts.

This brings us to the second question. What evidence is there in mammalian cells that glucose has the same effects on antioxidant enzyme patterns as in E. coli? The answer to this question is found in the Crabtree effect (34). It has been known for years that glucose inhibits respiration in some mammalian tissues. However, this effect is different from E. coli and other bacteria in two respects: (a) it does not occur

in all cells; (b) the effect is not total but respiration is inhibited only partially. These facts easily explain the lack of inhibition in regenerating liver because liver has a respiratory rate which is not affected by glucose (35). Thus, if the respiratory rate does not change, neither would the amount of O_2^- and Mn SOD. In contrast, the fibroblast studied by Yamanaka may exhibit a high Crabtree effect. Moreover, the effect of trypsin may be non-physiological so that much more glucose enters the cell than is normally seen. Loss of Mn SOD does not seem to be essential to activating normal cell division. In contrast, inhibition of Cu-Zn SOD seems to be usually necessary for normal cell division to occur. We have shown that Cu-Zn SOD is diminished in regenerating liver (33). We postulate that this occurs because of the burst of H_2O_2 produced after a signal for cell division arrives. H_2O_2 has repeatedly been shown to inactivate Cu-Zn SOD at concentrations as low as 4.2×10^{-5}M (36).

We believe the signal for cell division involves the increased intracellular H_2O_2 produced when catalase and peroxidase are inhibited. This may cause cell division by itself or by activation of soluble, intracellular guanylate cyclase. To clarify this issue, it would be of interest to see if there are two peaks of cGMP after initiation of cell division.

MODEL FOR STOPPING NORMAL CELL DIVISION

Our model for stopping cell division is shown in Figure 2. In this case, we postulate that the signal to stop cell division activates membrane bound adenylate cyclase. This leads to elevated levels of cAMP. Because of elevation of cAMP, the ratio of $\frac{cAMP}{cGMP}$ increases, cGMP is replaced from the cGMP-protein complex and a cAMP protein complex is formed. Then in analogy with E. coli, the cAMP-protein complex may increase the amount of mRNA corresponding to a lac like operon and thus promote the transcription of glucose-repressed operons (possibly via a protein kinase). Or it may simply cause suppression of the catalase and peroxidase inhibitors. In any case, catalase and peroxidase activities increase drastically. This leads to decreased intracellular H_2O_2 production. As discussed before, the fact that increased cAMP levels can lead to depressed H_2O_2 levels has been shown in rat adipocytes. Thus, dibutryl cAMP greatly inhibited H_2O_2 production stimulated by insulin, while nicotinic acid, an inhibitor of adenylate cyclase, stimulated H_2O_2 production (6). We hypothesize that elevated cAMP also leads to a microtubule-cAMP complex that inhibits NADPH oxidase activity and leads to lower O_2^- and H_2O_2 production in the plasma membrane. Recent work with polymorphonuclear leukocytes (PMNS) shows that such a system exists (37). Nakagawara and Minakami have shown that the release of O_2^- from human PMN's induced by cytochalasin E was greatly enhanced by the pretreatment of the cells with deuterium oxide or with concanavalin A. Colchicine and vinblastine, microtubule disrupting agents, as well as cAMP inhibited the release. The authors suggest the involvement of microfilament-microtubule system, which connects directly to the inner layer of the cell membrane, in the production and release of superoxide.

MODEL FOR REGULATION OF NORMAL CELL DIVISION - STOP SIGNAL

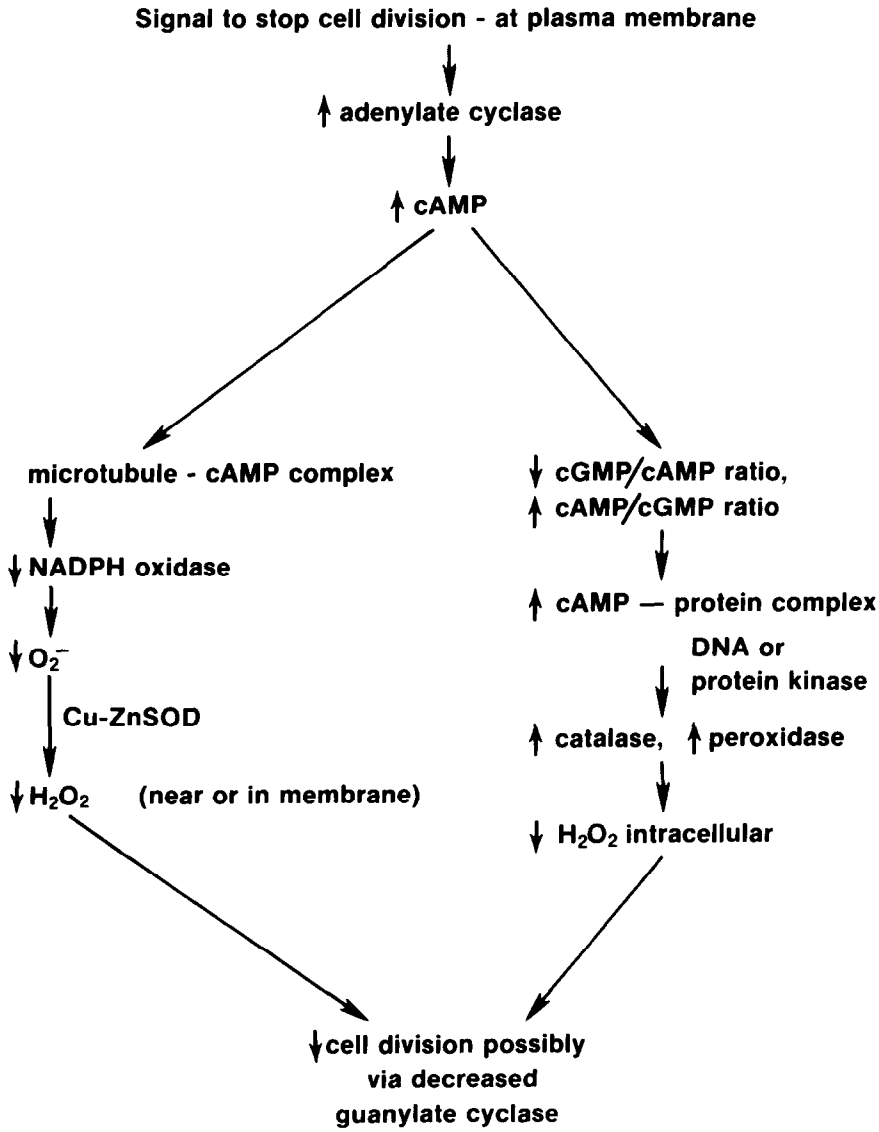

Figure 2. Model for regulation of normal cell division. Signal to stop division.

We believe the decreased membrane and intracellular H_2O_2 production lead to decreased cell division possibly due to decreased guanylate cyclase. Of course, if O_2^- alone activates guanylate cyclase, then the latter cannot be true.

SIMPLE MODEL OF CANCER

In this paper we will present two models of cancer, both of which may be true in different cancers. The first is the simpler model. This model is in essence what we have proposed in our earlier paper (1) and is shown in Figure 3. We hypothesize that a change in DNA or its expression causes a loss of Mn SOD (1). This leads to high levels of O_2^- (1). This O_2^- can in turn diffuse to the plasma membrane where it activates the glucose carrier by itself or through H_2O_2. This leads to elevated glucose and depressed cAMP, two common characteristics of tumors (38). This elevated glucose leads to the decreased catalase and peroxidase seen in tumors (39,40). Superoxide may also interact with the nucleus to cause either inhibition of cell differentiation or to cause cell proliferation (1). Thus, in this model, loss of Mn SOD would cause both the differentiation and proliferative changes seen in tumors.

EVIDENCE FOR MODEL

Evidence for each of the features of the model are presented in the above references. If our model for cancer is correct, one would expect that adding compounds with SOD activity to tumor cells would have a profound effect on their growth. Indeed, we have shown that copper coordination compounds with high SOD activity cause slower tumor growth and large increases in host survival in-vivo (41). Moreover, copper coordination compounds greatly slow down the growth of in-vitro neuroblastoma cells and cause large increases in cellular differentiation (42). Preliminary experiments with an iron compound with SOD activity also indicate that it slows down the growth of in-vivo tumor cells (Leuthauser, S.W.H.C. and Oberley, L.W., unpublished observations).

We would like to further discuss implications of the model. If we add cAMP to a tumor cell, it will allow catalase and peroxidase to be activated. This will allow the tumor cell to differentiate and stop dividing, as has been shown for many tumor cells (20). If the tumor cell produces O_2^-, then as soon as the cyclic AMP is removed, the tumor will start growing again as catalase and peroxidase will again be repressed. Thus, cAMP is totally reversible (43). Moreover, O_2^- is apparently formed in the nucleus and lack of Mn SOD there will most likely have some effect on growth (44).

One exception to this general picture is the case of neuroblastoma. Mammalian brain derives all of its energy from glucose metabolism (45). Thus, it is not altogether surprising that rat brain mitochondria have been reported not to produce superoxide as all superoxide production may be inhibited by glucose. Since O_2^- is not produced by brain mitochondria, it would not be surprising if these organelles contained no antioxidant

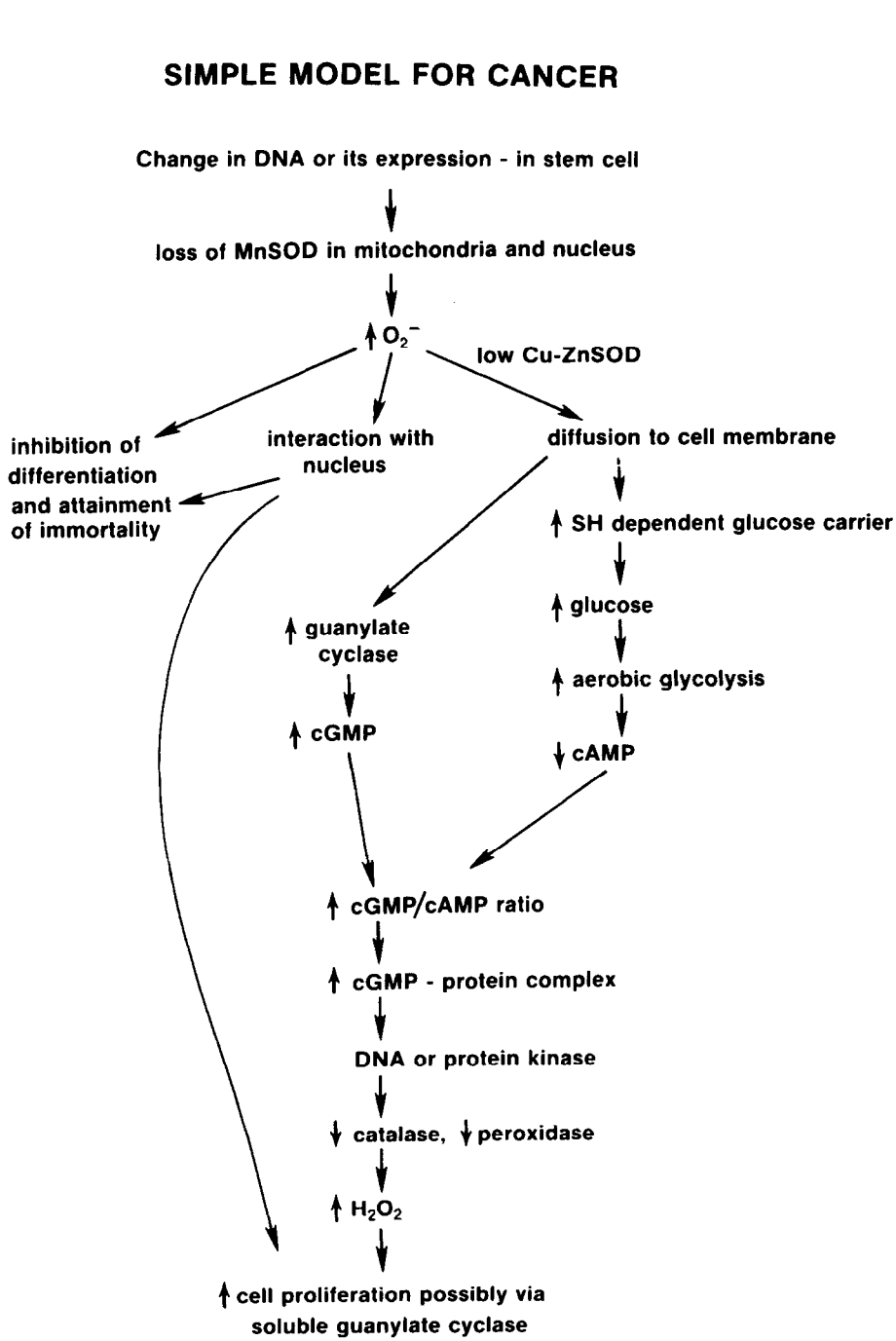

Figure 3. Simple model for cancer

enzymes. Normal mouse brain has Mn SOD, which is diminished in neuroblastoma, but it is not known if Mn SOD is mitochondrial in origin (46). Similarly, neuroblastoma cells contain glutathione peroxidase, but the subcellular localization is unknown (47). Catalase has been reported to be absent from rat brain mitochondria (48). In any case, cAMP causes irreversible differentiation of neuroblastoma cells (49). We hypothesize that it is irreversible simply because there is no superoxide present to reverse the effects of cAMP. That the differentiation of neuroblastoma cells is associated with a rise in antioxidant capacity has been shown by Arneson et al. (50). They have shown that cultured mouse neuroblastoma cells exhibit a striking increase in antioxidative capacity during the transition from logarithmically dividing cells to nondividing, neurite-bearing cells. This is manifested by a dramatic increase in resistance to lipid peroxidation. However, no one has measured catalase and peroxidase levels after differentiation.

It could be argued that a reverse sequence of these events are what occurs in malignancy. That is, a change in DNA or its expression causes a change in membrane structure which causes activation of the glucose carrier. This in turn increases glucose, depresses cAMP, and depresses Mn SOD, catalase, and peroxidase. There are two main arguments against this line of reasoning:

1. Glucose would not cause total repression of Mn SOD because the Crabtree effect is not absolute. Moreover, some tissues like liver, show no Crabtree effect (34).

2. In a recent comparative electron microscopic study of normal and tumor tissue, only mitochondrial and nuclear changes were noted in all tumor cells (51). The plasma membranes were apparently normal. Mitochondrial and nuclear damage may be caused by lack of Mn SOD coupled with O_2^- production, as argued in our recent paper (44). If membrane damage were primary, all membranes should be damaged. Of course, plasma membranes in tumor cells have repeatedly shown to be abnormal, but if the above report is believed, these abnormalities must not lead to structural damage.

The beauty of this model for cancer is that it explains the increased glycolysis seen in tumors. Many years ago, Warburg hypothesized that the increased glycolysis of tumors was caused by damage to the respiratory apparatus - i.e., the mitochondria (52). More recent evidence has implicated that the alterations in glucose metabolism in tumor cells are a consequence of a primary alteration in plasma membrane glucose transport (38). Our theory can unite these two disparate points of view. Mitochondria are damaged due to loss of Mn SOD, but this damage to the respiratory apparatus is not actually what causes the increased glycolysis. Rather, it is the increased flux of superoxide which diffuses to the plasma membrane and activates the glucose carrier that causes the increased glycolysis. Hence, both theories are in essence right.

SECOND MODEL FOR CANCER

The problem with the above model of cancer is that is assumes that superoxide can diffuse from the mitochondria to the plasma membrane.

This is at least partially feasible because superoxide has been shown to easily pass through cell membranes (53). However, diffusion can only take place if cytosol SOD is very low. Thus, in those tumors like the fast growing Morris hepatomas where both Cu-Zn and Mn SOD are very low, such a model may be valid (1). However, in most tumor cells, there is probably enough cytosol SOD to prevent much superoxide from diffusing from the mitochondria to the membrane. Thus, in those cases, we must seek a new model.

The simple model for cancer could hold if one of three conditions were met. First of all, if the Cu-Zn SOD is already low before transformation, superoxide could then readily diffuse across the cytoplasm. Second, if a second change at the nuclear level occurred, so that Cu-Zn SOD was low, then the above model would also be correct. Two changes in DNA or its expression would have to take place because Mn SOD and Cu-Zn SOD are coded for by genes found on different chromosomes (54). Third, the above model would hold if so much H_2O_2 were produced in the cell that most of the Cu-Zn SOD were inactivated. H_2O_2 could be produced from O_2^- in the mitochondria because of low Mn SOD, catalase, and peroxidase. One of these three mechanisms of Cu-Zn SOD loss must occur in most tumor cells because tumor cells are generally low in Cu-Zn SOD (1). However, exceptions have been reported to show loss of Cu-Zn SOD is not universally seen in tumors (55). It is possible that the H_2O_2 which inactivates tumor Cu-Zn SOD comes not from the mitochondria, but from the NADPH oxidase in the cell membrane via the start signal for cell division.

None of the above models explain recent data that shows that the sole transforming protein of the Rous sarcoma virus (coded by the src gene) is a cAMP independent protein kinase (56). Most likely this protein initiates cell division by generating the start signal by phosphorylating a protein(s). However, our theory and the data on the src gene can easily be reconciled into a unified theory. This theory is shown in Figure 4. We hypothesize in this model that here are two steps that must be fulfilled to lead to cancer. One step is the attainment of cell line immortality (involving Mn SOD) and the other is the loss of control of cell proliferation. The reasoning behind this is as follows. An in-vitro cell strain derived from normal cells is mortal, exhibits contact inhibition and anchorage dependence, and is non-tumorigenic. Sometimes during the culturing of a cell strain, a "crisis" will occur and the cell strain will turn into a normal cell line. This normal cell line is immortal. It, however, exhibits contact inhibition, anchorage dependence, and is non-tumorigenic. A tumor cell line by contrast is both immortal and does not exhibit contact inhibition or anchorage dependence.

We would argue that immortality in a cell line is caused by loss of Mn SOD coupled with superoxide production. This excess superoxide production leads to a cell which cannot differentiate (1). Thus, when a cell in a cell line divides, it gives rise to two cells which never fully differentiate and thus always have the capacity to proliferate.

TWO STEP MODEL FOR CANCER

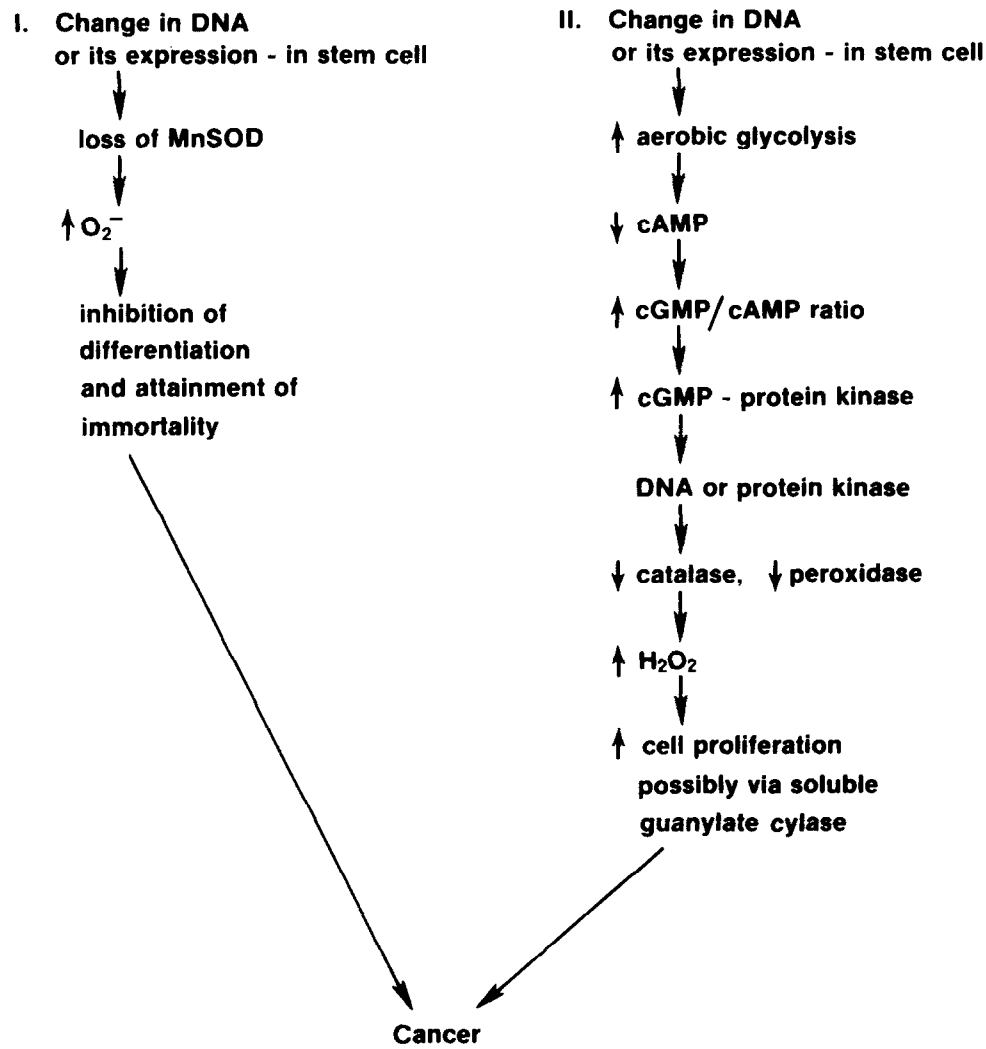

Figure 4. Two step model for cancer.

However, those cells are not tumor cells because they are contact inhibited and under growth control. Thus, in-vivo, they will not proliferate for a long time. A tumor cell, on the other hand, always has both the potential to divide and is not contact inhibited or anchorage dependent, so it can always divide. Lack of contact inhibition and anchorage dependence occurs because of change in the cell membrane so that a start signal for cell proliferation is always being sent.

Both immortality and loss of growth control are necessary for tumorigenicity; neither is sufficient by itself. Suppose a cell has lost its growth control so that it is always dividing. This cell is still not a tumor cell because it will only divide for a finite number of times and then will cease dividing. Conversely, suppose a cell has become immortal, but still has control of growth. Then if these cells are injected into an animal, they will not form a tumor because they will stop dividing as soon as a stop signal such as contact inhibition occurs. Thus, both loss of mortality and loss of growth control are necessary for tumorigenicity. It should be emphasized that when a cell line is used in the transformation experiment, only one gene product is necessary for transformation - that involving cell division. This is because the Mn SOD is already diminished in a cell line. On the other hand, in a cell strain, two transformation products are necessary - one for loss of Mn SOD and one for loss of cell division regulation. It should also be pointed out that we are not the first to propose a two step model for cancer. Studies involving tumor initiation and promotion have also suggested a two step model. It is tempting to speculate that initiation involves cell immortality via the stem cell, while promotion affects cell division. One should also realize that some diseases may involve only loss of growth control and not involve cell immortality. Benign tumors, psoriasis, and atherosclerosis are possibilities of diseases which affect only loss of growth control.

An alternative model is possible. In order to obtain immortality, it is only necessary that a stem cell on division produce two other stem cells, both of which do not differentiate. It is possible that this involves only a halt in the differentiation of a stem cell without involving Mn SOD. In this model, the Mn SOD and O_2^- levels would only reflect the levels found in the stem cell at the time of transformation. O_2^- levels would not continue to increase after transformation. If this model is correct, superoxide would be involved only in cell division and not in differentiation. However, this model is hard to reconcile with the data in the Morris hepatomas in which the capacity to produce superoxide in the slow growing Morris hepatomas was the same as in normal liver, yet the levels of Mn SOD are greatly lowered (44). Moreover, the two models already presented have the advantage that superoxide scavengers affect both differentiation and proliferation. It is often true that agents which affect differentiation affect proliferation and vice versa.

This theory can also explain observations on neuroblastoma cells. We believe neuroblastoma is derived from the brain stem cell. This is, of course, not a new hypothesis but explains the incidence of the disease

in young people only. When transformation occurs, it occurs in a neuronal stem cell which has low Mn SOD; in support of this concept is the fact that prenatal brain and neuroblastoma cells have low Mn SOD (46,55). The Mn SOD activity is thus fixed at a low level by transformation. However, the malignant phenotype does not occur because Mn SOD is unable to increase as in other cell lines. The malignant phenotype occurs because there is a defect in cell proliferation. Most likely this involves a membrane change that involves calcium, because phosphodiesterase, a calcium dependent enzyme, has been shown to be the likely cause of the increased growth due to its increased activity.(49). Differentiation in the case of neuroblastoma does not involve mitochondrial O_2^- because O_2^- is not produced by brain mitochondria (48). cAMP alone governs differentiation and cell division in this system. In support of this idea is the fact that dexamethasome and cAMP differentiate in-vitro neuroblastoma cells without causing any changes in Mn SOD, but changes are observed in Cu-Zn SOD (57). It should be emphasized that in this system, cyclic nucleotides must control both cell proliferation and differentiation. In other words, halt of cell proliferation by non-cAMP agents is not enough to cause differentiation. This has been shown by Prasad who has shown there can be halt in cell proliferation without differentiation (49).

It may seem difficult at first to reconcile either of our models with the fact that a copper coordination compound with SOD activity differentiates neuroblastoma cells (42). This is because if O_2^- has nothing to do with differentiation in this system how can an SOD be effective? The answer is that SOD affects two systems - that of differentiation and of proliferation. During proliferation, O_2^- is also produced, not in the mitochondria, but in the membrane. As outlined in Figure 1, during a signal for cell division, H_2O_2 is produced which inhibits Cu-Zn SOD. This allows O_2^- to arise, which can activate guanylate cyclase. But copper coordination compounds will keep O_2^- levels low and thus prevent this pathway from being activated. Thus, cell proliferation will be turned off and differentiation can occur. Moreover, this makes sense because the copper coordination compound we have tested is lipid soluble; it will thus most likely reside in the cell membranes. It is possible that copper coordination compounds work in all tumor systems by acting as Cu-Zn SOD and thus halting cell proliferation rather than acting as a Mn SOD and affecting differentiation.

This model also explains the data on the src gene. Transformation of chicken embryo cells by the Rous sarcoma virus is dependent on the expression of the src gene (58). The src gene product is a phosphoprotein, called $pp60^{src}$ which has cAMP independent phosphotransferase activity (59). Although other enzymic functions for $pp60^{src}$ have not been ruled out, it is likely that cellular transformation by Rous sarcoma virus can be attributed in part to about 100-fold higher levels of cAMP-independent protein kinase (56). One or more of the targets of $pp60^{src}$ appear to reside in the cytoplasm since enucleated cells can express the specific morphological transformation (60). A mainly cytosolic location of $pp60^{src}$ is suggested by experiments with immunofluorescence (61,62) and cell fractionation studies (63). It is of interest in this context that

the two key regulatory enzymes of glycolysis, namely, phosphofructokinase and pyruvate kinase, are also cytosolic and controlled by cAMP-independent protein kinases (64,65). However, membrane-bound forms of pyruvate kinase (66) and of $pp60^{src}$ have also been reported (67). Convincing evidence has recently been presented that pyruvate kinase enzyme type M_2 or one of its regulatory proteins are among the targets of $pp60^{src}$ (56). Since pyruvate kinase is a control point of glycolysis in tumor cells (68), it is possible that the altered kinetic properties of this enzyme in tumor cells (69) reside in alterations in the cAMP-independent protein kinase which controls pyruvate kinase and is responsible for the high aerobic glycolysis of tumor cells. This theory is consistent with our model. We believe the $pp60^{src}$ phosphorylates pyruvate kinases and this (plus other factors) turns on glycolysis and starts cell division. This activates the same pathway needed for normal cell division; i.e., in that case glucose is elevated and this turns on glycolysis and starts cell division. It should be emphasized that high aerobic glycolysis is probably not necessary in all tumor cells; it is simply necessary that cell division be turned on by activating any step in the pathway shown in Figure 4. Any alteration that can elevate H_2O_2 may cause cell division. Activation of the hexose monophosphate shunt might also stimulate cell division since this would cause elevated NADPH, the substrate for NADPH oxidase, and elevated levels of nucleic acid precursors. Elevated NADPH might then also lead to elevated H_2O_2 production.

Lastly, we would like to speculate on the control of cell differentiation. We have hypothesized that loss of Mn SOD is what prevents tumor cells from differentiating. At the same time, cAMP has been shown to promote the differentiated phenotype in a number of systems. How can these two views be reconciled? We would like to speculate that these two systems are involved in different differentiation pathways. O_2^- and Mn SOD control "stem cellness" (i.e., attainment of immortality) while cAMP controls cell proliferation. Thus, changes in either O_2^- or cAMP can affect differentiated functions, but different ones.

EFFECT OF TUMOR ON THE HOST

We have recently found that tissues other than the tumor have depressed SOD activities in mice bearing transplantable Ehrlich ascites carcinoma (Leuthauser, S.W.H.C. and Oberley, L.W., unpublished observations). Thus, liver, spleen, and lung showed depressed SOD activities, while the kidney showed only slight changes. Mn SOD seemed to be affected in all cases. Catalase in the host liver has also been shown to be depressed in animals bearing hepatomas (70). This has been shown to be due to an inhibitory substance produced by the tumor and carried by the bloodstream. This substance has been purified to some extent and found to be a substance of low molecular weight, stable to acids and alkalis which inhibits haemoprotein enzymes in-vitro by combining with the haem prosthetic group. A similar catalase inhibitor has been obtained from embryo extract (71). SOD in these tissues could be inhibited for a number of reasons. One possibility is the high blood glucose seen in tumor bearing hosts (72); however, others have reported low blood glucose in tumor bearing animals (73). High glucose can cause depression of Mn SOD

because of inhibition of respiration via the Crabtree effect. Alternatively, SOD may be low because these tissues are being starved due to the tumor and thus have little respiration. Catalase and peroxidase would be inhibited because of the haem inhibitor described above or alternatively by the glucose which gets into the cell.

These observations also partially explain the mechanisms of glucose inhibition of catalase activity described in Figure 1. Elevated glucose acts through cAMP to cause synthesis of the haem inhibitor described above. This inhibitor combines with the haem prosthetic group and prevents iron from binding to catalase. Similar mechanisms may prevent peroxidase from gaining activity.

SUPEROXIDE IN CELL PROLIFERATION

In our previous paper (1), we hypothesized that activated oxygen species halt normal cell division, whereas in the present work we are saying that it starts normal cell division. We now believe that oxygen species can do both depending on the amount and localization. In the previous work, we based our conclusion on the fact that increased levels of oxygen halt normal cell division in-vitro tissue culture. We pointed out that this most likely occurs because the dividing, "stem-cell" population was killed by oxygen. This is most likely true at high oxygen concentrations. Lower fluxes of superoxide delivered to the cell surface will cause cell division, not halt in cell proliferation via cell death.

CONCLUSION

A unified theory of cell division and two models of cancer dependent on this theory have been presented. Much of the theory is untested; the purpose of our model is to stimulate experimentation.

It should be emphasized that we have presented a general model. As an example, liver seems to be different from other tissues in a number of ways. cAMP increases not decreases with cell proliferation and no Crabtree effect is observed after glucose addition. These facts may all be a consequence of the fact that liver processes most of the glucose in the body and thus many changes may have to be made to compensate for the high levels of glucose in the tissue. Because of the high background levels of glucose in the liver, glucose may not be so important in regulating cell division in this system.

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