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Controlling Tumor Growth by Modulating Endogenous H_2O_2

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Abbreviations:

AT, 3-amino-1,2,4-triazole.
BCNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea
DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate
GPx, glutathione peroxidase.
GSSG, glutathione disulfide.
H_2O_2, hydrogen peroxide.
4-HNE, 4-hydroxy-2-nonenal
$O_2^{\bullet-}$, superoxide.
ROS, reactive oxygen species.
BSO, buthionine sulfoximine
CAT, catalase.
GSH, glutathione
GR, glutathione reductase
$HO^{\bullet}$, hydroxyl radical.
MDA, malondialdehyde
MnSOD, manganese-containing superoxide dismutase
SOD, superoxide dismutase

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Abstract

Reactive oxygen species (ROS) are generated during normal metabolic process in all of the oxygen-utilizing organisms, but may inflict cellular damage when generation is increased and antioxidant defense mechanisms are overwhelmed. Much experimental and epidemiological evidence indicate that ROS, especially H_2O_2 contribute to the initiation and promotion of carcinogenesis, so drugs or treatments aimed to reduce the tissue content of H_2O_2 can be chemopreventive and curative against cancer. The levels of H_2O_2 in cells are determined by their rates of generation from SOD and their removal by CAT or GPx. Higher concentration of H_2O_2 can suppress tumor growth. So modulation of endogenous H_2O_2 will play important role both in carcinogenesis and cancer prevention.

Introduction

ROS (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 (like superoxide, hydroxyl, and peroxyl radicals), but also non-radical molecules like singlet oxygen and hydrogen peroxide. ROS are generated by normal physiologic processes, including aerobic metabolism and inflammatory response in all of the oxygen-utilizing organisms. Significant fractions (approximately 2%) of oxygen are converted to the superoxide radical and its reactive metabolites (ROS) in and around mitochondria [1] ROS exert some functions necessary for cell homeostasis maintenance. ROS can react with various intracellular molecules, including lipids, proteins, and DNA. When produced in excess, ROS-induced damage can result in cell death, mutations, chromosomal aberrations, or carcinogenesis [2]. This review will focus on the ROS, particular H_2O_2 and carcinogenesis, role of endogenous antioxidant enzymes in carcinogenesis, and controlling tumor growth by modulating endogenous production of H_2O_2.

Reactive Oxygen Species and Carcinogenesis

ROS in metal induced carcinogenesis

Epidemiological data indicate that exposure to metal and metalloid species, including arsenic(III), chromium(VI), and nickel(II), increases the risk of cancer, particularly of the lung and skin [3]. Although the mechanisms of carcinogenesis caused by metals are still

unclear, accumulating evidence indicates that reactive oxygen species generated by metals may play an important role in the etiology of metal-induced carcinogenesis [4].

Metal-induced generation of reactive oxygen species:

1. Fenton-type reaction

One of the most important mechanisms of metal-mediated free radical generation is via a Fenton-type reaction (Reaction 1). In this reaction a transition metal ion reacts with H_2O_2 to generate $HO^{\bullet}$ radical and an oxidized metal ion:

$$\text{metal}^{n+} + H_2O_2 \rightarrow \text{metal}^{n+1} + {}^{\bullet}OH + OH^{-} \quad (1)$$

2. Haber–Weiss-type reactions

The Haber–Weiss-type reactions (Reactions 2, 3 & 4) are also important for metal-induced free radical generation. In these reactions, an oxidized metal ion is reduced by $O_2^{\bullet-}$, and then reacts with H_2O_2 to generate $HO^{\bullet}$ radical:

$$\text{metal}^{n+1} + O_2{}^{\bullet-} \rightarrow \text{metal}^{n+} + O_2 \quad (2)$$

$$\text{metal}^{n+} + H_2O_2 \rightarrow \text{metal}^{n+1} + {}^{\bullet}OH + OH^{-} \quad (3)$$

Overall,

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{metal}^{n+1}/\text{metal}^{n+}} {}^{\bullet}OH + O_2 + OH^{-} \quad (4)$$

3. Metals directly react with cellular molecules

Certain metal ions can react directly with cellular molecules to generate free radicals. For example, reaction of chromium (VI) with cysteine or penicillamine generates corresponding thiol radicals [5]. These radicals may cause direct cellular damage or may also react with other thiol molecules to generate (Reaction 5 & 6) $O_2^{\bullet-}$, which will be converted to H_2O_2 by endogenous antioxidant enzymes.

$$GS^{\bullet} + RSH \rightarrow RSSR^{\bullet -} + H^{+} \quad (5)$$

$$RSSR^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -} \quad (6)$$

Receptors and genes affected by ROS

ROS have been demonstrated to affect a number of receptors and genes, including growth factor receptors, src kinase, ras signaling, mitogen-activated protein kinases, and nuclear transcription factors NF-κB, AP-1, p53, NFAT, and HIF-1 (Table1) [4]. Here the effects of $HO^{\bullet}$, thiyl radicals, $O_2^{\bullet -}$, and H_2O_2 on signal transduction pathways associated with carcinogenesis will be discussed.

1. Hydroxyl radical

The $HO^{\bullet}$ has been implicated in MAPK signaling and gene expression in a variety of cell types. The increased p38 expression observed in the human small cell lung cancer line CL3 treated with Cr(VI) is hydroxyl radical-dependent [6].

2. Thiyl radicals

The $HO^{\bullet}$ can react with cysteine-containing proteins to form thiyl radicals. Equation (7) shows the reaction of a sulfhydryl-containing protein with $HO^{\bullet}$ to form a thiyl radical. The reaction is important because many proteins involved in signaling (EGF receptor, c-src, and phosphatases) possess sulfhydryl groups, which are available to react with ROS [3].

$$\text{protein-SH} + OH^{\bullet} \rightarrow \text{protein-S}^{\bullet} + H_2O \quad (7)$$

3. Superoxide and hydrogen peroxide

Superoxide may play a role in cancer through its ability to affect ras signaling and through its ability to liberate sequestered iron, which allows for additional ROS formation [7]. Hydrogen peroxide is believed to activate MAPK signaling

Table 1. Summary of Metal-Induced Proteins and Their Effectors

Protein	Classification	Role	Metal Inducers	Other Activators
EGF	Growth factor receptor, tyrosine kinase	*Normal:* cell proliferation and angiogenesis *Disregulated:* metastasis	As, Ni	ROS, oxidative stress
VEGF	Growth factor receptor, tyrosine kinase	*Normal:* cell proliferation and angiogenesis *Disregulated:* metastasis	As, Be, Co, Ni	ROS, oxidative stress
PDGF	Growth factor receptor tyrosine kinase	*Normal*: Cell proliferation. *Disregulated*: overexpressed in lung and prostate cancers	Co, Ni	ROS, oxidative stress
Src	Non-receptor tyrosine kinase	Initiates/activates MAPK, NF-kB, PI3K signaling	As, Cr	ROS, UV radiation
Ras	Membrane-bound G-protein	Regulates cell growth Opposes apoptotic effects	As, Be, Fe, Ni	ROS, cell stress, UV radiation. Mutation results in constant growth signals
MAPKs	Serine/threonine kinases	Transmits signals for apoptosis, differentiation, proliferation, cell morphology via phosphorylation	As, Co, Cr, Ni	Growth factor receptors, G-proteins, tyrosine kinases, NF-kB
PI3K/Akt	Membrane-associated kinases	Stimulates Inhibits apoptosis	As, Ca, V, Cu	ROS, src, MAPKs
NF-kB	Nuclear transcription factor	Once activated in nucleus, binds to DNA sequence of target genes involved in inflammation, carcinogenesis, apoptosis	As, Be, Cd, Co, Cr, Fe, Ni, Pb	ROS, MAPKs, cytokines
AP-1	Nuclear transcription factor	Binds to TPA response elements in DNA	As, Cd, Cr, Ni, V	ROS, MAPKs, cytokines, UV radiation
p53	Nuclear transcription factor	Can halt cell cycle and initiate apoptosis, involved in many forms of carcinogenesis	As, Cd, Co, Cr, Fe, Ni	ROS, MAPKs, PI3 kinase, DNA damage, oxidative stress
NFAT	Nuclear transcription factor	Involved in cytokine formation, angiogenesis, muscle growth, and differentiation	Fe, Ni, V	Phosphatase, calcineurin (Ca^{2+})
HIF-1	Nuclear transcription factor	Regulates expression of VEGF, erythropoietin, HO-1, aldose, enolase, and lactate dehydrogenase A	As, Co, Cr, Ni, Pb, V	Hypoxia, ROS, MAPKs, PI3 kinase

by deactivation of protein tyrosine phosphatases [8]. Transient oxidation of critical cysteines can inactivate phosphatases in vivo, allowing kinase signaling to continue until phosphatases are reactivated by reduction. It is possible that the rapid formation and disappearance of ROS also serve as “on and off” switches for cellular signaling [9]. From above research, the roles $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ are critical in the process of metal induced carcinogenesis.

ROS in nonmetal induced carcinogenesis

Szuster et al reported that the production of ROS, both spontaneous and phorbol 12-myristate 13-acetate (PMA)-induced $O_2^{\bullet-}$, was relatively higher in the patients with larynx carcinoma than in the healthy controls and increased parallel with the tumor stage (tumor, node, metastasis-TNM staging). After partial or total laryngectomy, a significant decrease in ROS production was observed [10]. The results indicate the existence of oxidative stress in the blood of patients with larynx carcinoma.

Suzuki et al measured ROS in thymocytes at the thymic prelymphoma stage in C57BL/6 mice. After radiation, a significant fraction of mice (11/13) bearing thymic prelymphoma cells exhibited elevated levels of ROS in thymocytes, which indicated that ROS may play an important role in radiation carcinogenesis [11]. In addition to traditional transition-type mutations at dipyrimidine sites, G:C to T:A transversions induced by 8-oxoguanine during DNA replication, are frequently observed in the ras oncogene and p53 tumor suppressor gene in human skin cancers of sun-exposed areas and in UV-induced mouse skin cancers [12].

Besides, upon UV-exposure, the natural cutaneous antioxidant defense is impaired. Increase ROS generation will enhance carcinogenesis, and supplementation with antioxidants can inhibit skin carcinogenesis, which provided a promising rationale for the development of powerful new antioxidant strategies in the prevention and therapy of skin cancer [13].

Imbalance of antioxidant mechanisms, role of H_2O_2 in Carcinogenesis

In the work done by Skrzydlewska et al [14], the levels of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) as well as copper and zinc-containing superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx) & glutathione reductase (GR) were significantly increased in cancer tissue compared to control group and were the highest in G3-grade adenocarcinoma and clinical IV stage of colorectal cancer. ROS mediated lipid peroxides are of critical importance because they participate in chain reactions that amplify damage to biomolecules including DNA, which may cause mutations in tumor suppressor genes or oncogenes [15].

Compared with control (normal colon mucosa), a decrease of catalase (CAT) activity, and reduced glutathione (GSH) & vitamins C and E were observed with progression of cancer [14]. One recently report by Tas et al gave similar results in breast cancer [16]. Reduced CAT activity and increased CuZnSOD activity pinpointed the importance of H_2O_2 in carcinogenesis [17]. Those results are in consistent with the report that various types of cancer examined to date manifest an imbalance in their antioxidant mechanisms to respect the primary cell [18].

In the work of Policastro et al, an increase in SOD activity and a decrease in the activities of H_2O_2-detoxifying enzymes, as a function of malignancy, coupled with a rise in H_2O_2 and a decrease in $O_2^{\bullet-}$ were demonstrated (Figure 1). Treatment of cells with exogenous CAT showed a dose-dependent inhibition of proliferation. This inhibition was

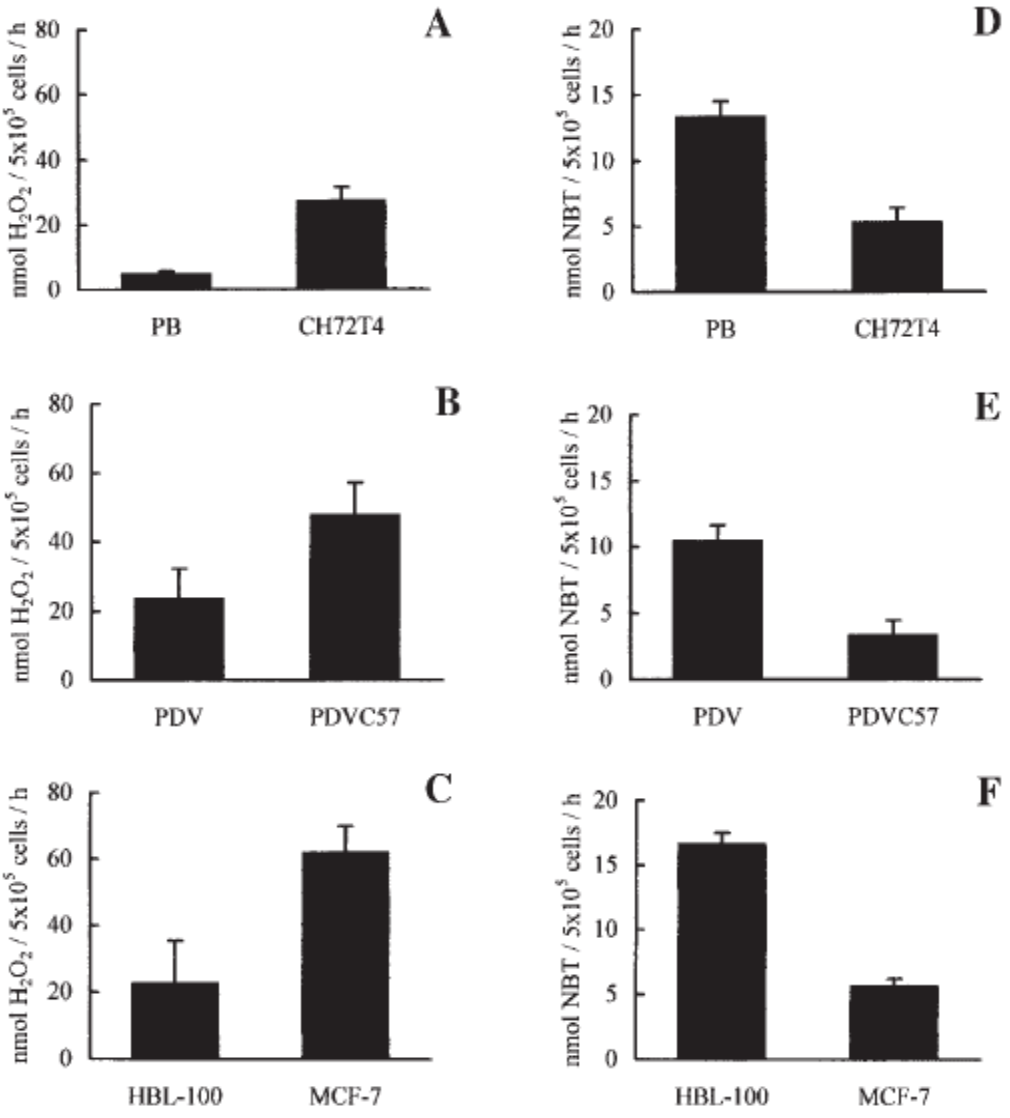

Fig 1. An increase in the constitutive levels of H_2O_2 and a decrease in the constitutive levels of $O_2^{\bullet-}$. Production of H_2O_2 by tumor epidermal cells measured by the scopoletin/horseradish peroxidase assay and expressed as nmol $H_2O_2/5x10^5$ cells/h (A, B, and C). Production of $O_2^{\bullet-}$ determined by the NBT reduction assay and expressed as nmol of reduced NBT/$5x10^5$ cells/h (D–F). PDV derived from newborn mouse epidermal keratinocytes treated in culture with the carcinogen, PDVC57 were obtained by explanting a tumor induced by PDV in a syngeneic mouse. PDVC57 cells are more tumorigenic and have more malignant phenotype features than PDV cells. PB and CH72T4 were originally derived from SENCAR mice. CH72T4 cells are more tumorigenic than PB. The human cell lines used were HBL-100 and MCF-7. HBL-100 cell presents markers of breast epithelium, and has been used widely as a near normal model for breast epithelial cells. The MCF-7 is breast carcinoma cell line. Data are expressed as mean ± SD [19].

also demonstrated in several cell lines of different tissue origin and species, suggesting a general role of H_2O_2 in cell proliferation. Moreover, stable expression of human CAT in MCF-7 cells inhibited proliferation and also reverted malignant features. So H_2O_2 played a crucial and general role in the regulation of carcinogenesis [19].

Superoxide and carcinogenesis

A number of tumors have been found to possess low levels of Manganese-containing superoxide dismutase (MnSOD), indicating that $O_2^{\bullet-}$ may play a role in carcinogenesis. MnSOD protein and activity deceased in a wide variety of tumor types when compared to an appropriate normal cell control [20-21]. Levels of immunoreactive protein and mRNA for MnSOD also appear to be lowered in tumor cells [22]. Because of the higher metabolic activity in tumor cells, more $O_2^{\bullet-}$ production coupled with diminished amounts of MnSOD might be a general characteristic of tumor cells, which would change the cell redox status and result in enhanced proliferation of tumor. In fact, the transforming capacity of $O_2^{\bullet-}$ is illustrated by the oncogenic transformation of NIH 3T3 cells by the NADPH oxidase homologue MOX-1, which induces the production of $O_2^{\bullet-}$ [23].

Tumor suppressive effects of MnSOD, role of $O_2^{\bullet-}$ in carcinogenesis

If antioxidant enzymes are important in cancer, then normalization of the levels of these enzymes should result in reversal of at least part of the cancer cell phenotype. This hypothesis was first suggested by Oberley and Buettner in 1979 [24] and has been tested with positive results in a number of publications.

Overexpression of MnSOD led to suppression of cell growth both *in vitro* and *in vivo*. Growth suppression was observed in human breast carcinoma MCF-7 [25], the plating efficiency and clonogenic fraction in soft agar culture were decreased after MnSOD cDNA transfection. When inoculated in nude mice, tumor growth was markedly inhibited in MnSOD overexpressing cells compared to wild type or plasmid control cells. These results supported the hypothesis that increased MnSOD expression suppresses the malignant phenotype of human breast cancer cells and suggested that the MnSOD gene is a tumor suppressor gene in human breast cancer.

When MnSOD cDNA was transfected into the SV40-transformed human fibroblast cell line WI-38, compared with the parental and neo (vector) control cells, the MnSOD-overexpressing clones had a slower growth rate, lower plating efficiency, increased anchorage dependence. These changes were correlated strongly with the level of MnSOD activity. The results suggest that an increase of MnSOD activity can reverse part of the malignant phenotype in SV40-transformed human fibroblast cells [26].

Clair et al put human MnSOD gene into mouse C3H 10T1/2 cells resulted in production of active MnSOD, which then was transported to mitochondrial. Overexpression of MnSOD protected cells from radiation-induced neoplastic transformation. This finding demonstrated that oxidative stress that occurs in the mitochondria plays an important role in the development of neoplastic transformation [27]. Besides, after overexpression of MnSOD, growth suppression was also observed in U118 human glioma [28], human pancreatic cancer [29], human oral squamous carcinoma SCC-25 [30], and human prostatic carcinoma DU145 cells [31].

A link between H_2O_2 & $O_2^{\bullet-}$ in carcinogenesis

Fibroblasts overexpressing Nox1 showed increased $O_2^{\bullet-}$ and remarkably, exhibited a transformed phenotype, including increased proliferation and aggressive tumor formation in athymic mice [23]. Arnold investigated the mechanism of Nox1 induces cell transformation and tumorigenicity. Compared with <2-fold increase in $O_2^{\bullet-}$, a 10-fold increase in concentration of H_2O_2 was observed in Nox1-expressing NIH 3T3 fibroblasts. When human CAT was expressed in Nox1-expressing cells, H_2O_2 concentration decreased, and the cells reverted to a normal appearance, the growth rate normalized, and cells no longer produced tumors in athymic mice. A large number of genes, including many related to cell cycle, growth, and cancer were expressed in Nox1-expressing cells, and more than 60% of these returned to normal levels on coexpression of CAT. The results indicated that H_2O_2 functions as an intracellular signal that triggers a genetic program related to cell growth triggeredd by Nox1 [32]. Strong evidence of H_2O_2 in carcinogenesis also comes from study on growth factors including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), both of them can trigger H_2O_2 production during downstream signaling transduction [33-34]. So the carcinogenesis role of $O_2^{\bullet-}$ seems to be mediated by H_2O_2.

Role of H_2O_2 in the mechanism of tumor suppressive effects of MnSOD

To investigate the mechanism by which MnSOD overexpression mediates this reversal, Melendez et al [35] established 29 independent, clonal MnSOD-overexpressing HT-1080 fibrosarcoma cells. They found MnSOD activity was inversely correlated with

cell proliferation. Both the parental and control transfectants showed similar population doubling numbers over 5 days of 4.68 ± 0.2 and 4.54 ± 0.1, respectively, whereas the MnSOD-overexpressing cells cell lines had doubling numbers of 1.81 ± 0.4 and 2.54 ± 0.3. This increase in doubling time of the MnSOD-overexpressing cells may account for at least 50% of the inhibition that is observed in both the cloning efficiency and cell growth assays. The significant point in their research is that, incubating cells in 3% oxygen counteracted the suppression of cellular proliferation mediated by MnSOD. If the proliferation inhibitory effects of MnSOD are dependent on $O_2^{\bullet-}$, decreased $O_2^{\bullet-}$ production should reverse any observed effects. The production of $O_2^{\bullet-}$ is dependent on cellular oxygen consumption and can be diminished by decreasing available oxygen. The result suggested that oxygen is an indispensable component of the MnSOD-dependent cellular proliferation inhibition. The oxidant-sensitive fluorescent dyes were used to assess the redox status of the cell. Compared with parental or control cell lines, a significant decrease in the rate of oxidation of the fluorophores in the MnSOD-overexpressing cell lines was found. A possible mechanism is that overexpression of MnSOD might alter the intracellular redox state by modulation of the balance of reactive oxygen species, which is closely associated with tumor regression in vivo and loss of the malignant phenotype in vitro by MnSOD.

Are the effects due to lowered $O_2^{\bullet-}$, increased H_2O_2? Is the change in the cellular redox status, especially change attributable to accumulation of H_2O_2 or other hydroperoxides (Figure2) a possible reason to explain the suppression of tumor growth observed in MnSOD-overexpressing cells?

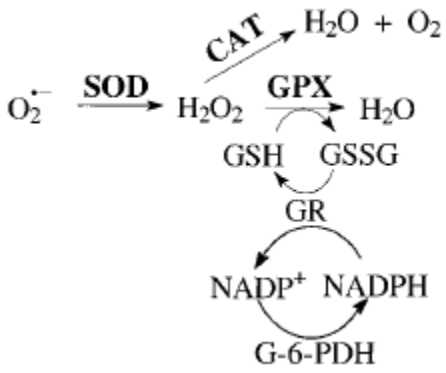

Fig. 2. Antioxidant enzyme system. The SODs convert $O_2^{\bullet-}$, into H_2O_2. The CAT and GPx convert H_2O_2 into water. In this way, two toxic species $O_2^{\bullet-}$ and H_2O_2 are converted into the harmless product water. GPx requires several secondary enzymes (GR and G-6-PDH) and cofactors (GSH, NADPH, and glucose 6-phosphate) to function. In this scheme, GR and G-6-PDH are considered secondary antioxidant enzymes, because they do not act on ROS directly but enable GPx to function [36].

After transfection of GPx cDNA into human glioma cells overexpressing MnSOD, it reversed the tumor cell growth inhibition caused by MnSOD overexpression [36]. The result suggests that H_2O_2 or other hydroperoxides appear to be key reactants in the tumor suppression by MnSOD overexpression. Because GPx can act on other hydroperoxides besides H_2O_2, so CAT transfection is needed to show which hydroperoxide is involved. Andres Melendez made mitochondrial CAT and showed it can inhibit the tumor suppressive effects of MnSOD [37]. The work strongly indicated that H_2O_2 cause the tumor suppressive effects of MnSOD.

Supporting evidence of H_2O_2 in tumor suppression also comes from treatment by anticancer drugs. Many anticancer drugs kill their target cells at least in part through the generation of elevated amounts of intracellular H_2O_2 [38-39]. H_2O_2 can stimulate proapoptotic signal molecules, such as apoptosis signal BAX [40], mitogen-activated

protein kinase, fas ligand expression [41] or engage the mitochondrial apoptotic cascade [42].

Unanswered Question:

Combine previous reports, H_2O_2 played a general and crucial role in the regulation of carcinogenesis [19], at the same time, H_2O_2 is the main reason of the tumor suppressive effects of MnSOD [36-37]. Paradoxically, How can H_2O_2 promote normal cellular proliferation and carcinogenesis, and can also induce suppression of tumor growth?

Hypothesis:

At low levels in normal cells, H_2O_2 promotes normal cellular proliferation and carcinogenesis. At higher concentration, H_2O_2 induce suppression of tumor cells growth.

Potential experiments related to cancer prevention

1. Compare the intracellular H_2O_2 levels of normal cells (fibroblast or epithelial cells) and tumor cells. Because cells in different tissues or tumors have different intracellular H_2O_2 concentration. So in order to get convincing evidence for this problem, I would like use normal cells, then transform them by UV/X-ray irradiation or SV-40 or chemicals. Once get malignant transformed phenotype, which will be demonstrated by growing in

soft agar and forming tumors in nude mice (inject subcutaneously into the flank region of female nude mice). Then remove the tumor from the nude mice, digest to get tumor cells. Be sure to include the nontransformed NIH 3T3 cells (as control for animal cells) or hTERT immortalized human cells (as control for human cells), and all kinds of tumor cells get by biopsy such as U118 human glioma, human pancreatic cancer, human oral squamous carcinoma SCC-25, and human prostatic carcinoma DU145 cells.

The level of intracellular H_2O_2 was assessed spectrofluorimetrically by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). H_2O_2 productions will be reported to the amount of proteins in each sample. Briefly, cells of both normal and tumor tissue are seeded in 96-well plates, and incubated for certain time after different treatment according to the requirement of experiment. Note here, when put the chemical for measuring intracellular H_2O_2, the cells should be at the same growth phase (Log or confluence).

2. Increase the intracellular H_2O_2 levels by MnSOD cDNA transfection (or mimics of superoxide dismutase such as manganese(III)tetrakis(4-benzoic acid) porphyrin, copper(II)(3,5-diisopropylsalicylate)2, or manganese dipyridoxyl diphosphate). Use the adenovirus to transfect cells because of its high efficiency in transfection. The purpose in this step is to get primary value of how long a certain amount MOI of adenovirus or certain dose of SOD mimics will last, or how much a certain amount of MOI adenovirus or certain dose of SOD mimics will increase intracellular H_2O_2. This experiment should be done in both normal cells and tumor cells.

3. Decrease the intracellular H_2O_2 by adenovirus CAT transfection or N-acetylcysteine. To get primary data about the amount of adenovirus CAT and doses of N-acetylcysteine

should be used in our experiment. Do the experiment both in normal cells and tumor cells. From this step, one can get how much effects the adenovirus CAT and N-acetylcysteine in decreasing the concentration of intracellular H_2O_2.

4. Transformation experiment. Seed a certain number of normal cells, treatment with carcinogens such as UV or X-ray. Compare the transformation efficiency of

normal cells

normal cells transfected with adenovirus MnSOD

normal cells transfected with adenovirus CAT

normal cells treatment by N-acetylcysteine

normal cells co-transfected with adenovirus MnSOD and adenovirus CAT

normal cells transfected with adenovirus MnSOD and N-acetylcysteine

The transformation efficiency will be determined by number of foci per survival cell after treatment by carcinogen (for animal cells), or how many colonies formed on soft agar per 2000 survival cells plated per well in six-well plate.

Before going on following experiment, one should get the idea whether modulation of endogenous H_2O_2 can change the process of carcinogenesis. Try to find how much range the value change of intracellular H_2O_2 concentration by different modulation. Does any intracellular H_2O_2 threshold exist to control the carcinogenesis?

5. Increase the intracellular H_2O_2 levels by MnSOD cDNA in real tumor cells such as U118 human glioma, human pancreatic cancer, human oral squamous carcinoma SCC-25, and human prostatic carcinoma DU145 cells. At this step, it is not possible to use the malignant transformed cells, because even being malignant transformed, some tumors regressed with the time. So in a sense, they are not real tumors.

Compare cell killing (%) with different treatments by Trypan blue staining. Compare the malignant phenotypes such as plating efficiency, growth in soft agar, cell numbers needed to form tumors in 50% of nude mice injected, tumor size (tumors size will be calculated from the following equation: $TV\ (mm^3) = (L \times W^2)/2$, where TV = tumor volume, L = length, and W = width) after injecting tumor cells of different modulation:

Tumor cells without modulation,

Tumor cells transfected with adenovirus MnSOD

Tumor cells transfected with adenovirus MnSOD, BSO, AT and BCNU (figure 3)

Tumor cells transfected with adenovirus CAT

Tumor cells treatment by N-acetylcysteine

Tumor cells co-transfected with adenovirus MnSOD and adenovirus CAT

Tumor cells transfected with adenovirus MnSOD and N-acetylcysteine

Finally, a curve will be drawn to show the trend of tumor growth with the function of time.

6. Statistical analysis

Analysis of variance (ANOVA) will be performed for multiple comparison of each dependent variable. P value < 0.05 is considered to be statistically significant. All data is presented as mean $\pm$ SD.

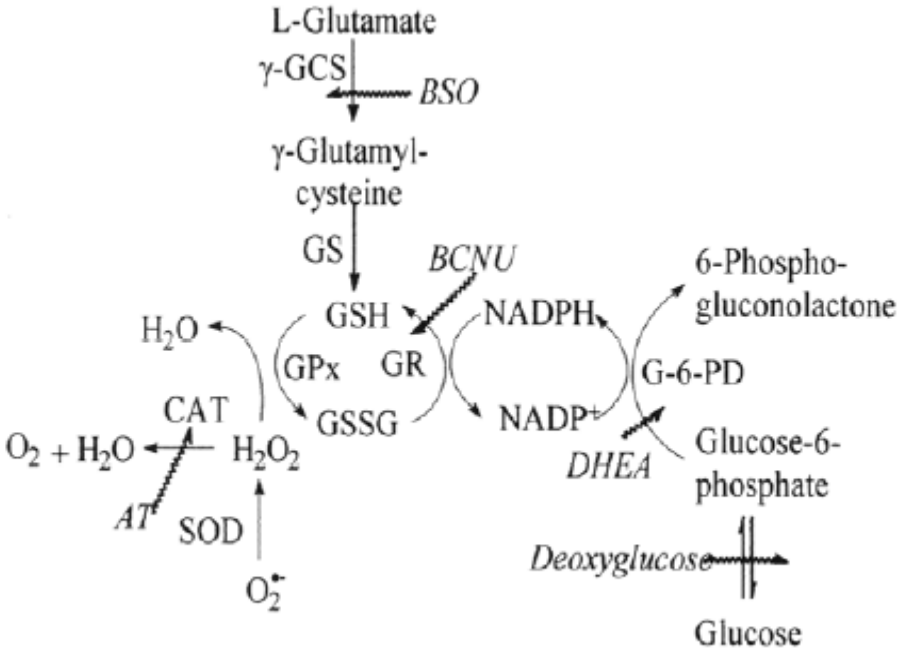

Fig. 3. Antioxidant enzymescheme [43]

Summary

ROS are generated by normal physiologic processes in all of the oxygen-utilizing organisms. ROS, particular H_2O_2 exert some functions necessary for cell homeostasis maintenance. Hydrogen peroxide can promote normal cellular proliferation and carcinogenesis, it can also lead to tumor growth suppression. Modulating endogenous antioxidant enzymes in tumor cells can compensate the resulting changes in the levels of H_2O_2 to control the malignant growth.

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