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Reactive Oxygen Species and Breast Cancer Carcinogenesis

by

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Abbreviations:

2-OHE	catechol estrogen 2-hydroxyestrone
4- OHE	catechol estrogen 2-hydroxyestrone
8-OHdG	8-hydroxy-2'deoxyguanosine
16α-OHE	16α-hydroxyestrone
ADH	alcohol dehydrogenase
AOX	aldehyde oxidase
BaP	benzo[a]pyrene
CATs	catalases
CYP	cytochrome P450
DMBA	7,12-dimethylbenz [a]anthracene
E_2	17 β-estradiol
GPx	glutathione peroxidase
MDA	malondialdehyde
NADH	nicotinamide-adenine dinucleotide
PAH	polynuclear aromatic hydrocarbon
PMA	phorbol 12-myristate 13-acetate
PUFAs	? -polyunsaturated fatty acids
ROS	reactive oxygen species
SODs	superoxide dismutases
XOR	xanthine oxidoreductase

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Abstract

Breast is the first most frequent female cancer and the second most frequent cause of female cancer death. About one-third of all cases of breast cancer can be attributed to recognized risk factors, such as estrogens, obesity, alcohol consumption, smoking, chest radiation therapy history and family history. Reactive oxygen species-induced damage is involved in most of the mechanism of all these risk factors. This paper will focus on the effects of reactive oxygen species and antioxidant on the carcinogenesis of breast cancer. Finally, future experiments were designed to investigate the tumor-initiation and –promotion effect of estrogen and its metabolites and the counteract effect of antioxidant on them.

Introduction

Breast cancer is a malignant tumor that has developed from cells of the breast. With 1 million new cases in the world each year, breast cancer is the commonest malignancy in women and comprises 18% of all female cancers. Many research show that environmental factors would be related to breast cancer, including diet with high animal protein, estrogenic food additives, alcoholic beverage consumption, contact of chemical carcinogens and exposure to radiation. All these risk factors have a close relationship with the production of reactive oxygen species (ROS), indicating that ROS play an important role in the carcinogenesis of breast cancer. This paper will focus on the mechanism of the ROS production from these risk factors and their effect on breast cancer happening.

Epidemiology of Breast Cancer

Breast cancer is the most common cancer among western women. It is also the second leading cause of cancer death in women, after lung cancer. The American Cancer Society predicts that there will be about 182,800 new cases of invasive breast cancer in the year 2001 among women in the United States and about 40,800 deaths from the disease. A woman in the United States has a 12.5% lifetime risk of developing breast cancer. Breast cancer also occurs occasionally in men. About 1,400 cases of breast cancer are expected to occur among men in the United States in 2001 [1].

All women are at risk for breast cancer. Until now, it is not known exactly what causes breast cancer, but there are certain risk factors are linked to the disease. About 30% of women diagnosed with breast cancer have one or several risk factors. The biggest risk factors is aging. Other risk factors include: 1) personal or family history of breast cancer, 2) history of non-cancerous but proliferative breast disease, 3) having early onset of menstrual periods or late

menopause, 4) recent use of oral contraceptives or post-menopausal estrogens, 5) never having children or having first child after age 30, 6) chest radiation therapy as a child or young adult, 7) obesity, especially after menopause, 8) alcoholic abuse.

General Effects of Reactive Oxygen Species and Antioxidants on Cancer

1) Reactive Oxygen Species and Cancer

Reactive oxygen species within cells can be generated from various sources. The exogenous sources include redox-cycling substances (for example, paraquat, diquat), drug oxidations, cigarette smoking and ionizing radiation. Oxygen radicals can also be generated in biological systems during enzymatic oxidation and autooxidation, such as mitochondrial electron transport chain, phagocytic cells and autoxidation reaction of metals. Reactive oxygen species derived from oxygen are involved in both tumor initiation and promotion and metastasis.

Ray *et at* [2] reported that compared to age- and sex-matched patients who had no history of any neoplastic or breast disorders, breast cancer patient has a significantly higher rate of $O_2^{\bullet -}$ production ($p < 0.001$), irrespective of clinical stages and menopausal status. Similarly, H_2O_2 production was significantly higher in breast cancer patients, especially in stage III and postmenopausal groups, as compared to the respective controls. There was also a significantly elevation of the concentration of malondialdehyde (MDA), the index of lipid peroxidation, in stage II ($p < 0.001$), stage III ($p < 0.01$), postmenopausal ($p < 0.005$), and premenopausal ($p < 0.02$) group as compared to their corresponding controls. Also, in breast carcinoma, there is a substantially elevated level of serum and breast ferritin [3]. Iron can catalyze the conversion of H_2O_2 into $^{\bullet}OH$ and has been recognized as an important risk factor for breast cancer.

Oxidative DNA modification is random, and it seems that there is no specific genetic targets for ROS generated mutation in carcinogenesis in breast. ROS, especially $^{\bullet}OH$, induce DNA

strand breakage and modification. Hydroxyl radical induced DNA modification includes single base deletion, base ring opening, single and double strand breakage and adduction of bases. Among all four bases, guanine is the most susceptible DNA target for oxidative reactions mediated by $^{\bullet}OH$ and other free radicals. 8-hydroxy-2'-deoxyguanosine (8-OH-dG), which is an indicator of oxidative stress and links to the G::C to T::A transversion mutation, has been reported as a key biomarker related to carcinogenesis [4]. In breast cancer, there is a markedly elevation of 8-OH-dG. Some key genes that contribute to breast cancer progression, such as p53, BRCA1 and BRCA2 gene, have been identified undergo ROS-induced mutation [5].

Besides DNA, lipids and protein are also attacked by ROS. In a lipid-rich environment, the radical chain reactions result in the production of hydroperoxides; these hydroperoxide residues change the hydrophobic interaction between adjacent chains of phospholipids allowing water molecules to penetrate the biological bilayer easily. The altered water gradient across the membrane consequently changes the substructure of membrane and leads to the degradation of lipids and proteins. Significant changes in structure and function of membranes cause cell apoptosis.

2) Antioxidant and Cancer

Living cells develop efficient defense and repair mechanisms to protect against oxidant species. The antioxidative defense system is composed of methods to 1) transfer sensitive material to compartments better protected from the action of reactive species, 2) chelate transition metals and make them unreactive, thus decrease the potential source of electrons, 3) inhibit vulnerable processes such as DNA replication, 4) repair damaged molecules, 5) initiate apoptosis, 6) activate antioxidant enzymes and 7) use a variety of direct free radical scavengers [6].

Three major enzymes involved in antioxidative defense, they are superoxide dismutases (SODs), catalases (CATs) and glutathione peroxidase (GPx). Ray *et al* [2] found that SOD and GPx activities significantly raised in all stages of breast cancer patients ($p < 0.001$), except the GPx activity was found a smaller alteration in stage IV ($p < 0.02$). On the contrary, CAT activity was found significantly depressed in all the study groups and the maximum depression was observed in stage II (-61.8%). They proposed that lower CAT activity that was found in their study might be the effect of high production of ROS, particularly $O2^{\bullet -}$ and $^{\bullet}OH$. The relatively higher SOD and GPx may be due to the response of increased ROS production in the blood. However, the higher SOD and GPx activities were inadequate to detoxify high levels of H_2O_2 into H_2O leading to the formation of the most dangerous $^{\bullet}OH$ radical followed by MDA.

If increased activity of antioxidant were still not enough to counteract the toxicity of ROS, oxidative stress would cause carcinogenesis. Then what will happen when the level of antioxidant activity is low? Manganese superoxide dismutase (MnSOD) has been proposed to function as a tumor suppressor gene [7], its abnormal expression can be found in almost 100% cancer cells and in 90% cancer cell line, there is a low level of MnSOD. Reconstitution of MnSOD expression in several human cancer cell lines leads to reversion of malignancy. By transfection MnSOD cDNA in to a human breast cancer cell line (MCF-7), Li *et al* [8] found that the plating efficiency and clonogenic fraction of MCF-7 was decreased and the tumor growth in nude mice was markedly inhibited. MnSOD expression acts by alteration the activity of several redox-sensitive transcription factors, including AP-1 and NF-kappa B [9]. However, the signaling pathways downstreaming MnSOD are far from clear.

Carcinogenesis of Breast Cancer

1) Estrogen

The risk of developing breast cancer is closely linked to reproductive events, implying a role of endogenous estrogens in breast cancer development. Cumulative lifetime exposure to bioavailable 17 β-estradiol (E_2) is the common linkage of most known risk factors (except radiation) to breast cancer. Naturally occurring or synthetic xenohormones may affect the process of tumorigenic transformation at both genotoxic and epigenetic levels.

Estrogens can cause cancer by stimulating cell proliferation and causing genotoxic damage. Estradiol metabolize through two competing, mutually exclusive enzymatic pathways: the first pathway inserts a hydroxyl group at the C2-position and yields the catechol estrogen 2-hydroxyestrone (2-OHE_1); the second pathway adds an OH at the C16α position and yields 16α-hydroxyestrone (16α-OHE_1). 16α-OHE_1 is the potent estrogenic metabolite that covalently binds to estrogen receptor. Estrogen receptors, acting as a transcription factor, bind to appropriate DNA response elements, enhance transcriptional activation. There are several estrogen-responsive genes such as c-*fos*, c-*jun* and c-*myc*, which code for positive growth regulatory nuclear proteins [10].

During metabolism, steroidal estrogen E_2 also undergoes a one-electron oxidation reaction to produce its reactive phenoxyl radical intermediate; the phenoxyl radical metabolite abstracts hydrogen from reduced glutathione generating the glutathione thiol radical, which subsequently consumes molecular oxygen *via* a series of reactions, or the phenoxyl radical abstracts hydrogen from reduced beta-nicotinamide-adenine dinucleotide (NADH) to generate the NAD radical; the NAD radical then reduces molecular oxygen to the superoxide radical. The superoxide may either spontaneously dismutate to form hydrogen peroxide or react with another NADH to form NAD radical, thus propagating a chain reaction leading to oxygen consumption and hydrogen peroxide accumulation [11]. The substrate for redox cycling and free radical generation may be

4-hydroxoestradiol, a potent and long-acting estrogen, which generates free radicals through metabolic redox cycling and the chemically reactive estrogen semiquinone /quinone intermediates; these metabolic intermediates can damage DNA, induce cell transformation and initiate carcinogenesis. 4-hydroxoestradiol also completes the carcinogenic process by stimulating receptor-mediated proliferation [12]. Figure 1 shows the impact of steroid hormones on interacting genetic pathways critical for breast carcinogenesis. There are several types of direct and indirect free radical-mediated DNA damage that are induced by estrogens *in vitro* and *in vivo*, including DNA single strand breaks, 8-hydroxylation of guanine bases (8-OHdG), and DNA adduct formation by malondialdehyde. Using immunoslot blot assay, Musarrat *et al* [13] found that the amount of 8-OHdG in invasive ductal carcinoma is 9.76-fold higher than that in normal tissue and there was a significantly elevated level (3.35-fold higher) of 8-OHdG in estrogen receptor-positive compared with estrogen-negative malignant tissues, suggesting a positive relationship between 8-OHdG formation and estrogen responsiveness.

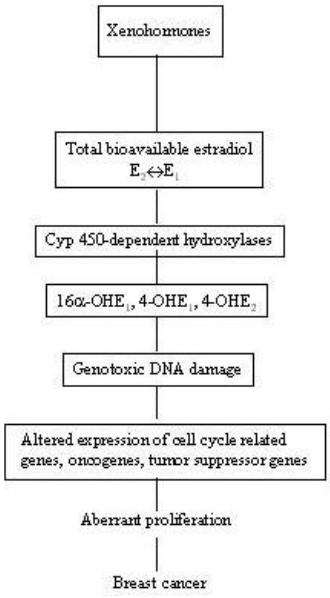

Figure 1. Impact of steroid hormones on genotoxic pathways of breast carcinogenesis. In the genotoxic pathway, bioavailable 17beta-estradiol is converted via Cyp450-dependent hydroxylases to 16alpha-OHE_1, 4-OHE_1, or 4-OHE_2. These metabolites, by virtue of their direct effect on DNA, cause genotoxic DNA damage. This damage changes the expression of cell cycle related genes, oncogenes, and tumor suppressor genes leading to aberrant proliferation and breast cancer development. Adapted from [14]

2) Lipid Dietary

Most human diets contain a variety of saturated fatty acids of different chain lengths. The major saturated fatty acid in the diet is palmitic acid (C16:0); the major monounsaturated fatty acid in human diets is oleic acid (*cis*-C18:1 ?-9) and the principal polyunsaturated fatty acid is linoleic acid (*cis,cis*-C18-2 ?-6). Soybeans, corn oils are rich in linoleic acid. ?-3 fatty acid (eicosapentaenoic and docosahexaenoic acid) is found in deep cold-water fatty fish. Both ?-6 and ?-3 fatty acids are essential and must be provided in diet.

The effect of high-fat diet on the development of breast cancer is supported by international data showing a strong correlation between fat intake and breast cancer rates and a modest positive association with high-fat diet in case-control studies [15]. Studies in animal models and recent observations in humans have provided evidence that a high intake of ?-6 polyunsaturated fatty acids (PUFAs), stimulates several stages in the development of mammary and colon cancer, including the increase in oxidative DNA damage, stimulating cell proliferation, up-regulating free estrogen levels and increasing hormonal catabolism [16]. The hypothesis that consumption of a high fat diet during gestation increases the incidence of carcinogen-induced mammary tumors has been demonstrated in animals. Hilakivi-Clarke *et al* [17] feed 7,12-dimethylbena[a]anthracene-pretreated rat with either low fat (16% calories) or high fat (43% calories) diet throughout gestation. The fat source was corn oil, which is rich in ?-6 PUFAs. They found the incidence of mammary tumors in the group given the high fat diet was significantly higher (40%) than that in the low-fat-diet group.

Dietary fats, specifically ?-6, directly or indirectly affect a variety of steps in the multistage carcinogenesis process [18]: 1) peroxidation of conjugated double bonds in PUFAs, causing persistent oxidative stress and generation of reactive lipid peroxidation products, which can

induce DNA damage; 2) conversion of essential fatty acid to eicosanoids, short-lived hormone-like lipids derived primarily from dietary linoleic acid; 3) interaction of fatty acids with signal transduction pathways leading to altered gene expression; 4) effects on unbound estrogenic hormone concentrations; 5) effects on membrane (lipid)-bound enzymes such as cytochrome P450 (CYP) that regulate xenobiotic and estrogen metabolism; 6) structural and functional changes in cell membranes resulting in alterations in hormone and growth factor receptors. High intake of ?-6 PUFAs enhanced activities of protein kinases, such as protein kinase C in rodent mammary gland and an increased number of estrogen receptor binding sites [19]. Here I will focus on the relation of high fatty acid diet and lipid peroxidation and oxidative DNA damage.

The oxidizability of PUFAs linearly depends on the number of *bis*-allylic methylenen positions. Peroxidation of conjugated double bonds in PUFAs, leading to persistent oxidative stress and generation of several reactive a,ß-unsaturated aldehydes, such as malondialdehyde, and *trans*-4-hydroxy-2-nonenal, which moderately increased the frequency of malonaldehyde-derived DNA adducts in human cells (Figure 2). One of the major lipid peroxidation products of the oxidation of linoleic acid is *trans*-4-hydroxy-2-nonenal; *trans*-4-hydroxy-2-nonenal then can be easily oxidized to form 2,3-epoxy-4-hydroxynonanal, which can react with DNA to yield etheno and other base adducts. Etheno adducts are highly miscoding lesions in mammalian cells and are thought to contribute to the carcinogenic process by specific point mutation [20]. To support the fact that lipid peroxidation-derived products and oxidative DNA base damage increase breast cancer risk, Boyd et al [21] measured the malondialdehyde in urine and found that in pre-menopausal women with mammographic dysplasia, the malondialdehyde level was approximately double that in women without these radiological change.

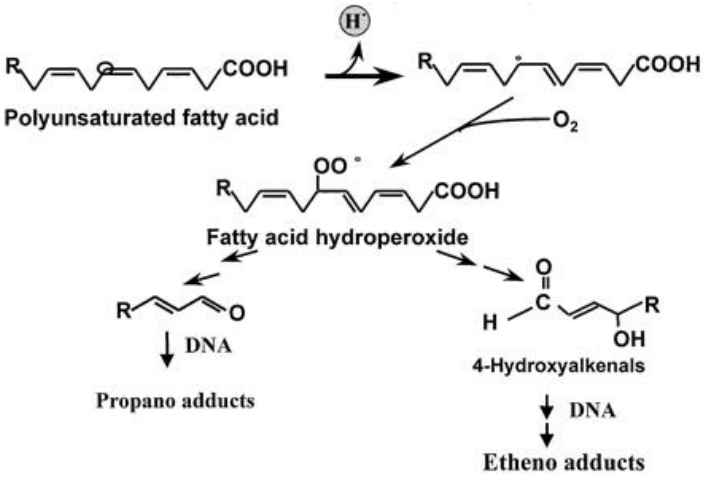

Figure 2. Suggested mechanism for the formation of exocyclic propane and etheno adducts from DNA nucleosides and lipid perosidation products of polyunsaturated fatty acids. Adapt from [18]

Taken together, there is lots of experimental evidence that ?-6 fatty acids enhance the risks for breast cancers. So it is important for us to moderately restrict the total fat consumption. It has been recommended that common people consume 30% of their total energy intake from dietary fat.

3) Alcohol

Many studies in the past twenty years have shown that an increased breast cancer risk is associated with chronic alcohol intake in women and that the risk increases with increasing alcohol consumption. According to the survey of 322,467 women, including 4,335 cases of invasive breast cancer, alcohol consumption increased the incidence of breast cancer by 41% [22]. And the relative risk tended to increase with daily alcohol consumption greater than 40 g of alcohol [23].

Alcohol, or ethanol can be metabolized to acetate by a two-step reaction: first alcohol dehydrogenase (ADH) converts alcohol to acetaldehyde and then the molybdenum hydroxylase enzymes, xanthine oxidoreductase (XOR) and aldehyde oxidase (AOX), produce acetate from acetaldehyde. During the second step, ROS was generated. XOR can generate ROS through the metabolism of both acetaldehyde and NADH, which was formed stoichiometrically during the

conversion of alcohol by ADH. All of the three partial reduction products of oxygen are formed in this process: H_2O_2 and $O_2^{\bullet-}$ are generated directly from substrate reduced enzymes when diatomic oxygen acquires reducing equivalents from a reduced flavin site and $^{\bullet}OH$ is produced secondarily via Fenton reaction of iron or copper [24]. Figure 3 shows the mechanism of alcohol induced ROS generation by XOR.

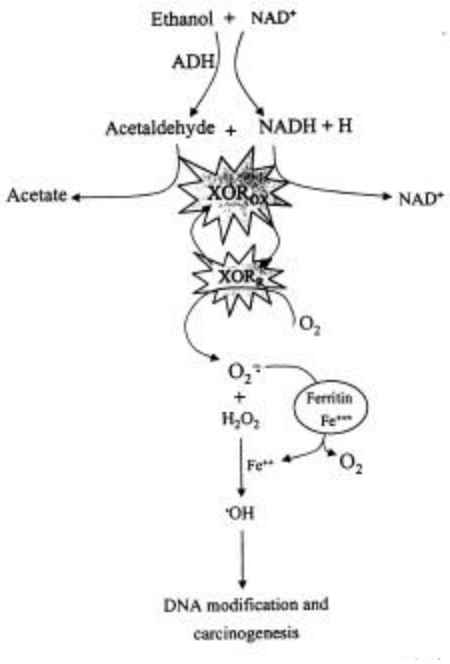

Figure 3. The XOR hypothesis. The action of ADH on ethanol in the breast produces acetaldehyde and NADH both of which can be substrates for XOR. Reduction of XOR by either acetaldehyde or NADH results in ROS formation when XOR is subsequently oxidized by molecular oxygen. The resulting superoxide anion and hydrogen peroxide can participate in Fenton chemistry yielding hydroxyl radical that produces DNA damage and mutation. Adapt from [24]

Acetaldehyde itself also is a mutagenic and carcinogenic chemical, which is known to be able to inhibit O^6-methyl guanine demethylase, which is known to be critical for the repair of O^6-methyl guanine lesions in DNA caused by *N*-nitrosodimethylamine and of other equivalent alterations resulting from other alkylating carcinogens [25].

To support this mechanism, ADH and XOR or AOX must be present in breast tissues in a sufficient amount to promote ROS formation form alcohol. It has been measured that the activity of ADH in normal human breast is 23.8 IU/gram of tissue and there is no remarkable change in carcinoma [26]. Also ADH gene can be induced by alcohol consumption [27]. And further

research shows that XOR and ADH genes contain the same recognition sites for transcription factor supporting the possibility that ADH and XOR genes share some common modes of induction related to alcohol consumption [28].

The other possible mechanism of alcohol-induced breast cancer carcinogenesis include that 1) alcohol can increase estrogen concentrations, 2) alcohol cause increase in lipid peroxidation, and 3) alcohol can act as a solvent to enhance the absorption of other carcinogens.

4) Smoking & Chemical Carcinogen

Endogenous or exogenous chemicals potentially play important roles during the multistep development of mammary tumors. Animal models have demonstrated the several different classes of chemicals act as initiators and induce mammary cancer in rodent models. This paper will focus on polynuclear aromatic hydrocarbon (PAH) carcinogens, such as 7,12-dimethylbenz [a]anthracene (DMBA), benzo[a]pyrene (BaP), and N-methylnitrosourea. PAHs have been shown to induce mammary cancer in rodent models [29].

PAHs are routinely identified as by-products of combustion and in cigarette smoking, and these compounds are also formed in cooked foods. The carcinogenic activities of PAHs are not associated with the parent hydrocarbons but result from the conversion of PAHs into ultimate carcinogenic metabolites, which alkylate or covalently bind to DNA. Metabolic activation of PAHs can be divided into two main pathways [30]: one-electron oxidation to yield reactive intermediate radical cations and monooxygenation to produce bay-region diol epoxides. Some PAHs are activated exclusively by one of these mechanisms and others are activated by a combination of both, as shown for benzo [a]pyrene in figure 4. The one-electron oxidation of PAHs is a coherent mechanism of activation that can account for the binding of the most potent PAHs to DNA and presumable their carcinogenic activity. Cytochrome P450 not only converts

PAHs to form their oxygenated metabolites by removing a p electron, but also catalyses the covalent binding of PAHs to DNA. PAH-DNA adducts are either remaining intact in the DNA during normal conditions of purification, or released from DNA by cleavage of the glycosidic bond. Overall, the depurinating adducts is the predominate product and the unrepaired apurinic sits is correlated with mutations in the *ras* oncogene [31].

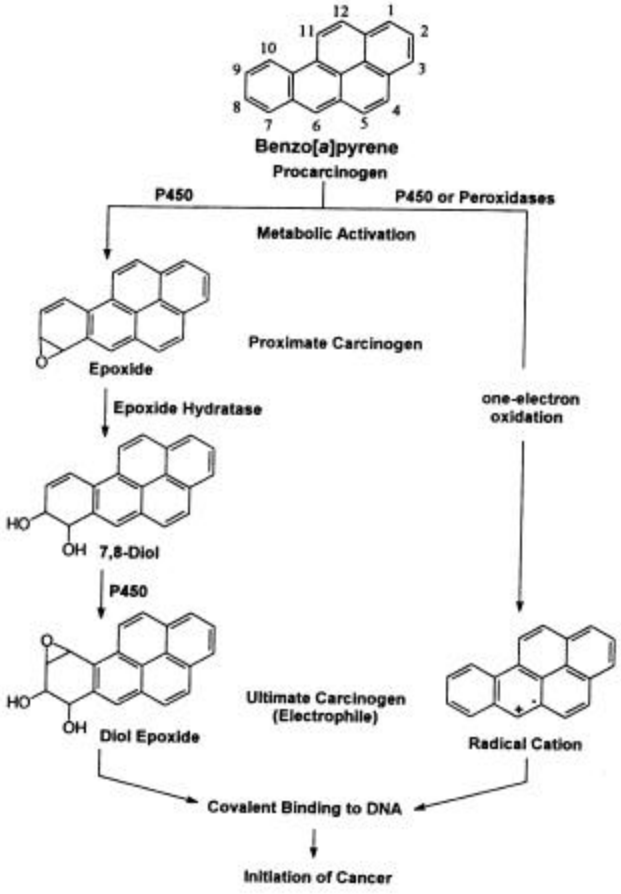

Figure 4. Overall view of metabolic activation of benzo [a]pyrene. Adapted form [32]

As a major endogenous antioxidant enzyme, SOD activity mediates the susceptibility of cells to PAHs and prevents PAHs-induced mammary carcinogenesis. Compared with DMBA alone, DMBA + Manganese-Desferrioxamine, a mimic of MnSOD, can significantly decrease the mammary tumor incidence ($p < 0.02$), tumor size ($p < 0.05$) and tumor cell proliferation index ($p < 0.05$) in rats [33].

5) Ionizing Radiation

Ionizing radiation cause direct and indirect damage in human tissues. For low LET radiation, such as x- or ?-ray, biological damage is due to indirect reaction of radiation and biological targets. Radiation causes the radiolysis of water to produce e_{aq}^-, $^{\bullet}OH$ and $H^{\bullet}$. About 60%-70% of cellular DNA damage produced by ionizing radiation is estimated to be caused by $^{\bullet}OH$ [34]. All biological molecules are targets for $^{\bullet}OH$, and the rate constant of the reactions is at a very high level, exceeding $10^9\ M^{-1}s^{-1}$. The interaction of ionizing radiation with living cells induces a variety of reaction products and a complex chain reaction in which many macromolecules and their degradation products participate.

The carcinogenic effects of ionizing radiation and the sensitivity of breast tissue to radiation are well established. Although it is difficult to evaluate the health effects associated with low exposures, single large-dose exposures to ionizing radiation from the atomic bomb blasts in Hiroshima and Nagasaki, and repeated exposures from therapeutic regimes, such as treatment for tuberculosis, postpartum mastitis, and cervical cancer, are an established cause of breast cancer. The determinants of the incidence rate ratio of breast cancer after exposure of the breast to ionizing radiation include total dose, age at first exposure, and time since first exposure. In a cohort study that consisted of 1,216 women who received radiation therapy (mean dose 5.8 Gy, range from0.003 Gy to 50.1 Gy) for benign breast disease and 1,874 women who has the same diagnosis but did not receive radiation therapy, the radiation-associated incidence rate ratio was 3.58 and the dose-response gradient was statistically significant ($p < 0.01$). The data also showed that the incidence rate ratios decreased starting about 25 years after first exposure but were at increased levels throughout the entire follow-up period (60 years) [35].

An *in vitro* experiment showed that exposure to ionizing radiation with a cumulative dose of 54–70 Gy increased the ratio 2-deoxy-7-dihydro-8-oxoguanosine/2'deoxyguanosine (8-oxodG/dG) from 1.73×10^{-5} to 3.33×10^{-5} [36].

Ionizing radiation causes oxidative damage to radiosensitive tissues within an extremely short period, and possible protection against it would require the rapid transfer of an antioxidant to the target cells. N-acetyl-5-methoxytryptamine (Melatonin), a free radical scavanger that can cross all biological membranes with easy, effectively protects DNA, lipids and proteins from ionizing-induced free radical damage. Melatonin scavenges $^{\bullet}OH$ to form its stable metabolite form, cyclic 3-hydroxymelatonin. This reaction happens quickly with a rate constant that is roughly on the order of 2.7×10^{10} $M^{-1}s^{-1}$ [37]. Melatonin can also influence the activities of all main antioxidant enzymes, including SOD, CAT, GPx, glutathione reductase and glucose-6-phosphate dehydrogenase. There is evidence showed that melatonin increases tissue mRNA levels of MnSOD and CuZnSOD [38].

Melatonin is used in chemoprevention of radiation-induced cancer. Mockova *et al* [39] analyzed the possible properties of melatonin in a combined model of radiation plus chemocarcinogen-induced mammary carcinogenesis. They continuously irradiated virgin female rats with daily dose 96 mGy of gamma rays up to 15 days. At the end of irradiation, between 52-60 postnatal days, 7,12-dimethylbenz(a)anthracene was administered by gavage, in three 10 mg/rat consecutive doses. A part of animals drank melatonin in a concentration 100 μg/ml of tap water continuously from the beginning of irradiation and 26 weeks after its end. In this trial melatonin decreased markedly the volume of mammary tumors, but did not influence any other tumor characteristics.

Future Research

Although there are many experiments shown that free radicals, especially ROS plays an important role in the carcinogenesis of breast cancer, there are still controversy about it and many problems remain unsolved. To better understand the relationship between the risk factors mentioned above and breast cancer carcinogenesis, further research is needed.

Among all these risk factors mentioned above, cumulatively exposure to E_2 links them (except radiation) for breast cancer. It has been proposed that a high ω-6 PUFAs linoleic acid and arachidonic acid intake can inhibits the detoxification of estrogens by 2-OHE and increases 16α-OHE, resulting in metabolites that will undergo redox cycling and produce hydroxyl radicals [40]. The carcinogenesis effect of alcohol consumption also relates to the level of estrogen in circulation, especially to postmenopausal woman. Ginsburg *et al* [41] reported that for postmenopausal woman who receiving estrogens and consuming alcohol regularly, the blood estrogen level is raised to values characteristic of the periovulatory peak in the menstrual cycle, which may be above the threshold of breast cancer and result in the increase of breast cancer risk. Furthermore, smoking changes estrogen production mostly in a qualitative fashion, thus altering the total estrogenic effect. Prolonged smoking intensifies 2-hydroxylation of estrogens thus prevents the inactivation of other catecholestrogens (4-hydroxyestrone and 4-hydroxyestradiol), which are powerful genotoxicants and carcinogens [42].

Based on the information reviewed above, it is reasonable to hypothesis that the metabolites of estrogen cause breast cancer by causing genotoxic damage and stimulating cell proliferation. The genotoxic effect of estrogen is due to the ROS produced by reactive estrogen semiquinone /quinone intermediates of 4-hydroxoestradiol.

1) To testify the role of catechol estrogens in tumor initiation, we need to examine the DNA damage induced by it.

i) Measure the effect of estrogen on hydroxy radical formation.

Choose estrogen-dependent MCF-7 human breast cancer cells and incubate the cells with different concentration of E_2, 2-OHE and 4-OHE at 37°C for 1-2 hour. Use spin traping to measure the level of free radicals produced.

ii) DNA damage induced by estrogen.

There are several ways to measure the DNA damage induced by estrogen. First, I will measure the 8-OHdG formation by estrogens: The amount of 8-OHdG was measured by a modification of the method of Kasai *et al* [43]. The reaction mixtures containing 100 μM/base calf thymus DNA fragments, catechol estrogen (2-OHE_2 or 4-OHE_2) or estradiol, 100 μM NADH and 20 μM $CuCl_2$ in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 hr at 37°C. After ethanol precipitation, DNA was digested to the nucleosides with nuclease and calf-intestine phosphatase and analyzed with an HPLC.

Second, I can detect the damage to radioactive-labeled DNA fragments: Coincubate catechol estrogen (2-OHE_2 or 4-OHE_2) or estradiol, ^{32}P-DNA fragment and calf thymus DNA together. After incubation for 2 hr at 37°C, use a laser to measure relative amounts of oligonucleotides from treated DNA fragments [44].

2) To investigate whether estrogens and their metabolites are important in tumor promotion, I will examine the effects of estradiol and catechol estrogens on proliferation of cells derived from human breast cancer. Here I will choose MCF-7 human breast cancer cell lines, because it expresses estrogen-receptor on its surface. Seeding a certain number of cells in the plate. After

the cell attach to the plate, incubate the cell with E_2, 2-OHE or 4-OHE respectively for 6 days, then harvest cells and count them.

These experiments will clearly prove that estrogen, especially 4-OHE induces carcinogenesis through ROS-induced DNA damage. So my next hypothesis is that antioxidants can inhibit the tumor-initiation and -promotion effect of estrogen. To prove this hypothesis, following experiments are needed.

1) Evaluate the protective effect of antioxidant in estrogen-induced DNA damage *in vivo.*

Use hamsters in this experiment. Randomly divide the hamsters into several groups: group I keeps as control without receiving either E_2 or antioxidant; the animals in other groups are injected with E_2; Groups III–IV were injected with different antioxidants, including *N*-acetylserotonin and ascorbic acid. Hours later kill the hamsters and collect their organs (kidneys, liver). Isolate and purify the DNA, then measure the level of 8-OHdG.

2) Evaluate the protective effect of antioxidant on tumor-promotion effect of estrogen *in vivo.*

Use homozygous female nude mice in this experiment. Ovariectomize all animals before cell inoculation. ZR-75-1 cells were inoculated in the flank of animals. Randomly divide the hamsters into several groups: group I is control mouse that receive E_2 only, the other group mice receive E_2, at the same time, inject different concentration of lipsomal MnSOD into the tumor. Measure the tumor volume every two days and compare the effect.

Summary

Breast cancer, the most common cancer among women, is a complex and important disease. There are several risk factors that are associated with the initiation and promotion of breast cancer. Among all factors, estrogens play a predominant role in breast cancer development. Many of these factors are known to cause their effects by producing reactive oxygen species.

Reactive oxygen species can cause DNA damage and their effect can be counteracted by antioxidant.

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