Free Radicals: The Pros and Cons of Antioxidants

Executive Summary Report[1]

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Greetings and Opening Remarks

Harold Seifried, Program Director, Division of Cancer Prevention, Nutritional Sciences Research Group, National Cancer Institute; Margaret Chesney, Deputy Director, National Center for Complementary and Alternative Medicine, NIH; Paul Coates, Director, Office of Dietary Supplements, NIH

Harold Seifried, Program Director, Division of Cancer Prevention (DCP), Nutritional Sciences Research Group (NSRG), National Cancer Institute (NCI), welcomed participants to the conference and characterized the conference as an informative and broad presentation of the state of the science in antioxidant research. The field of antioxidant research is controversial and confusing to many clinicians because the results of some studies conflict with others, making simple conclusions as to efficacy and safety difficult. At a very recent conference in Paris, France, the results of a 7-y study involving a prospective intervention with β-carotene, vitamins E and C, zinc, and selenium were reported. The study found that antioxidants had no apparent effect on heart disease. However, in males, total cancers and incidence of mortality decreased by 31 and 38%, respectively. No such effect was observed in females, perhaps due to better diet.

Margaret Chesney, Ph.D., Deputy Director, National Center for Complementary and Alternative Medicine (NCCAM), NIH, welcomed participants and described the NCCAM mission, which is to investigate complementary and alternative medicine (CAM) therapies and approaches and to educate both the public and the healthcare community regarding their effectiveness and safety. Recent surveys indicate that 31 to 84% of cancer patients use CAM. Most use CAM in addition to conventional treatment. More research is needed to understand CAM and cancer.

NCCAM supports research on the use of hyperbaric oxygen chambers to improve healing after surgery in cancer patients and of acupuncture, healing massage, and other approaches to ameliorate the side effects of chemotherapy. NCCAM also is working with the NCI and the ODS to improve the portfolio for cancer research. Antioxidants are of great interest to NCCAM, and Dr. Chesney expressed the hope that this conference will shed some light on the areas of research that NCCAM should be considering in the future.

Paul Coates, Ph.D., Director, Office of Dietary Supplements (ODS), NIH, welcomed participants and provided background on the ODS. He described supplements as falling under regulatory guidelines similar to those for foods and explained that supplements cannot be marketed for disease treatment or disease prevention. Health promotion is the primary mode of marketing for dietary supplements, although use transcends some of the regulatory barriers. To make the right decision on research needed to better understand dietary supplements, it is important that the NIH gather information from many sources, and this meeting was designed to be a primary resource.

Free Radicals, Cancer Prevention, and Therapy: Delaying the Oxidative Mitochondrial Decay of Aging

Bruce Ames, Children's Hospital and Research Institute at Oakland

Bruce Ames, Professor and Senior Scientist, Department of Biochemistry and Molecular Biology, University of California—Berkeley, discussed the effect of oxidants on aging and metabolism, strategies for preventing oxidants from being produced during aging, and his perspective on the manner in which the scientific community views oxidants and antioxidants (1).

Research over the past 40 y has led to a greater understanding of the aging process. Energy production occurs in the mitochondria, and these energy generators become less efficient as we age, producing greater numbers of mutagenic oxygen radicals. Experimental studies indicate that there is a decrease in the level of cardiolipin, a key lipid in the mitochondrial membrane, responsible for the membrane's electrical potential, causing reduced utilization of oxygen and increased production of oxygen radicals. Studies in rats show that young rats have ~24,000 oxidative lesions in DNA per cell, increasing to ~67,000 oxidative lesions per cell in older rats.

Animal studies from Italy report that old rats fed acetyl carnitine (ALC), the transporter that carries the fatty acid "fuel" into the mitochondria, have less mitochondrial damage and less DNA damage than old rats not fed ALC. Dr. Ames's

[1] Presented as part of the conference "Free Radicals: The Pros and Cons of Antioxidants," held June 26–27 in Bethesda, MD. This conference was sponsored by the Division of Cancer Prevention (DCP) and the Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH, Department of Health and Human Services (DHHS); the National Center for Complementary and Alternative Medicine (NCCAM), NIH, DHHS; the Office of Dietary Supplements (ODS), NIH, DHHS; the American Society for Nutritional Science; and the American Institute for Cancer Research and supported by the DCP, NCCAM, and ODS. Guest editors for the supplement publication were Harold E. Seifried, National Cancer Institute, NIH; Barbara Sorkin, NCCAM, NIH; and Rebecca Costello, ODS, NIH.

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0022-3166/04 $8.00 J. Nutr. 134: 3143S–3163S, 2004.

research group repeated and extended this experiment using isolated hepatocytes and confirmed the earlier findings. Cardiolipin levels and mitochondrial membrane potential in old rats fed acetyl carnitine remained as high as in young rats, although the production of oxygen radicals remained elevated. To address this conundrum, old rats were fed the oxidized form of lipoic acid (LA), a coenzyme for mitochondrial enzymes, as a potential mitochondrial antioxidant, and the number of oxygen radicals decreased as a result. In addition, vitamin C and glutathione levels also increased. ALC and LA may also diminish the effects of aging on the immune system and brain function. Both old and young rats fed ALC and LA showed increased T-cell stimulation, a positive sign for improved immunity. In tests of spatial memory and ambulatory activity, rats fed ALC and LA did better than rats fed a standard diet. Other research groups have conducted numerous human trials on ALC for Alzheimer's disease and cognitive impairment and on LA for diabetes. Meta-analyses of the overall study results show improvement among patients administered ALC or LA.

As life expectancy increases, there will be pressure on the scientific community to address the causes of aging with better treatments and, ideally, prevention of the aging process. Metabolic changes and the biochemistry involved in aging are emerging fields in science, but much remains to be learned. Diet and lifestyle can be modified to decrease the effects of aging. Diet in America has received a lot of attention in the past few decades. Much of this research shows that micronutrient deficiencies accompany caloric excess in this country. Obesity is of great concern because of the negative effects of this condition on the economy and the medical system. Obese individuals tend to be deficient in dietary micronutrients, including zinc, iron, and calcium. Vitamin and mineral deficiencies adversely affect general biochemistry. For example, 25% of menstruating women in the United States consume <50% of the recommended daily allowance (RDA) of iron. Iron deficiency destroys mitochondria, increases oxidant levels, and accelerates the aging process. Conversely, men may consume too much iron because they eat a lot of meat. Deficiencies of zinc, vitamins B-6 and B-12, and folate also are marked in the United States and can lead to chromosome breakage just as severe as that caused by radiation exposure.

Diet has been the focus of many large epidemiologic studies, and many (but not all) show that fruits and vegetables have a protective effect against cancer. In comparisons of the quartiles of the population that consume the fewest versus the most fruits and vegetables, 24 of 25 studies show that the group that consumes the fewest servings of fruits and vegetables per day has double the risk of developing cancers of the lung, oral cavity, larynx, and esophagus. Even among cigarette smokers (smoking accounts for 90% of lung cancers), consuming fruits and vegetables cuts the risk by half. Smoking puts a tremendous amount of oxidative stress on the body because cigarette smoke is full of nitrogen oxides, which are powerful oxidants, and evidence indicates that oxidants produced by smoking lower the body's levels of vitamin C, which leaves the cells less well defended against oxidants. *Helicobacter pylori* infection, which can cause stomach cancer, also reduces vitamin C levels. Fruits and vegetables may afford some protection by reducing the levels of oxygen radicals produced by the infection.

Mitochondrial damage is associated with an array of chronic diseases that are related to dietary deficiencies. A wealth of information shows that methyl-group deficiencies are a major contributor to DNA damage and that folate deficiency is a major cause. For example, folate and vitamin B-12 deficiency increases the level of homocysteine, which is associated with damage to endothelial cells and consequent heart disease. Biotin deficiency is associated with an increase in oxidants, and zinc deficiency is associated with chromosome damage by oxidation.

There is a need to conduct small intervention studies on antioxidants to address many of the questions remaining about the role of diet and dietary factors on cancer and oxidative stress. The type of study envisioned would be similar to the recent study conducted in Washington State in a collaboration of the Bruce Ames group with the Terry Shultz group, which investigated whether vitamin B-6 deficiency causes chromosome breaks. This study found that there is a level of deficiency (i.e., ~50% of the RDA for vitamin B-6) that is associated with chromosome breaks. These types of studies are difficult to conduct, but they offer a level of control of the diet that cannot be satisfied in epidemiological studies.

Discussion. A participant asked whether the ALC used in the Italian studies was the same as that offered over the counter in the United States to lower lipids and triglycerides. Dr. Ames responded that the ALC used in the Italian studies was not the same as carnitine used as a dietary supplement, although both substances have benefits. He noted that ALC tends to cross the brain barrier better than carnitine.

A participant asked whether studies have examined the effects of nutrients on brain development during gestation. Dr. Ames explained that recent studies indicate that a third of the DNA damage occurs during fetal growth, a third during the growing years, and the last third during the rest of the life span. There is a large literature base that shows iron, folate, B-12, and B-6 deficiencies can damage the brain, especially early in life.

A participant asked whether the human body can acclimate to the presence of substances such as carnitine or flavonoids, thus reducing the influence of free radical generation over time as a function of the dose, and what role genetics plays in this process. Dr Ames responded that the human body can adapt but that the body pays a price for long-term deficiency.

In response to a question about induction of specific enzymes by foods to reduce toxicity and correct nutritional deficiencies, Dr. Ames said that induction of phase II enzymes is of significant benefit for cancer prevention. He added that it is important to remember that humans evolved with a diet that is very different from the diet normally consumed today.

A participant commented that the brain, retina, and neuronal tissues are very high in docosahexaenoic acid (DHA), which is a very long chain polyunsaturated lipid. He asked whether lipid peroxidation was monitored when there were DHA deficiencies in any of the studies discussed. Dr. Ames responded that malondialdehyde (MDA) was measured by mass spectrometry and that MDA levels increase with age due to peroxidation. Thirty percent of human fatty acids are made up of DHA and long-chain (n-3) fatty acids, and it is clear that there are deficiencies in the diet.

A participant asked whether DNA strand breaks correlate with lower numbers of mitochondria or other indices (e.g., homocysteine levels) that could be used as biomarkers for different types of cancer. Dr. Ames responded that there has been very sparse research in this field to date, only small studies on human cells and limited intervention studies. The

area of biomarkers needs more attention to identify endpoints (e.g., mitochondrial decay or DNA damage) related to antioxidant intake.

SESSION 1: OXIDATIVE STRESS—POSITIVE AND NEGATIVE ASPECTS

Session Chair: *Steven Zeisel, University of North Carolina—Chapel Hill*

Antioxidants Can Be Prooxidants When You Least Expect That to Be So

Frank Meyskens, University of California—Irvine

Frank L. Meyskens, M.D., Professor, Department of Medicine and Biological Chemistry, Cancer Center, University of California—Irvine, presented information on antioxidants behaving as prooxidants. The antioxidant/prooxidant issue was highlighted recently by the results of the Alpha-Tocopherol and Beta-Carotene (ATBC) Cancer Prevention study, which reported that β-carotene caused an increase in lung cancer among heavy smokers. This was contrary to predictions from epidemiologic studies in the 1980s that reported an inverse dietary relation between many epithelial cancers and β-carotene. There were, however, experimental studies and mechanistic data showing that β-carotene could be a prooxidant at high oxygen concentrations and under special circumstances, which could have helped to predict the adverse effects noted in the more recent trials.

A further look at the data from the β-Carotene and Retinol Efficacy Trial (CARET) showed similar unexpected results. Participants had a significant smoking history or were prior smokers and were randomly assigned in a 2 × 2 factorial design to treatment with β-carotene, retinol, a combination of both, or placebo. The cumulative incidence of lung cancer was greater among those treated with β-carotene, compared with placebo. In addition, the incidence of cardiovascular disease was greater for those treated with β-carotene than for those treated with placebo. However, there was a nonsignificant decrease in lung cancer in nonsmokers, which was teased out by later analysis. This study supported the earlier overall results of the ATBC study, and all the β-carotene clinical studies being conducted at that time were stopped.

The question remains as to whether β-carotene causes or stimulates lung cancer. One possible explanation for the difference in results between epidemiological studies and the ATBC and CARET trials is that the dose of β-carotene in the epidemiological studies ranged from 6 to 89 mg and the dose of β-carotene in the ATBC and CARET trials was 25 to 30 mg. The high doses selected in the trials using β-carotene supplements probably play a role in lung cancer development because they produce high serum concentrations of the vitamin that are not physiologic. These higher levels may be in the range to act oxidatively.

Another recent trial investigated the separate effects of β-carotene, vitamins C and E, and β-carotene plus vitamins C and E on the recurrence of colorectal adenomas. Results indicate protection among nonsmokers and nondrinkers but an increase in similar carcinogenic and cardiovascular adverse effects in smokers and drinkers as seen in the earlier lung cancer trials. This is a very important observation, supporting a marked difference in response in active smokers to β-carotene specifically.

One potential mechanism to explain β-carotene becoming a prooxidant is that at high doses, and in the presence of high oxygen tension, β-carotene produces free radicals. Evidence also suggests that peroxyl radicals form after autooxidation and the consumption of β-carotene. This produces an additional prooxidative event because another free radical is generated. The prooxidative and antioxidative effects of many compounds, including β-carotene, are highly dependent on the underlying redox milieu of the tissue in which they take place. Another explanation may be the effect of β-carotene on other carotenoids. There is some evidence that the uptake of oxycarotenoids from the gut is negatively affected by too much β-carotene in the system. Cytokines may also interact with β-carotene to produce oxidative stress and cause an unexpected prooxidative effect. Some people think that high levels of β-carotene can enhance Phase I enzymes, so the compound may function as a cocarcinogen for some procarcinogens under certain conditions. More recently, there is evidence that β-carotene can suppress RAR-β, one of the more important retinoic acid receptors in epithelial tissue.

Melanin, contained in melanocytes in the skin, is a redox-active polymer that can serve as an antioxidant in most circumstances but as a prooxidant in others. It also can bind metals and functions as a stable semiquinone and as a free radical. Dr. Meyskens noted that most of his research on melanin has been on eumelanin, the form of melanin responsible for black and brown hair. A model of early melanoma progression has been developed that includes the production of high concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Melanin usually serves as an antioxidant, which helps to decrease the concentration of ROS. In the intracellular milieu, there are sufficient amounts of enzymatic and nonenzymatic antioxidants to lower the overall levels of ROS. The intracellular milieu also maintains the appropriate control of transcription factors and stress responses, as well as a very strong antiapoptotic mechanism. Redox cycling of melanin may be one mechanism for reducing the potential prooxidative effects of melanin. Other antioxidants also may help to decrease ROS levels in melanocytes and slow or stop progression to melanoma, although a study including large doses of vitamin C in melanoma patients showed explosive tumor growth. A downstream effect of some of the cardiovascular drugs is to lower ROS levels, which might explain some of the epidemiological findings of lovostatin, for example, which seems to protect against melanoma. It may be that the timing of antioxidant administration provides a benefit, although this needs to be investigated in much more detail.

Dr. Meyskens noted that to avoid some of the pitfalls recognized in the β-carotene saga, there must be an assessment of all the factors that might lead to an adverse event in Phase III trials. Mechanisms that determine when an antioxidant becomes a prooxidant are largely unknown, and these need to be established before recommending nutritional or nutritional/pharmacologic interventions. Doses are important and the underlying oxidative properties of the tissue being looked at are extremely critical.

Signaling Pathways Activated by Oxidative Injury and Their Roles in Determining Cell Fate

Nikki Holbrook, Yale University School of Medicine

Nikki Holbrook, Ph.D., Professor, Department of Internal Medicine, Geriatrics Section, Yale University School of Medicine, Cambridge, MA, discussed signaling pathways activated by oxidative injury and their roles in determining cell fate. Historically, ROS have been viewed in a negative light; both their generation and targets were presumed to be indiscrimi-

nate and random, and their consequences entirely detrimental. We now know that ROS serve some very important physiologic functions as second messengers in a variety of different signal transduction pathways (most notably proliferative signaling pathways). They also provide host-defense mechanisms against microbial invaders. In these instances, the generation of ROS is both purposeful and necessary. However, ROS also produce a number of undesirable effects that are believed to contribute to disease and aging, including damage to DNA, proteins, and lipids and the inappropriate activation of some signaling pathways. Many of the ROS are produced as byproducts of normal metabolism, but excessive ROS levels also occur as a consequence of environmental exposure. Certain toxins themselves behave as oxidants, whereas others trigger ROS production as the cell attempts to detoxify or eliminate them. Hence, for cells living in an aerobic environment, ROS constitute a double-edged sword. Researchers need to know how antioxidants can be used to prevent the undesirable effects of ROS without compromising normal physiologic functions.

We know that ROS can elicit a plethora of responses ranging from proliferation, to growth arrest (transient or permanent), to senescence, to cell death (through either an apoptotic or necrotic mechanism). Lower doses of oxidants are generally associated with mitogenesis, moderate doses with growth arrest, and higher doses with cell death. Other factors that determine oxidative effects include the nature of the ROS and the type of cell in which it is operating. On the positive side, certain ROS-activated pathways are important for normal cell growth and may be protective in cases of acute oxidative injury such as reperfusion injury. However, in the long run, they may promote tumor growth. Moreover, necrosis and apoptosis may cause the loss of physiologic function, which is considered a negative consequence, but the removal of damaged cells is the same process the body uses for tumor suppression. It is important to know what determines the effects that are seen, and understanding the affected signal pathways may help explain what happens in the cell. Notably, however, the same signaling pathway can be beneficial in one instance of oxidative stress and harmful in another.

The extracellular signal-regulated kinase (ERK) activation pathway serves as an example to emphasize this point and to illustrate the complexity of the response to oxidative injury. ERK is activated in response to both oxidant exposure and growth factor treatment, with similar mechanisms serving to activate the pathway in each case. In acute oxidative injury, ERK activation generally blocks apoptosis and promotes survival. The short-term beneficial effects are the prevention of tissue loss and the enhancement of host survival, but in the long term, it could lead to tumorigenesis or affect therapeutic drug sensitivity. In other situations, however, ERK activation promotes apoptosis. For example, ERK activation increases the sensitivity of cells to cisplatin treatment and promotes apoptosis in response to the drug in many cell types. ERK activation in response to oxidative injury decreases with aging, and this contributes to the reduced tolerance of old cells to oxidative stress. Restricting energy intake can delay the onset of many characteristics of aging. Accordingly, cells from animals fed an energy-restricted diet do not show the attenuated activation of the ERK pathway as a function of age and exhibit greater tolerance to acute oxidative injury. It remains to be determined what downstream targets of ERK might account for these effects.

Mechanisms of Pro- and Antioxidation

Homer Black, Baylor College of Medicine

Homer Black, Ph.D., Professor, Department of Dermatology, Baylor College of Medicine, Houston, TX, presented information on the mechanisms of pro- and antioxidation (2). Oxidation related to diet has been studied for >60 y. Dietary energy restriction reduces cancer at many sites but the mechanisms for such protection are not completely understood. Studies on (n-6) fatty acids (PUFAs) show that they increase free radical reactions, and these reactions can be exacerbated by UV light to increase the likelihood of carcinogenesis. It is assumed that a process of lipid oxidation occurs with polyunsaturated fats, in which a radical attacks a polyunsaturated fatty acid to produce free radicals. It has been assumed that supplementation with one or more free radical reaction inhibitors, such as antioxidants, would prevent lipid oxidation.

Antioxidant function, however, is much more complex than just radical scavenging. To illustrate, animal studies show that the phenolic antioxidant BHT reduces the rate of tumor growth. It may be that the mechanism by which BHT exerts its anticarcinogenic activity involves the quenching of lipid-soluble radicals and ROS. Animals fed a high-fat diet supplemented with BHT exhibit a significant lengthening of the tumor latency period compared with animals fed a diet without BHT. As the dietary lipid level is reduced, so is the effect of BHT. At the lowest lipid level, the protective effect of BHT is almost nonexistent. This suggests that the exacerbative effect of increasing lipid levels on UV carcinogenesis, and presumably lipid peroxidation, are important parts of the carcinogenic process and that BHT is effective in blocking that process. In addition, the skin of animals fed a diet without BHT allows ~65% more UV light through the stratum corneum, which may also promote UV carcinogenesis.

β-Carotene does not affect epidermal absorption through the stratum corneum, and although earlier studies reported that it has a photoprotective effect, this photoprotection was based on the carotenoid-specific capacity to quench singlet oxygen and other oxy-radicals. Under certain dietary conditions, β-carotene exacerbates UV carcinogenesis. Supplementing even a semidefined diet containing β-carotene diminishes the tumor latency period and increases tumor multiplicity. β-Carotene can act as a prooxidant at high oxygen concentrations and under oxidative stress conditions. Many of the oxidizing species, especially peroxyl radicals, convert this carotenoid to the 1-electron oxidized form, yielding a β-carotene radical cation.

Studies show that β-carotene reacts not with the α-tocopherol radical but with the α-tocopherol radical cation to produce a carotenoid radical cation. This radical cation can be repaired with ascorbic acid, producing an ascorbate radical. To explore the role of ascorbate on β-carotene radical repair, animals were fed a semidefined diet (i.e., casein, corn oil, and cornstarch or corn sugar) supplemented with β-carotene and either no extra ascorbate or a 6-fold increase in ascorbate. The level of ascorbate did not influence the exacerbative effect of the carotenoids. These findings weaken the argument that ascorbate can repair the β-carotene radical, which leaves it in a prooxidative state.

Before recommending that individuals take antioxidants for chemoprevention, a better understanding of free radical–mediated damage must be considered.

Iron, Free Radicals, and Oxidative Injury

Joe McCord, University of Colorado

Joe McCord, Ph.D., Professor, Department of Medicine, Webb-Waring Institute, University of Colorado—Denver, discussed the role of iron, free radicals, and oxidative injury (3). Iron has been studied as a human micronutrient since ancient Greece. Although iron is essential, it also may be toxic in certain forms and at high doses. The relation between iron and free radicals has been studied in many disease types because of their ability to damage cellular components and processes (i.e., DNA, proteins, aberrant signaling). Iron can undergo single-valence changes in both directions, and, like copper and other transition metals, can interface very easily with free radical reactions because these reactions typically involve the transfer of single electrons. If available, iron can greatly amplify the damage caused by free radical generation. There is an ongoing discussion within the scientific community concerning whether there is a healthy level of iron stores in the body.

One potential negative effect of increased iron stores is their ability to react with superoxide to form iron-loaded ferritin, which is reduced from ferric to ferrous valence and then released to participate in redox reactions. Ferritin only binds ferric iron, but it binds it so strongly that the iron is redox inactive. When the iron is released, it becomes redox reactive and can react with hydrogen peroxide to generate another secondary radical, the hydroxyl radical, which is the second most potent oxidizing species.

Ferritin is relatively harmless until disease strikes, when the excess iron becomes a significant liability, increasing the damage caused by heart attack or stroke and increasing the likelihood of cancer. It is estimated that most Americans are iron loaded as a result of food supplementation. In addition, ~14% of Americans carry a mutant *HFE* gene, which causes hemochromatosis. Humans accumulate iron as they age, which may contribute to and amplify disease processes.

An *HFE* mutation has been introduced in a mouse knockout model to produce a model of human hemochromatosis, which is extremely useful. These mice accumulate iron in their tissues just like humans with hemochromatosis. Even when fed an extremely iron-restricted diet, the *HFE* knockout mice accumulate more iron in the heart than do wild-type mice fed a normal diet. When the heart is subjected to ischemia reperfusion (triggering a heart attack in this laboratory model), it is apparent that the heart damage is in direct proportion to the amount of iron the heart has stored. Lipid peroxidation can be used as the index of damage; after a heart attack, wild-type mice increase their lipid peroxidation 4-fold, but the *HFE* knockout mice increase theirs by 10 to 15 times.

HFE is a transcriptional factor that induces the production of the hormone hepcidin, which regulates iron uptake if both of these gene products are present. The mutant *HFE* that produces hemochromatosis transcribes little or no hepcidin. In an unregulated system—one that is homozygous for hemochromatosis—iron is actively absorbed from the gut and appears in the bloodstream as iron-loaded transferrin. Iron-loaded transferrin is detected by a receptor on the surface of liver cells (the TF2 receptor), which normally binds HFE protein and β-2 microglobulin to the cell membrane. When the liver cell detects adequate iron in the system, it releases HFE from its membrane. HFE then translocates to the cell nucleus, where it upregulates the production of hepcidin secreted by the liver cell into the bloodstream. The hepcidin goes to the intestinal cells, which have a hepcidin receptor, and shuts off the absorption of iron from the gut. This feedback system controls the absorption of iron.

A recent study in *Nature Genetics* reported that constitutive hepcidin expression in transgenic mice can prevent iron overload in *HFE* knockout mice (a better model for studying hemochromatosis). Theoretically, recombinant hepcidin may restore the normal regulation of iron in patients with hemochromatosis, although too much hepcidin may shut down iron absorption completely, which would lead to anemia.

Oxidative Stress and Human Genetic Variation

Ralf Morgenstern, Karolinska Institutet

Ralf Morgenstern, Ph.D., Professor, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, presented information on oxidative stress and human genetic variation (4). Single nucleotide polymorphisms (SNPs) are the most common single-base-exchange genetic variations in humans. There are 3 billion bases in the human haploid genome, and most of the variants are SNPs, but there also are insertions and deletions. It is estimated that humans currently have 1 genetic variant per 100–300 base pairs, which means there are 10 million possible sites of such genetic variation in a typical human. Most of the variation and allelic frequency of these variants are the result of drift; these random events may have occurred in very small tribes during human evolution. The NCI has developed a database of SNPs (see http://www.ncbi.nlm.nih.gov/SNP/), many of which are involved in oxidative stress. There also is a database of validated SNPs at the NCI Cancer Genome Anatomy Project website (see http://www.nih.gov/science/models/mouse/resources/cancer_genome.html) that includes oxidative-stress-related genes. One example of an oxidative-stress-related gene is the catalase gene, which has been mapped. Expression analysis of variants of the catalase promoter region (C/T) shows that the variants affect transcription factor binding sites. Carriers of the T allele have markedly more catalase than carriers of the C allele. This shows that the genetic variant affects human catalase levels.

Studies of genes for glutathione peroxidase I show that a particular amino acid alteration is present in samples collected from participants in past studies, but there is no correlation between the SNP and glutathione peroxidase I levels. However, this variant correlates to lung cancer in association studies. In addition, blood glutathione peroxidase concentration is a biomarker for selenium status.

There are many variants that may have potential applications as markers of oxidative stress, including single amino acid alterations, alterations that affect the intracellular targeting of mitochondrial superoxide dismutase, and numerous variants of glutathione. There is a lack of strong evidence from association studies to show that genetic variants are directly related to disease or disease processes. In addition, there may be a lack of real benefit in studying variants that have low penetrance in the population, especially if they have no apparent negative effect on health.

There is, however, a need to find out whether genetic variants are related to cancer, and this may be one of the greatest research needs for the future. In vitro models are needed, but there also is a need for human studies. Even if the effects of a variant are small and the effect on a population is small, there may be improved statistical methods in the future that can help address the underlying questions about genetic status and oxidative stress. One example of a gene that could be studied at the present time is the 8-hydroxy deoxyguanosine (*8OHdG*) repair gene.

Discussion Session 1

Dr. Zeisel asked participants what type of request for application (RFA) they would like the NCI to write to help researchers address some of the issues presented in this session. A participant responded that it is important to remember that the goal of research is to develop some practical application for patients. Any research on antioxidants or oxidative stress should have in mind the ultimate application. Specifically, research on the processes that occur to change antioxidants to prooxidants is one area that needs clarification through carefully designed research. Animal models may be very useful in this research.

Dr. Holbrook added that there is a need to show that what happens in terms of blocking oxidative stress in vitro can also be accomplished in vivo in an effective manner. Dr. Zeisel asked how a model could be designed to determine what perturbations in redox status affect different pathways and lead to different outcomes. Dr. Holbrook agreed that it would be important to understand the effect of antioxidants on various pathways.

Dr. McCord said that an RFA could be issued that addressed the role of hepcidin in iron regulation, and the general area of free radical metabolism.

Dr. Ames commented that there may be a problem in spending huge amounts of money on minor hypothetical risks and not putting the money where it is needed, such as in the areas of obesity, bad diet, and general nutrition. Dr. Morgenstern agreed that priorities are important, that learning about oxidative stress genes and genetic variations is a worthwhile goal, and that there is a lot of knowledge to be gathered, so some money could be directed there. Learning more about genetic variations also could yield benefits.

Dr. Zeisel added that there are many layers of study after genetics, including epigenetics, proteomics, lipidomics, and metabolomics. Each area will have some answers to the issues of the pros and cons of antioxidants. There also will always be a need to understand the mechanisms involved in each of these layers regarding pro- or antioxidants.

A participant commented that it is important to take into consideration the endpoints of antioxidant use and oxidative events and how they affect very specific genes or very specific proteins. The important aspect is that the event must change the biochemical and proliferative events of the tumor cell itself. Dr. Zeisel added that lipids also should be studied, as should signaling pathways.

A participant from the Annie Appleseed Project, an organization for patients interested in complementary or alternative therapies, commented that chemotherapy can have adverse consequences for patients and that it will be important to move from research to translation to the patient level.

One participant commented that intervention studies seem to be trying to find the "magic pill" or combination that will lead to good health. The issue of healthy diet should be the focus of research. Diet seems to be the problem; for example, a high-fat diet and obesity are proinflammatory, which induces oxidative stress.

A participant commented that it is important to devote resources to two things: measurement and functional consequences. Measurement of oxidant activity and markers of oxidant activity in humans, and what constitutes a measure of exposure to oxidants and therapy, are some of the main issues that need to be addressed. Hypertension researchers have made these discoveries and do very well at treating the disease.

SESSION 2: ANTIOXIDATIVE EFFECTS—PROS AND CONS

Session Chair: *Richard Rivlin, Institute for Cancer Prevention*

Richard Rivlin, M.D., Senior Vice President, Medical Affairs, Naylor Dana Chair in Nutrition, Institute for Cancer Prevention, New York, NY, introduced the session on the pros and cons of antioxidative effects. One of the most troubling aspects of antioxidant research is that clinicians do not have the information they need to make recommendations to patients regarding antioxidant supplementation. There are very strong market forces that tell the public about supplements, but there is very little reliable advice about them. In addition, the amount of contradictory advice is confusing to consumers. Also, physician training is inadequate on this issue; only one-quarter of the nation's medical schools have required courses in nutrition, so most physicians have no training in this area. It is important that we understand the factors that regulate the serum levels of endogenous antioxidants and learn more about herbal products. Other areas for research include making cancer therapy more effective and safer, and understanding the dose-response relation with respect to the efficacy and toxicity of antioxidants.

Phytochemical Effects beyond Antioxidation

David Heber, University of California—Los Angeles

David Heber, M.D., Ph.D., Director and Professor, Department of Medicine, Center for Human Nutrition, David Geffen School of Medicine, University of California–Los Angeles, discussed phytochemical effects beyond antioxidation (5). There may be as many as 25,000 phytochemicals in the human diet, with many having physiological antioxidative effects, but these effects are not directly related to their many other effects on cellular signaling pathways, gap junctions, and metabolic enzyme induction, which often do not follow their antioxidative potencies in rank order of comparison. Phytochemicals occur in families; they are usually present in plants as complex mixtures and not as single purified compounds. Moreover, members of the same family of compounds may act through different mechanisms.

Phytochemicals will interact with cells in unique ways: synergistically with related compounds as they occur in nature, with unrelated compounds, and through the activation of metabolic enzymes. What the pharmaceutical literature calls *drug-metabolizing enzymes* actually are *phytochemical-metabolizing enzymes* (i.e., Phase I and Phase II enzymes). Humans evolved without drugs per se, but used the environment (e.g., plant and animal products and minerals) to treat medical conditions.

Lycopene is a phytochemical antioxidant with no pro–vitamin A activity that is found in tomatoes, which have only been widespread in the human diet for ~500 y. Having the highest antioxidative activity among all carotenoids, lycopene exists in tomatoes and derived products as one of numerous phytochemicals, many with similar structures and properties. Epidemiological data suggest that lycopene may reduce the risk of prostate cancer. When consumed, phytochemicals enter the cells, where they interact with very low affinity, high-capacity receptor molecules that trigger various intracellular actions and cell signaling pathways, as well as stimulating the metabolism of these compounds. They do not act on a single pathway, but in concert with many other pathways. If supplemental lycopene is added to a cell culture of prostate or breast cancer cells and tested against tomato oil, the complex product is more effective than lycopene alone. This argues strongly for

not simply studying single compounds when exploring the mechanisms behind epidemiological observations.

When phytochemicals are consumed, some of them are absorbed intact, but many are metabolized in very subtle ways. For example, lycopene is metabolized to form the *cis*-metabolite, which is found in larger amounts in the bloodstream than in the tomato product consumed. In addition, the amount of lycopene that gets to a specific cell is often very different from what is found in the blood or the food itself. This is true for ascorbic acid as well.

Tumor-Suppressing Effects of Antioxidants from Tea

Roderick Dashwood, Linus Pauling Institute, Oregon State University

Roderick Dashwood, Ph.D., Chief, Cancer Chemoprotection Program, and Professor, Linus Pauling Institute, Oregon State University—Corvallis, provided information on the tumor-suppressing effects of antioxidants from tea (6). In human colon cancer, the β-catenin/Tcf signaling pathway is activated by mutations in *APC* or *β-catenin*, which cause overexpression of downstream targets such as c-*myc*, c-*jun*, *cyclin D1*, PPAR-δ, and *matrix metalloproteinase-7*. Research shows that epigallocatechin-3-gallate (EGCG), an antioxidative polyphenol in tea, can inhibit the activity of the β-catenin/Tcf signaling pathway in vitro. More than 80% of human colon cancers have a mutation in the *APC* gene, and those that do not have mutations in *β-catenin*.

To investigate diet and its effect on the genetic processes that lead to colon cancer, in vitro studies were conducted in human embryonic kidney cells transfected with *β-catenin* and *TCF-4*. A reporter (Top Flash) was introduced into this model because it binds to *TCF-4* and *β-catenin*. Adding purified EGCG to the mix inhibited reporter activity in a concentration-dependent manner. Adding tea with EGCG more effectively inhibited reporter activity; Sulindac, a nonsteroidal anti-inflammatory drug (NSAID), had no effect at the doses tested in vitro.

In vivo studies in a mouse model using an oncogenic form of *β-catenin* under the control of the A33 antigen promoter were conducted to determine whether EGCG or Sulindac could reduce the formation of colon polyps. Mice were pretreated with a colon carcinogen and then exposed postinitiation to white tea or Sulindac. There was no reduction in aberrant crypt foci; however, a combination of white tea and Sulindac caused a significant reduction in tumor volume, tumor number, and tumor size.

Further studies of molecular changes in the polyps showed that *β-catenin* was more strongly expressed in the polyp than in the adjacent normal-looking tissue from the same mouse. The mice fed Sulindac expressed much lower levels of *β-catenin*. Looking at downstream targets of β-catenin/TCF-4 signaling, the polyps had much higher levels of the target proteins than the adjacent normal-looking tissue. Sulindac alone reduced expression of β-catenin protein, as well as downstream targets, either in polyps and/or in the adjacent normal-looking tissue around the polyps. These results support the view that a drug and diet combination may be more effective against colon cancer than single treatment with tea or an NSAID alone.

Antioxidants Suppress Apoptosis

Steven Zeisel, University of North Carolina—Chapel Hill

Steven Zeisel, M.D., Ph.D., Professor and Chair, Department of Nutrition, Associate Dean, Research School of Public Health, University of North Carolina—Chapel Hill, discussed antioxidants and the mechanisms for suppressing apoptosis (cell suicide) and apoptotic signaling (7). There is a growing body of evidence that there are signaling systems that physiologically use ROS as intermediate signals. ROS not only regulate the signaling for apoptosis, but are capable of activating apoptotic pathways upstream, and many of the drugs and treatments used to kill cancer cells (chemotherapy and radiation) work by generating ROS to activate apoptotic pathways and kill cells. These pathways involve activation of a caspase upstream, a mitochondrial depolarization that generates ROS, which can then activate the caspase, as well as activation of downstream signals that end in final common pathways for cell suicide.

Choline deficiency involves an apoptotic pathway that uses ROS as an intermediary message and a nuclear factor κ-B (NFκB) signal downstream. If there is little antioxidant content in liver cells that are also choline deficient, apoptosis is induced. If an antioxidant is added, such as *N*-acetylcysteine, apoptosis is inhibited by blocking the ROS signal. *N*-acetylcysteine also blocks transforming growth factor β-1 (TGF-β-1)–induced apoptosis, which also uses a ROS to produce an intermediary signal from the mitochondria during the signaling cascade for apoptosis.

There is a lot of research ongoing involving ROS and apoptosis, including research showing that the activation of caspase-9, which has a cysteine-cysteine bond that is sensitive to redox state, causes apoptosis. In addition, *p53* activation increases ROS production and induces apoptosis. ROS production also causes induction of cytochrome-C, which activates the caspase-3 signaling pathway. The key question is still how to make cancer cells undergo apoptosis without affecting normal cells.

Studies using a mouse model with a mutated retinoblastoma (Rb) protein show that mice fed a diet low in vitamin E and other antioxidants have higher rates of apoptosis and decreased tumor volume. Other researchers report that antioxidants such as vitamin E and *N*-acetylcysteine delay and inhibit apoptosis in a number of models, including pancreatic cells and PC-12 cells. There are some data in the literature to suggest that the effective mechanism in killing cells with chemotherapy or radiation is the generation of excess levels of ROS that then induce cell death. Administration of antioxidants during these treatments would reduce the amount of cell death produced.

Studies have investigated the effects of antioxidant supplementation on cancer therapy. Studies on cisplatin indicate that it kills breast cancer cells by apoptosis and necrosis, and that the addition of vitamin E blocks much of the apoptotic process. High-dose vitamin E reduces the efficacy of cisplatin, although the normal cells involved would be protected by vitamin E. Lymphoma cells treated with 5 Gy of radiation die or stop dividing, but if *N*-acetylcysteine is added to the media, the lymphoma cells keep growing. Vitamin E succinate also protects cells against the effects of radiation in vitro.

There is no conclusive evidence to show which antioxidant doses or mixtures protect cells against DNA damage and lipid and protein oxidation but do not interfere with apoptosis signaling pathways. There may be a threshold beyond which DNA is protected against oxidants because the ROS oxidants produced are quenched and there may be a higher dose needed to suppress signaling. Oversupplementation may actually produce an environment that is beneficial to the tumor and allow it to survive.

Green Tea Polyphenols: Antioxidative and Prooxidative Effects

Chung S. Yang, Rutgers, The State University of New Jersey

Chung S. Yang, Ph.D., Professor and Chair, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey—Piscataway, discussed the antioxidative and prooxidative effects of green tea polyphenols (8). Green tea and green tea polyphenols inhibit tumorigenesis at different organ sites, including the skin, lung, oral cavity, esophagus, stomach, liver, pancreas, and prostate. Studies on skin and lung demonstrate that tea is an effective inhibitor when given to animals at the initiation, promotion and progression stages of carcinogenesis.

There is a presumption that the active ingredient in tea is EGCG, but much is unknown about the specific mechanisms involved. Other tea constituents (such as caffeine) could also be important. EGCG is a strong antioxidant, and its antioxidative activity is stronger than that of vitamins C and E in vitro. However, the importance of such antioxidative activity in vivo after tea consumption, has not been fully established.

Much of the published mechanistic information on the action of EGCG was obtained from studies in cell culture. When EGCG is added to different cell lines, it can inhibit growth and/or induce apoptosis, but the results need to be interpreted with caution, because the concentrations of EGCG used are usually much higher than those that can be reached through systemic distribution. EGCG enters the cell through passive diffusion, is methylated and glucuronidated, and is pumped out of the cell by multidrug resistance associated proteins (MRPs). In addition, EGCG can be oxidized to form dimers and produce H_2O_2. EGCG can induce apoptosis at concentrations of 10 μmol/L (micromolar), and this activity becomes more prominent at 30 and 100 μmol/L. This proapoptotic activity is at least partly mediated by H_2O_2, because catalase blocks apoptosis completely in some cells and partially in others. The addition of EGCG to cultured cells causes the overexpression of many genes, and some of these genes are not activated in the presence of catalase.

It is reported that EGCG inhibits the epidermal growth factor (EGF)-induced signal transduction pathways. Many of these experiments require a preincubation period. During this period of time, a large part of the added EGCG has been oxidized (to form dimers and other derivatives). Superoxide is believed to be involved in mediating the autooxidation, because EGCG is stabilized by the addition of superoxide dismutase (SOD). SOD also prevents the inhibition of EGF-induced signaling pathways by EGCG. It is possible that the superoxide generated during autooxidation of EGCG contributes to the inactivation of EGF receptor and thus inhibits the signaling pathway. In the presence of SOD, the cell growth inhibition effects are enhanced, suggesting that the growth inhibition is caused by EGCG, not mediated by H_2O_2.

Many research groups report the inhibition of MAP kinases by EGCG (possibly through competition for the binding site with protein substrates). The inhibition of other protein kinases such as IKK and cyclin-dependent kinases as well as proteinase activities such as the chymotryptic activity of 20s proteosomes and matrix melalloproteinases (MMP2 and MMP9) could also be important mechanisms. These activities do not appear related to the antioxidative activity of EGCG.

In summary, there is only a moderate increase in antioxidant capacity after tea consumption because the bioavailability of tea polyphenols is low. Although antioxidative and prooxidative activities can be demonstrated in vitro, other mechanisms may be important in the anticancer activity of tea and EGCG in vivo.

Rationale for Using High-Dose Multiple Antioxidants as an Adjunct to Radiation Therapy and Chemotherapy

Kedar Prasad, University of Colorado Health Sciences Center

Kedar N. Prasad, Ph.D., Professor, Department of Radiology, University of Colorado Health Sciences Center, Denver, presented information on the use of high-dose multiple antioxidants as an adjunct to radiotherapy and chemotherapy (9). The use of antioxidants in cancer therapy is driven by two opposing hypotheses. One hypothesis states that the use of dietary multiple antioxidants and micronutrients improves the efficacy of treatment; the opposing hypothesis states that the use of antioxidants and micronutrients protects cancer cells against free radical damage. These opposing hypotheses have grown out of generalized experimental data.

No data exist to clearly show that antioxidants protect cancer cells at doses that reduce the growth of the tumor cell but not the growth of the normal cell. At these doses, there is a selective effect of antioxidants on growth inhibition, apoptosis, or cell differentiation in cancer cells but not in normal cells. Given these facts, it seems that antioxidants might enhance the effects of radiation and chemotherapy on tumor cells but not on normal cells, but supporting data are scant.

There is a difference between dietary antioxidants and endogenous antioxidants. Studies indicate that endogenous antioxidants, such as glutathione-elevating agents and *N*-acetylcysteine or α-lipoic acid, always protect both normal cells and cancer cells. Thus, there should not be a recommendation to supplement endogenous antioxidants or compounds that will increase the levels of endogenous antioxidants. In addition, there are data that show that cancer cells transfected with the SOD enzyme become resistant to radiation and therefore should not be used as an adjunct in radiotherapy. When dietary antioxidants are used at low doses, they do not affect the growth of either cancer cells or normal cells. It is not recommended that low doses of antioxidants be given in any therapeutic situation.

Some experimental studies provide information on the use of antioxidants and cancer therapy. In a rat melanoma cell line, cells treated with vitamin E succinate converted to a normal phenotype. Studies in human melanoma cell lines indicate that vitamin E succinate just inhibits growth or induces apoptosis.

In a study of hormone-insensitive breast cancer cells pretreated with vitamin E succinate, the cells became hormone sensitive after radiation treatment. Vitamin E succinate did not affect the mitotic accumulation of human fibroblasts in vitro, but slowed down the cell cycle.

Human parotid acinar carcinoma cells exposed to vitamin C do not show growth inhibition, but conversely, human melanoma cells respond to vitamin C. Acinar carcinoma cells are extremely sensitive to β-carotene, but the vitamin has no effect on melanoma cell proliferation. These effects can be dose dependent. Often, different cell lines require different doses to respond. Vitamin E succinate also affects one of these cell lines and not the other, as does retinoic acid. Determining the dose of a nutrient that is necessary to produce an inhibitory effect is very important before beginning a clinical trial. At certain doses, nutrients can enhance the growth of cancer cells instead of inhibiting it.

Although it is difficult to extrapolate from one experimental condition to another or from one dose to another, it is clear that dose is important. In a human neuroblastoma cell line, 2 μg of vitamin E succinate does not affect growth, but 20 μg markedly inhibits growth. The gene expression profile is also

entirely different between the two doses, which should be an area of interest to clinicians and cancer researchers.

Experimental studies also show that combinations of antioxidants often are more effective than single antioxidants. A single antioxidant did not affect the growth of human melanoma cells, but when a combination of antioxidants was added to the media, it inhibited the growth of the cell line by 50%. Increasing the dose of vitamin C in this mixture from 50 to 100 μg enhanced the inhibitory effect dramatically, even though vitamin C by itself did not affect growth.

In another experiment, vitamin E succinate inhibited the growth of neuroblastoma cells more effectively than radiation, but the two together produced an even more powerful effect. In addition, a water-soluble preparation of vitamin E inhibited the growth of colon cancer cells transplanted to athymic mice better than 5-fluorouracil, but the two together produced almost no growth. Vitamin C enhances the effect of 5-fluorouracil on cancer cells but not on normal cells, and enhances the effect of adriamycin on HeLa cells but not on normal cells.

Discussion Session 2

A participant asked about receptor-mediated pathways and whether low doses of phytochemicals could be rendering the cells susceptible to physiological inducers of apoptosis. A panel member responded that there are tests currently being proposed to look at combinations of things such as green tea and tamoxifen, or lycopene and vitamin D.

A participant asked what form of α-tocopherol is able to enter the cell and whether this form reduces the therapeutic potential of α-tocopherol. Dr. Prasad responded that to be effective, α-tocopherol succinate must be an intact molecule. It is metabolized to α-tocopherol in vivo; therefore it must be administered intravenously. When the ester is given intravenously, 70% of the tumor can be reduced. A similar dose of unesterified α-tocopherol reduces tumor growth by only 35%. This is why the dosage formulation is so important. If the in vivo dose level is high enough, α-tocopherol can have anticancer activity that may be related to its antioxidant activity. The succinate ester itself has no antioxidant activity.

A participant asked whether the in vitro effects of green tea extract were due to hydrogen peroxide or to the inhibition of catalase. Dr. Yang responded that it is probably due to a similar type of peroxidant mechanism.

A participant commented that the use of the term *antioxidant* might have outlived its usefulness because of the chemical heterogeneity of these compounds. He suggested that a new term, such as *reductants* be considered. A panel member suggested that this might be a good idea, but that antioxidants have many other functions in addition to acting as reducing agents.

A participant asked for a clarification on the use of antioxidants with radiation therapy and whether there are ongoing clinical trials addressing this issue. Dr. Rivlin responded that the real question may be whether we are scientifically and ethically ready for randomized, controlled, double-blind studies of antioxidants, radiation, and chemotherapy. Another panel member added that at the Henry Ford Hospital, Detroit, MI, in a randomized trial of patients with stage 0–III breast cancer, 25 patients received radiation therapy alone and 22 patients received micronutrients, including high doses of antioxidants and their derivatives. A median follow-up at 2 y showed that 2 new tumors developed in the radiation group but no tumors developed in the combination group. Antioxidants also inhibit the repair of radiation-induced damage. This is one reason why antioxidants can enhance the effects of radiation, if given before and/or after treatment, but may not interfere with the life of normal cells.

A participant commented that there might be a U-shaped dose-response curve; when α-tocopherol concentrations are high enough there may be an apoptotic effect. At 15 μmol/L of vitamin E, tumor cells survive and are resistant to an apoptotic signal. At 50 μmol/L of vitamin E, those cells die.

A participant asked what to do about patients who are already taking antioxidants and are scheduled for chemotherapy or radiotherapy. A panel member stated that this is the key issue facing this conference.

SESSION 3: BIOMARKERS

Session Chair: *Steven Clinton, Ohio State University, Arthur G. James Cancer Hospital*

Biomarkers of Oxidative Stress: Fact or Artifact?

James Swenberg, University of North Carolina

James Swenberg, D.V.M., Ph.D., Professor, Department of Environmental Science and Engineering, University of North Carolina—Chapel Hill, described research on biomarkers of oxidative stress. Oxidative damage is the most common form of DNA damage, with $\sim 1 \times 10^6$ nucleotides damaged by oxidation at any one time. Damage can arise from both endogenous and exogenous sources, which can complement each other. Most studies in the literature focus on adducts of *8OHdG*, although there are newer studies focused on oxidized bases, oxidized abasic sites, and cyclic DNA adducts. Slot blot electrophoresis is used to analyze intact DNA, and mass spectrometry is used to analyze nucleosides and bases.

One of the most complex issues regarding antioxidants is the dose response to hydrogen peroxide. At low concentrations, iron associates with DNA around the *N*-7 position of guanine, which is readily available for Fenton chemistry and is responsible for a steep increase in oxidation early in the process. Iron also associates with the deoxyribose moiety of DNA, where it is tightly bound and it is not readily available for Fenton chemistry, which results in a descending slope of activity. This decrease in oxidation damage is seen because hydrogen peroxide is not only a prooxidant; it can also be an antioxidant under certain conditions.

The efficiency of oxidant-induced DNA damage is highest at low concentrations, such as 0.6 μmol/L. As the concentration goes up, there is a smaller effect on the DNA per unit of exposure, which is not the dose response that is normally seen in the laboratory. Using base-excision repair enzymes, it is possible to look at oxidized purines, such as 8-hydroxydeoxyguanosine (*8OHdG*), and pyrimidines as targets of oxidation. From 1983 to 2003, the same complex dose response for hydrogen peroxide was seen with 4 different endpoints being measured. This is the result of iron being present in different intracellular pools, with differential availability for Fenton chemistry, and hydrogen peroxide acting as both an anti- and a prooxidant.

In experiments using *8OHdG* formation as an endpoint biomarker, it is important to avoid artifacts, which can change the results of the experiment. The use of TEMPO, a free radical trapping agent, or desferal, an iron chelator, during the tissue workup helps to reduce artifacts. It is also important to understand the amount of background *8OHdG* found in the specific tissues and cells used in specific experiments. A large study conducted by the European Standards Committee on Oxidative DNA Damage (ESCODD) found that the average amount of *8OHdG* present in the lymphocytes of a normal,

healthy, 25- to 30-y-old is ~0.6 to 6 per 10^6 guanines, depending on the method used for isolation and analysis. Additional assays are being developed to improve the accuracy of measurements of direct oxidative damage and lipid peroxide-induced DNA damage, and to determine whether adducts are produced from exogenous or endogenous exposure.

Plasma Antioxidant Measurements

Ronald Prior, Arkansas Children's Nutrition Research Center

Ronald L. Prior, Ph.D., Research Chemist and Nutritionist, Agricultural Research Service, USDA, Arkansas Children's Nutrition Research Center, Little Rock, discussed plasma antioxidant measurements (10). There are several antioxidant defense mechanisms, such as free radical–scavenging enzyme systems and nonenzymatic systems that include antioxidant compounds, compounds that are active in the lipid domain, water-soluble compounds, flavonoid compounds, the carotenoids, uric acid, and plasma proteins. Antioxidant capacity assays essentially are inhibition methods. A free radical species is generated, and the inhibition of the free radical action by an added antioxidant is designated as the antioxidant capacity. Antioxidants can produce either a total inhibition of free radical action that is detected as a lag phase or a partial inhibition of free radical action, in which no lag phase will be detected unless a very high concentration of free radicals is involved. Inhibition of free radical action by an antioxidant has 2 components: the inhibition time and the degree of inhibition.

Measures of in vivo antioxidant status are important in understanding the role of oxidative events in the initiation and progression of numerous diseases, including cancer, atherosclerosis, and diabetes. Measurement of individual plasma or tissue levels of antioxidants such as vitamin C, vitamin E, or the carotenoids can assess in vivo antioxidant status. However, it is a much more difficult task when one considers the numerous other compounds, including flavonoid and polyphenol-like compounds, that may influence in vivo antioxidant status. In this case, measures of antioxidant capacity are an important tool in the assessment of antioxidant status. Numerous techniques, often utilizing quite different free radical sources, have been developed and used to assess antioxidant capacity (AOC) in plasma. AOC is evaluated in terms of the levels of low-molecular-weight antioxidants in plasma or tissue. However, AOC assays do not address the role of various antioxidant enzymes in protecting against free radical action. Advantages of AOC measurement are that individual analysis of each antioxidant component is not necessary and an estimate of the total AOC can be obtained. However, until recently, no true measure of total AOC (hydrophilic and lipophilic AOC) was available. A useful assay should be able to use a biologically relevant radical source, such as peroxyl, hydroxyl, or singlet-oxygen- and peroxynitrate-containing compounds. Radicals from different sources produce different estimates, so it is important which radical is utilized. The ideal assay should measure both lipophilic and hydrophilic antioxidants and measure both inhibition time and the degree of inhibition.

The oxygen radical absorbance capacity (ORAC) assaycan determine antioxidant activity against peroxyl radicals (ROO) and measures most of the well-known antioxidants, including ascorbic acid, glutathione, bilirubin, α-tocopherol, β-carotene, uric acid, melatonin, and flavonoids. The ORAC assay measures a total of the antioxidants that are present in a sample, which can be from biological fluids, or tissue, or food. The advantages of ORAC is that it takes into account the time and degree of inhibition. ORAC can be adapted to analyze both hydrophilic and lipophilic antioxidants and can be automated for large studies in which a large number of samples must be processed.

A limitation of ORAC is that it often is restricted to measurement of events in the blood, and these measurements may not reflect what is happening in the target tissue. Another potential question revolves around the response to oxidative stress. Immediately after exposure to an oxidative stressor, there is a decrease in antioxidant capacity using the available antioxidants, but over time there may be a response in the tissue, so that antioxidant capacity increases. This complicates interpretation of the results. The assay gives a snapshot in time of the potential antioxidant status.

The literature reports lower antioxidant capacity in preterm infants, patients with HIV (37% reduction), Alzheimer's patients (24% reduction), patients with sepsis, and patients with diabetes (50% reduction).

Preliminary studies indicate that the intake of total antioxidants from the diet is reflected in higher serum concentrations of antioxidants. For example, in a study of antioxidant levels after consuming a meal high in antioxidants, serum levels increased after consumption of blueberries, strawberries, spinach, red wine (phenolics), and ascorbic acid. Further analysis of the meal with blueberries showed that the hydrophilic antioxidant levels peaked at 2 h and then declined, but the lipophilic antioxidants peaked at 2 h and remained stable. Cherries produced an oxidative effect for hydrophilic antioxidants, but also produced a very strong rise in the level of lipophilic antioxidants. It is not clear why most fruits have relatively low lipophilic components. Prune juice contains a high level of antioxidants, but very low levels of them were found in serum. All of these fruits have high antioxidant levels but very different phytochemical composition, absorption profiles, and metabolism, which produce highly varied in vivo responses.

A European study used the ferric-reducing antioxidant power (FRAP) assay to investigate serum antioxidant levels after consuming cranberries, blueberries, and control foods. Results indicate that serum concentrations were highest with cranberries and lowest with the controls.

Plasma antioxidant measurements seem to be stable over a 2- to 3-mo period, and certain clinical disease states can alter plasma antioxidant capacity. Increased consumption of fruits and vegetables can increase antioxidant levels, depending on the food's metabolism and absorption profile. This elevation is transient and returns to baseline in 4 to 6 h after the meal.

Measurement of serum antioxidant capacity is a research tool that can be used to assess oxidative stress. A battery of assays should be performed, not just one, to fully explore antioxidant status. Relations between dietary antioxidant intake and plasma antioxidant capacity, and how they affect cancer and other health problems, remain to be determined.

DNA Oxidation Products, Antioxidant Status, and Cancer Prevention

Henry Thompson, Colorado State University

Henry Thompson, Ph.D., Professor and Director, Cancer Prevention Laboratory, Colorado State University—Fort Collins, discussed the challenges encountered when laboratory data on DNA oxidation products are used to design clinical intervention studies on antioxidant status and cancer risk (11). The direct evidence of an antioxidant effect on DNA oxidation is inconsistent at best, although most researchers

think that antioxidants should affect DNA oxidation based on indirect evidence.

Several gaps in knowledge appear critical in addressing the antioxidant–cancer prevention hypothesis, which can only be resolved through well-designed studies to specifically answer this question. The study should last at least 2 to 8 wk and include specific markers of oxidative stress, such as *8OhdG*. It should also use both direct and indirect measurement approaches. The tissue of choice could be the lymphocyte, although some have considered urine and specific target organs. Measurements should be taken at weekly intervals to assess differences in treatment effects, and attention should be paid to the methods used for sample collection, storage, and the number of analytical runs necessary to produce good data. Measurements of antioxidant concentrations, scavenging activity, ROS, and oxidation products (DNA, lipids, proteins, or downstream events) would make the study valuable in addressing the many questions that still remain about oxidative stress and antioxidants.

To address the need to correlate this research with cancer risk factors, DNA oxidation could be assessed, because the oxidation of bases produces promutagenic events, promutagenic events increase the potential for mutagenic events, and more mutagenic events increase the risk of cancer. Although *8OHdG* would be the oxidative marker of choice, as it seems to be the most prevalent, other markers should be simultaneously assessed. There are many methods to measure oxidation products; LC tandem mass spectrometry is used in many studies, as is HPLC.

There must be some method to minimize ex vivo DNA oxidation, such as isolation of the nuclei, adding an iron ion chelator, or using a precipitating agent such as sodium iodide. The results from different laboratories encompass differences of several orders of magnitude if these methods are not used. Variance and random error are other methodologic barriers to consider.

In a study of an intervention on women at risk for breast cancer, 50% of the participants had *8OHdG* levels that were higher than the target value. To address this finding, participants were randomly assigned to a lower– or higher–fruit and vegetable diet group (3.5 vs. 12 servings/d, respectively, for 2 wk). Levels of *8OHdG* decreased 16% in the high–fruit and vegetable diet group.

The neutral single-cell microgel electrophoresis assay (comet assay), a simple, rapid, sensitive, indirect technique that can be performed on hundreds of cells at a time, can be applied to investigate oxidized pyrimidines using endonuclease-3 and oxidized purines using formamidopyrimidine *N*-glycosylase (Fpg). The assay uses a small sample and the cells can be frozen for months, which is an advantage when conducting large-scale experiments or complex human trials. However, intralaboratory variability is high with this qualitative assay, and intraindividual variability is equal to the variability between different individuals. Calibration of interlaboratory data is also difficult due to the semiquantitative nature of the assay.

ELISA can also be used to assess urinary *8OHdG*, although there are problems with correlation to intake of antioxidants, as there are with almost all methods described in this presentation.

There are many gaps in our knowledge on this topic. Questions about the relation between different target tissues and appropriate markers of DNA, lipid, and protein oxidation remain unanswered. Markers need to be validated to improve correlation so information gained in these studies can be used to tailor antioxidant treatment to individual genotypes. The overall question remains, however, about what role DNA oxidation plays with specific genetic mutations and how this affects carcinogenesis.

Use of Biomarkers of Oxidative Stress in Research Studies

Jeffrey Blumberg, Tufts University

Jeffrey B. Blumberg, Ph.D., Professor, Friedman School of Nutrition Science and Policy and Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, discussed the use of biomarkers of oxidative stress in research studies (12). Biomarkers can be employed to reflect environmental prooxidant exposures and dietary antioxidant intake or serve as a surrogate measure of a disease process. To be truly useful, the biomarker must have some degree of predictive validity, but full substantiation of this relation is still lacking. A number of challenges must be overcome to obtain not only a better understanding of the contributions of reactive species to the carcinogenesis process but a rational application of biomarkers of oxidative stress to observational studies and clinical trials of antioxidants and cancer. Nonetheless, without measuring parameters relevant to the status of antioxidant defenses and oxidative stress, it is not possible to determine whether the selection, dose, and duration of an antioxidant intervention achieves its intended biochemical or physiological endpoint or whether the enrolled subjects even present with oxidative stress.

Exposure to endogenous and environmental carcinogens causes DNA damage indicative of oxidative stress, with consequences for cytotoxic and mutagenic activity, as well as aberrant changes to cell cycle progression and replication. Moreover, oxidation of cellular lipids and proteins can adversely affect several steps of the carcinogenic process through changes in a variety of cell regulatory functions, including signal transduction and gene expression. Thus, biomarkers of oxidative stress have the potential to help establish pathogenic stages of and risk for disease and should be employed to inform the design and outcome measures of clinical trials. Identification and application of suitable biomarkers should shorten the time it takes to demonstrate that an agent has a beneficial, untoward, or null effect on health promotion and disease prevention or a therapeutic value in disease treatment. However, some proposed biomarkers of oxidative stress might prove simply to be general markers of oxidative damage and relatively poor indicators of disease process and outcome.

New research studies must address whether and how biomarkers adequately measure relevant physiologic functions or relate to established pathological signs, particularly with regard to their accuracy, precision, and reliability. Such efforts must consider the potential for artifacts produced during sample collection, processing, storage, and instrumental analyses, as well as confounding by the presence of related factors such as the status of facets of the antioxidant defense network that are not under direct study. The validation of biomarkers must include an assessment of the degree of bias in their measurement, especially the characterization of their prevalence and variability within large-scale population studies. An important issue for study will be determining whether specific biomarkers reflect short- or long-term exposure to an antioxidant status or oxidative stress.

When establishing the Dietary Reference Intakes, the Institute of Medicine (IOM) used biomarkers of oxidative stress to define dietary antioxidants. The IOM definition of dietary antioxidant includes the ability to significantly decrease the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiologic function in humans.

However, it is not clear whether sufficient scientific agreement yet exists regarding the validity of these biomarkers as a reflection of the action and efficacy of dietary antioxidants. This issue is confused by the apparent difficulty, in many studies, of demonstrating an antioxidant effect unless oxidative stress is first markedly elevated, as found, for example, in smokers or patients with marked inflammatory conditions.

One common working definition of oxidative stress is the disturbance in the prooxidant–antioxidant balance, in favor of the former, leading to potential cellular damage. However, measuring oxidative stress can be difficult due to the presence of complex endogenous systems for correction and repair, as may occur, for example, when a brief elevation in oxidative stress rapidly induces various antioxidant defenses, particularly antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, that quickly reduce the stress and limit the researcher's ability to detect a change. Oxidative stress can result from diminished antioxidant protection as well as increased free radical production. Therefore, investigating antioxidant depletion as a biomarker of oxidative stress may involve determining decreases in concentrations of antioxidants or increases in levels of their metabolites. However, such changes may not reflect a clinically significant or pathogenic event but merely indicate that the antioxidant defense system is functioning.

DNA, lipid, and protein oxidation products provide an extensive and growing array of potential biomarkers, although our understanding of the relation between their status in cells and tissues, including plasma and urine, remains to be elucidated. Development of a broader panel of biomarkers to examine both pro- and antioxidant reactions should be pursued. This might include the capacity of a biological sample to resist oxidation in vitro or ex vivo and modulation of redox-sensitive transcription factors or related alterations in signal transduction pathways.

In practice, single elements or combined parameters from these approaches are currently employed, although, not infrequently, only one analyte is measured. The conclusion may then be incorrectly drawn that the single measurement satisfactorily reflects overall oxidative stress. Although the capability to adequately assess genomic factors relevant to antioxidant defenses and oxidative stress is limited, this facet added to new research approaches will become increasingly important in determining which individuals are most likely to respond to antioxidant interventions. Further elucidation of the relation between antioxidants and cancer risk will require validation of existing biomarkers of oxidative stress as well as the creation of new indices and their further evaluation.

There may come a time when entry into a clinical trial could be determined by biomarkers of oxidative stress, although that is not now possible. An even better approach would be to develop the ability to stratify individuals at risk in clinical trials through a panel of biomarkers.

Manganese Superoxide Dismutase: Genetic Variation and Regulation

Daret St. Clair, University of Kentucky

Daret St. Clair, Ph.D., Professor, Department of Toxicology, University of Kentucky—Lexington, discussed manganese superoxide dismutase (MnSOD) and genetic variation and regulation (13). MnSOD is the primary antioxidant essential for the survival of aerobic life. Its relation to cancer is well documented in cell cultures and animal models. MnSOD activity is altered in cancer cells compared to normal cells, which makes a difference in cell function because MnSOD reduces radiation-induced neoplastic transformation, protects against the cytotoxicity of chemotherapy, promotes cellular differentiation, suppresses cancer phenotypes and metastatic potential, and alters the expression of oncogenes.

The gene that regulates MnSOD is a single-copy gene consisting of 5 exons and 4 introns. One of the unique characteristics of this gene is its intronic enhancer element, which is absolutely essential for the induction of the gene by cytokines and tumor necrosis factors. The promoter region is extremely G-C rich, and consists of a cluster of Sp1 and AP2 transcription factor binding sites, which overlap extensively.

Sp1 upregulates the transcription activity of the human MnSOD promoter, and mutations to the Sp1 binding sites decrease *MnSOD* transcription. AP2 downregulates the transcription activity of the human MnSOD promoter, and mutations of the AP2 binding sites increase such transcription and promoter activity. A set of 3 unique mutations that cause these problems occur in the promoter region of *MnSOD* in many types of cancer.

Polymorphisms in the leader sequence of *MnSOD* are associated with an increased risk of lung and breast cancer. Premenopausal women who are homozygous for the AA allele have a 4-fold greater risk of developing breast cancer than women with 1 or 2 V alleles. The risk is greatest among women who consume low levels of dietary antioxidants. Women who consume high levels of antioxidants have a minimal risk of developing the disease. Menopause does not alter these risks. Polymorphisms in exon 3 of the mature protein are associated with an increased sensitivity to, and inactivation by, thiol reagents.

Discussion Session 3

A participant asked what biomarker would be used to investigate coenzyme Q-10 [CoQ (10)] in studies with outcomes or endpoints of cancer. A panel member responded that it would depend on the body compartment that is most likely affected by CoQ (10). If DNA damage is the target, it may be best to choose lymphocytes.

A participant commented that they work with women who have rheumatoid arthritis, very high levels of C-reactive protein, and higher rates of cardiovascular disease. There are very few studies on oxidative damage in this population. Some studies show that CoQ (10) may be able to repair mitochondrial damage associated with disease. A study at Cornell, using a mouse model of amyelotrophic lateral sclerosis, showed that CoQ (10) can repair the results of genetic defects.

A participant asked whether protein or lipid parameters have the same variability as that seen at major sites of oxidative stress. Dr. Thompson responded that all of these assays have problems; in some instances they are different problems from those seen at major sites. Another panel member added that some biomarkers have not been characterized as well as others. Because proteins are not subject to repair like DNA, they integrate the amount of oxidative damage over their life span. If the protein is an albumin, the life span will be ~21 d. If the protein is hemoglobin, the life span will be 120 d, which makes hemoglobin a better choice for study because of its longer life span. It was also noted that studies on *8OHdG* found that it is formed endogenously and during sample preparation. This means it is being formed while it is being studied and may not prove to be a useful biomarker unless endogenous and postsampling formation is tightly controlled.

A participant asked about the effects of age on the use of biomarkers. A panel member responded that age groups are heterogeneous, being affected by a myriad of different factors,

and that it makes more sense to look at people by considering genetic variability and biomarker status rather than age itself. It may be possible to use a panel of biomarkers to stratify people into biological age groups and use those results rather than chronological age.

A participant asked where most of the biomarkers discussed in this session occur and whether it is possible to have a mild inflammatory response in arteries over a lifetime with very few biomarkers of oxidative stress appearing in plasma. A panel member responded affirmatively and said that there is some evidence of this phenomenon (the absence of oxidative biomarker response) in patients with ischemic stroke. There are, however, other factors that could amplify the biomarkers and confound their predictive validity.

A participant asked whether it is possible to perform plasma antioxidant level–reducing equivalence evaluations on stored or frozen specimens. Dr. Prior responded that plasma has been successfully stored at ~−70 °C over a period of several months.

A participant asked whether there is any information on the use of sulfhydryl oxidation in protein biomarkers rather than carbonyl oxidation. A panel member responded that there has been extensive investigation on sulfhydryl damage. Evidence from clinical trials on the protective effects of grapes, lycopene, and flaxseed indicates that sulfhydryl damage to protein is an effective serum biomarker.

A participant commented that proteins are found in the plasma and cytoplasm rather than the nucleus of the cell, so they might possibly be oxidized sooner than DNA. It is possible for methionines in proteins to be repaired without being removed from the protein matrix. The participant asked whether this is true for carbonyl groups and what is known about the turnover of carbonyl. Dr. Clinton responded that many researchers think proteins are more stable and might be better biomarkers of some states, but proteins are also changed, altered, and damaged and can have different polymorphisms. When this occurs, they have very different degradation patterns and half-lives, and it is not known whether proteins that are damaged by oxidative stress will have the same half-lives as proteins that are not damaged. Another panel member added that protein oxidation may just be long-lasting, posttranslational change.

A participant commented on the ORAC assay. A study on diet using ORAC and FRAP showed that when subjects are given fructose, their rate levels increase substantially because fructose is very rapidly metabolized by fructokinase. This leads to a decrease in ATP and the breakdown of uric acid. This suggests that results from ORAC and FRAP may not be showing that the levels of flavonoids are responsible for changes in total antioxidant capacity. A panel member agreed but added that in the study described, uric acid levels were only measured beforehand.

SESSION 4: CLINICAL ASPECTS OF ANTIOXIDANT USAGE

Session Chair: *Rebecca Costello, Office of Dietary Supplements, NIH*

Rebecca Costello, Ph.D., F.A.C.N., Deputy Director, Office of Dietary Supplements, NIH, Rockville, MD, presented goals for Session 4. The session served to set the stage and describe what is known about dietary supplement use among Americans as well as by individuals at risk for or diagnosed with cancer. Topics included types of supplements, frequency of consumption, how they are consumed (i.e., single or multiple supplements), and how they are purchased (i.e., over the counter or prescription). In addition, behaviors and motivations related to supplement use were discussed. The session presented information on oxidative biomarkers of disease, the utility of biomarkers in studying population groups, and the methodological approaches currently available for assessing exposure to antioxidant nutrients for epidemiologic research, with an emphasis on the use of biomarker-based approaches.

Consumer Perspectives about Antioxidants

Cheryl Toner, International Food Information Council

Cheryl Toner, M.S., R.D., Director of Health Communications, International Food Information Council (IFIC), Washington, DC, discussed consumer perspectives about antioxidants (14). IFIC conducted telephone surveys in 1998, 2000, and 2002, of 1004 adults in the United States. Results of the survey indicated that Americans have a positive attitude about nutrition and health and want to know more about the health benefits of food, with an increasing need for information on "functional foods." *Functional foods* were defined as foods that may provide a health benefit beyond basic nutrition, and may include fortified foods as well as fruits and vegetables. In general, consumers are focused on the role of foods in cardiovascular disease and cancer and are becoming more aware of the associations between specific food health claims and disease. For example, in 2002, 79% of consumers were aware of the association between calcium and osteoporosis; 54% for antioxidants and cancer; and 35% for soy protein and heart disease.

According to the survey, consumers tend to increase consumption of foods that carry a health claim if they believe in the efficacy of the claim. For antioxidants, consumers understand the health claims but are not sure which foods contain the antioxidants that could be beneficial. In addition, a significant barrier to increasing use of antioxidants for health benefits may be a lack of confidence in the claims; many consumers do not seem convinced that antioxidants are as great a health benefit as reported in the media.

Factors for health message effectiveness include the seriousness of the disease expressed in the claim (e.g., cancer is more serious than osteoporosis), the knowledge of the component (e.g., fruits and vegetables have many benefits), the association with supplements (e.g., consumers do not know what foods contain vitamin E), and the availability of the component (e.g., calcium is perceived to be in many foods; therefore, supplements are not as necessary).

When consumers were asked whether they had heard of an individual's genetic variability as a factor to consider when determining the right foods or supplements to use for health benefits, they expressed some awareness of the concept, but were cautious about privacy concerns surrounding information on their personal genetic makeup. The word *nutrigenomics* was not perceived to be a consumer-friendly term, and researchers should be aware of the sensitivity of such technical terms.

Antioxidant Supplement Use in Cancer Survivors and the General Population

Cheryl Rock, University of California—San Diego

Cheryl Rock, Ph.D., R.D., Professor, Department of Family and Preventive Medicine, University of California—San Diego, reported on antioxidant supplement use in cancer survivors and the general population (15). Approximately one-half of the general population takes dietary supplements, and use is higher among individuals with health concerns, especially

those diagnosed with cancer. Researchers have suggested that there may be both beneficial and adverse effects of supplement use among individuals diagnosed with cancer. Two recent studies, the Olestra Post-Marketing Surveillance Study (OPMSS) and the Women's Healthy Eating and Living (WHEL) Study, collected data on supplement use. Results from OPMSS indicate that predictors of antioxidant use are age, education, sex, and region of the United States. OPMSS participants reported taking multivitamins (41%), vitamin E (9.5%), vitamin C (17%), and β-carotene (2%). Among users of supplements, the median dosage of vitamin C was 500 mg and that of vitamin E was 34 mg; Dr. Rock commented that each of these represents relatively modest intake from supplements.

The WHEL study examined data on women diagnosed with stage I, II, or IIA invasive breast cancer within the past 48 mo and after treatment. Results indicated that at baseline (1995–2002), there was a wide range of antioxidant dietary supplement use. At baseline, 91% of women used dietary supplements. Specific antioxidant dietary supplements used included multivitamins (59%), antioxidant mixtures (9.8%), selenium (10.1%), vitamin A and carotenoids (10.6%), vitamin C (41.6%), and vitamin E (45.8%). Positive predictors of supplement use included older age (vitamins E and C), high levels of physical activity (vitamins E and C and multivitamins), and education and stage at diagnosis (multivitamins). Negative predictors of supplement use included race or ethnicity and BMI (vitamins E and C and multivitamins) and time since diagnosis (vitamins E and C). Motivations for supplement use included beliefs that vitamin C and E increase general health, that vitamin E decreases menopausal symptoms, and that vitamin C improves immune functions.

Conclusions drawn from these studies suggest that the prevalence of antioxidant dietary supplement use is higher among cancer survivors than among the general population, and that the supplements being used are increasingly complex mixtures of ingredients.

Efficacy of Dietary Antioxidants to Prevent Oxidative Damage

Balz Frei, Linus Pauling Institute, Oregon State University

Balz Frei, Ph.D., Director and Endowed Chair, Linus Pauling Institute, and Professor, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, presented data on antioxidant protection and oxidative damage in human plasma (16). He reviewed the levels of defense against oxidative damage, which can be categorized into proteinaceous and small-molecule antioxidants. There are antioxidant proteins that are nonenzymatic defense systems (e.g., iron- and copper-binding proteins such as transferrin, ferritin, and albumin), which prevent metal ions from producing free radicals in solution. Antioxidant enzymes, including superoxide dismutase, catalase, and peroxidases, are primarily intracellular enzymes and are absent or found only in small concentrations in extracellular fluids. Among the small-molecule water-soluble antioxidants in plasma, urate is found in high concentrations, followed by vitamin C (ascorbate) and bilirubin, whereas glutathione is present only in low concentrations, usually <2 μmol/L. Lipid-soluble small-molecule antioxidants in plasma include α-tocopherol, which is present at a level of about 10 molecules per low-density lipoprotein particle, and β-carotene, lycopene, and other carotenoids and oxycarotenoids, which are found in considerably lesser amounts.

To investigate the relative importance of the endogenous antioxidants in plasma, fresh human plasma was exposed at 37°C to 50 mmol/L 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), which causes oxidative stress by producing aqueous peroxyl radicals at a rate of $\sim 3\ \mu mol \cdot L^{-1} \cdot min^{-1}$; the consumption of endogenous antioxidants in relation to the formation of lipid hydroperoxides was measured as a marker of oxidative damage. Vitamin C is the first line of defense and is used up in the first 60 min of the experiment; no lipid peroxidation occurs during this time. After the vitamin C is consumed, different classes of lipid hydroperoxides are formed, and bilirubin, urate, and α-tocopherol are consumed, in that order. These data suggest a defined sequence of antioxidant defense.

Further studies of other types of oxidative stress, (e.g., activated neutrophils, the gas phase of cigarette smoke, superoxide radicals and hydrogen peroxide generated by the xanthine-xanthine oxidase system, and excess copper or iron), show that vitamin C always forms the first line of antioxidant defense and is the only antioxidant in plasma that can completely prevent lipid peroxidation. Interestingly, copper or iron ions and ascorbate act as prooxidants in vitro because ascorbate reduces the metal ions, leading to production of hydroxyl radicals from hydrogen peroxide. This does not, however, happen in biological systems such as plasma, where ascorbate appears to act only as an antioxidant, even in the presence of excess copper or iron and hydrogen peroxide.

Identified biomarkers of oxidative stress in vivo include F_2-isoprostanes, 8-oxo-2′-deoxyguanosine, and protein carbonyls. For example, F_2-isoprostanes are validated biomarkers of lipid oxidative damage. F_2-isoprostanes are oxidation products of arachidonic acid and are elevated in humans with many conditions, including Alzheimer's disease, hepatic cirrhosis, and atherosclerosis, and with coronary risk factors such as cigarette smoking, diabetes, obesity, hypercholesterolemia, and hyperhomocysteinemia. A gap in knowledge exists because of the lack of prospective studies and clinical trials to clearly establish a link between oxidative stress (assessed by validated oxidative biomarkers) and increased disease risk.

Studies in healthy subjects indicate that there is no relation between vitamin C dose and lower F_2-isoprostane levels in urine. In addition, vitamin E supplementation does not appear to affect urinary levels of F_2-isoprostanes in healthy subjects. In patients with elevated F_2-isoprostane levels at baseline, however (e.g., smokers), vitamin C (2.0 g for 5 d) markedly decreases F_2-isoprostane levels. Very similar results are reported in patients with liver cirrhosis administered vitamin C supplementation (2.5 g for 10 d). Furthermore, patients with type 2 diabetes have elevated levels of F_2-isoprostanes, which are markedly decreased by vitamin E supplementation (0.6 g/d for 2 wk).

The European Prospective Investigation of Cancer (EPIC) Trial, a multinational survey on diet, lifestyle, and physical activity involving >500,000 volunteers, found a strong inverse association between plasma vitamin C levels and total, cardiovascular, and cancer mortality. However, whether this is a beneficial effect of vitamin C itself or fruit and vegetable consumption in general (for which plasma vitamin C serves as a marker) remains unclear. The MRC/BHF Heart Protection Study, a large secondary prevention trial in patients with cardiovascular disease or diabetes, found that a daily cocktail of 250 mg vitamin C, 600 mg vitamin E, and 20 mg β-carotene does not reduce mortality from, or incidence of, any type of vascular disease, cancer, or other chronic disease. In addition, pooled analyses of prevention trials of vitamin E and β-carotene did not show significant benefits for these antioxidants with respect to cancer, cardiovascular disease, or all-cause mortality. In fact, the results of the ATBC and CARET trials

showed that β-carotene supplements have a markedly adverse effect among active smokers.

The question regarding the role of oxidative stress in chronic disease remains unanswered. Clinical trials have not investigated oxidative stress, nor has the relation between risk factors for disease and oxidative stress been adequately addressed. It is not known whether certain risk factors increase disease through oxidative stress mechanisms or whether oxidative stress increases independently of disease risk or as a consequence of disease. Future clinical trials need to assess oxidative stress (i.e., validated oxidative biomarkers) before and after antioxidant supplementation; otherwise, it is impossible to know whether antioxidants fail to prevent or treat disease because they do not lower oxidative stress or because oxidative stress does not a causal role in disease.

Biomarkers for Assessing Antioxidant Nutrient Intakes and Status

Susan T. Mayne, Yale University School of Medicine

Susan Mayne, Ph.D., Associate Professor, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT, discussed the challenges in assessing antioxidant intake and status (17). She focused on exposure assessment for carotenoids, vitamin E, vitamin C, and selenium.

Although there are >600 identified carotenoids, the following carotenoids account for most intake in humans: α- and β-carotene, lycopene, lutein and zeaxanthin, and β-cryptoxanthin. Food-composition data for these compounds have improved in the past decade, which is a benefit to epidemiologists attempting to estimate intake of these compounds. The database developed by the USDA is available online [www.nal.usda.gov/fnic/foodcomp]. Researchers also can use HPLC technology to assess carotenoids in plasma and in other tissues. Dr. Mayne discussed issues regarding the best method to quantify nutrient status and how this can be related to health messages for the public were discussed. She also presented emerging noninvasive technologies, including skin reflection spectrophotometry and Raman resonance spectroscopy (RRS). Carotenoids accumulate in skin, which makes these methods attractive. Validation studies of these technologies are underway.

Vitamin E includes 4 tocopherols and 4 tocotrienols, although the USDA food-composition database uses α-tocopherol equivalents as the standard metric for intake assessment. Bioequivalency in the current USDA database is based on activity in the rat fetal resorption assay. A 2000 IOM report concluded that vitamin E forms are not interconvertible in humans and recommended the use of 2*R*-stereoisomeric forms of α-tocopherol to meet vitamin E requirements in humans. With this change, food-composition databases and nutrient requirements are based on different systems. Biochemical assessment of the various vitamin E forms (e.g., HPLC) is another approach for exposure assessment. The α:γ tocopherol ratio can be used as a biomarker of vitamin E supplement use.

Plasma ascorbate can also be measured by HPLC as a biomarker for vitamin C status, although these values may not correlate with intake estimates at very high levels of intake. Dietary estimates of selenium based on food-composition data are not accurate because the amount of selenium in food varies as a function of the selenium content of the soil, which differs markedly geographically. Biomarkers of selenium intake, such as selenium content in toenail clippings and plasma selenium levels, are preferred to assessments of dietary intake, given the uncertainties in intake assessment.

Most epidemiologic studies of antioxidant nutrients focus on one or two specific compounds, with little research on synergism and interactions among antioxidants. There is a need to develop more comprehensive indices of antioxidant nutrient intake and status. Dr. Mayne discussed various approaches for creating indices, such as principal component analyses of dietary antioxidants, or using ORAC scores to weight foods based on their antioxidant score. All of the more comprehensive approaches require further development and validation prior to routine use in human studies of antioxidant nutrients.

Discussion Session 4

A participant asked whether a hypothetical study of vitamin E and selenium for cancer risk reduction should also include vitamin C. In response, Dr. Rock noted that a factorial design should be proposed for such a study, but it would increase the cost dramatically because of the increased number of participants needed. In addition, there is little evidence that adding vitamin C to a regimen of vitamin E and selenium would offer greater protection from disease. Dr. Seifried commented on recent results (not yet published) from a trial in France that included vitamin C as part of a 5-component supplement, which showed a marked decrease in cancer incidence and mortality in males but not in females. Another participant added that in the next few weeks, there would be a news release warning consumers against β-carotene, which is a miscommunication of what research studies have actually shown to date. Consumers who smoke should be warned against taking high-dose supplements of β-carotene. Another participant added that studies in ferrets show that high doses of β-carotene, but not dietary intakes, are apparently harmful and produce lung changes when administered along with cigarette smoke, suggesting caution in the use of high-dose supplements. It is difficult to achieve tissue levels as high as are implied by intake levels; a 30-mg intake of β-carotene may cause a 10-fold greater concentration in serum than in tissue.

Dr. Toner commented that there is a need for greater translation of research results for the public and media to try to dispel some of the myths about antioxidants and disease prevention. People get their information from a variety of sources, and the media have a difficult time providing the proper context for health messages. A participant commented that the media need to describe the population studied in a reported trial so the public can decide if they will have the same risk as that population. For example, in CARET, ~20% of the subjects were asbestos-exposed workers who had a mean smoking history of 55 pack/y; the remainder were current or former smokers with no asbestos exposure. Few people in the general population would be similar to those in this asbestos-exposure group. Dr. Milner added that the difference in lung cancer risk in the ATBC study was ~20% for those exposed to β-carotene, but the real risk was only a difference of 4/1000 versus 5/1000; this could not be shown in a small study. This further supports the need for larger studies.

A participant commented that he was struck by the data presented by Dr. Rock on the number of patients taking dietary supplements and the fact that >80% of people take supplements that contain herbal constituents, although very little is known about their mechanisms. For example, indole-3-carbonol, found in cruciferous vegetables, is a strong anti-initiation agent against cancer, but in animal models it acts as a tumor promoter if taken after an initiating agent. It is a challenge for health professionals to clearly and succinctly present these findings in lay terms.

SESSION 5A: CHEMOTHERAPY-ANTIOXIDANT INTERACTIONS

Session Chair: *David Rosenthal, Harvard University Health Sciences Center*

Cancer Chemotherapy and Antioxidants: An Overview

Kenneth Conklin, David Geffen School of Medicine, University of California—Los Angeles

Kenneth A. Conklin, M.D., Ph.D., Clinical Professor, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California—Los Angeles, discussed the controversy surrounding the use of antioxidants during chemotherapy (18). Approximately 300 to 400 preclinical studies have been published on this topic, and most show that antioxidants do not interfere with the mechanism of action of therapeutic agents. However, too few clinical studies have been done to draw any definitive conclusions.

Antioxidants (e.g., vitamins C and E) act as reducing agents to neutralize free radicals. If a therapeutic agent works by releasing free radicals, it is possible that antioxidants may interfere with its action. Some antioxidants are also strong nucleophiles (e.g., GSH, *N*-acetyl cysteine, and α-lipoic acid), and they may interfere with the anticancer effects of platinum coordination complexes (e.g., cisplatin and carboplatin) and alkylating agents.

Doxorubicin (Adriamycin), a very versatile antineoplastic agent, is an anthracycline that is reduced to a semiquinone that can generate superoxide radicals. Anthracyclines are important in the study of antioxidant effects because they form large amounts of free radicals and induce oxidative stress. Regarding the mechanism of action of anthracyclines, the most compelling evidence shows that at clinically relevant concentrations they intercalate with double-stranded DNA and inhibit the function of topoisomerase II. Free radicals produced by doxorubicin may play a role in cancer therapy, but preclinical studies suggest that antioxidants do not interfere with the anticancer actions of the drug.

The side effect of greatest concern when doxorubicin is administered is cardiac toxicity. In heart cells, doxorubicin forms a deoxyaglycone that can replace coenzyme Q10 (CoQ10) in the electron transport chain and act as an electron acceptor. This disrupts the energetics of cardiac mitochondria, leading to reduced generation of ATP, and accounts for the commonly seen side effects of acute cardiac toxicity (arrhythmias and reduced ejection fraction). The effect of doxorubicin on cardiac mitochondria is uniquely different from its effect on mitochondria of other cells. This is most likely due to the unique structure of cardiac mitochondria, which contain an NADH dehydrogenase on the outer surface of the inner membrane; this organization is not found in noncardiac mitochondria. Animal studies show that doxorubicin generates ROS in cardiac mitochondria for at least 1 wk after the drug is administered. This causes the formation of mitochondrial DNA-adducts that can suppress gene expression and reduce synthesis of critical components of the mitochondrial electron transport systems. This may irreversibly damage cardiac mitochondria and be responsible for the development of chronic cardiac toxicity (congestive heart failure that is not responsive to digitalis).

The platinum coordination complexes also generate free radicals that can damage the kidney. Animal model studies with antioxidants (reducing agents) show that they do not reduce the toxicity of platinum drugs such as cisplatin. However, several studies suggest that the nucleophilic antioxidant GSH, administered intravenously, ameliorates renal toxicity without interfering with the antineoplastic action of the drug.

Oxidative stress induced by many antineoplastic agents may interfere with cell-cycle progression and reduce the cytotoxicity of drugs that exhibit activity on specific time points in the cell-cycle phase. The aldehydes generated from PUFAs during oxidative stress may also interfere with drug-induced apoptosis, possibly by interference with death receptor pathways or by inhibition of caspase activity.

Future studies could include CoQ10 supplementation as a potential means for reducing cardiac toxicity of anthracyclines and study of certain nucleophilic antioxidants such as GSH for lowering or preventing toxicity of platinum coordination complexes. Such studies may lead to valuable information on reducing toxicity without interfering with the action of these widely used drugs.

Redox and Vitamin C in Cell Signaling and Genomic Protection

David Golde, Memorial Sloan-Kettering Cancer Center

David W. Golde, M.D., Enid A. Haupt Chair in Hematologic Oncology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, reviewed basic laboratory data on vitamin C. Ascorbic acid is oxidized to dehydroascorbate (DHA) outside cells and then transported intracellularly by the facilitative glucose transporters. Inside the cell, DHA is quickly reduced back to ascorbate, where it serves as an antioxidant to protect the cell from oxidation. Another mechanism for the cell to accumulate vitamin C is through the sodium-ascorbate cotransporter system, which allows vitamin C to be directly transported into the cell. Studies in mice show that vitamin C crosses the blood-brain barrier in the form of DHA through the glucose transporters. The therapeutic importance of this finding is that DHA injections provide significant neuroprotection in a stroke model in mice.

Experimental studies with vitamin C show that it inhibits signaling pathways associated with human granulocyte macrophage colony-stimulating factor (GM-CSF). At the molecular level, loading the cells with vitamin C inhibits GM-CSF phosphorylation of mitogen-activated protein (MAP) kinase, JAK-2, and βGMR. Likewise, vitamin C loading suppresses TNFα-induced NfκB activation. A study in human monocytes shows that DHA inhibits FAS-mediated apoptosis and the amount of ROS generated in the cell as a result of signaling. The mechanism acts largely through inhibition of caspase-8 activation. The implication is that vitamin C may enhance the immune system because it may increase the life of FAS-sensitive cells.

Cancer cells have an increased number of glucose transporters; therefore, intracellular vitamin C can protect them from hypoxia as well as increase their resistance to chemotherapy and radiation. In addition, vitamin C can reduce apoptosis, increase resistance to oxidative stress, inhibit mutation, and influence signaling involving ROS.

Drugs, Glutathione, and ROS in Regulation of Proliferation

Kenneth Tew, Fox Chase Cancer Center

Kenneth Tew, Ph.D., D.Sc., Chair, Department of Pharmacology, Fox Chase Cancer Center, Philadelphia, PA, presented data on a 10-y drug development project related to phase II detoxification enzymes (19). The glutathione *S*-transferase (GST) family is very commonly seen in many solid tumors and is overexpressed in drug-resistant tumors that have been exposed to toxic anticancer drugs. Dr. Tew said it would be accurate to extend the definition of GSTs as ligand-binding

proteins in addition to enzymes and that human polymorphisms will undoubtedly influence the drug resistance phenotype.

A drug to inhibit GSTπ, designated TLK199, was studied in animals. Results show that TLK199 treatment increases white blood cell levels in animals. TLK199 is designated a small-molecule myeloproliferative agent. Mechanisms were investigated and there is a connection between GSTπ and c-Jun-*N*-terminal kinase (JNK), in which the GST acts as an endogenous suppressor of kinase activity. Treatment of cells with TLK199 causes a disassociation of the protein:protein interaction with subsequent activation of the kinase cascade.

Another novel drug is TLK286, which is a GSTπ-activated prodrug. Although less is known about the mechanisms of TLK286, preliminary investigations identified DNA-dependent protein kinase as a potential target for the drug. Studies in human ovarian cancer cell lines indicate that TLK286 has significant activity in cisplatin-resistant cells, which makes this a promising drug for phase II and III clinical trials in this disease.

Discussion Session 5A

A participant asked what preclinical studies would help in understanding the relation between antioxidants and chemotherapy. To answer this question, the panel agreed that there is a need to be able to measure interference of antioxidants on oxidative stress and to identify targets of both the antioxidants and drugs (e.g., caspases and other biomarkers).

One participant noted that broccoli contains GST and other phase II enzyme inducers and asked whether this raises concerns about broccoli in the diet of patients undergoing chemotherapy. The panel agreed that broccoli consumption does not affect chemotherapy.

The panel discussed the need for clinical trials on antioxidants and chemotherapy, such as cisplatin. However, it may be difficult to determine endpoints because so little research has been conducted in this area.

Dr. Tew was asked whether there is an increase in JNK that GSTπ is trying to neutralize in cancer cells. He responded that his research did not address timing, but this may be a matter of compensation.

SESSION 5B: RADIOTHERAPY-ANTIOXIDANT INTERACTIONS

Session Chair: *Norman Coleman, Center for Cancer Research, NCI*

Antioxidants and Radiation Therapy

Carmia Borek, Tufts University School of Medicine

Carmia Borek, Ph.D., Professor of Community Health, and Director, Nutrition and Infectious Diseases, Tufts University School of Medicine, Boston, MA, reviewed background material on antioxidants and radiation therapy (20). Approximately 60% of cancer patients in the United States receive radiotherapy, mostly ionizing radiation (IR) or to a lesser extent particle beam radiation (PR). Radiotherapy is a local treatment confined to the area of affected cells and activates a variety of genes, including NfκB, thereby activating cytokines and causing inflammation. An important goal of radiotherapy is to administer enough radiation to kill tumor cells without killing adjacent normal cells. DNA is the primary target of radiotherapy; damage to DNA occurs through a direct effect but mostly (two-thirds of damage) through an indirect effect, by free radicals [superoxide, hydroxyl radical (the most toxic), and nitric oxide metabolites]. Cells are most sensitive to radiation damage in the G1-mol/L phase of the cell cycle; oxygen concentration and cyclins will modify radiation response. Irradiation of nondividing or slow-dividing cells causes apoptotic death.

Antioxidants, including vitamins, help normal cells to withstand oxidative stress and may modify tumor-cell response to radiation. Depending on the tissue and the presence and level of free radicals, specific vitamins may be of greater benefit. To illustrate, β-carotene is an effective antioxidant at low levels of pO_2, and vitamin E is more effective at high levels of pO_2. Radiation reduces tissue antioxidant levels; in animals, radiation exposure reduces vitamin E levels in cells. In other studies, bone marrow vitamin C and E levels are reduced, and in breast cancer studies, vitamin A, C, and E and selenium levels fall during cancer radiotherapy. Whether supplementing antioxidants during radiotherapy is beneficial to cancer patients or has an adverse effect is not known.

Studies of antioxidants in radiation therapy provide interesting insights into the amount of protection that is possible with supplementation. Selenium increases the number of antioxidant enzymes in normal cells but not in cancer cells. A study using ultraviolet light as the source of radiation on human cell lines indicates that functional *p53* increases, causing increased DNA repair in these cells.

Vitamin E protects cells from radiation-induced chromosome damage, reduces side effects of radiotherapy, reduces the expression of *ras* oncogenes, prevents apoptosis in normal cells by increasing *bcl2* and decreasing *bax*, and induces apoptosis in cancer cells. In the brain, vitamin E plays an important role in protecting neurons and acetylcholine receptors from free radical damage and prevents apoptosis in neural cells. In glioblastoma, vitamin E may help increase apoptosis and activates capsase-3 enzyme activity.

Cancer is generally an age-related disease, and plasma antioxidant levels decrease with age; it is important to take this into consideration during radiotherapy. Recent human experiments show that plasma proteins containing thiols are radioprotective and there is an inverse relation between plasma radioprotective ability and age.

Phytochemicals found in fruits and vegetables include many with antioxidant potential (e.g., flavonoids and carotenoids) that also have an important role in reducing oxidative stress. For example, *S*-allyl cysteine is a water-soluble compound in garlic that increases glutathione in cells and stimulates apoptosis in prostate cancer cells. A trial in England is investigating a compound in grape-seed extract that may protect against fibrosis after radiation treatment for breast cancer. Tea compounds are also a rich area of investigation in the search for agents to increase apoptosis in cancer cells.

It is important to remember that radiation does cause cancer and that vitamin E and selenium protect against radiation-induced malignancy in vitro. This may be the direction of future research because radiotherapy will remain a part of cancer therapy. The use of antioxidant combinations can help decrease damage expected from radiotherapy, especially high-dose radiotherapy.

Novel Functional Imaging for Tissue Oxygen Concentration and Redox Status

James Mitchell, Center for Cancer Research, NCI

James B. Mitchell, Ph.D., Branch Chief, Radiation Biology Branch, Center for Cancer Research, NCI, NIH, Rockville, MD, discussed noninvasive functional and molecular imaging and what they can tell us about the role of antioxidants (21). Electron paramagnetic resonance (EPR) has been used in

investigations of free radical chemistry for some time; however, new techniques are being developed with the potential for imaging electrons. Nitroxides—stable free radicals—are used as the contrast agents in EPR. Tempol and Tempol-H are the nitroxides that act as antioxidants and protect against superoxide, pO_2, and hydroperoxide-induced cytotoxicity. Investigations are under way to determine whether these agents can be used with EPR imaging.

One study investigating whether Tempol protects tumor cells, as it does normal cells during localized radiation, found no protective effect on tumor cells. Analysis of the results suggests that tumor cells reduce the nitroxide at a faster rate than normal tissue. A trial at the University of Pennsylvania is investigating whether timing is important in the topical application of nitroxide. Results indicate that nitroxide applied 15 min before radiotherapy decreases hair loss. If these results can be applied to humans, it may be possible to limit hair loss in patients undergoing full-brain radiotherapy.

Animal studies are testing the use of nitroxides in redox imaging. It appears that redox rates differ between normal and tumor cells, which leads to some interesting questions related to radiotherapy timing. It is possible to develop a redox image based on these relative redox rates. An experiment on redox rates in mouse tumors indicates that glutathione slows reduction rates, suggesting that glutathione has a role in the tissue's ability to reduce nitroxide. Because tumors are hypoxic (i.e., low in oxygen), they represent a reducing environment compared with normal tissue. To test this hypothesis, mice were placed in an atmosphere of 95% oxygen; redox maps showed that this treatment impaired the ability of the tumor to reduce oxygen.

Oxygen imaging is of interest because cancer researchers have long known that tumors create a hypoxic environment. Noninvasive low-intensity magnetic resonance (LIMR) with a free radical contrast agent is being used to develop oxygen maps. Assessments using microelectrodes show correlations between LIMR images and pO_2.

This imaging may be used to select patients with low pO_2 levels, evaluate the effectiveness of molecular targeted reagents, and see changes in tumor and normal tissue during cancer treatment. It may also be useful for angiogenesis inhibitor assessment, stroke assessment, and cardiac ischemia assessment.

Oxidative Genome Damage and the Pathogenesis of Prostate Cancer

William Nelson, Johns Hopkins University School of Medicine

William Nelson, M.D., Ph.D., Professor, Departments of Oncology, Urology, Pharmacology, Pathology, and Medicine, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, presented information on oxidative damage and prostate cancer (22). An environment of elevated ROS characterizes early pathogenesis of prostate cancer. The target cell for ROS is the prostate epithelial cell; it becomes crippled by a lack of protection from chronic oxidative damage, but the disease takes many years to develop. This makes prostate cancer a good candidate for prevention and early intervention.

All prostate cancers have many somatic genome abnormalities, although the abnormalities are heterogeneous. A common abnormality is the presence of prostate cells that have lost the ability to use the GSTπ expression system, causing hypermethylation and leading to a loss of transcriptional ability. These cells are targeted for neoplastic transformation and may have unbridled JNK signaling that gives them a growth advantage. Studies of *RNASEL*- and *MSR1*-knockout mice suggest that infection (and the resulting inflammation) may be the initiating event for prostatic lesions, although more study of familial clusters is needed to verify this.

Studies of diet and prostate cancer are equivocal on an association with fat and meat consumption. There is evidence, however, that cooking red meat at high temperatures (blackened or well done) creates heterocyclic aromatic amine carcinogens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Male mice fed PhIP have higher rates of prostate cancer; female mice have higher rates of breast cancer. Cell studies show that PhIP exposure increases formation of DNA adducts, but the addition of GSTπ1 reduces the number of adducts, detoxifying the metabolically activated *N*-hydroxy PhIP. The pathway that operates in the intact prostate may be an ATP-dependent pathway, specifically in the absence of GSTπ1.

Dr. Nelson suggested that oxidation damage in the prostate might arise from infection. Epidemiologic studies have not been totally successful in connecting inflammation from prostatitis with prostate cancer, although there are many other infections that also exist at lower levels in the prostate. Recent studies report epithelial damage in prostate epithelium, possibly caused by inflammation. For example, almost all prostate cancer cells present GSTπ1 silencing; almost 70% of samples from prostatic intraepithelial neoplasia show silenced GSTπ1. Early in the neoplastic process, proliferative inflammatory atrophy (PIA), a lesion characterized by GSTπ1 silencing, exists in ~10% of cells. The appearance of PIA cells may indicate the potential for subsequent cancer or an early stage of the disease, although more research is needed to confirm this finding.

Case-control studies on the use of selenium to prevent prostate cancer show a clear association between higher levels of selenium and reduced risk of prostate cancer. Further analysis of these studies shows that plasma levels of selenium tend to decrease with age; this parallels the time when prostate cancer rates increase. This suggests that selenium supplementation for older persons may help prevent prostate cancer.

Anti-inflammatory agents also may help prevent prostate cancer. The therapeutic target may be a COX2 enzyme, and a clinical trial to test this hypothesis is planned. The most promising targetable stage of prostate neoplastic progression appears to be in PIA, where COX2 is expressed at high levels. Another possible target is inducible nitric oxide synthase, which also is found in PIA.

Redox-Sensitive Signaling Factors and Antioxidants: How Tumor Cells Respond to Ionizing Radiation

David Gius, Center for Cancer Research, NCI

David Gius, M.D., Ph.D., Chief, Molecular Radiation Oncology Branch, Center for Cancer Research, NCI, NIH, Rockville, MD, discussed the molecular aspects of redox signaling and the role of antioxidants in this process regarding radiation exposure (23). There is a paradigm that ionizing agents induce the expression of prosurvival genes and that activation of these genes can alter phenotypes in cells. A model utilizing AP-1 DNA-binding transcriptional complex, containing a protein from the *fos* family and a protein from the *jun* family, was used to illustrate this paradigm. Various outside factors that produce oxidative stress, such as ionizing radiation, activate this complex. Hydroxyl radicals produced from water by ionizing radiation probably act as a signal that the cell has been initiated by oxidative stress.

Many signaling cascades that redirect metabolism in response to stress are thought to sense changes in cellular oxidation–reduction (redox) status through redox-sensitive thiol-

containing proteins [such as thioredoxin (TRX), Ref-1, and AP-1]. These redox-sensitive signaling proteins and downstream transcription factors might therefore play a central role in maintaining the steady-state intracellular balance between prooxidant production, antioxidant capacity, and the repair of oxidative damage. Ionizing radiation (IR) causes the formation of reactive oxygen intermediates that are thought to initiate several redox-sensitive signaling cascades in response to the damaging and cytotoxic effects of IR. Because IR appears to activate redox-sensitive signaling factors, it is logical to hypothesize that critical cysteine residues contained in thioredoxin and thioredoxin reductase (TRX/TR) might mediate these signaling pathways.

Antioxidants and oxidative stress activate proteins such as thioredoxin and thioredoxin reductase Ref-1 through modification of sulfur atoms on cysteines, primary targets for redox reactions. The critical redox-sensitive signaling proteins and their cysteines transport a signal from the cytoplasm to the nucleus to turn on the transcription factor. For example, thioredoxin interacts in the nucleus with a second signaling protein, Ref-1 (i.e., an endonuclease), a protein that has a 5′-critical cysteine that is necessary for its signaling activity. Investigations confirm this observation. There is a physical interaction between Ref-1 and the *fos* and *jun* proteins of the transcriptional complex of AP-1, and the DNA-binding activity of the complex increases, as does transcription.

Hydrogen peroxide stimulates many cytoplasmic signaling factors (e.g., *erk* families, *p38*, *ras*, and *raf*). Hence, it seems logical to determine whether activation of these factors by hydrogen peroxide and ionizing radiation is important in the response to the damaging effects of oxidative stress. To address this issue, cell lines that overexpress wild-type or cysteine mutant forms of TR were used. The mutant form of TR lacks critical *N*-terminal cysteine residues that presumably are involved with the transfer of electrons from NADPH to TRX, effectively inhibiting the ability of TR to reduce TRX. The results of the experiments with cells that overexpress wild-type TR demonstrated constitutive increases in AP-1 DNA-binding activity and reporter gene expression (relative to vector controls), with little further induction after exposure to IR. In contrast, cell lines that overexpress mutant TR showed no increase in constitutive AP-1 DNA-binding activity and reporter gene expression (relative to vector controls), as well as no induction after IR. In addition, very similar results were observed with the permanently transfected cell lines expressing the wild-type and mutant TRX genes. Interestingly, this observed increase in AP-1 DNA-binding activity is independent of increased total TRX or c-Fos and c-Jun protein levels. Finally, preliminary results suggested that TR may regulate AP-1 activity by a mechanism involving the regulation of TRX subcellular localization. The results of these experiments, combined with earlier results, strongly support the hypothesis that after exposure to IR, TR mediates an alteration in the redox state of TRX that participates in the activation of AP-1 DNA binding activity and gene expression. In addition, it appears that the critical cysteines in TR and TRX are targets for this signaling process, further suggesting a mechanism involving alternations in the redox status of these proteins.

Based on these results, it is appealing to hypothesize that TR is a signaling factor in a cascade that begins with IR-induced free radicals in the cytoplasm, then activates transcription factors in the nucleus, which, in turn, regulate downstream genes that protect the cell from the oxidative stress induced by free radicals. This raises several interesting questions regarding the mechanisms involved in cytoplasmic signaling cascades activated by H_2O_2 or IR as well as the specific factors that pass the signal from the cytoplasm to the nucleus. The results of these studies identify the cysteine residues located in the *N*-terminal regions of TR and TRX as critical for IR-induced activation of AP-1 activity. Thus, it would appear that these critical cysteine residues are targets for the passage of redox-sensitive cellular signals to transcription factors in response to stress. In this model, subtle changes in cellular redox potential induced by a stressing agent could alter the flow of electrons through the cysteine residues of TR and TRX, causing profound changes in protein activity. These critical cysteines would appear to act as redox-sensitive "sulfhydryl switches" that reversibly modulate protein activity and allow signal transduction cascades to redirect metabolism in response to radiation-induced stress using redox-sensitive transcription factors.

To summarize the model, hydrogen peroxide and ionizing radiation produce free radicals; the NADP level is altered in cytoplasm and mitochondria (not proven); thioredoxin reductase is activated and passes the signal on to thioredoxin, which is transported into the nucleus; thioredoxin forms a physical interaction with REF1; REF1 passes the signal to the AP-1 transcriptional complex, which is composed of *fos* and *jun*, each of which have critical cysteine in the DNA-binding domain; and DNA-binding activity increases.

Discussion Session 5B

Dr. Coleman asked whether gene transcription ends once oxidative stress is no longer present. Dr. Guis responded that over time, thioredoxin, for example, goes from a reduced to an oxidized state and is pumped back out of the nucleus into the cytoplasm, thus ending transcription.

A participant commented that many people are confused because thioredoxin and thioredoxin reductase contain antioxidant-response elements in their regulatory region, and asked about the mechanism for balance between AP-1 binding and NERF2 binding. Dr. Guis said he is not sure of this mechanism, although these questions are being investigated.

A participant commented that hydrogen peroxide, in some cells, activates the JNK and P38 pathways and asked whether *jun* is phosphorylated by some of the more conventional pathways upstream leading to AP-1 activation. Dr. Guis responded that many pathways are activated, and it is hard to know what percentage of each pathway is activated, although in his investigations he did not find increased phosphorylation in *jun*. This may be unique to the experiments being conducted in cervical cells, which are constituitively phosphorylated at a higher rate than normal cells.

Another participant asked whether radiation induces glutathylation of cysteines on either thioredoxin or RAF1. Dr. Guis responded that this is being pursued, but there are no results yet.

A participant commented that irradiated fibroblasts turn off ROS for a long period of time and asked whether anything is known about potential postexposure interventions that can counter tissue damage. Dr. Nelson responded that research is looking at the possibility that a single photon can cause irradiation damage; if so, this may lead to a clearer understanding of this issue. Dr. Mitchell responded that sublethal radiation damage can occur through constitutive enzymatic processes, and there may be no reason to interfere in these processes. Dr. Guis added that this might be due to posttranslational modification of signaling proteins.

A participant asked the panel what clinicians should do when patients say they are taking antioxidants before radiotherapy. Dr. Coleman responded that because of the lack of

data, it is prudent to recommend that the patient stop taking antioxidants. Dr. Milner added that this conundrum is the issue that has led to this meeting and that the NCI is looking at this issue.

Many participants stated that there is a need to develop models and asked which models are most appropriate to test the hypothesis that antioxidant supplementation either accentuates or inhibits the efficacy of radiotherapy. Dr. Mitchell responded that an animal model might be useful to see whether fractioned radiation therapy with supplement use affects tumor growth and tumor cure rates. Most people in radiation research agree that mouse and rat tumor models are not reflective of human tumor models with regard to radiation response. A xenograft human tumor-cell model should be considered.

SESSION 6: WRAP-UP, SUMMARY, OPPORTUNITIES, AND CHALLENGES

Session Chair: *John Milner, DCP, NCI*
Panel Members: *Steven Clinton, Steven Zeisel, Richard Rivlin, David Rosenthal, Susan Mayne, and William Nelson*

Dr. Milner began the session by restating the purpose of the meeting. The main purpose was to assist clinicians and researchers regarding recommendations on diet, including the use of dietary supplements, for minimizing the risk of cancer through prevention efforts and by working in concert with other types of therapy. It is clear that interindividual variability is a large issue, as is the definition of *antioxidant*. The NIH needs suggestions on how to move forward on these issues. He also noted that Dr. von Eschenbach, the new director of NCI, has requested research on discovery, development, and delivery in all areas of cancer research

Dr. Rosenthal said that, as a clinician, it is difficult to make any recommendation to a patient because the answers are not clearly known. There is a need to assimilate the available information before clinical trials are carried out. Standard measurements of intake must also be done first, which may involve the use of biomarkers.

Dr. Zeisel said that oxidants and antioxidants act in many ways, via a series of signaling pathways, as well as by causing a series of structural effects (e.g., damage to proteins, lipids, and DNA). He suggested that cell culture methods might be a place to gain information to use in the development of model systems before human trials are planned. The NIH should invest in a model system with outcome markers for each of the important signaling pathways and functional outcomes (e.g., apoptosis, necrosis, and structural damage) so that they can be measured accurately. Once the model system is implemented, dose and combinations of antioxidants can be investigated.

Dr. Clinton commented that there is a lot of information already known, although clinicians do not have the guidance necessary to make the decisions they must make. An investment in translational animal studies seems necessary to determine the outcome of antioxidant use.

Dr. Mayne reaffirmed the comments made by Dr. Clinton but added that there is a need to determine what antioxidants people are taking through data collection. In addition, there is a need to validate biomarkers and collect them for populations through observational studies.

Dr. Rivlin said that there are many challenges for scientists (e.g., understanding the mechanisms and basic science), clinicians (e.g., letting patients know how complex the issues are), and consumers who want good information.

Dr. Rosenthal added that looking at the past should be a lesson in how we could move forward in the future. The pharmaceutical industry, in the current environment, has no incentive to do any specific research in the antioxidant arena. Because natural products are not patentable in the usual sense, it is all the more important for the NCI to step in to fund development of infrastructure to conduct research and trials in order to answer many of these questions. This will give some direction as to what the industry is allowed to do and say regarding health claims it would like to make for dietary supplements.

Dr. Milner informed participants that a U54 mechanism initiative (collaborative research) will be issued soon that will ask for research applications from those interested in investigating the nutritional modulation of genes associated with cancer. There will be $28 million in funding for this collaborative research, which could be used to investigate free radicals. In addition, there is existing RO3 and R21 funding that can be used for this research.

Key comments made by participants during this session included the following:

- It is difficult to make the leap from experimental studies to human clinical trials because studies in cell lines show very different responses to antioxidant exposure with regard to gene expression as opposed to responses in animals or in situ tumors in humans.
- It may be hard to recruit eligible patients to clinical trials of antioxidant use because many people are already taking antioxidants. For example, at least one-half of the participants in the PCPT trial were already taking selenium and vitamin E, and many would not agree to stop taking them to participate in the SELECT trial.
- If it is advisable to discontinue antioxidants before radiotherapy, is it not logical that we also tell patients to reduce their intake of fruits and vegetables that contain antioxidants? It was stated that this comment was only meant to highlight the lack of information regarding antioxidants and radiotherapy or chemotherapy. In response, Dr. Mayne commented that the bioavailability of β-carotene in supplements is 10 to 20 times that of β-carotene normally found in food, implying that one must also consider the dose.
- There are antioxidant enzymes that are highly efficient at removing ROS, such as superoxide and hydrogen peroxide. These are catalytic reactions to antioxidants. It may be important to understand the effects of chemotherapy and radiotherapy on antioxidant enzymes (as well as phase I and phase II enzymes), which may play a more important role in reducing oxidative stress than antioxidant compounds

Recommendations

Recommendations were developed from comments made by the panel members, speakers and from the audience during the comment period.

- There is a critical need to use an evidence-based approach for summarizing data, drawing conclusions, and making recommendations about antioxidant usage and efficacy. An advisory panel approach may need to be developed to assist with this process while research continues.
- This evidence-based approach should focus on the need to answer the myriad questions surrounding pro- and antioxidative mechanisms of action associated with antioxidant use, and what effects dose and environment have on these mechanisms before recommending nutritional or nutritional-pharmacologic interventions.

- There is a need for additional short- and longer-term clinical trials (both small interventions with specific agents as well as larger placebo-controlled double-blind studies) that build on the substantial in vitro and preclinical data.
- Biomarkers, including biomarkers of oxidative stress and damage as well as markers of exposure and consumption, must be identified and validated.
- There is a need to study the relation of genetic variants to specific cancers and develop in vitro models systems to study them.
- It is important to evaluate molecular targets and signals to explain what happens in the cell and why the same signaling pathway can be beneficial in one instance of oxidative stress and harmful in another.
- A central database will allow easier comparison of research results related to antioxidants and help with data-mining activities and the design of probing studies.
- Among those engaged in antioxidant research, there is a need to investigate, develop, and cultivate collaborations with existing NCI programs, such as the Early Detection Research Network and the Mouse Models for Human Cancers Consortium, as well as other institutes and industrial research organizations.

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