NUTRITIONAL GENOMICS AND PROTEOMICS
IN CANCER PREVENTION
CONFERENCE

September 5-6, 2002
Masur Auditorium
NIH Campus
Bethesda, Maryland

The Nutritional Genomics and Proteomics in Cancer Prevention Conference was sponsored by the Center for Cancer Research, National Cancer Institute (NCI); the Division of Cancer Prevention (DCP), NCI; the National Center for Complementary and Alternative Medicine, National Institutes of Health (NIH); the Office of Dietary Supplements, NIH; and the Office of Rare Diseases, NIH. The conference, which brought together expert scientists from various relevant disciplines, was organized by Young S. Kim, Ph.D., Program Director, Nutritional Science Research Group, DCP, NCI.

Session I: Opening Remarks and Welcome: Young S. Kim, Alan S. Rabson, Peter Greenwald, and John A. Milner

The conference was opened by Dr. Kim at 8:20 a.m. on September 5. She welcomed participants to the conference and thanked them for attending. She acknowledged the support provided by the conference sponsors and thanked the Conference Planning Committee for its contributions. Dr. Kim explained that this conference would highlight molecular mechanisms in nutritional genomics and proteomics to enhance understanding of nutritional constituents in cancer biology, and to foster free exchange of insight, ideas, and knowledge in this important research area.

Alan S. Rabson, M.D., Deputy Director of the NCI, emphasized that Dr. Andrew von Eschenbach, the new Director of the NCI, is very interested in this conference. He said that the NIH originally had two areas of interestCinfectious diseases and nutrition in health. Although NIH research moved away from nutrition, there is now renewed interest in nutritional science research, which is recognized as an important component of a health-related research program.

Peter Greenwald, M.D., Dr.P.H., Director of DCP, NCI, explained that nutritional science can be viewed as three overlapping research areasCbasic research, epidemiology, and eating behaviorCand that epidemiology is responsible for bringing the field of nutritional science to the forefront. Expanded efforts are needed in basic research in nutritional science and in the conduct of small clinical-metabolic intervention studies to build depth in these areas. Dr. Greenwald stated that it is necessary to take advantage of the new technologies and to integrate research in nutritional sciences with research in molecular biology. He affirmed that the outcome of this conference will help to guide future research efforts.

John A. Milner, Ph.D., Chief of the Nutritional Science Research Group, said that this conference would be exciting and stimulating. He commented that it appears that all dietary changes are not appropriate for all people. Diet and genetic expression influence each other, although their interactions are not yet understood. A need exists to identify the molecular targets of dietary constituents. Dr. Milner stated that a need also exists to identify people who will benefit most from dietary intervention strategies and suggested that future strategies for cancer prevention might move toward a tailored medical intervention approach for individuals, as opposed to a public health approach.

Session II: Setting the Stage (Moderator: Young S. Kim)

Diet, Individual Responsiveness, and Cancer Prevention: Michael J. Wargovich

Michael J. Wargovich, Ph.D., F.A.C.N., Director for Basic Research and Professor in the Department of Pathology at the University of South Carolina School of Medicine, presented general information on diet and cancer, emphasized some questions associated with the responses of individuals to dietary interventions, highlighted the relationship between inflammation and cancer risk, and introduced the “thrifty gene hypothesis.” Risks for various types of cancer vary around the world. For example, regions with some of the highest cancer incidence include: United States, colon; Canada, leukemia; United Kingdom, lung; Brazil, cervix; China, liver; Japan, stomach; Australia, skin. Studies of people who migrated between countries were among the first studies to suggest that changes in diet influenced cancer risk. Diet may account for 10-70 percent of cancers; it is a source of compounds that may prevent cancer, as well as compounds that may cause cancer. In the 1980s, based on epidemiologic and animal studies, Americans were advised to eat less fat, eat more fiber, eat more vegetables and fruit, modify (reduce) alcohol intake, and use salt moderately. The 5 A Day Program, which recommends eating a total of five or more vegetables and fruits daily, has increased public awareness of the desirability of high vegetable and fruit consumption. Many Americans, however, continue to consume a high-calorie, unbalanced diet and, in 1999, an estimated 66 percent of U.S. adults were overweight (BMI >25) and 27 percent were obese (BMI>30).

Several large dietary intervention studies have been conducted that tested hypotheses stemming from epidemiologic data. These included the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study, the Beta-Carotene and Retinol Efficacy Trial (CARET), the Arizona Wheat Bran Fiber Trial, and the Polyp Prevention Trial (PPT). None of these trials found a benefit. Unexpectedly, in both the ATBC Study and CARET, supplementation with ∃-carotene increased the incidence of lung cancer in the high-risk study populations (smokers). Neither the Arizona Trial (high fiber, from wheat bran) nor the PPT (low fat, high fiber, high vegetables/fruits) found any effect on adenomatous colon polyp recurrence in the study population.

Dr. Wargovich pointed out that studies at the Apopulation@ level may not hold for particular individuals. For example, individual polymorphisms in genes that code for metabolic enzymes (e.g., cytochome P450s, glutathione-*S*-transferases, *N*-acetyltransferases) likely also play a role in determining a person's cancer risk. To illustrate, the risk for colon cancer can be increased by polymorphisms in the genes *MTHFR* (folate metabolism) and *NAT1*, *NAT2* (metabolism of heterocyclic aromatic amines in meats cooked at high temperatures). Dr. Wargovich presented an overview slide that indicated genetic polymorphisms related to metabolism also have been reported to influence the risk of lung, breast, gastric, bladder, and cervical cancers. He explained that, overall, individual responsiveness to the effects of dietary components on cancer risk may be influenced by genes governing metabolic activation and detoxification pathways; DNA repair capacity accounting for susceptibility to different kinds of dietary mutagens; oxidative status (i.e., presence of adequate antioxidants); and confounding variables such as smoking or the presence of other disease.

Dr. Wargovich proposed a number of possible targets for genomic/proteomic research in diet and nutrition, including the pathways associated with signal transduction, inflammation, apoptosis, DNA repair, folate/vitamin D effects, and metabolism of hormones and phytohormones. Using inflammation as an example, he highlighted its possible relationship to cancer risk as well as the use of anti-inflammatory compounds, including dietary constituents, that may reduce cancer risk. Dr. Wargovich showed the pathway for the biosynthesis of prostanoids (e.g., prostaglandins, thromboxanes, leukotrienes) from arachidonic acid (AA). Prostanoids have been linked to inflammation, angiogenesis, cytoprotection, and renal function, and may play a role in carcinogenesis. He described the characteristics of cyclooxygenase 1(COX-1) and cyclooxygenase 2 (COX-2), which are the enzymes that convert AA to prostaglandins and thromboxanes. COX-2 is not expressed in normal tissue; however, COX-2 expression is common in colon, breast, and other epithelial tumors. Cells that overexpress COX-2 have altered adhesion properties and resist apoptosis. Dr. Wargovich showed the multistep pathway to colon cancer and the points at which chemopreventive agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) might affect the process, possibly in part because they inhibit COX-2. In epidemiologic studies, habitual use of aspirin (an NSAID) has been linked to reduced colon cancer risk. Numerous edible plant products contain phytochemicals that have anti-inflammatory properties. Examples include ginger, ginseng, grape seed extract, green tea, licorice, and milk thistle. Green tea has been associated with reduced risk for some cancers, and animal data suggest that tea may inhibit skin, lung, and colon cancers. Tea may affect the cell signaling pathways.

Dr. Wargovich posed the question, "What are the biological consequences of the switch from cycles of feast and famine to constant "feasting," accompanied by the disruption of diurnal/seasonal rhythms (resulting from availability of electricity)?" He then presented information related to the "thrifty gene hypothesis." This hypothesis asserts that the human genome still contains late Paleolithic hunter-gatherer genes that result in more calories being converted to adipose tissue during "feasting" to help ensure survival during "famine." During

the last 100 years, food in the developed nations has become plentiful and affordable. Thus, people are in a state of constant "feasting," and, according to the hypothesis, molecular pathways leading to adipose tissue remain "on," resulting in increased obesity, heart disease, Type 2 diabetes, and increased risk for breast, colon, prostate, and pancreatic cancers.

Dr. Wargovich suggested the following future directions for research:

- Develop methods for rapid assessment of polymorphisms linking diet and cancer risk.
- Explore the effects of individual and combinations of dietary elements that might influence molecular pathways of cancer prevention.
- Determine the feasibility of genomic/proteomic profiling to construct dietary recommendations pertinent to risk for cancer and other diseases.
- Explore the ramifications of changes in diurnal rhythms and the molecular consequences of "feast versus famine."

The Use of Genetically Altered Mice for Nutrition Studies: Jeffrey E. Green

Jeffrey E. Green, M.D., Head of the Transgenic Oncogenesis Group in the Laboratory of Cell Regulation, Center for Cancer Research, NCI, discussed the selection of genetically engineered mouse models for studies in cancer prevention, highlighted chemoprevention research conducted in C3(1)/Tag transgenic mice (a model for breast cancer), and presented information relevant to a genomic "systems biology approach" for identifying potential molecular targets for chemoprevention. Dr. Green pointed out that in people, environmental influences and genetic predispositions result in genetic alterations that lead to cancer. In genetically engineered mice, however, the genetic alterations are introduced into the mice. When selecting an animal model, it is very important to assess whether the model is a good representation of what is happening in the human disease. Researchers must compare the biology, pathology, detection/prediction, and genomic alterations of the disease in the human versus the animal model. Analysis of genomic alterations in mouse models indicates cancer can develop by different paths, depending on whether an oncogene is overexpressed or a tumor suppressor gene is lost. Of these two possibilities, it appears that loss of suppressor genes (i.e., p53, BRCA) results in genomic alterations in mouse tumors more similar to human tumors. Dr. Green emphasized that the specific research question and the molecular targets associated with that question determines the most appropriate mouse model to use for an experiment. He used an illustration of a cell to highlight some of the more important chemoprevention targets. Specific pathways targeted by chemopreventive agents, including nutrients, are: growth factors/receptors (vitamin D, insulin-dependent growth factor [IGF]; cell cycle (genestin, selenium); apoptosis (genestin, selenium, sodium butyrate); differentiation (retinoic acid [RA], dehydroepiandrosterone [DHEA]); cyclooxygenase (COX-2 inhibitors, selenium); angiogenesis (endostatin, 2-methoxyestradiol [2-ME]); and metabolism of carcinogens and antioxidants (selenium, difluoromethylornithine

[DFMO]). An important advantage of animal models is that they can be used to study stage-specific molecular events that occur throughout the carcinogenic process.

Dr. Green presented the results of chemoprevention experiments conducted using C3(1)/Tag transgenic mice. These mice exhibit T-antigen (Tag)-induced changes in cell cycle regulators, resulting from inactivation of tumor suppressor genes (p53, Rb). Mammary lesion progression in this model mimics that observed in humans. However, loss of estrogen receptor (ER)- occurs fairly early in this (and other) models, a disadvantage if studying hormone responsiveness is of interest. Specific results include the following:

- 9-cis RA significantly decreased the number of tumors developed per animal (3.43 vs. 1.13). A more selective retinoid X receptor (RXR) also suppressed tumorigenesis. Both 9-cis RA and the RXR inhibit differentiation.
- DHEA modestly inhibited the incidence of tumorigenesis (-15 percent). However, the number of tumors per animal decreased approximately 50 percent. DHEA inhibits differentiation.
- COX-2 expression increases in early-stage disease in this model and persists through invasive carcinoma, mimicking human breast cancers. COX-2 inhibitors (e.g., celecoxib, indomethacin) had a moderate effect on tumor multiplicity.
- DFMO is an irreversible inhibitor of ornithine decarboxylase (ODC), a compound important in tumor promotion. DFMO only modestly decreased tumor incidence, but decreased tumor multiplicity by approximately 50 percent.
- Endostatin, an antiangiogenic agent, demonstrated stage-specific inhibition of progression from the preinvasive lesion to invasive carcinoma. Recombinant murine endostatin, recombinant mutated human endostatin, and adenoviral delivery of endostatin ("gene therapy") were effective stage-specific inhibitors.
- Another antiangiogenic agent, 2-ME, which is a metabolite along the estrogen hormone pathway, significantly inhibited both tumor number and tumor volume.
- Vascular epithelial growth factor (VEGF) is proangiogenic. Compounds that inhibit the VEGF receptor through phosphorylation have been effective in decreasing tumor multiplicity and in extending survival.
- Preliminary data suggest that lovastatin may inhibit the development of early lesions. (Note: All of the other compounds tested and mentioned above appear to affect later stages of tumorigenesis).

Dr. Green referred to the "treasure chest" of genomics and indicated that a genomic "systems biology approach" can be helpful in trying to understand the simultaneous multiple pathways involved in carcinogenesis. He discussed research using cDNA microarrays that was aimed at examining how different initiating oncogenic events affect the gene expression profiles in various mammary tumor models. Approximately 900 genes (-8 percent of the total genes), were differentially regulated among the models; these genes were considered to be the "tumor signature genes." The function of more than 57 percent of these genes is unknown. The tumor

signature genes clustered into three separate groups, based on whether the initiating oncogenic event involved myc, Tag, or Her2/neu and ras. The Tag group showed the highest number of tumor signature genes; a number of them are involved in calcium binding and signaling pathways. Dr. Green said that data from this research has shown that the IGF pathway, calcium-regulated pathways, and pathways involving selenoproteins may be important therapeutic targets in these models. He mentioned that new research is using laser capture microdissection to study stage-specific gene expression changes.

In summary, Dr. Green stated that he believes genetically engineered mice are valuable tools for studying particular chemopreventive compounds and pathways, but that pathways need to be validated in the various models. He emphasized that nutritional intervention or chemoprevention may be stage-specific and that all stages of carcinogenesis might be affected, from initiation through invasion and metastasis. Furthermore, Dr. Green suggested that, rather than using single compounds in chemoprevention, it is time to combine compounds that may inhibit different pathways involved in tumor progression, as is done in chemotherapy. He added that gene expression profiling is helping to identify new targets and may suggest new models for translational research in nutrition and cancer prevention.

Application of Gene Expression Profiling to Colon Cell Maturation, Transformation, and Chemoprevention: Leonard H. Augenlicht

Leonard H. Augenlicht, Ph.D., Professor, Medical and Cell Biology, and Director, Molecular Oncology, at the Albert Einstein Cancer Center, provided some historical information on gene expression profiling, followed by an overview of several gene expression profiling projects conducted in his laboratory, including research on the butyrate "paradox," sulindac chemoprevention, absorptive cell differentiation, goblet versus absorptive cell lineages, cell sensitivity to chemotherapy, novel mechanisms of *c-myc* regulation by nutritional factors, and dietary/genetic modulation of tumor formation. He stated that his laboratory first performed gene expression profiling in the early 1980s. In 1984, collaborating with Argon National Laboratories, they developed the first computerized scanning and image-processing systems. In 1991, they published the first example of using gene expression profiling in a clinical setting; gene expression in colonic mucosa clearly differed for patients at high risk versus low risk for colon cancer.

Dr. Augenlicht explained that the "butyrate paradox" refers to the fact that butyrate promotes the growth of normal intestinal epithelial cells, but promotes the death (apoptosis and cell cycle arrest) of transformed epithelial cells. Data showed that, in the colon cancer cell line HCT116, a *ras* mutant allele renders these cells highly sensitive to and is necessary for butyrate-induced apoptosis. Gene expression profiling in this system found that the gene for gelsolinCa binding protein that has a role in regulating the response to apoptotic stimuliCwas downregulated in the cells that contain the *ras* mutation (butyrate-sensitive cells). Further research using a retroviral

system to modulate gelsolin expression confirmed that butyrate responsiveness increases when gelsolin is downregulated.

In chemoprevention research using sulindac, “before and after” gene expression profiles were developed for the rectal mucosa of three subjects who received sulindac for 1 month. Dr. Augenlicht reported that, by comparing databases, they found that only 0.1 percent of the genetic sequences changed the same way in the subjects and *in vitro*. Expression of *p21*, a cell cycling dependent kinase inhibitor, was induced. They investigated this finding further in a mouse model that was a cross between an APC mouse (which had an initiating mutation for intestinal tumorigenesis) and a *p21* knockout mouse. Adding sulindac to the diet of *p21* wildtype mice resulted in inhibition of tumor formation. However, the loss of even one *p21* allele in the model resulted in loss of responsiveness to sulindac. Later data showed that the model contained only one *p21* allele, explaining why the loss of a single allele eliminated responsiveness.

Next, Dr. Augenlicht focused on absorptive cell differentiation in the Caco-2 colon cancer cell model. In this model, the cells undergo a G0/G1 arrest and a consequent decrease in proliferation. The cells also undergo absorptive cell differentiation. Research showed that the promoters for the genes involved in spontaneous Caco-2 differentiation showed increased activity during differentiation. Data indicated that downregulation of ∃-catenin-TCF signalling was partly responsible for the activation. Array gene expression analysis by a method called “functional group analysis” was used to further explore the complex process of differentiation (Mariadason, et al., *Cancer Res* 2002;62:4791-4804). This approach divides the genes in the data set into different functional groups and facilitates interpretation of changes in gene expression. Dr. Augenlicht showed a slide that summarized the data and pointed out the various functional groups, including cell cycle, DNA synthesis and repair, absorptive cell or membrane transport, and nucleotide metabolism. He commented that sometimes it is difficult to interpret the data biologically, because some genes in a group may be upregulated, and others may be downregulated. For example, this occurs in genes involved in protein synthesis, drug metabolism, and cell matrix interaction.

Other research in Dr. Augenlicht’s laboratory compared gene expression profiles for two cell lines developed in Paris about a decade ago that differentiate along a goblet cell lineage and an absorptive cell lineage. Gene expression in differentiation in these goblet cells is of interest, because early aberrant foci in the intestinal mucosa are decreased in this particular lineage. Using a knockout mouse model that lacked the *muc-2* gene (no mucin-B secretion, no goblet cell development), data showed that the mice spontaneously developed invasive adenocarcinomas in the duodenum, colon, and rectum (first example of rectal tumor mouse model). Although these mice did not synthesize mucin, they did express other markers of the goblet cell phenotype. Thus, it is unclear to what extent the goblet cell lineage is really perturbed in this model and to what extent the mucin alone is important to tumor development. Gene expression profiling that compared goblet cell differentiation to absorptive cell differentiation found that the qualitative changes in gene expression were very similar for these two types of cells, but there were

quantitative differences. Dr. Augenlight proposed that there is a quantitative element to the change in gene expression that leads to one phenotype versus another phenotype.

Dr. Augenlicht discussed the use of gene expression profiling to identify patients who do not respond to chemotherapy. His laboratory has developed extensive gene expression databases for 30 colon carcinoma cell lines that have been characterized in terms of their response to the chemotherapy agent, 5-fluorouracil (5-FU). Gene expression data is log transformed and then analyzed using the "jack-knife" approach. This approach entails performing iterative analyses on data for the 30 cell lines, selecting the N "best" genes that show correlation with phenotype and extracting the principal componentsCthat is, the 10 principal components that represent 70 percent of the variance for the N genes. Then, predictor values are determined and a gene list is developed that shows how frequently the genes are represented among the iterative comparisons for the 30 cell lines that are initially completed in the jack-knife approach. Dr. Augenlicht showed an example of a gene list developed in this manner. He said that arithmetical experiments in his laboratory have determined an "N" of approximately 50 results in the best correlational analysis. Current research is investigating the functions of the selected genes.

Dr. Augenlicht presented information on novel mechanisms by which nutritional factors can regulate *c-myc*. He began by pointing out that there is no "one" cell cycle pathway. Both butyrate and sulindac give a G0/G1 cell cycle arrest that appears similar in terms of cell biology. However, butyrate and sulindac show different patterns of changes in cell cycle gene expression. For example, *c-myc* is repressed by butyrate, but induced by sulindac. By using a new method of transcriptional imaging, it was found that both butyrate and sulindac increase the number of *c-myc* transcriptional sites, but butyrate also causes a transcriptional block, thereby repressing *c-myc*. Sulindac does not cause a transcriptional block; thus, *c-myc* is induced. Dr. Augenlicht noted that transcriptional imaging is an important methodology because it allows researchers to investigate transcription sites for multiple genes simultaneously to help determine how nutritional agents like butyrate can regulate target genes. He stated that this technique is an important complement to gene expression profiling.

To conclude his presentation, Dr. Augenlicht described a project using four mouse models (wild-type, APC+/-, Msh2 Mut, Muc2-/-) that investigates genetic/dietary interactions in the development of colorectal cancer. Animals are fed either the standard control AIN-76A diet or a Western diet, which is high in fat and phosphate, and low in calcium, vitamin D, choline, methionine, and fiber. To date, studies have only been done with wild-type mice. These mice develop tumors on the Western diet after approximately 1 year; tumor formation can be reversed by adding calcium to the Western diet. Gene expression analysis for control mice versus test mice, using a 27,000-member cDNA microarray, showed a one-fold difference in expression for 1,828 genes, a two-fold difference for 415 genes, and a three-fold difference for 43 genes. The gene expression pattern for test mice moved back towards the control when calcium and vitamin D were added back to the Western diet. Dr. Augenlicht added that a huge amount of research

will need to be done to determine how the genetic components interact with the dietary components.

Session III: Nutritional Genomics in Cancer Processes

A. Apoptosis/Cell Cycle (Moderator: Jeffrey E. Green)

Oncogenic Transformation as a Cause of Apoptosis: Yuri Lazebnik

Yuri Lazebnik, Ph.D., from Cold Spring Harbor Laboratory, provided some background information on apoptosis and then presented the results of research in his laboratory aimed at elucidating the mechanisms involved in apoptosis, to help determine how apoptosis can be induced selectively in cancer cells, while normal cells remain intact. He explained that the general concept of apoptosis was derived from two simple observations: (1) a dead cell is often surrounded by a vast number of normal cells, indicating that cell death is an "inside job," the result of endogenous cell machinery; and (2) cells from a variety of tissues and species all look similar when dead, indicating that the machinery is the same across cell types. He said that research during the past 5 years has revealed that apoptosis is executed by caspases, a family of proteases that disassemble a cell. The caspases are activated through various pathways, depending on the specific cytotoxic stimuli. For example, in one proposed model, DNA damage initiates signaling pathways that activate the Bcl-2 family of proteins; this can result in mitochondrial permeabilization and, subsequently, in cytochrome C being released from the mitochondria. Cytochrome C, in a complex with the cytoplasmic protein Apaf-1, activates caspase-9, which in turn activates caspase-3, the caspase that cleaves the majority of caspase substrates during apoptosis. Thus, mitochondrial permeabilization is required for initial caspase activation. Dr. Lazebnik said that the drug etoposide, which causes DNA damage by inhibiting topoisomerase, induces apoptosis in cancer cells. He emphasized that such drugs can adversely affect normal cells through the same mechanisms. Dr. Lazebnik added that, in another model, caspases can be activated by cytokines; the cytokines assemble receptor complexes that activate caspases directly. In this case, the subsequent mitochondrial permeabilization accelerates apoptosis by amplifying caspase activity; thus, mitochondrial permeabilization is not required for initial caspase activation.

Dr. Lazebnik reported results from studies of oncogene-dependent apoptosis, conducted to find clues as to how to kill cancer cells selectively. Comparing normal human fibroblasts with fibroblasts transformed with the adenoviral oncogene *E1A* determined that *E1A* sensitized cells to cytotoxic drugs by promoting activation of Bax (a proapoptotic protein), release of cytochrome C, and the subsequent activation of caspase-9. This data fit the current view that cytotoxic stress induces apoptosis through regulation of mitochondrial permeability (in which caspases are activated *after* mitochondrial permeabilization).

Dr. Lazebnik next presented research results (*Science* 2002;297:1,352-1,354) that seem to be contrary to the current view. Findings indicated that cytotoxic stress caused activation of caspase-2, a caspase that has been implicated in apoptosis, but whose exact structure and function remain unknown. This caspase, however, appeared to be activated early in the pathway that links DNA damage and apoptosis (*before* mitochondrial permeabilization) and to be required for mitochondrial permeabilization. The research approach involved using small interfering RNA (siRNA) to silence the expression of various proteins by RNA interference (RNAi) in human fibroblasts transformed with the adenoviral oncogene *E1A*. Initial data indicated that either caspase-2 was required for apoptosis or the caspase-2 siRNA interfered with the expression of other proteins. To test whether apoptosis actually did require caspase-2, efforts were made to restore sensitivity to cytotoxic agents by expressing caspase-2 ectopically. To prevent destruction of the ectopic caspase-2 mRNA by the caspase-2 siRNA, two silent mutations were introduced into the region of the caspase-2 cDNA that is complementary to the caspase-2 siRNA. Data showed that the ectopically expressed caspase-2 restored the sensitivity of cells to etoposide, indicating that the effect of the caspase-2 siRNA could be explained by inhibition of caspase-2, rather than inhibition of other proteins. Further data demonstrated that when cells lacking caspase-2 were exposed to etoposide, cytochrome c remained in the mitochondria. In addition, data suggested that caspase-2 was activated before or independently of caspases 9, 3, and 7. The last two observations indicated that caspase-2 was required to permeabilize mitochondria in these cells. Dr. Labeznick stated that results of this research imply that both stress-induced and cytokine-induced apoptosis can be executed through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation. He noted that more research is needed to investigate what factors mediate activation of caspase-2.

Dr. Lazebnik speculated as to whether the energy of cancer cells could be rerouted, using some type of experimental system, to learn how that energy could be used to destroy the cancer cells. He compared the cell to a transistor radio that has about 100 components and converts electromagnetic waves to sound waves, suggesting that the radio works as a signal transduction pathway. Dr. Lazebnik added that his logic using this radio metaphor to describe the current approach to understanding signal transduction pathways (including apoptosis) is presented in a letter he wrote (*Cancer Cell* 2002;2:179-182). In the letter, he concluded that, unless experimental biologists adopt a more formal, systematic approach to research and share a common formal language, significant progress in elucidating biological processes (including apoptosis) will not be possible.

Prostate Cancer Chemoprevention by Green Tea (Cell Cycle Dysregulation, Induction of Apoptosis, and Inhibition of Metalloproteases): Hasan Mukhtar

Hasan Mukhtar, Ph.D., Evan P. Helfaer Professor and Director of Research, Division of Dermatology, at the University of Wisconsin Medical Science Center, defined cancer

chemoprevention as the use of natural or synthetic agents to reverse, block, or suppress the process of carcinogenesis, and defined its goals as regulation of growth and differentiation (cellular level), reversal of premalignant lesions (tissue level), and reduction of cancer development (clinical level). Dr. Mukhtar emphasized that diet is a “mixed bag” that contains both carcinogens and anticarcinogens, and it is difficult to identify the active components. Much research has been conducted on the effects of green tea on cancer risk. In animal studies, green tea has been reported to reduce risk for cancers of the skin, lung, stomach, esophagus, duodenum, liver, pancreas, breast, colon, and prostate. Dr. Mukhtar focused his presentation on green tea and prostate cancer. Prostate cancer incidence in China, where tea is consumed daily, is the lowest in the world. Further, two epidemiologic studies indicate that people who regularly consume green tea have reduced prostate cancer risk. The chemopreventive effects of green tea are primarily a result of its major polyphenolic constituent, (-) epigallocatechin-3-gallate (EGCG). *In vitro* data have shown that EGCG treatment of human prostate cancer cells (DU145) results in cell cycle disregulation and induction of apoptosis.

Dr. Mukhtar presented the results of research conducted in his laboratory (Gupta, et al., *Proc Natl Acad Sci* USA 2001;98:10,350-10,355) using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, in which mice develop prostate cancer spontaneously at puberty. Between 8 and 32 weeks of age, the test group of mice was fed GTP (a decaffeinated, polyphenolic fraction isolated from green tea, 0.1 percent in drinking water); the control group was fed plain water. The GTP dose was equivalent to a human dose of six cups of green tea daily. Compared with controls, the GTP-fed mice had lower IGF-1 levels, higher insulin-dependent growth factor binding protein (IGFBP)-3 levels, significantly lower prostate and genitourinary weight, significantly longer tumor-free survival (median survival of approximately 40 vs. 20 weeks), significantly longer overall survival (median survival of approximately 68 vs. 42 weeks), and significantly greater apoptosis of prostate cancer cells. Furthermore, in terms of molecular targets, feeding GTP at this dose to TRAMP mice significantly inhibited the expression of matrix metalloproteases (MMP-2 and MMP-9), the ratio between MMP and the tissue inhibitors of matrix metalloproteases (TIMPs), urokinase plasminogen activator (uPA), and vascular endothelial growth factor (VEGF).

In other research that identifies molecular targets for prostate cancer prevention by green tea, Dr. Mukhtar and colleagues used a cDNA microarray approach. They found that *in vitro* EGCG treatment of human prostate cancer cells (LnCaP) induced a subset of genes that functionally exhibit inhibitory effects on cell growth (e.g., tyrosine receptor kinase type E). Many of the genes repressed by ECGC belong to the G-protein signaling network (e.g., protein kinase C-∀). Overall, data suggest that multiple targets exist for green tea. Dr. Mukhtar predicted that, in the future, chemopreventive strategies using multiple agents (cocktails) would be custom designedCusing knowledge gained through genomics and proteomics and taking into account an individual=s risk factorsCand that chemoprevention would have an integral role in cancer management.

Role of Mitochondria and Caspases in Vitamin D-Mediated Apoptosis of MCF-7 Breast Cancer Cells: JoEllen Welsh

JoEllen Welsh, Ph.D., Professor in the Department of Biological Sciences at the University of Notre Dame, presented background information on vitamin D and described both *in vitro* and *in vivo* studies aimed at elucidating the cancer preventive mechanisms of vitamin D in breast cancer. Vitamin D, a steroid, can either be obtained from the diet or produced in the skin as a result of exposure to sunlight. Vitamin D, an inactive molecule, undergoes two hydroxylation steps to form 1,25-dihydroxyvitamin D_3 (1,25D_3), the active compound that interacts with the vitamin D receptor (VDR) and mediates the biological effects attributed to vitamin D. These effects include effects on calcium and bone metabolism, the immune system, the central nervous system, maintenance of skin and hair, and secretion of certain hormones not involved in calcium homeostasis. The VDR acts a ligand (i.e., 1,25D_3)-dependent transcription factor to regulate tissue-specific gene expression; it is expressed in a variety of normal tissues and transformed cells, including those from prostate, breast melanoma, and leukemia.

In vitro studies were conducted in MCF-7 breast cancer cells. Findings indicated that 1,25D_3 induced apoptosis in MCF-7 cells by disruption of mitochondrial function, which was associated with Bax translocation to mitochondria, cytochrome c release, and production of reactive oxygen species (ROS). In addition, data showed that Bax translocation and mitochondrial disruption did not occur after 1,25D_3 treatment of MCF-7 cells selected for resistance to 1,25D_3-mediated apoptosis, indicating that 1,25D_3 somehow is triggering a change in Bax expression and/or subcellular localization. Moreover, neither mitochondrial disruption nor cell death induced by 1,25D_3 can be blocked by caspase inhibition, suggesting that the commitment to 1,25D_3-mediated cell death is caspase-independent. Furthermore, results from studies conducted in transformed cells derived from VDR knockout mice demonstrated that the VDR was essential for 1,25D_3-mediated apoptosis.

Dr. Welsh reported that *in vivo* studies in wild type and VDR knockout mice were carried out to investigate the effects of 1,25D_3 on the mammary gland. Data showed that the VDR was, in fact, expressed in the normal (wild type) mammary gland. VDR expression was lower in proliferating cells and higher in mature differentiated cells, suggesting that the VDR might be involved in growth regulation in the normal mammary gland. Comparison of ductal development of the mammary gland in wild type versus VDR knockout mice found that development was accelerated in the knockout animal. Also, the reduction in size of the mammary gland that takes place after lactationCa process of natural apoptosisCoccurred more slowly in the knockout animal, suggesting that the VDR might influence apoptotic pathways *in vivo*.

In addition, *in vivo* studies in wild type and VDR knockout mice are being carried out to investigate the effects of 1,25D_3 on the sensitivity of the mammary gland to tumorigenesis. Preliminary data unexpectedly showed that treatment with 7,12-dimethylbenzanthracene

(DMBA) resulted in high incidence of skin tumors in VDR knockout mice, but not in wild type mice. Dr. Welsh noted that these results have not yet been explained. Although incidence of mammary tumors was similarly high (>90 percent) in both wild type and knockout mice, the histological appearance of the tumors differed between these two types of mice. In other ongoing research, VDR knockout mice have been crossed with MMTV-neu transgenic mice, which develop mammary tumorigenesis and metastasis. Preliminary data showed an increase in preneoplastic lesions in the VDR knockout mice and an increase in tumor development in the VDR heterozygous mice.

In conclusion, Dr. Welsh proposed that $1,25D_3$ and the VDR regulate differentiation, proliferation, and apoptosis of mammary epithelial cells, and that dysregulation of $1,25D_3$-regulated genes might enhance the susceptibility of the mammary gland to either hyperplasia or transformation. She suggested that the VDR is a nutritionally modulated target in the mammary gland and that continued research may lead to dietary guidelines and/or use of $1,25D_3$ analogs in either prevention or treatment of breast cancer.

Translocation of Bax to Mitochondria Induces Apoptotic Cell Death in Indole-3-Carbinol (I3C) Treated Breast Cancer Cells: Fazlul H. Sarkar

Fazlul H. Sarkar, Ph.D., Professor in the Department of Pathology at the Wayne State University School of Medicine, began by providing some background information on breast cancer and indole-3-carbinol (I3C). In 2002, 203,500 new cases of breast cancer and 39,600 deaths from breast cancer were reported. He emphasized that, because 25 percent of women diagnosed with breast cancer will not be cured by current therapies, it is also necessary to focus on prevention. “Prevention” includes primary prevention in the general population (dietary and lifestyle changes); primary prevention in high-risk individuals (interventions); and secondary prevention to prevent tumor recurrence (interventions). I3CCa bioactive compound found in Brassica vegetables such as cabbage, broccoli, and Brussels sproutsChas received special attention as a chemopreventive agent. Data from experimental studies, supported by epidemiologic data, suggest that I3C may contribute to reduced cancer risk, possibly through multiple biological effects. For example, when breast cancer cells are exposed to I3C, alterations in the regulation of apoptosis-related genes have been observed, leading to the induction of apoptosis.

Dr. Sarkar described research in his laboratory to test the hypothesis that the induction of apoptotic processes by I3C is a result, in part, of Bax translocation to the mitochondria, leading to cytochrome c release in the cytoplasm. Breast epithelial cells, both nontumorigenic (MCF-10A cell line) and tumorigenic (cancer cell line derived from MCF-10A), were exposed to I3C, and growth inhibition, apoptosis, and expression of genes involved in apoptotic processes were measured. Translocation of Bax to the mitochondria, mitochondrial potential, and cytochrome c release also were measured. Data showed that I3C inhibited the growth of breast cancer cells with G1 arrest of the cell cycleCalready published data demonstrated that I3C induces the

proteins p21 and p27, affecting the G1 phaseCand induced apoptosis in these cells. This chemopreventive agent also downregulated the Bax family of proteins, which are important in apoptotic processes. I3C (60 μM) resulted in significantly greater apoptotic cell death in cancer cells than in MCF-10A cells (80 percent vs. 16 percent). Data demonstrated that I3C induced the translocation of Bax to the mitochondria in both cancer cells and MCF-10A cells, but concomitant loss of mitochondrial potential and release of cytochrome c were observed only in cancer cells. Further research in Dr. Sarkar's laboratory established that I3C is a potent inhibitor of signaling by AKT (a kinase) and NF- B (a transcription factor), which are both important regulators of cell growth and cell death; thus, this inhibition may contribute to I3C-induced cell death. Based on data that show that AKT activates NF- B in breast cancer cells, Dr. Sarkar hypothesized that I3C might initially inhibit AKT activity, which might facilitate the translocation of Bax to the mitochondria. Subsequently, the decreased NF- B activity resulting from decreased AKT activity might downregulate genes such as Bcl-2 and Bax that are associated with enhanced cell survival. He pointed out, however, that this is only a hypothesis and that research in this area is continuing.

Discussion

Dr. Green, the moderator of Session III.A., requested that the speakers from this session take the stage to respond to questions from the audience. The following points were made during the discussion:

- In response to a question about the mechanism by which diindolemethane (DIM), a metabolite of I3C, inhibits the kinase AKT, Dr. Sarkar said that either I3C or DIM affects key kinases in the PI3 kinase pathway. He speculated that this might initiate a cascade of signals leading to inactivation of AKT, but added that the details of the mechanism are not known. In response to a question about whether I3C could modulate components of the IGF pathway, he said that research in his laboratory found that epidermal growth factor (EGF)-induced AKT activation was completely abrogated by I3C. He suggested that EGF and IGF might operate through the same pathway, implying that I3C also might be able to affect IGF.
- One participant suggested that it is important to involve patients in nutrition and cancer studies. Dr. Sarkar indicated that a current intervention in his laboratory is administering I3C to breast cancer patients for 3 weeks prior to surgery to determine if the intervention results in any observable changes in biomarkers.

B. Cell Signaling (Moderator: Stephen Hursting)

Signal Transduction by the JNK (Role of JNK in Tumor Development): Roger J. Davis

Roger J. Davis, Ph.D., F.R.S., affiliated with the Howard Hughes Medical Institute and Professor at the University of Massachusetts Medical School, began by presenting background information about signal transduction and the c-Jun amino terminal kinase (JNK) group of kinases. He then described research using Jnk knockout mice aimed at determining the role of JNK in signal transduction, and studies that focused on the relationship between JNK and transformation of the oncogene Bcr/Abl, which causes leukemia. The transcription factor activating protein -1 (AP-1) refers to a complex of protooncogenes (proteins) of the Jun and Fos families that function as transcriptional regulators in signal transduction processes leading to cell proliferation and transformation. Many of these protooncogenes are early gene products and are regulated at the level of protein expression. However, they also can be modified by covalent modification. For example, the Jun family members (c-Jun, Jun-d, and Jun-b) are phosphorylated by JNK kinases, a group of 10 protein kinases that phosphorylate the NH_2-terminal activation domain, causing increased transcriptional activity.

Three genes, two ubiquitous (*Jnk1*, *Jnk2*) and one that is brain-specific (*Jnk3*), encode JNK. Knockout mice lacking only one of these genes (i.e., *Jnk1 -/-*, *Jnk2 -/-*, *Jnk3 -/-*) were crossed to obtain mice that completely lacked *Jnk* gene expression (*Jnk -/-*). Cell cultures were developed from *Jnk -/-* embryos (the embryos could not survive) to study the role of JNK in signal transduction and tumor development. Investigation of the selective killing of *Jnk -/-* cells by various means, with subsequent study of cytochrome C release in *Jnk -/-* cells, led to a model that proposes cellular stresses can activate JNK, leading to cytochrome C release, caspase activation, and cell death. Although the mechanism by which JNK leads to cytochrome C release is unknown, Bax and Bak have been identified as two of the proteins in this pathway. Data indicated that JNK is upstream of Bax and is required for Bax activation. It was hypothesized that JNK acts in a death signaling pathway that leads to Bax activation and mitochondrial dysfunction; studies are underway to identify the direct targets of JNK in this process. Additional research found that JNK was required for cell survival in early forebrain development in mice; *Jnk -/-* embryos, which lack JNK, exhibited ectopic caspase-3 activation and widespread apoptosis in the developing forebrain. Thus, JNK appears to play a role in both cell death and cell survival.

Dr. Davis next focused on the potential role of JNK in tumor development. He pointed out that many studies implicate AP-1 transcription activity (and thus JNK) in tumor development and that usually it is assumed to have a proliferative role. The data in this presentation, however, indicate that cell survival and apoptosis also may be relevant. Dr. Davis explained that he and his colleagues have examined various tumor models to determine which role of JNKCthat is, in proliferation, cell survival, and/or apoptosisCis most important in tumor development. They found that the role of JNK is different in different types of tumors. Dr. Davis discussed their research on leukemia, which is caused by a single oncogene, *Bcr/Abl*. This oncogene regulates many signal transduction pathways in the cell, including the JNK pathway. Bone marrow was extracted from wildtype (WT) mice and *Jnk1 -/-* mice that had only partial loss of JNK function; the bone marrow was then infected with a virus that carried *Bcr/Abl* and was used for *in vitro* and

in vivo experiments. *In vitro*, the WT cells (*Bcr/Abl* transformed) showed a large amount of JNK activity and greatly increased proliferation, whereas the *Jnk1 -/-* cells (*Bcr/Abl* transformed) did not, indicating that JNK is important to *Bcr/Abl* transformation. To investigate leukemia *in vivo*, infected bone marrow was transplanted into irradiated mice (WT and *Jnk1 -/-*). Leukemia occurred in both types of animals. However, malignant infiltration of peripheral organs occurred in the WT mice, but not in the *Jnk1 -/-* mice, indicating that JNK also is important to *Bcr/Abl* transformation *in vivo.* Additional data, however, showed that JNK deficiency did not decrease proliferation *in vivo*. Dr. Davis explained that this suggests JNK deficiency increases apoptosis *in vivo*. He postulated that JNK regulated leukemic cell survival through substrate phosphorylation and effects on gene expression, possibly on *Bcl2*, which codes for a family of antiapoptotic proteins. Studies found that JNK deficiency resulted in decreased *Bcl2* expression by leukemic cells. Further studies found that the effects of JNK deficiency (e.g., increased apoptosis, lack of malignant infiltration of peripheral organs) can be "rescued" by transgenic expression of *Bcl2*. He concluded that JNK is necessary for *Bcr/Abl* transformation, is required for cell survival, is not required for cell proliferation, and that Bcl2 proteins play a role. He emphasized that these data apply only to leukemia.

Resveratrol (A Nutritional Suppressor of EGFR-Dependent Erk1/2): Catherine A. O'Brian

Catherine A. O'Brian, Ph.D., Professor in the Department of Biology at the University of Texas M.D. Anderson Cancer Center, briefly reviewed some characteristics of resveratrol, which is a candidate of nutritional substance for prostate cancer chemoprevention. Resveratrol is one of many dietary polyphenols that show cancer preventive activity and is the most notable of the healthful dietary stilbenes (biphenyl compounds with a methylene bridge). It is highly abundant in the diet; resveratrol, which is nontoxic, constitutes up to 10 percent of grapeskin biomass. Resveratrol is an antagonist of tumor initiation, promotion, and progression. It inhibits COX-1 and COX-2, and thus has anti-inflammatory properties, and it also inhibits phorbol ester-responsive protein kinase C isoenzymes. In addition, resveratrol has protective cardiovascular effects.

Dr. O'Brian showed *in vitro* data that indicated resveratrol is a potent inhibitor of growth in human prostate cancer PC-3 and DU145 cell lines, and that resveratrol inhibits DNA synthesis in the PC-3 cell line. She proposed that resveratrol may prevent prostate cancer by: inhibiting multistage carcinogenesis; scavenging incipient populations of androgen-dependent prostate cancer cells (inhibits androgen receptor function and expression); and scavenging incipient populations of androgen-independent prostate cancer cells (short circuits EGRF-dependent autocrine loops in prostate cancer cells). Dr. O'Brian presented research that was conducted in her laboratory aimed at testing the hypothesis that resveratrol affects EGRF autocrine loops driving Erk1/2 activation in androgen-independent prostate cancer cells, and thus impedes EGRF-dependent proliferative signaling in human prostate cancer cells. Results showed that resveratrol disrupts EGRF-dependent proliferative signaling by two mechanismsCinhibition of

EGRF transactivation and inhibition of EGRF postreceptor signaling. Data demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Erk1/2 activation in PC-3 cells occurs via EGRF transactivation and is PKC dependent; resveratrol suppressed the TPA-induced Erk1/2 activation. Data also demonstrated that the inhibition of PKC isozymes by resveratrol is a possible mechanism for this suppression. In addition to antagonizing EGRF transactivation, data showed that resveratrol also suppresses EGRF postreceptor signaling in PC-3 cells.

Vitamin E Reduces Chromosomal Damage and Inhibits Hepatic Tumor Formation in a Transgenic Mouse Model: Snorri S. Thorgeirsson

Snorri S. Thorgeirsson, Chief of the Laboratory of Experimental Carcinogenesis at the Center for Cancer Research, NCI, presented background information on human hepatocellular carcinoma (HCC), discussed experimental mouse models and the possible role of ROS in hepatocarcinogenesis in these models, and proposed that vitamin E, which is nontoxic to humans, can protect against oxidative stress in liver tissue and reduce cancer risk. HCC is the fifth most common cancer worldwide. The highest incidence is in sub-Saharan Africa and southeast Asia; incidence in the United States and western Europe is on the rise. Most cases of HCC are a result of either chronic infection by hepatitis B virus (HBV), or hepatitis C virus (HCV) and/or exposure to aflatoxin B1 (AFB1). The disease has a predictable natural history. Viral infection and/or AFB1 exposure cause chronic hepatitis and, in some cases, cirrhosis; these lead to phenotypically altered hepatocytes (preneoplasia, 10-30 years; dysplastic hepatocytes (dysplasia, 3-5 more years); and finally HCC (neoplasia, <5 more years). Dr. Thorgeirsson explained that two major categories of HCC have been hypothesized: disease which is characterized by an accumulation of multiple genomic alterations (mutator phenotype); and disease that is characterized by a "relatively" stable genome (activation of ∃-catenin pathway). The latter form of HCC generally has a more favorable prognosis.

Dr. Thorgeirsson said that experimental animal models are valuable in helping to define the molecular pathways and, by extension, the genes responsible for HCC. Several mouse models have been developed, including the transgenic lines *c-myc*, c-myc/TGF∀, E2F-1, and c-myc/E2F-1, in which the transgenes are overexpressed. The coexpression of c-myc and either TGF-∀ or E2F-1 has synergistic effects on hepatic tumorigenesis. Double transgenic mice exhibit shorter latency, higher multiplicity, and larger tumors. The chronic activation of mitogenic signaling induced by overexpression of c-myc and TGF∀ transgenes in the mouse liver induces a state of oxidative stress, characterized by greater metabolic generation of ROS. Dr. Thorgeirsson proposed that these enhanced levels of ROS may be responsible for the early appearance of extensive chromosomal damage and the generation of initiated cells in the c-myc/TGF∀ transgenic mouse model.

Dr. Thorgeirsson pointed out that the antioxidant vitamin E, an integral component of plasma membranes that scavenges oxygen radicals and lipid peroxyl radicals, is present at reduced levels

in diseased livers. He continued by describing research in his laboratory that aimed to determine whether supplemental vitamin E can protect against the formation of ROS and reduce risk for hepatic tumorigenesis in the c-myc/TGF∀ transgenic mouse model. The mice were fed a vitamin E-supplemented diet, starting at the time of weaning. The levels of hepatic injury, ROS production, and chromosomal and mtDNA damage were estimated at 4 weeks and 10 weeks of age. At 26 weeks of age, a histopathological analysis of tumor burden was done. Dr. Thorgeirsson reported that vitamin E supplementation corrected the dysregulated liver physiology induced by the transgenes, reduced genomic damage, inhibited preneoplastic lesions, and prevented malignant conversions. Specific effects of vitamin E supplementation, compared with controls, included the following:

- Enhanced cell survival (prevention of excessive membrane damage and increases in both yield and viability of isolated hepatocytes).
- Normalized liver mass (by 10 weeks of age, reversal of absolute and relative liver weight in transgenic mice to levels found in wild type mice).
- Increased antiproliferative activity (blocking of increases in both cell proliferation and apoptosis).
- Decreased severity of liver lesions.
- Decreased production of ROS (as determined by fluorescence).
- Decreased mtDNA damage and chromosomal damage.
- Decreased tumor multiplicity and size.
- Inhibited tumor formation (control vs. vitamin E:adenomas, 15/15 vs. 7/20; carcinomas, 4/15 vs. 0/20).

Dr. Thorgeirsson suggested that the ROS generated by the overexpression of c-myc and TGF∀ in the liver are the primary carcinogenic agents in this animal model, and that the data demonstrate that dietary supplementation with vitamin E can effectively inhibit liver cancer development. In conclusion, he indicated that more research is needed on characterization of the oxidative DNA adducts that are formed in a state of oxidative stress; analysis of the DNA repair pathways; sources of ROS (e.g., nicotinamide adenine dinucleotide phosphate [NADPH] oxidase, inducible nitric oxide synthase [iNOS]); and cancer prevention using antioxidant therapy.

Discussion

Stephen Hursting, Ph.D., M.P.H., Head, Nutrition and Molecular Carcinogenesis Section, Laboratory of Biosystems and Cancer, DCP, NCI, the moderator of Session III.B., requested that the speakers from this session take the stage to respond to questions from the audience. The following points were made during the discussion:

- Individual antioxidant compounds have unique characteristics; replacing one with another should not be expected to give the same effect. For example, even the isoforms

of vitamin E have different effects. Also, the actions of an antioxidant may depend on the specific physiologic milieu. For instance, clinical trial data showed that the antioxidant ∃-carotene enhanced, rather than inhibited, the risk for lung cancer in aged smokers. Thus, the potential of antioxidants for cancer prevention should not be accepted or rejected as a group.

● Antioxidants are contraindicated for patients undergoing chemotherapy or radiation therapy, which aim to induce free radical generation.

● Antioxidants, including vitamin E, might inhibit carcinogenesis through mechanisms other than their antioxidant activity, and research in this area should be encouraged.

● A delicate balance exists between peroxidation and antioxidation in the cell; maintaining this balance is critical to conducting physiologic processes in an optimal manner.

< ● Oxidizing events with the cell can damage DNA, which has significant consequences, but these events also can have serious consequences by damaging proteins. For example, damage to a transcription factor could have adverse effects on many pathways simultaneously. Little study has been done in the area of protein oxidation.

● Protein *S*-glutathiolation, an oxidative post-translational modification, has been investigated for its potential contribution to the anticancer effects of glutathione. Glutathione, through *S*-glutathiolation, can activate protein kinase C-) (a proapoptotic compound) and thus enhance cancer-preventive activity. At the same time, *S*-glutathiolation decreases the activity of protein kinase C isoenzymes that are either oncogenic or conducive to proliferation.

● Phytochemicals can be metabolically modified; thus, the compound tested *in vitro* may not be the compound in the cells of treated animals. For example, when genistein is administered in an inflammatory model of atherosclerosis, 90 percent of the genistein found in lung tissue is in the form of nitrogenistein, which has an estrogenic effect two orders of magnitude different than genistein. Very little is known about the metabolism of most nutrients and their predominant form in various tissues. Fundamental studies are needed in this area.

● It is clear that nutrients, including phytochemicals, have various potent effects on biological systems. Combining nutrients to take optimal advantage of their multiple effects for cancer prevention presents a major challenge for the scientific community. It is critical to address questions about how the results of studies focused on single nutrients and either one pathway or one mechanism fit into the overall cancer prevention picture. For example, how can findings be compared among separate studies that investigate nutrient effects on the JNK pathway, the Erk pathway, and the IGF pathway, especially when the key steps in these pathways have not yet been identified? Consider the complexity of the JNK pathway. JNK is required to regulate AP-1 activity. However, no gene is regulated by AP-1 alone; AP-1 regulates genes in combination with various other transcription factors. When JNK activates AP-1, the specific genes expressed might depend on the specific transcription factors present, which in turn might depend on the

type of cell. Thus, the effect of nutrients on JNK and subsequent gene expression might exhibit tissue specificity.
● The ongoing Selenium and Vitamin E Clinical Trial (SELECT), which will recruit 32,000 men, has been designed to test the effectiveness of selenium and vitamin E, individually and in combination, in preventing prostate cancer. This trial is taking advantage of the fact that vitamin E and selenium, a component of selenoproteins (e.g., glutathione peroxidase), have different mechanisms-of-action.

C. Nuclear Factors (Moderator: Young S. Kim)

Nuclear Receptors and Lipid Physiology: Steven Kliewer

Steven Kliewer, Ph.D., Professor in the Department of Molecular Biology at the University of Texas Southwestern Medical Center, first presented some background information on nuclear receptors and lipid physiology and then focused on the Pregnane X Receptor (PXR, named on the basis of its activation by natural and synthetic C21 steroids (pregnanes)). Members of the nuclear receptor family of ligand-activated transcription factors play important roles in protecting the body against the accumulation of potentially toxic concentrations of lipophilic chemicals, including cholesterol, bile acids, and fatty acids. The human genome contains 48 nuclear receptors; some are "classical" receptors (e.g., estrogen [ER], androgen [AR], vitamin D [VDR], *trans*-retinoic acid [RAR]) and some are "orphan" receptors (e.g., PXR, retinoid X receptor [RXR], peroxisome proliferation activation receptor [PPAR]). The orphan nuclear receptors regulate lipid homeostasis.

In humans, PXR is expressed primarily in the liver, but also in the colon and small intestine. It binds to the xenobiotic response elements (XREs). PXR regulates numerous genes that are involved in the metabolism and excretion of toxic substances from the body, including those encoding phase I oxidation enzymes (e.g., cytochrome P450 [CYP] enzymes), phase II conjugation enzymes, and various transporters. Although PXR helps to protect the body from toxins, it also can be activated by drugs, sometimes resulting in drug-drug interactions; for example, one drug can accelerate the metabolism of a second drug and thereby reduce the second drug's efficiency. Dr. Kliewer explained that, in addition to being activated by several widely used prescription drugs (e.g., tamoxifen, phenobarbital, ethinylestradiol, taxol), PXR also is activated by hyperforin, the psychoactive constituent of St. John's Wort, an herbal antidepressant.

Dr. Kliewer described the research in his laboratory that is aimed at elucidating the ligand-binding properties of PXR. In x-ray crystallography studies performed in collaboration with Dr. Matt Redinbo (University of North Carolina), the ligand binding pocket of PXR was determined to be very large (>1300 cubic angstroms) and elliptical in shape. The pocket can change shape to

accommodate different ligands. Hyperforin, for instance, brought about a change in the pocket=s structure. These features of PXR distinguish it from other nuclear receptors.

Dr. Kliewer pointed out that PXR is an interface between mammals and their environment, that PXR causes hepatomegaly in rodents (increased proliferation, decreased apoptosis), and that PXR agonists are nongenotoxic hepatocarcinogens. He concluded by posing the question, “Does PXR activation actually contribute to cancer risk?”

Indole-3-Carbinol Signaling Controls Cell Cycle Gene Transcription in Human MCF-7 Breast Cancer Cells by Regulating Promoter-Sp1 Transcription Factor Interactions: Gary L. Firestone

Gary L. Firestone, Ph.D., Professor in the Department of Molecular and Cell Biology, University of California, Berkeley, provided some background information on the relationship between I3C and breast cancer and described the research in his laboratory that is aimed at elucidating the mechanism by which I3C inhibits breast cancer cell growth. Epidemiologic data suggest that consumption of cruciferous vegetables, rich in I3C, is associated with reduced risk for reproductive cancers, including those of the breast and prostate. Cancer preventive effects of I3C in animal models support the epidemiologic findings. Dr. Firestone said that, in earlier studies, he and his colleagues found that I3C dramatically inhibited DNA synthesis in ER-positive MCF-7 breast cancer cells. He added that tamoxifen, an antiestrogen, also had a potent inhibitory effect in MCF-7 cells, and that the combination of I3C and tamoxifen inhibited cell growth synergistically. I3C also inhibited the growth of ER-negative breast cancer cells under conditions in which tamoxifen had no effect, suggesting that the two agents had different mechanisms-of-action.

Dr. Firestone reported that further research showed I3C could induce a G1 cell cycle arrest in MCF-7 breast cancer cells that was accompanied by the selective inhibition of *cyclin dependent kinase 6* (*CDK6*) gene expression and stimulation of *p21*$^{Waf1/Cip1}$ gene expression. Construction and transfection of a series of promoter-reporter plasmids demonstrated that the I3C-regulated changes in CDK6 and p21$^{\mathrm{Waf1/Cip1}}$ levels were a result of specific effects on their corresponding promoters. Dr. Firestone said that his laboratory was the first to clone the *CDK6* promoter; he explained that it was a difficult, year-long task, because the gene had tiny exons (−100 base pairs) separated by very large intervening sequences (50,000-60,000 base pairs). Also, he pointed out that tamoxifen did not regulate the *CDK6* promoter, providing evidence that tamoxifen and I3C act through different mechanisms. Studies using mutagenic analysis revealed that I3C signaling targeted a composite transcriptional element in the *CDK6* promoter that required both Sp1and Ets transcription factor elements for transactivation function. Disruption of the composite element by I3C resulted in loss of transcription by the *CDK6* promoter and consequent loss of CDK6 protein activity.

Dr. Firestone explained that, because the lack of CDK6 alone is not enough to cause a G1 cell cycle arrest, additional studies were done. Findings showed that I3C also inhibited the activity of the protein cyclin dependent kinase 2 (CDK2); the combined loss of CDK6 activity and CDK2 activity was enough to cause a G1 cell cycle arrest. Dr. Firestone said that, in I3C-treated cells, 30 percent of the I3C was converted to DIM, which accumulated in the cell nucleus, suggesting that DIM also may have a role in the transcriptional activities of I3C. Findings showed that DIM had no effect on the *CDK6* promoter. However, mutagenic analyses of the $p21^{Waf1/Cip1}$ promoter demonstrated that, in transfected MCF-7 cells, DIM (as well as I3C) stimulated $p21^{Waf1/Cip1}$ transcription through an indole-responsive region of the promoter that contains multiple Sp1 consensus sequences. Consequently, the levels of protein p21 (a CDK2 inhibitor) increased, leading to reduced CDK2 activity.

In summary, Dr. Firestone proposed that the results demonstrated that both the *CDK6* and $p21^{Waf1/Cip1}$ promoters were downstream targets of the indole-signaling pathway, and that the observed transcriptional effects resulted from a combination of the cellular activities of I3C and DIM. The particular nuclear target proteins for I3C and DIM are not yet known. Dr. Firestone suggested, however, that researchers are very close to identifying the specific pathway by which extracellular dietary indole exerts a specific effect on cell cycle genes.

Carcinogens Interfere With the Expression of Retinoic Acid Receptors: Luigi De Luca

Luigi De Luca, Ph.D., Chief, Differential Control Section, Laboratory of Cellular Carcinogenesis and Tumor promotion, NCI, presented data that demonstrate retinoids, important for controlling differentiation in epithelial cells, can inhibit epithelial carcinogenesis (lung adenomas and skin tumors) in mouse model systems. He also presented data relevant to the mechanism(s) by which retinoids may act as chemopreventive agents.

Dr. De Luca explained that the exposure of respiratory epithelial cells to the carcinogens benzopyrene (BP) or DMBA mimics the effects of vitamin A (retinol) deficiency; these include formation of squamous metaplasia (a cancer precursor) and expression of new keratin synthesis, particularly K6 and K13. These changes can be prevented *in vivo* and *in vitro* (e.g., hamster trachea cells) by using very low concentrations of retinoic acid (RA), a metabolite of vitamin A. Some studies using orally administered 13-*cis* RA reduced second cancers, including lung tumors, in head and neck cancer patients, but side effects were problematic. Research in Dr. De Luca's laboratory tested the hypothesis that epithelial delivery of 13-*cis* RA by inhalation is an effective way to prevent lung cancer. Delivery was accomplished using an electrohydrodynamic inhalation apparatus developed by Battelle that allowed a very fine dispersion of 13-*cis* RA to reach the alveolar region of the lung where adenoma formation takes place. Inhaled 13-*cis* RA significantly inhibited lung adenomas in strain A mice exposed to either BP, urethane, or (4-methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK). Furthermore, lung (but not liver) retinoic acid receptors (RARs; ∀, ∃, () were upregulated in mice treated with 13-*cis* RA and might serve

as a biomarker for inhaled retinoid effects. In this study, dietary RA had no effect on lung adenoma formation or lung biomarkers, most likely because the drug never reached the target tissues. However, dietary RA did upregulate liver RARs and transglutaminase II (TgaseII), underscoring the importance of delivery method on agent effectiveness.

Dr. De Luca next described research that investigated the chemopreventive effectiveness of RA in mice in a two-stage model of skin carcinogenesis in which DMBA was the initiator (normal cell   initiated cell) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was the promoter (initiated cell   papilloma, which eventually converts to cancer). This study found no effect of RA on the formation of papillomas; however, RA (30μ/g diet, fed from birth) inhibited the conversion from papilloma to carcinoma by approximately 65 percent, compared with mice fed 3μ RA/g diet from birth. To determine whether this inhibition was "remembered" by the cell, the diets of two groups of mice were switched at 20 weeks of age; one group went from 3μ RA/g diet to 30 RA/g diet; the other group went from 30μ RA/g diet to 3μ RA/g diet. At 40 weeks of age, similar inhibition was observed for both groups (the 3/30 mice showed slightly higher carcinoma yield than the 30/3 mice). It is noteworthy that the 30/3 mice had the same low carcinoma yield as mice that were fed 30μ RA/g diet continuously from birth, indicating that the cells "remembered."

Dr. De Luca also discussed experiments designed to determine whether or not retinoid signaling was impaired during malignant progression in skin cancer. Data showed that mRNA for RAR((the most highly expressed RAR in this skin tissue) was reduced in mouse skin papillomas and disappeared in carcinomas. In addition, overexpression of RAR∀ in papilloma cells, brought about by introducing RAR∀ (carried by a retrovirus) into the cells, enhanced cell growth inhibition by RA, underscoring the importance of retinoid signaling. Further research showed that expression of the RAR∀-dominant-negative has the opposite effect; it renders the cells insensitive to RA.

Additional studies were conducted to investigate the interactions between carcinogens (e.g., BP, DBMA) and their receptors. Dr. De Luca explained that *Ahr*-/- mice (which lack the aryl hydrocarbon receptor) show the following phenotype: liver fibrosis; accumulation of liver retinoids (e.g., retinol, retinyl palmitate, RA); induction of TgaseII; increased expression of TGF∃s; and accumulation of collagen. Data demonstrate that RA metabolism is reduced in microsomes from livers of *Ahr*-/- mice. Dr. De Luca pointed out that the amount of oxidized RA (particularly 4-hydroxretinoic acid) is significantly lower in these mice. He suggested that *Ahr*-/- mice most likely have a deficiency of some CYP450 enzyme that depends on an aromatic hydrocarbon receptor (AHR) for activation. Further research showed that inducing vitamin A deficiency in *Ahr*-/- mice by dietary manipulation reversed the characteristics of the phenotype: liver fibrosis was prevented; liver retinoid levels were depleted; expression of TGF∃s returned to normal; and collagen deposition returned to normal. Dr. De Luca stated that being able to reverse the phenotype of *Ahr* deficiency by dietary manipulation of vitamin A was an exciting finding.

p53-Independent Cell Cycle Arrest and Apoptosis via Butyrate-Inducible ZBP-89: Juanita L. Merchant

Juanita L. Merchant, Ph.D., M.D., Associate Professor in the Departments of Internal Medicine and Physiology at the University of Michigan, provided background information on the protein ZBP-89 (a Krüppel-type zinc transcription factor) and presented data from research that investigated the effect of ZBP-89 on butyrate regulation of $p21^{Waf1}$ in mutated *p53* cells, the relationship between ZBP-89 and chromatin in mutated *p53* cells, and the interaction of ZBP-89 with wild type *p53*. ZBP-89 binds to the same guanine/cytosine (GC)-rich gene elements as the transcription factor Sp1, and can both activate and repress gene function. ZBP-89 is expressed ubiquitously; it is overexpressed in gastrointestinal cancers. Overexpression of ZBP-89 can arrest cell growth at the G1 phase and also can induce apoptosis. ZBP-89 has the ability to bind to and regulate numerous genes, including *gastrin*, $p21^{Waf1}$, and genes involved in immune regulation.

Dr. Merchant explained that, considering ZBP-89 and Sp1 bind to similar GC-rich sequences and that Sp1 binds to GC-rich sequences on $p21^{Waf1}$ (thereby helping to mediate transcription), it was postulated that ZBP-89 also might activate $p21^{Waf1}$. A study in HT-29 colon cancer cells found that, although overexpression of ZBP-89 did not increase $p21^{Waf1}$ expression, ZBP-89 potentiated the ability of butyrate to increase $p21^{Waf1}$ expression. In addition, data showed that ZBP-89 potentiated butyrate activation of the $p21^{Waf1}$ reporter. Using DNA-protein interaction assays (e.g., footprinting) and mutations of ZBP-89, analyses demonstrated that the N-terminal domain (first 100 amino acids) of ZBP-89 was required to potentiate the action of butyrate. Because data also indicated that ZBP-89 binding on the $p21^{Waf1}$ promoter was not stimulated in the presence of butyrate, coprecipitation studies were completed to determine if ZBP-89 might potentiate butyrate activation of $p21^{Waf1}$ through an effect on histone acetylase activity. Results showed that the N-terminal of ZBP-89 binds to p300 (a histone acetyltransferasease). Dr. Merchant proposed that ZBP-89 recruits p300 to the $p21^{Waf1}$ promoter; this action, combined with the inhibition of histone deacetylases by butyrate (which enhances $p21^{Waf1}$ activation), could potentiate the effect of butyrate. Dr. Merchant pointed out that *p53* was mutated (and inactivated) in the HT-29 colon cancer cell line; thus, the effect of ZBP-89 in this cell line was *p53*-independent.

Dr. Merchant also presented results of research that investigated the relationship between ZBP-89 and chromatin in mutated *p53* cells. Data indicated that, in mutated *p53* cells, ZBP-89 moved to heterochromatin (where gene silencing occurs) at the nuclear periphery; this relocation of ZBP-89 could inhibit its activity. Data also showed that butyrate increased the acetylation of ZBP-89 and decreased the association of ZBP-89 with heterochromatin. Dr. Merchant proposed that these results may help to explain the potentiating effect between ZBP-89 and butyrate in mutated *p53* cells.

Additional research was conducted using wild type *p53* cells to determine whether ZBP-89 could regulate cell growth through $p21^{Waf1}$ promoters by its ability to interact with *p53*. Dr. Merchant reported that ZBP-89 interacted, through its zinc finger binding domain, with the C-terminal domain of *p53;* that ZBP-89 stabilized wild type *p53*; and that *p53* stabilization by ZBP-89 stimulated $p21^{Waf1}$ expression, possibly by enhancing *p53*-dependent induction of the $p21^{Waf1}$ promoter. She suggested that ZBP-89 may stabilize *p53* by preventing nuclear export of *p53*. Data indicated that transcriptionally active *p53* was predominantly nuclear. Research using colon tumors that contained various *p53* mutations demonstrated that ZBP-89, even though it interacted with mutant *p53,* did not stabilize mutant *p53.*

D. Hormonal Regulation (Moderator: James A. Crowell)

Pathways of Vitamin D Action To Inhibit Prostate Cancer Cell Growth: David Feldman

David Feldman, M.D., Professor in the Department of Internal Medicine, Division of Endocrinology at the Stanford University School of Medicine, began by showing the metabolic pathways of vitamin D and highlighting vitamin D biochemical actions. Humans acquire vitamin D from food and by exposure to sunlight. Vitamin D is an inactive molecule that requires two hydroxylation steps to form 1,25-dihydroxyvitamin D_3 ($1,25D_3$), the active compound. Although the kidney is the main source of $1,25D_3$, it also is formed in bone and in the prostate. $1,25D_3$ binds with the vitamin D receptor (VDR), dimerizes with retinoid X receptors (RXR), and turns on target genes. The classical view of vitamin D actions asserts that it maintains mineral homeostasis and is necessary for normal bones. An expanded view of vitamin D actions proposes that it also is antiproliferative, promotes differentiation, and is immunosuppressive.

Dr. Feldman briefly discussed the links between vitamin D and prostate cancer. Epidemiologic data indicate that prostate cancer risk is inversely related to sun exposure and increases with vitamin D deficiency. Also, certain VDR polymorphisms can increase prostate cancer risk. *In vitro* data shows that the VDR is present in prostate tissues, and that $1,25D_3$ inhibits prostate cell proliferation. A dose-response inhibitory effect of $1,25D_3$ on proliferation rate was observed in LNCaP (biggest decrease) and PC-3, but not in DU-145 (very small decrease; essentially resistant) cancer cell lines. Proposed mechanisms for the anticancer activity of $1,25D_3$ include: G1/G0 cell cycle arrest; apoptosis; differentiation; regulation of tumor suppressors/oncogenes; antiangiogenesis; and inhibition of invasion and/or metastasis.

Dr. Feldman presented information on several newly considered pathways through which $1,25D_3$ activity might be modulated. One such pathway is the inhibition of 24-hydroxylase, the enzyme that converts $25(OH)D_3$ to $24,25(OH)_2D_3$ or $1,25D_3$ to $1,24,25(OH)_3D_3$, an inactive precursor and inactivation of the active molecule into a form destined for excretion. DU-145 cells, which

are resistant to the antiproliferative actions of $1,25D_3$, exhibit exuberant 24-hydroxylase induction. However, LNCaP cells, which are very sensitive to $1,25D_3$, exhibit very low levels of 24-hydroxylase induction. It has been postulated that 24-hydroxylase might inactivate $1,25D_3$, resulting in the apparent resistance of DU-145. Data show that liarozole, an inhibitor of P450 enzymes, inhibits 24-hydroxylase activity, and thus prolongs the half-life of $1,25D_3$, and leads to VDR upregulation. Data show that a combination of $1,25D_3$ and liarozole renders DU-145 cells sensitive to the antiproliferative activity of $1,25D_3$, a result of the increased level of $1,25D_3$ and increased VDR abundance. Thus inhibition of 24-hydroxylase sensitizes the resistant DU145 cells and allows the antiproliferative activity of $1,25D_3$ to become apparent.

The second proposed pathway for modulation of $1,25D_3$ activity is through the stimulation of IGFBP-3 expression. IGFBP-3 is the major circulating binding protein for IGF-1, but also is expressed in some target cells, including prostate. The actions of IGFBP-3 are antiproliferative; it binds and sequesters the mitogen IGF-1, and its actions are independent of IGF-1. Epidemiologic studies link high levels of IGFBP-3 with reduced prostate cancer risk. Data from *in vitro* studies indicate that IGFBP-3 is induced by $1,25D_3$ in LNCaP cells in a dose-dependent manner. Furthermore, inhibition of IGFBP-3 by immunoneutralization or antisense nucleotides blocks $1,25D_3$ antiproliferative activity in LNCaP cells, indicating that induction of IGFBP-3 is essential for growth inhibition. In addition, although both $1,25D_3$ and IGFBP-3 induce the cell cycle inhibitor p21, immunoneutralization of IGFBP-3 blocks $1,25D_3$ induction of p21. Thus, it is possible that the antiproliferative activity of $1,25D_3$ is mediated by p21 via IGFBP-3. Dr. Feldman and colleagues have used cDNA microarray analysis to identify target genes that are upregulated and downregulated by $1,25D_3$ in normal and LNCaP prostate cancer cells. Interestingly, the highest induction was observed for the IGFBP-3 gene. Dr. Feldman emphasized that the target genes for $1,25D_3$ are dependent on the type of cell. That is, microarray data for LNCaP prostate cells may not be applicable to other prostate cancer cell types.

The third hypothetical pathway for modulation of $1,25D_3$ activity involves 1-hydroxylase; it was recently found that this enzyme is expressed in prostate cells and converts $25D_3$ to $1,25D_3$. However, although high levels of 1-hydroxylase activity are expressed in normal prostate cells, only relatively low levels are expressed in prostate cancer cells. In addition, although $25D_3$ is as effective as $1,25D_3$ in growth inhibition of normal prostate cells, it is less effective in prostate cancer cells. Based on the limited findings, Dr. Feldman speculated that administration of $25D_3$ to patients is unlikely to be effective in treating established prostate cancer. He suggested, however, that either $25D_3$ or $1,25D_3$ might be useful as a chemopreventive agent locally within the normal prostate.

Dr. Feldman described a human trial of calcitriol ($1,25D_3$) conducted at the Stanford University School of Medicine. The trial was conducted in seven prostate cancer patients (mean age: 71.7 years) for whom there was minimal chance of harm with delay of alternative therapy. Entrance criteria were: Stage A or B prostate cancer; minimal recurrent disease after radical

prostatectomy or radiation therapy; prostate specific antigen (PSA) post-treatment nadir totally suppressed; three or more rising PSA assays; normal calcium balance and renal function; negative bone scan and/or magnetic resonance imaging (MRI). The protocol included: dietary calcium <800 mg/day; a calcitriol starting dose of 0.5 µg/day; a calcitriol dose escalation of 0.25 µg weekly; monitoring urinary and serum calcium; and having a kidney ultrasound (for stones) every 3 months. The results of this study indicated that calcitriol slows the rate of rise of PSA in early recurrent prostate cancer. Dr. Feldman pointed out that the findings suggested, but did not prove, that tumor growth was slowed. Also, he noted that hypercalciuria limits the amount of calcitriol that can be safely administered, and that calcitriol analogs that are not hypercalcemic are needed.

In conclusion, Dr. Feldman felt his data and data from other labs, indicates that $1,25D_3$ will be a useful drug in the treatment and/or the prevention of prostate cancer. In the future, less calcemic analogs of $1,25D_3$ are likely to be the drugs that are used clinically. These analogs cause less hypercalcemia while being more antiproliferative, thus having an improved therapeutic ratio.

Molecular Determinants for the Tissue Specificity of SERMs: Myles Brown

Myles Brown, M.D., Associate Professor in the Department of Medical Oncology at Dana-Farber Cancer Institute, presented background information on nuclear receptors and their link to cancer; described basic research investigating the role of coregulators in determining the spectrum of transcriptional responses to ER and its ligands in various target tissues; and addressed the role of coregulators in determining the tissue specificity of selective estrogen receptor modifier (SERM) action. Various types of nuclear receptors exist, such as receptors for steroids (e.g, ER, AR), vitamins (e.g.,VDR, RAR), thyroid hormone (e.g., TR), eicosanoids (e.g., PPAR), and xenobiotics (e.g., PXR); this is by no means an inclusive list. Certain nuclear receptors have been linked with cancer development and look attractive as potential targets for chemoprevention; examples of cancer types and associated nuclear receptors are breast (ER∀, pregesterone receptor [PR]), endometrium (Er∀, PR), prostate (AR, Er∃), colon (PPAR(, Er∀), and liposarcoma (PPAR(). EstrogenCwhich has various target organs, including breast, uterus, ovary, cardiovascular system, bone, and brainCand the ER have been a major research focus.

Dr. Brown stated that certain observations on ER∀ and breast cancer made over the past 20 years provided a basis for the hypothesis that coregulators play an important role in determining the spectrum of ER activity. For example, Er∀ is the critical target for both endocrine therapy and chemoprevention for breast cancer and its presence is necessary for positive response to these treatment approaches. In addition, at relapse from endocrine therapy, Er∀ is rarely mutated, suggesting that other mechanisms contribute to treatment response. Furthermore, although tamoxifen functions as an estrogen antagonist in the breast, it is a partial agonist in the uterus. The sum of these observations led to the hypothesis that cell type, ligand, and gene-specific

differences in steroid receptor signaling are mediated by the differential action of coactivators and corepressors recruited by the receptor to target gene promoters.

Dr. Brown explained that the search for coregulators began a decade ago by looking for proteins associated with the receptors, continued with genetic approaches, and led to an explosion in the number of molecules now known to play an important role in ER signaling. He showed slides that listed 10 examples of nuclear receptor coactivators (e.g., the p160 family, which includes SCR-1, GRIP-1, and AIB-1), two examples of nuclear receptor corepressors (e.g., N-coR, SMRT), and illustrated a schematic of proposed complicated model showing how these coregulators might interact with the nuclear receptors as well as with histone complexes. In the proposed model, histone acetylation is associated with gene activation and histone deactylation with gene inactivation. Dr. Brown next described the sensitive technology, chromatin immunoprecipitation (ChIP), that his laboratory uses to examine the transcription complex assembly on endogenous ER target genes in different cell typesCthat is, what factors actually are bound by a certain promoter in a certain cell type. This research has shown that the ER occupied its promoters only in the presence of a ligand, and the ER was able to recruit the coregulator. Also, various coregulators appeared to act at different steps during the process of gene activation, indicating that the transcription complex formed is not static. Data demonstrated that tamoxifen was able to induce recruitment of the ER and two corepressors (N-coR, SMRT), but not the coactivators. Furthermore, data from knockout mice suggest that tamoxifen cannot have antagonist activity in the absence of corepressors. Additional studies were conducted to investigate whether changes in levels or activity of coregulators can change tamoxifen from an antagonist to an agonist. Using reversed pharmacology coactivators (coactivators that bind to the ER-corepressor site), data showed that, in MCF-7 cells, tamoxifen stimulated both the recruitment of the reversed coactivators and cell growth.

Dr. Brown described studies that focused on the role of coregulators in determining actions of SERMs, including tamoxifen and raloxifene; unlike tamoxifen, raloxifene is not an agonist in the uterus. He summarized the findings as follows:

- Tamoxifen and raloxifene both act as antagonists in breast cancer cells and recruit corepressor complexes to both the *cathepsin D* (*CATD*) and *c-myc* promoters.
- Tamoxifen acts as a partial agonist in endometrial cancer cells activating *c-myc*, but not *CATD* transcription.
- Consistent with its partial agonist activity, tamoxifen recruits coactivator complexes to the *c-myc* promoter in endometrial cancer cells.
- SRC-1 is highly expressed in the endometrium compared with the breast.
- SRC-1 is both sufficient and necessary to support the partial agonist activity of tamoxifen.
- Overall, results suggest that cell type and promoter-specific differences in coregulator recruitment are determinants of the spectrum of responses to SERMs and form one basis for SERM action.

Throughout his presentation, Dr. Brown emphasized that coregulators represent only one possible mechanism that contributes to the action of SERMs, and that additional mechanisms likely play important roles.

Indole-3-Carbinol Is a Negative Regulator of Estrogen: Karen J. Auborn

Karen J. Auborn, Ph.D., Head of Phytochemical Research and Professor in the Research Department of the North Shore-Long Island Jewish Research Institute, described the research in her laboratory that examined opposing activities of I3C and estrogen, involvement of specific genes, and a synergistic effect of I3C and genistein. She explained that because I3C can be biologically converted to DIM, some studies were conducted using both I3C and DIM. Dr. Auborn reported that epidemiologic cohort studies have shown that diets rich in cruciferous vegetables, which contain I3C, are associated with decreased risk for breast cancer. In addition, laboratory and animal studies indicate that I3C prevents breast, endometrial, and cervical cancers. Furthermore, translational studies have demonstrated the efficacy of I3C for treatment of precancerous lesions of the cervix.

The K14-HPV 16 transgenic mouse model, which expresses papillomavirus E6 and E7 under a keratin 14 promoter, was used in this research. This transgenic model develops cervical cancer when given estrogen chronically, in contrast to a "normal" mouse, which develops only cervical dysplasia when given estrogen chronically. Data showed that feeding the transgenic mice a diet supplemented with I3C inhibited the development of cervical cancer, and that dietary I3C decreased the proliferation of cervical epithelium in both transgenic and normal mice. Also, I3C induced apoptosis in cervical epithelium, in contrast to estrogen, which inhibited apoptosis. Thus, interplay can occur between I3C and estrogen. Dr. Auborn pointed out that I3C/DIM can influence estrogen metabolism favorably by binding to the AHR, and thus inducing some CYP450 enzymes that result in increased formation of 2-hydroxyestrone and decreased formation of 160hydroxyestrone. 2-Hydroxyestrone is further metabolized to compounds that are antiangiogenic, antiproliferative, and proapoptotic, whereas 16-hydroxyestrone binds to the ER and has a prolonged estrogenic effect and increases tumor growth. Dr. Auborn commented that I3C/DIM also could influence cancer risk through other mechanisms. For example, *in vitro* findings showed that a combination of DIM and genistein (a soy phytoestrogen) acted synergistically to decrease estrogen signaling driven by ER-∀.

Microarray studies in various cervical cancer cell lines indicated that DIM abrogates the induction of gene expression by estrogen for more than 100 known genes, including genes involved in cell cycle and proliferation, transcription and signaling, and growth. Furthermore, DIM regulated the expression of many genes independent of estrogen; the majority of genes were upregulated. Dr. Auborn said that studies are focusing on the *GADD*s, a series of unrelated genes named for their ability to bring about "Growth Arrest in response to DNA Damage." The

GADDs, which are robustly upregulated by DIM, induce growth arrest upstream to the induction of apoptosis, as part of the metabolic stress response. Dr. Auborn explained that tumor cells *in vivo* are stressed and, in their metabolic response to this stress, many genes are upregulated; some increase tumor cell survival, but someCsuch as the *GADD*sCincrease growth arrest and apoptosis. She proposed that stressed tumor cells *in vivo* might be more sensitive than normal cells to killing by I3C/DIM because of the additional upregulation of *GADD*s caused by I3C/DIM, and that combinations of certain nutrients might be synergistic in killing tumor cells. In support of this hypothesis, data demonstrated that using a combination of DIM and genistein synergistically induced GADDs and also synergistically increased apoptosis in cervical cancer cells *in vitro*.

Dr. Auborn also briefly reported data on the effects of I3C/DIM on the *BRCA1* gene. *BRCA1* induces genes involved in DNA repair and apoptosis, upregulates *GADD*s, and is a negative regulator of Er-∀ signaling. Data show that I3C/DIM increased the expression of *BRCA1* and that I3C and *BRCA1* work together to abrogate gene expression driven by ER

Dr. Auborn concluded by emphasizing that powerful tools exist for implicating genes whose involvement in carcinogenesis has not been suspected, and that these tools should be used to explore the effects of nutrients. She acknowledged that the challenge will be to fit the genes identified into a meaningful biological context.

Discussion

James A. Crowell, Ph.D., Chief, Chemopreventive Agent Development Research Group, DCP, NCI, the moderator of Session III.D., requested that the speakers from this session take the stage to respond to questions from the audience. The following points were made during the discussion:

- I3C is not a ligand for the AHR and DIM has only a very low affinity for this receptor. However, indolo[3,2-*b*]carbazole (ICZ), also a metabolite of I3C, is a ligand for AHR, raising the possibility that ICZ, rather than I3C or DIM, is responsible for the regulatory effects of I3C on estrogen.
- It is not yet known if genistein and DIM upregulate GADDS through the same or different mechanisms.
- The classical pharmacological approach to determining the effect of drugs on cancer and other diseases is not appropriate for nutrients, which are normally delivered by eating a whole food. Individual nutrients may not have the same potency that they have in mixtures, as exemplified by the synergism between genistein and I3C.
- At low concentrations (<1 μM), genistein acts as an ER agonist; however, at concentrations higher than 1μM, it generally acts as an ER antagonist. Dr. Auborn said that the presence of estradiol in a system can affect this balance. She reported that, in her laboratory research, 5 μM genistein could be an agonist when estradiol was present. A

participant pointed out that information gained through DNA microarrays and proteomics most likely would result in redefining terms, such as agonists and antagonists. For example, even though tamoxifen mimics estradiol, tamoxifen upregulates only a subset of the same genes; also, it upregulates some genes that estradiol does not.

- Very little work has been completed in the area of phytoestrogens, with regard to their ability to recruit coactivators and/or corepressors. Some work has used PC-SPES, a mixture of eight herbs, which is used to treat prostate cancer. *In vitro*, certain fractions of PC-SPES have very potent estrogenic activity and are able to recruit coactivators. Dr. Brown pointed out, however, that dietary agents with estrogenic or antiestrogenic effects could act through various mechanisms involving the ligands, receptors, and/or coregulators.
- Nutrients may have different effects in normal cells and in stressed cells, such as tumor cells, that are stressed in various ways; that is, the physiological context may influence the action of a nutrient. Dr. Auborn noted that mice given I3C throughout their lifetime simply live a little longer, and that I3C seems to have no effect on normal cells.

Session IV. Nutritional Proteomics in Cancer Prevention

A. Technology Issues and Approaches (Moderator: Sharon Ross)

New Approaches to Protein Profiling of Cancer: Emanuel F. Petricoin

Emanuel F. Petricoin, Ph.D., Department of Therapeutic Proteins, Center for Biologics Evaluation and Review, Food and Drug Administration (FDA), and Co-Director of the FDA/NIH Clinical Proteomics Program, provided background information on proteomics and described ongoing cancer-related research aimed at using proteomics in molecular diagnostics, molecular-targeted therapeutics, and early detection using protein pattern diagnostics. Dr. Petricoin emphasized that, unlike the human genome, a unique human proteomeCthat is, all of the proteins present at a particular timeCdoes not exist. In reality, the proteome is a constantly changing response to a person's environment (e.g., food eaten, time of day, drugs taken). The proteomic networks that drive biology exist both within and outside of the cell, and help cells and organ systems communicate. Dr. Petricoin said that, although genomics and genetic mutations underpin cancer, cancer is a proteomic disease at the functional level. He underscored the importance of studying cancer-related proteomics in tissues rather than in cell culture models. Research focuses on protein expression patterns and interactions within the cell and outside of the cell at the tumor-host interface, the pathogenic role of either dominant or deranged signaling pathways inside evolving cancer cells, and protein patterns in blood serum, which can reflect organ disease states.

Using prostate cancer as an example, Dr. Petricoin explained that the different types of cells (e.g., stromal, luminal, epithelial) present in a tissue sample have a unique proteome, and that

combining the cells by grinding up the tissue, as is done in traditional laboratory procedures, only leads to confusion. Thus, laser capture dissectionCa technology developed by Lance Liotta, M.D., Ph.D., Co-Director of the FDA/NIH Clinical Proteomics ProgramCwhich allows individual cells to be removed from the tissue and then analyzed, is widely used in proteomics. It is used as a starting point for two-dimensional (2D) gel differential display, protein arrays, and protein pattern diagnostics.

Dr. Petricoin reported that studies using cells from breast, prostate, ovarian, and esophageal cancers identified more than 400 differentially expressed proteins by 2D gel differential display/mass spectrometry. Proteins were both overexpressed and underexpressed in cancer cells compared with normal cells. However, differentially expressed proteins may or may not be important to the cancer process. Thus, before such proteins can be considered to be legitimate targets for cancer prevention or therapy, their relevance must be validated. Dr. Petricoin stated that one of the best ways to validate protein targets is by using protein microarrays. He cautioned, however, that protein arrays present a problem in that approximately 10^8-10^9 log orders exist between the most abundant protein and the least abundant protein. Also, he noted that because proteins cannot be amplified, either the proteins must have a tag (e.g., fluorescent, radiometric) or some molecule that specifically recognizes the proteins must be tagged, as in enzyme-linked immunosorbent assay (ELISA). He advised that anyone considering using protein arrays should approach them with both caution (because they offer danger) and excitement (because they offer opportunity).

Dr. Petricoin described studies using a reverse phase protein array developed in his laboratory. This approach arrays denatured lysates from microdissected cells on the slide; they are then analyzed directly as in a Western blot or an ELISA. The lysates are arrayed in miniature dilution curves so that direct comparisons can be made among samples. This approach was used to look at signaling activation in prostate cancer. Findings showed that as cells progressed from normal to prostatic epithelial neoplasia (PIN) to cancer, apoptosis was inhibited as a result of changes in the activation of several apoptosis-related proteins. Dr. Petricoin added that this technique also was used for validation of a target protein (Rho G-protein dissociation inhibitor [Rho GDI]) in ovarian cancer cells that was identified by 2D gel differential display/mass spectrometry. Lysates of microdissected cells from 50 different invasive cancer cases, compared with lysates of cells from 50 cases with low malignant potential (LMP), confirmed that Rho GDI was uniquely overexpressed in invasive ovarian cancer, and thus had potential as an immunotherapy (vaccine)-based target.

Dr. Petricoin reported that his laboratory has been making protein arrays of both normal and tumor cells from vital organs, including brain, lung, heart, kidney, liver, prostate, and testes. They can now probe these arrays (e.g., with Rho GDI) and look at the relative protein expression between cancer and normal cells, with the aim of validating targets for vaccine-based therapies. Other ongoing research using the reverse phase protein array includes studies of certain proteins (e.g., caspases) in the apoptosis pathway, and investigation of human B cell lymphoma cells

treated *ex vivo* with various chemotherapeutic compounds to determine if the compounds individually, or in combination, could affect these proteins and cause apoptosis. He stated that the ultimate goal for molecular-targeted therapeutics is to use protein arrays to profile complete signaling pathways (pathways governing cell survival and cell death) for a patient at one time. Protein arrays have been used to examine changes in protein expression (e.g., AKT, Erb2) in signaling pathways in breast cancer, in cells that range from normal to premalignant to invasive cancer. Dr. Petricoin hypothesized that, based on results from a herceptin-taxol trial in breast and ovarian cancer patients who were biopsied before and after therapy, herceptin reduces the AKT prosurvival pathway by disengaging the receptor (i.e., the Erb1/Erb2 ligation). This leads to increased sensitivity to apoptosis-inducing therapy such as taxol. He pointed out that cluster analysis can be conducted using protein arrays, and that data from before and after treatment may link with biologically significant correlates.

At present, early detection of ovarian cancer is difficult, because no single, reliable biomarker is available. Dr. Petricoin reviewed current research that examines serum protein patterns in women with ovarian cancer and in normal women, using the Surface Enhanced Laser/Desorption Ionization (SELDI) chip process, in combination with mass spectrometry and intelligence-based bioinformatic tools. Using this methodology, knowing the identity of the individual proteins is not necessary; rather, the proteomic "fingerprint" or "signature," which can be considered to be a biomarker, is the important element. Dr. Petrocoin noted that this approachCfocusing on a pattern instead of on specific proteinsCrepresents a paradigm shift in biomarker research. He explained that earlier studies at the NIH using SELDI showed that protein signatures of microdissected cells from prostate tissues changed in specific ways, going from normal to premalignant to cancer. Using the protein signatures from study sets of microdissected cells, it was possible to determine the disease state of the cells. This type of researchCmatching tissue protein patterns to different types of cancerCis still being conducted. Dr. Petricoin acknowledged that serum-based studies are more difficult than tissue-based studies, because the serum proteome can be extremely heterogeneous among individuals. In addition, the SELDI technology generates about 15,000 data points; thus, sophisticated bioinformatic analysis is required.

Dr. Petricoin briefly discussed two types of bioinformatic data-mining approachesCsupervised and unsupervised learning. A supervised learning system requires a body of data for which the classification outcome is known, and this then trains a system (e.g., regression models, genetic algorithms). An unsupervised learning system clusters groups or records without previous knowledge of outcomes (e.g., Euclidean distance, nearest neighbors). The challenge in proteomics is to use these data-mining approaches to determine which proteins characterize the biological state and which are background noise. Dr. Petricoin said that, for the ovarian cancer studies, they are using a combined approach, which consists of a genetic algorithm to optimize the training/feature set (normal patients vs. cancer patients), along with unsupervised modules as the fitness test for the genetic algorithm. The result is a model that uniformly groups data into homogeneous clusters of normal women and cancer patients. Applying this approach to 50

women with ovarian cancer and 50 healthy women resulted in a model that had four "normal" clusters and three "cancer" clusters. Serum from new patients can be analyzed, compared with the existing data clusters, and determined to be benign (normal), cancerous, or "no match." Dr. Petricoin indicated that this approach also can be used to determine clusters of protein patterns for prostate cancer, as well as for benign prostate conditions.

Dr. Petricoin reported that they recently began using the "QSTAR" for higher resolution mass spectrometry analysis of samples on a SELDI chip and have achieved both 100 percent sensitivity and specificity in a larger study set; QSTAR yields about 900,000 data points versus the 15,000 generated by the Cyfergen mass spectrometric equipment used earlier. Dr. Petricoin said that data from new patients will be continually added to the training set to improve its accuracy. He reported that NCI clinical trials using protein pattern diagnostics to discriminate different types of cancer cohorts will begin in 3 to 6 months.

B. Translational and Post-Translational Modifications Influenced by Nutrients (Moderator: Paul Coates)

Role of Selenium-Containing Proteins in Health: Dolph L. Hatfield

Dolph Hatfield, Ph.D., Chief, Section on the Molecular Biology of Selenium, Basic Research Laboratory, Center for Cancer Research, NCI, reviewed the health benefits of selenium, discussed the molecular biology of selenium, and described research using transgenic and knockout mouse models to study the role of selenoproteins in health. Dr. Hatfield said that selenium is one of the most promising cancer chemopreventive agents. He noted that the NCI is sponsoring the ongoing Selenium and Vitamin E Clinical Trial (SELECT); this trial, which will recruit 32,000 men, is designed to test the effectiveness of selenium and vitamin E, individually and in combination, in preventing prostate cancer. In addition, he reported that selenium may have a role in preventing heart disease and other cardiovascular and muscle disorders, and may have a role in male reproduction, mammalian development and immune function. Furthermore, this element serves as an antiviral agent, and may delay the onset of AIDS in HIV patients and delay the aging process. Dr. Hatfield showed a slide from an earlier study by other investigators of mice fed selenium-deficient versus selenium-supplemented diets to illustrate the health benefits of selenium. Within 25 days, the selenium-deficient mice manifested severe, stunted growth and developed cataracts, muscular and skeletal disorders, and other maladies. It has been proposed that selenium may affect health through the actions of selenoproteins and/or small molecular weight selenium compounds, many of which are found in food (e.g., garlic, Brazil nuts).

Dr. Hatfield explained that selenium is incorporated into proteins through the amino acid selenocysteine (Sec), the 21st amino acid in the genetic code. Sec is inserted into polypeptide chains in response to the codon UGA, which requires a Sec-specific tRNA (the tRNA has two

isoforms that differ by a single methyl group) and a specific elongation factor (EFsec). He noted that Sec mRNAs have a unique stem-loop structure, designated the Sec insertion sequence (SECIS) element, that is located in the 3' untranslated region of mammalian Sec mRNAs; this structure is essential for insertion of Sec in response to UGA. A specific SECIS binding protein (SBP2) binds to the stem loop structure and forms a complex with Sec tRNA and EFsec. This complex allows Sec to be inserted into the nascent selenopeptide, after which the elongation factor and the deaminoacylated tRNA are released for recycling, while SBP2 remains attached to the stem-loop structure. Dr. Hatfield pointed out that some selenoprotein mRNAs terminate in UGA. He said that the distance between the stem-loop structure and the UGA codon in Sec mRNA is critical for UGA to act as a Sec codon rather than a termination codon.

Dr. Hatfield described the research in his laboratory that used transgenic mice to study the role of selenoproteins in health. He stated that since Sec tRNA governs the synthesis of the entire class of selenoproteins, perturbation of this tRNA provides a powerful research tool to study selenoprotein expression. Transgenic mice, carrying mutant Sec tRNA transgenes lacking isopentenyladenosine (i^6A) in the anticodon loop, were developed. Studies examined selenoprotein synthesis in mice carrying multiple copies of the wild type or mutant Sec tRNA transgene. Findings showed that overexpression of wild type tRNA did not affect selenoprotein synthesis. However, in mice expressing i^6A-deficient tRNA, levels of numerous selenoproteins decreased; these decreases varied in a selenoprotein and tissue specific manner. For example, the level of glutathione peroxidase 1 (GPX1) and thioredoxin reductase 3 (TR3) were the most and least affected selenoproteins, while the overall reduction in selenoprotein expression was most and least affected in liver and testes, respectively. Dr. Hatfield stated that a cancer driver gene (*TGFα*) has been introduced into this mouse model and that studies investigating the effects of selenium deficient diets on these animals are underway.

In addition, Dr. Hatfield's laboratory is conducting studies on changes in selenoprotein expression using conditional knockout mice carrying a floxed Sec tRNA gene (i.e., a conditional knockout of the Sec tRNA gene), whose deletion is dependent upon Cre recombinase transgenes that are under the control of organ specific promoters. The Sec tRNA gene is selectively knocked out in breast, prostate, liver, or other tissues using Cre recombinase under the control of promoters specifically expressed in these tissues. Dr. Hatfield presented data that reflected changes in several selenoproteins (e.g., GPX1, GPX4, TR1, Sep15) in various tissues. For example, TR1 increased slightly in mammary epithelium, and dramatically in skin, while GPX1 was reduced in these tissues.

Dr. Hatfield reported plans to cross the conditional knockout mouse with a transgenic mouse carrying an SV40 T-antigen, which is known from studies in Dr. Jeffrey Green's laboratory to target breast and prostate tissues, in order to create a mouse model that has an altered selenoprotein population and may develop malignancies earlier than control mice carrying the SV40 T-antigen alone. Such a model should provide insight into the role of the selenoproteins in cancer prevention.

Relationships Between Chromatin Organization and DNA Methylation: Peter Jones

Peter A. Jones, Ph.D., D.Sc., Director at the Norris Comprehensive Cancer Center, University of Southern California, provided background information on DNA methylation, discussed the relationship of abnormal DNA methylation to cancer, and presented the results of several studies aimed at discovering why abnormal DNA methylation occurs. In DNA methylation, a methyl group is added to a cytosine base that is part of a CpG dinucleotide; approximately 4 percent of cytosine bases are methylated. The methyl groups serve as tags on DNA and change the interaction of proteins with DNA. The patterns of DNA methylation, which can be inherited, represent an "information coding system." Some CpG sites (normally methylated) are spread out in the genome, whereas other sites are clustered in "CpG islands." Dr. Jones explained that analysis of the sequences of human chromosomes 20, 21, and 22 showed that CpG islands occur within the 5' region of genes (often in promoters), in exons (a gene sequence that usually contains the protein-coding information), and in repetitive DNA. In the normal genome, CpG islands in the repeat sequences are methylated; however, CpG islands in the 5' regions tend to be unmethylated.

Dr. Jones pointed out that normally gene expression is controlled without methylation changes. He also stated that gene silencing by DNA methylation is a normal biological process with regard to X chromosome inactivation, imprinting, and intragenic parasites. In cancer (and in aging), abnormal methylation occurs. CpG island methyl groups move in the genome from the repetitive DNA to the exons (no consequent change in gene expression) and to the 5' regions, where methylation in the CpG island of a promoter results in the gene being permanently silenced. Many tumor suppressor genes have CpG islands within the promoters; these genes can be switched off by abnormal methylation, thus increasing cancer risk without the presence of a mutation. Dr. Jones used a slide to illustrate that numerous genes associated with pathways thought to be important for cancer development have been shown to be subject to abnormal methylation and silencing. He emphasized that abnormal methylation is a very common way for tumor suppressor genes to be switched off.

Dr. Jones summarized data from a study on the *p15* tumor suppressor gene and the *p16* gene, both of which are involved in the retinoblastoma (*Rb*) gene pathway, and the *PAX6* gene, a gene not related to cancer that was used as a control. Very little CpG island methylation in these genes was found for people who did not have cancer. However, in patients with either myeloid dysplastic syndrome (MDS) or acute myelogenous leukemia (AML), the exonic regions of all genes and the promoter of *p15* were methylated. In patients with colorectal cancer, exonic methylation was common even in the "normal" epithelium adjacent to the tumor. In colorectal tumors, however, *p15* was unmethylated, whereas *p16* was abnormally methylated. Dr. Jones presented a possible model of how these gene and methylation changes occur during aging and cancer development. He proposed that transcription factors block the access of methyl

transferase to the promoter region. Therefore, the exonic regions of genes, downstream of where transcription starts, are abnormally methylated first. If the transcription factors are then displaced, abnormal methylation also occurs in the promoter region, silencing the gene. Dr. Jones presented data obtained by Jean-Pierre Issa and colleagues illustrating the direct linear relationship between age and the level of abnormal methylation in CpG islands in the *ER* gene in colonic epithelium of people without cancer (age 25 years, −5 percent; age 65 years, −15 percent).

Dr. Jones introduced an important model developed about 5 years ago that shows how DNA methylation can change chromatin structure. Methylation encourages the binding of proteins to DNA; one of these proteins is methyl-CpG-binding protein 2 (MeCP2). The bound MeCP2 encourages the binding of corepressors and HDACs to the promoter region, resulting in loss of acetylation and consequent compaction of chromatin and repression of transcription. Dr. Jones explained that more recent research shows that methylation of the lysine residues of histone (e.g., lysine 9 of histone H3) can make acetylation of the histone more difficult to achieve, leading to more compact chromatin. Moreover, the methyl group on the lysine can act as a tag for binding proteins, which also leads to more compact chromatin. It has been postulated that methylation of the lysine residues of histone actually attracts CpG methylation to the promoter region, and that this works in concert with the fact that methylation of CpG islands attracts the binding of HDACs to the promoter region, resulting in more lysine methylation. Thus, these two processes may reinforce each other to keep chromatin in the compact inactive configuration.

Dr. Jones discussed results of a study in four different human cancer cell lines that each have three CpG islands in the *p14* and *p16* genes. Data showed that the level of CpG methylation tracked completely with the levels of histone lysine methylation and histone acetylation. That is, whenever CpG methylation occurred, binding of MeCP2 and methylation of lysine 9 occurred to the same degree. Using an “open, shut, and locked door” analogy to describe promoter “lockdown,” Dr. Jones speculated that methylation of the lysine residues of histones may occur first in this process (shuts the door). Then, CpG methylation takes place (locks the door). The binding of proteins to the DNA (e.g., MeCP2) and the reinforcing processes described above keep the door locked (compact chromatin), ultimately leading to gene silencing. He stated that gene silencing resulting from abnormal methylation most likely contributes strongly to cancer development. Dr. Jones suggested that it may be possible to reverse methylation changes through nutritional or pharmacological approachesCfor example, levels of S-adenosyl-methionine (SAM) might influence methylation.

Inhibition of Histone Deacetylase Activity by Butyrate: Jim R. Davie

Jim R. Davie, Ph.D., Professor in the Manitoba Institute of Cell Biology at the University of Manitoba, presented background information on both butyrate and histone acetylation and described studies in his laboratory that investigated the dynamics and consequences of histone

acetylation and the role that butyrate can play. In the mid-1970s, it was reported that butyric acid was a potent inducer of erythroid differentiation in cultured erythroleukemic cells. Other data indicated that, in cultured mammalian cells, sodium butyrate could inhibit proliferation, decrease DNA content, modify morphology, and either increase or decrease production of specific enzymes. Also, it was found that butyrate caused histone modification in HeLa and erythroleukemia cells and inhibited histone deacetylation in cell cultures. Histones are acetylated on their N-terminal regionsCthat is, on the histone "tails" that emanate from the nucleosome. Acetylation of the histone tails prevents the chromatin fibers from interacting with each other, resulting in a less compact chromatin structure. When tails are not acetylated, the more compact chromatin structure is relatively "invisible" to transcription. Histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs); in mammalian cells, three classes of HDACs exist. Butyrate is the most effective of the fatty acids in inhibiting HDACs.

Dr. Davie showed some data related to the global dynamics of histone acetylation. He reported that treatment with butyrate increased the level of acetylated histones, with 60-70 percent of the histones being engaged in dynamic acetylation in ER-negative breast cancer cells, but only 2 percent in avian immature erythrocytes. He said that the mechanism for inhibition of histone deacetylation by butyrate is not yet known. A study in ER-positive breast cancer cells found that estradiol slightly increased histone acetylation (2-3 percent); further investigation determined that estradiol did not affect acetylation rates (two rates exist: rapid, $t_{1/2}$ = 8 min; slow, $t_{1/2}$ = 320-350 min), but the rate of deacetylation was inhibited in the presence of estradiol.

Dr. Davie noted that sodium butyrate can lead to either repression (e.g., *cyclin D1*) or induction (e.g., *IGFBP-3*, *p21*$^{Waf1/Cip1}$) of gene expression; the genes affected by butyrate often have a butyrate response element, and the genes can be grouped by response element. For example, *IGFBP-3*, *p21*$^{Waf1/Cip1}$, *galectin 1*, and *Gai2* all have an Sp1/Sp3 response site in their butyrate response element. Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function either as activators or repressors. Research has shown that Sp1 forms multimers at its response site. To determine whether Sp3 interacts with Sp1 in the multimers, Dr. Davie and his colleagues developed an approach to release Sp1 and Sp3 from the nucleus of T5 breast cancer cells, followed by biochemical fractionation using immunoprecipitation. They found that Sp3 did not form complexes with Sp1. Further data indicated that HDAC activity associated with Sp3 was higher than HDAC activity associated with Sp1; both Sp3 and Sp1 formed complexes with HDAC1 and HDAC2, but not with HDAC3 (these are all class I HDACs). In addition, the HDAC2 bound to Sp1 and Sp3 was associated with protein kinase CK2 and was modified by this kinase through phosphorylation. This phosphorylation appears to be important for HDAC2 activity.

Dr. Davie proposed that butyrate inhibits Sp1/Sp3-associated HDAC activity, resulting in histone hyperacetylation and, consequently, a more open chromatin structure, leading to transcription of the *p21* gene. Using a slide illustrating the factors/pathways that contribute to cell cycle

progression from the G0/G1 phase to the S phase, he explained that *p21* activity (and p21 protein level) initially increase and then later decrease due to transcriptional repression of the *p21* gene during the progression from G0/G1 to S. The decrease in *p21* activity leads to the activation of other genes involved in S phase and cell cycle progression. If butyrate is added to the cell system and fosters *p21* transcription, the decrease in *p21* activity does not take place, and the normal progression from G0/G1 phase to S phase can be disrupted.

Genistein (GEN) Targeting: DINGG Proteins in Cancer and Osteoarthritis (Use of Intelligent Proteomics): Stephen Barnes

Stephen Barnes, Ph.D., Professor in the Department of Pharmacology and Toxicology at the University of Alabama, noted that several dietary polyphenols, including resveratrol, catechin, quercetin, and genistein, may reduce cancer risk. Genistein, the focus of his presentation, is an isoflavone found in soy that may influence risk for breast and prostate cancers. Epidemiologic data indicate that countries with high soy consumption have lowered risk for breast and prostate cancers. *In vitro*, genistein has been shown to inhibit growth factor-stimulated cell growth. In an animal study, genistein administered in the diet of rats perinatally and prepubertally reduced the number of chemically induced mammary tumors. The early life effects of genistein were very important for reducing risk. Dr. Barnes pointed out that genistein (and various other test substances) inhibits chemically induced mammary tumors in rats when given in a chow diet but not when given in a purified diet. He indicated that the mechanism(s)-of-action by which genistein might have a chemopreventive effect are not yet known. One possibility is that it acts as an estrogen.

Dr. Barnes briefly presented the results of a recent experiment (Naciff, et al., *Toxicol Sci* 2002;68:184-99) that used microarray analysis to determine changes in patterns of gene expression in the developing uterus of rats exposed to either genistein, 17∀-ethynyl estradiol (EE), or bisphenol A (BPA). Genistein brought about expression changes (dose dependent) in more genes (227, many downregulated) than either EE (26) or BPA (35). In previous studies, genistein both upregulated genes (e.g., progesterone receptor, interleukin [IL]-4 receptor) and downregulated genes (e.g., retinol binding protein, protocadherin-5).

To help determine how research should proceed in view of the knowledge that genistein can regulate gene expression, Dr. Barnes suggested that basic biochemistry provides an approach. That is, genes are transcribed to mRNA, MRNA is translated into proteins, and the proteins do the work in the cell. Therefore, for genistein to have a mechanistic role in the cell, genistein must have "target proteins," such as a receptor, a transcription factor, or an enzyme. Dr. Barnes next described the proteomics approach that he and his colleagues used to isolate a protein from human breast cancer MCF-7 cells that is a protein target of genistein. They built an affinity matrix in which genistein (as genistein-2-carboxylic acid) was covalently bound to agarose, while preserving all of genistein's hydroxyl groups. Then, MCF-7 cells were grown in culture

and lysed, the lysate was centrifuged to remove cell debris, and the supernatant (containing the protein) was passed over an anion-exchange column (Sephadex-DEAE) and adsorbed onto the 2-carboxygenistein-agarose affinity matrix. After careful washing, the bound protein was eluted with 1 mM 2-carboxygenistein and was then purified using an SDS-PAGE gel. The resulting purified protein, p38, was subjected to chemical N-terminal sequencing, and available databases were searched to identify related proteins. Related proteins (DING proteins) include: p205, in synovial fluid, is a T-cell attractant and stimulates fibroblast proliferation; p40, in neurons, binds cotinine (a nicotine metabolite) suggesting a signaling role; and p40, in turkey lung, is involved in bacterial adhesion to epithelial cells. The genes corresponding to these proteins have not been located. Dr. Barnes concluded by stating that the human genome database, although enormously helpful, does not tell the whole story, and that intelligent proteomics has a role in revealing the effects (some likely unexpected) of nutritional components. He commented that "it's amazing what we don't know."

Panel Discussion: Opportunities and Challenges for Future Nutrition Research in Cancer Prevention

Co-Chairmen: John A. Milner and Richard G. Allison
Panel Members: James G. Elliott, Vay Liang Go, Gary A. Miller, Cheryl Rock, Rabindra Roy, and Michael J. Wargovich

The Co-Chairmen, John A. Milner, Ph.D., Chief of the Nutritional Science Research Group in the Division of Cancer Prevention and Richard G. Allison, Ph.D., Executive Officer of the American Society for Nutritional Sciences, declared that the area of nutrition research in cancer prevention is an incredibly complex story and that even defining terms is difficult. They pointed out, however, that it is necessary to start somewhere. They introduced the panel members. Each panel member gave a brief presentation that outlined their individual recommendations regarding opportunities and challenges in cancer-related nutrition research. Following completion of all presentations, the Co-Chairmen, panel members, and the audience participated in a group discussion.

James E. Elliot, Ph.D., Research Leader, Human Nutrition Research Department, Roche Vitamins, Inc., pointed out that cancer is not a practical clinical endpoint; therefore, biomarkers for cancer prevention (and diagnosis) are needed. He focused on the role of genomics and proteomics in the discovery of biomarkers. Currently, biomarkers are used in the areas of environmental exposure, polymorphism identification, risk profile screening, nutrient-gene interactions, molecular epidemiology, disease mechanisms, early cancer diagnosis, and chemoprevention (as surrogate endpoints for cancer). Most of these biomarkers, however, have limitations. For example, specific biomarkers may be too far along the disease pathway; may not be in the direct pathway of disease (e.g., PSA); may have a low predictive value as a result of low specificity (e.g., PSA, colon polyps); and/or may not be sufficiently characterized or

validated for routine use. Dr. Elliott suggested that the synergy of genomic and proteomic technologies offers the potential to discover nutrient-gene interactions, as well as early biomarkers of disease for diagnosis and clinical trials. He used an overview slide to illustrate the possible relationships between nutrients (e.g., folic acid, selenium, lycopene, genistein, and others), dietary regulation, gene expression, metabolism, sophisticated analytical techniques, and biomarkers. Dr. Elliott said that a genomic approach to biomarker discovery can proceed along two pathways: it can focus on the disease state, identify the earliest genes involved, and use the genes as targets to identify nutritional agents capable of modifying their expression; or, it can focus on the healthy condition, examine effects of dietary components on global gene expression, and seek links between patterns of gene expression and disease development processes. He suggested that panels of biomarkers, rather than single biomarkers, may provide the best approach. Dr. Elliott proposed that the identification and validation of cancer-related biomarkers that are modulated by nutrients should be a major future research effort.

Dr. Wargovich suggested that the NCI should fund animal and clinical research that looks at the complexities of diet, including the interactions of dietary components. He commented that much of the discussion in this meeting was from a reductionist viewpointCthat is, focused on individual nutrientsCand expressed concern that research was moving away from the fundamental idea that diet is a complex interaction of nutrients. Dr. Wargovich said that he strongly favored using genomics/proteomics to identify cancer-susceptible animals or persons who are presymptomatic so that interventions could be tailored based on the individual risk profiles.

Vay Liang W. Go, M.D., F.A.C.P., Professor, Center for Human Nutrition, University of California, Los Angeles, showed a slide that illustrated the course of cancer over 30 years, from prevention (0-20 years) to treatment (20-30 years). He emphasized that the nutrient requirements for cancer prevention may differ from the nutrient requirements after cancer develops and then metastasizes. Dr. Go stated that it is important to characterize nutrient effects, including effects on gene expression, at different stages of the life cycle (e.g., *in utero*, adolescence) and in normal versus abnormal tissues from various stages of cancer. Dr. Go pointed out that cancer cells have limitless replicative potential, are self-sufficient with regard to growth signals, are insensitive to antigrowth signals, evade apoptosis, are able to invade tissue and metastasize, and sustain angiogenesis. He said that information is needed about how particular nutrients affect all of these cancer cell characteristics. Dr. Go added that the study of metabolism, including nutrient metabolism, is key to clarifying the nutrient-cancer relationship; cellular metabolic events may vary according to organ tissue type and cell status (normal vs. cancer). He indicated that reductionist approaches are very important, but that the knowledge gained from these approaches must be brought back to the human level. For example, instead of focusing only on soy (or genistein) in the Asian diet, it is important to remember that the Asian diet also contains rice, green tea, and is combined with exercise.

Rabindra Roy, Ph.D., Research Scientist, Department of Carcinogenesis and Molecular Epidemiology, American Health Foundation, focused on the role of DNA repair in cancer susceptibility and initiation. He said that ROS are constantly causing DNA damage and if the damage is not repaired, transcription may be inhibited. Dr. Roy briefly presented research in his laboratory, using a rat model for hepatocarcinoma in which a genetic mutation causes copper and iron to be accumulated in the liver, resulting in ROS generation. All of these animals develop hepatitis at early age (16-18 weeks), and develop hepatocarcinoma a year later. He said that the base excision repair (BER) capacity for oxidative DNA damage is significantly reduced in the acute hepatitis period of these animals; this results in the formation of DNA mutations. Dr. Roy proposed that the BER pathwayCthat is, changes in associated protein levels and protein-protein interactionsCcould be used as a biomarker pathway for early detection of cancer, as well as for chemoprevention. In addition, he proposed that nutrients could be used to target DNA repair. He added that his laboratory planned to test various nutrients (e.g., phenethyl isothiocyanate [PEITC], tea polyphenols, garlic, organoselenium compounds, ∀-tocopherol, curcumin, carotenoids) to determine if they can induce DNA repair capacity back to normal levels in the hepatocarcinoma rat model.

Cheryl L. Rock, Ph.D., R.D., Professor, Department of Family and Preventive Medicine, University of California, San Diego, began by quoting Dr. GreenwaldC"People eat food, not nutrients or dietary constituents." She stressed the importance of continuing to conduct clinical dietary intervention studiesCboth small studies and large-scale trialsCin cancer prevention and control, as well as the need to incorporate mechanistically oriented research into these studies. She highlighted specific issues associated with mechanistic research in clinical studies: methodologies must be sufficiently robust to be usable with stored samples; the tissue/sample measured must be feasible for inclusion in human studies; and, a demonstrable relationship should exist between the measured tissue/sample and the target tissue of interest. Dr. Rock reinforced Dr. Go's earlier statements that the timing of an effect in the continuum of cancer is of crucial importance with regard to testing and interpretation in human studies, and that it is important to consider whether a particular nutrient intervention is appropriate at any given stage of carcinogenesis. Also, she agreed that understanding the metabolism of compounds of interest is essential. In addition, Dr. Rock stated that the concentrations of nutrients used in cell culture studies and the relative degree of nutrient deficiencies in animal studies required to bring about an effect must be relevant to what the human body can achieve; otherwise, results are difficult to interpret and to translate to human cancer research.

Gary A. Miller, Ph.D., an independent consultant, stated that a dual approach to genomics and cancer prevention must be undertaken: with a public health perspective that focuses on helping the population-at-large manage cancer risk; and, programs that focus on helping individuals identify and deal with their particular cancer risk profiles. He agreed with Dr. Rock that a major challenge in nutrition and cancer prevention research is interpreting data from *in vitro* and animal studies in terms of its applicability to humans. He said that the prevailing clinical trials paradigm, is best suited to testing compounds that have big effects over short periods i.e. drugs.

However, foods and nutrients do not necessarily have rapid, large effects, rather they often have mild effects that are more likely to provide changes in the metabolic milieu conducive to prevention of disease. Dr. Miller suggested that NIH should try to develop a more appropriate clinical trial design that would facilitate testing and understanding the long-term effects of diet on cancer. Dr. Miller also agreed that it is essential to understand metabolism and the metabolic role of nutrients. He stated that studies in metabolomics are critical to an understanding of diet and health. He proposed that a future conference might include discussion of nutrition and metabolomics in cancer prevention.

Dr. Milner opened the group discussion by reiterating several major research needs identified by the panel participants. These included:

- Identification and validation of biomarkers for cancer;
- Investigation of the temporal relationship between nutrients and cancer risk; and
- Examination of possible tissue specificity of nutrients with regard to cancer.

After reminding the audience that an objective of the NCI is to help move the field of nutrition and cancer prevention forward by fostering research, Dr. Milner said that, unfortunately, the NCI is not receiving applications for probing types of studies. He indicated that this is a serious issue.

The following points were made during the remainder of the group discussion:

- The University of California, Davis, is creating a Center for Nutrigenomics in collaboration with the Children's Hospital Research Institute. The Center has a Web site (http://nutrigenomics.ucdavis.edu) and has started a listserv to help create a community of scientists interested in nutrigenomics. Individuals can register on the Web site if they wish to receive information (e.g., press releases) from the Center.

- The scientific review panels at the NIH currently are very "reductionist," which makes it difficult for studies in systems biology to receive approval. The participant suggested that a different type of review panel is needed for these studies.

- Clinical-metabolic studies are needed not only to address basic questions, but also to help integrate information from animal and epidemiologic studies. Many epidemiologic studies now include a biomarker component; clinical-metabolic data are necessary to test and validate these various biomarkers.

- Cancer survivors and patients are available and very willing to participate in studies that examine cancer-related effects of nutrients that are not cytotoxic.

- One participant commented that the NCI should foster funding that requires integration across departments. Dr. Milner responded that both a recent NCI Request for

Application (RFA) on molecular targets for nutrients and an upcoming RFA on DNA methylation include a requirement for collaboration between nutritionists and individuals with expertise in genetics/DNA methylation. Another participant commented that a single institution may have only one outstanding group within the institution, making integration across departments difficult. She proposed that the NIH should consider the possibility that a "virtual college" of groups around the country could be a way to satisfy the collaboration requirement for future RFAs. This type of approach currently is used for clinical trials.

● Although strong epidemiologic evidence supports a relationship between consumption of vegetables and fruits—particularly those rich in carotenoids—and reduced risk for cancer and heart disease, little evidence exists regarding which components of the vegetables and fruits are the active compounds. Scientists hypothesized that carotenoids (specifically, ∃-carotene) might be responsible for the beneficial effects; nevertheless, findings in intervention trials did not support this hypothesis. The participant who raised this point argued that a need exists to prepare extracts of whole plant foods, fractionate the extracts, and do high-throughput screening with activity-based assays of all the chemical constituents. Detailed mechanistic studies then could be conducted on the probable active constituents.

● Another participant pointed out that the issue of identifying active dietary constituents was raised in the 1980s, but was not received favorably by the NIH. He suggested that the issue needs to be revisited. He pointed out that vegetables and fruits have multiple species, and nutrient concentrations can vary among species. In addition, growing conditions can affect nutrient content; for example, catechin concentrations in teas grown in northern versus southern Japan can vary up to 10-fold. As another example, research has shown that 90 percent of the I3C content of cruciferous vegetables is determined by the growing conditions; only 10 percent is determined by the genes of the plant.

> ● Several participants raised the issue that research to determine all of the probable bioactive compounds in vegetables and fruits would be very expensive. Also, analysis of the enormous amount of data generated by this approach would be an issue. The United States Department of Agriculture (USDA) has been developing methods to analyze foods for many years. It was suggested that the food industry also needs to play a role in determining food composition; the tea, wine, and chocolate industries are actively seeking ways to fulfill this. A concerted, joint effort between government agencies and the food industry will be required.<
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> ● Not all participants agreed that the reductionist approach of conducting detailed studies on individual dietary constituents is the correct approach; they commented that research should focus on the total diet. As a compromise approach, one

participant suggested that mixtures of compounds could be screened for activity in high-throughput assays, and promising mixtures could be tested in clinical intervention studies. Another participant said that his research program already is investigating the effects of mixtures of terpenes. Data showed that genistein mixed with terpenes resulted in a strong, synergistic increase in apoptosis. Examination of the signaling pathways indicated that significant crosstalk was present among the compounds, emphasizing the importance of examining nutrient interactions.

- One participant noted that the definition of a nutrient as being "anything that causes a biological response" is too general; he commented that he was not sure how to define the term.

- Another participant suggested that imaging systems and computer modeling have potential applications in nutrition and cancer prevention research.

Closing Remarks and Adjournment: Young S. Kim

Dr. Kim thanked all of the participants for making the conference an overwhelming success. She announced that the proceedings of the conference would be published in the near future. The conference was concluded at 4:20 p.m. on September 6.