Diet, Epigenetic Events, and Cancer Prevention Symposium
September 26-27, 2007
Gaithersburg Marriott Washingtonian Center

Introductory Remarks

Sharon Ross, Ph.D., Program Director
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The Division of Cancer Prevention (DCP), National Cancer Institute (NCI), and the Office of Dietary Supplements (ODS), Office of the Director, NIH, sponsored the symposium *Diet, Epigenetic Events, and Cancer Prevention*, held September 26-27, 2007. This symposium represented a continuation of a previous trans-Department of Health and Human Services workshop on diet, methylation, and health, held in August, 2001. This earlier workshop gave rise to a number of publications as well as Requests for Applications (RFAs) that led to the funding of 10 projects in collaboration with ODS. Active funding opportunities are currently active in the areas of diet, epigenetic events, and cancer prevention.

The goal of this meeting was to discuss and critically evaluate the evidence for the impact of diet and bioactive food components (BFCs) on epigenetic processes (including DNA methylation, histone modifications, chromatin remodeling factors, and noncoding regulatory RNA), implications for cancer prevention, as well as next steps for advancing diet, epigenetic events, and cancer prevention research.

Peter Greenwald, M.D., Dr. P.H., Director
Division of Cancer Prevention
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The field of epigenetics and cancer prevention is an emerging one, although the idea of epigenetic aspects in cancer development is generally accepted. There is solid evidence that histone modification and other epigenetic processes affect gene expression. There also is evidence that food and dietary supplements influence gene expression as well as an individual's risk of developing cancer. Further understanding of the interaction between diet, genetics, and critical times for exposure during development and throughout the entire lifespan is needed. Animal models suggest that epigenetic effects during pregnancy can have an impact on the offsprings' cancer risk later in life. Bioactive food components (BFCs) are known to exert their effects over an individual's lifespan, with implications for effective use of these compounds for cancer prevention. The information presented at this meeting will help improve progress in basic, translational, and clinical research related to the use of BFCs in cancer prevention, and will provide information on new tools to facilitate epigenetic research.

Paul Coates, Ph.D., Director
Office of Dietary Supplements
Office of the Director
National Institutes of Health

Dietary supplements are usually versions of BFCs found in food, so similar questions arise concerning the effects of supplements on cancer risk. Information concerning the potential impacts of BFCs, both positive and negative, on health is needed because many people in the United States use significant amounts of dietary supplements. Accurate information is needed to fully inform consumers, healthcare providers, and researchers on the effects of these supplements. NIH funding of dietary supplements research is growing and now includes projects on epigenetics, nutrigenomics, and other relevant technologies and research areas. Since ODS exists within the Office of the Director of the National Institutes of Health (NIH), rather than within a distinct institute, it lacks direct funding authority. However, ODS partners with the NCI and DCP and other groups to support new research initiatives and co-fund grants relevant to research on dietary supplements.

John Milner, Ph.D., Chief
Nutritional Science Research Group
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Today, funding of epigenetics research has increased from approximately $500,000 to more than $13 million. Nonetheless, publications describing the relationship between diet, epigenetics, and cancer are few. Understanding epigenetics will help to define this relationship and explain an individual's response to diet, particularly in terms of cancer prevention. The effects of folate on methylation and epigenetics have been described, and other compounds likely modify the epigenome as well. Researchers in this field were asked to identify priority research areas and tools necessary to advance the study of BFCs and their effects, epigenetic or otherwise, on cancer prevention. Areas to address to clarify the effects of diet on cancer prevention include identification of relevant compounds, issues related to dose and timing of exposure, and nutrigenomics. Whether the effects of BFCs are independent or dependent on genetic polymorphisms remains to be determined. Epigenetic research also may help to identify individuals likely to develop cancer and those likely to benefit from nutritional cancer prevention strategies.

Epigenetic Events and Cancer Prevention: Changes in Early Neoplasia
James G. Herman, M.D.
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Changes in methylation, particularly in promoter regions, are commonly observed in a number of cancers. Transcriptional silencing associated with promoter methylation or histone deacetylation can have the same effect as coding mutations on crucial tumor suppressor genes. The enzymes that mediate hyper- or hypomethylation of DNA and modification of histone proteins are potential targets for cancer prevention by BFCs and dietary supplements.

A recent analysis of the Nurses' Health Study suggests that the BFC folate affects cancer development. In this study, a small decrease in colorectal cancer incidence was observed for women with increased dietary or supplemental folate intake. The duration of exposure was proportional to the degree of protection. Data from the Netherlands Cohort Study, which examined the effects of dietary folate and alcohol intake on colorectal cancer incidence, found slightly increased methylation of six genes known to be involved in colorectal cancer in the tumors of participants with low methyl donor intake.

In lung cancer, an increasing number of genes have been found to be hypermethylated in their promoter regions, leading to gene silencing. The gene p16, which regulates RB and the cell cycle, shows increased methylation associated with progressive histologic changes leading to squamous cell lung cancer. A distinct form of lung cancer, adenocarcinoma of the lung, is increasing in frequency but is less associated with smoking than is squamous cell carcinoma of the lung. Similar to squamous cell lung cancer, increased methylation of the promoter regions of 17 genes, such as p16, MGMT, APC, and RUNX3, was observed to be associated with progression from normal tissue to atypical adenomatous hyperplasia (AAH), believed to be a precursor to adenocarcinoma, to adenocarcinoma of the lung. The frequency of methylation was proportional to the degree of malignancy. Multifocal AAHs from the same patients showed distinct patterns of promoter hypermethylation, indicating a divergent epigenetic field effect.

Many non-squamous cell lung carcinoma cell lines have evidence of active Wnt signaling. Antagonists of Wnt activity such as APC, DKK1, and RUNX3 were silenced, which correlated with promoter hypermethylation. Promoter hypermethylation increased with degree of malignancy, and thus could be predictive for lung cancer progression. A similar analysis showed that methylation of the p16 and APC loci appear to be predictive for progression of Barrett's esophagus to esophageal cancer.

Promoter methylation is a common event in cancer and is related to tumor progression. Genes involved in regulation of the cell cycle, apoptosis, DNA repair, relevant signaling pathways, and invasion are differentially methylated during progression from normal tissue to carcinoma. The frequency and timing of the methylation changes suggest that they may be useful for early detection or for risk assessment and may also be related to dietary or environmental exposure.

Discussion

Dr. Herman explained that in the lung cancer studies, differences in methylation between lung adenocarcinoma samples from smokers versus nonsmokers could not be determined because this work used archived samples. However, previous studies have shown that p16 is more highly methylated in smokers and is increasingly methylated in squamous cell lung cancer.

Dr. Asad Umar (DCP, NCI) asked why p16 and APC but not MGMT show methylation changes in Barrett's esophagus, given that dietary and environmental factors likely affect its development. Dr. Herman answered that MGMT shows early methylation changes that are not related to progression. Methylation changes at the MGMT locus may permit mutations to occur in other genes, such as K-ras or p53. MGMT methylation is not predictive because this event

occurs early and events secondary to MGMT methylation may be needed for cancer to develop. The mechanism by which this occurs is currently unknown.

Dr. Mukesh Verma (Division of Cancer Control and Population Sciences [DCCPS], NCI) asked why reports on hypomethylation are not common. Dr. Herman explained that global loss of methylation at repetitive elements could lead to aberrant expression of retroviral genes, but it can be difficult to determine the impact of global hypo- or hypermethylation on cancer. Genome-wide approaches will help link hypomethylation events to cancer.

Dr. Steven Belinsky (Lovelace Respiratory Research Institute) noted that recent epidemiological studies have shown that adenocarcinoma incidence is increasing and the link to smoking is growing stronger. Despite observing methylation changes at 50 genes, no "magic target" indicative of cancer development in non-smokers has been identified. More information about the etiology and risk factors for people who have never smoked is needed.

Dr. Roderick Dashwood (Oregon State University) asked Dr. Herman to comment on the apparent ability of folate to enhance growth of established tumors, yet function protectively in a primary prevention manner, in the absence of precancerous changes. This suggests opposing beneficial *vs* deleterious effects for folate in primary *vs* secondary prevention. Dr. Herman speculated that the impact of folate on cancer development may depend on which hypo- or hyper-methylation events have already occurred. If carcinogenesis has progressed sufficiently, folate may not be protective.

A participant asked for information on the proportion of cells in a tumor that are hypermethylated. Dr. Herman answered that most lesions are not pure but instead contain normal tissue, from which most of the signal is derived. Methylation is associated with gene silencing; clonal selection may contribute to heterogeneity in tumors.

Dr. Milner (DCP) asked whether the cells surrounding the tumor represented a second target for cancer prevention. Dr. Herman acknowledged that field defects were common in lung cancer and that second primary cancers are the leading cause of death for lung cancer patients. To develop a tissue-specific surrogate marker, different samples (i.e., tissues, fluids, etc.) likely will be needed to identify field defect changes or other events.

Session I: Are There Critical Times of DNA Methylation and Potential Modification by Dietary Factors?
Moderator: Johanna Dwyer, D.Sc. (ODS, NCI)

Maternal Nutrient Supplementation Counteracts Bisphenol A-Induced DNA Hypomethylation in Early Development
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Epigenetic mechanisms can mediate gene-environment interactions that allow environmental factors to affect health and disease. Epigenetic variability, genetics, and environment likely interact to have an impact on disease susceptibility in complex disorders such as asthma, cardiovascular disease, and schizophrenia. Environmental factors such as diet can cause epigenetic changes. Determining critical windows of vulnerability to relevant environmental exposures is crucial to understanding the full impact of these factors on health.

The viable yellow agouti (A^{vy}) mouse model can serve as an epigenetic biosensor to determine how maternal exposures affect the fetal epigenome. Coat color variation in these mice is related to epigenetic marks established early in development. Yellow mice are completely unmethylated at the A^{vy} locus, which allows ectopic expression of the agouti locus throughout life, giving rise to a variable yellow coat color and increased rates of diabetes and tumors. If completely methylated, ectopic expression of A^{vy} does not occur and the mice are brown in color with decreased risk of obesity, diabetes, and cancer. A^{vy} is a metastable epiallele, which is an allele that is variably expressed in genetically identical individuals due to epigenetic modifications that are established early during development. Metastable alleles show potential for transgenerational inheritance in addition to mitotic inheritance and are targets for environmentally induced epigenetic modifications of the fetal epigenome.

Genistein is a plant phytoestrogen found in soy and soy products that has mixed pro- and anti-estrogenic effects depending on tissue, dose, and timing of exposure. The putative protective effective of soy against cancer in Asian populations is thought to be due to the isoflavones, including genistein. To determine whether genistein exerts protective effects by modifying the fetal genome, non-agouti female mice were fed a genistein-supplemented diet 2 weeks prior to mating with heterozygous agouti males. Offspring of female mice fed the genistein-supplemented diet showed a shift toward the brown coat color. Site-specific methylation analysis showed an increase in methylation at six CpG sites at the A^{vy} locus. Methylation at CpG site 4 was principally responsible for the effects of genistein supplementation on coat color. DNA methylation profiles across a number of tissue types and times of development showed that the effects of genistein occurred before germ layer differentiation. Analysis of body weight found that the offspring of the unsupplemented females were more likely to be obese.

The effects of maternal exposure to bisphenol A (BPA), an endocrine active compound commonly found in polycarbonate plastics, also was analyzed using the A^{vy} model. Similar to the genistein study, female mice were fed a diet containing high amounts of BPA prior to mating. Offspring of mothers fed the BPA diet showed a shift toward yellow coat color. Mice exposed to BPA *in utero* also had decreased methylation at the A^{vy} locus. Methylation rates were highly correlated across germ layers, indicating that BPA exerts its effect early in development. Maternal exposure to BPA also decreased methylation at another metastable epiallele, the CDK5 activator protein $Cabp^{IAP}$, indicating that BPA promotes hypomethylation at multiple metastable epialleles. Analysis of methylation changes at other metastable epialleles is ongoing.

To determine whether exposure to methyl donors such as folate could counteract the effects of BPA, mice were fed a methyl donor-supplemented diet along with BPA. There was no difference in coat color distribution or DNA methylation between control animals and those fed BPA plus methyl donors, indicating that methyl donor supplementation can counteract the

effects of BPA. Feeding mice a genistein-supplemented diet together with BPA also resulted in a coat color distribution and DNA methylation pattern similar to the control animals. Parental nutritional supplementation thus could be considered as a preventive intervention approach for counteracting deleterious environmental influences on the fetal epigenome. The A^{vy} model also might be useful as a biosensor for other environmental exposures, including other BFCs, low-dose radiation, environmental tobacco smoke, or pharmaceuticals and anesthetic agents.

Discussion

A participant asked about the timing of genistein supplementation. Dr. Dolinoy clarified that the mice were fed the supplemented diet from 2 weeks before mating until after weaning. Thus, the critical days of exposure are now known, but because methylation changes are observed across all germ layers, the effect likely occurs early in development.

Another participant asked whether BPA had dose-response effects. Dr. Dolinoy explained that these experiments used only a single dose of BPA. The dose used was significantly higher than that at which low-dose BPA effects are observed; the experiment will be repeated to determine the effects of lower doses of BPA.

Dr. Robert Waterland (Baylor College of Medicine) asked Dr. Dolinoy to describe approaches for finding metastable epialleles in humans. Dr. Dolinoy explained that a genomic microarray approach is underway to identify additional metastable epialleles in mice. Genes with agouti expression profiles but high variability across individuals have been identified. It will be difficult to identify metastable epialleles in humans, although there appear to be many candidates that may show imprinting or parent-of-origin effects.

Dr. Cornelia Ulrich (Fred Hutchinson Cancer Research Center) asked Dr. Dolinoy to describe potential molecular mechanisms that could explain the effects of genistein or BPA on methylation. Dr. Dolinoy answered that the link between BPA exposure and changes in methylation is currently unknown. Non-phytoestrogen estrogens appear to cause DNA hypomethylation, in contrast to genistein which has estrogenic activity. The effects may occur through scavenging of free radicals rather than through the estrogen pathways.

A participant asked whether color changes could be observed in yellow mice fed a methyl-deficient diet. Dr. Dolinoy answered that changes in coat color would not be observed but changes in obesity and cancer rates could occur. Brown mice fed a methyl-deficient diet could be examined for changes in methylation at the A^{vy} locus or for changes in bodyweight.

A participant asked whether multiple pathways are affected by genistein in this model. Dr. Dolinoy answered that changes in methylation at the A^{vy} locus result in changes in gene expression. Expression of other genes probably is affected, but genome-wide approaches will be needed to determine this.

Effects of methyl donors on the germline epigenetic stability of the A^{vy} allele

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Epigenetics mediates interactions between genes and the environment; naked DNA does not act on its own. Although methylation at CpG islands is a common focus of epigenetic studies because it is a covalent modification of DNA and thus convenient to analyze, this is only one of a number of epigenetic modifications that work in concert with modification of histones and other chromatin proteins. Changes in the structure of chromatin have profound affects on gene expression and a number of elaborate modifications ensure that transcription initiation is normally suppressed, except at certain sites. CpG methylation recruits histone deacetylase (HDAC) activity, which is dependent on methylation of histone H3 lysine 9 and is strongly associated with suppression of transcription initiation.

Controlling elements are transposable elements that are subject to epigenetic effects and can disrupt normal gene regulation in the vicinity of their integration sites. The epigenetic state of a controlling element determines its ability to disrupt regulation of a nearby gene; usually the active state produces disruption. Disruption may involve either inappropriate activation or suppression of the controlled gene. Because the controlling element is subject to epigenetic silencing, these elements can act in mosaic, tissue-specific, heritable, or inducible patterns. Mammalian genomes contain many potential controlling elements, such as A^{vy}, which may affect phenotypic variation. The variation in coat color observed in otherwise isogenic A^{vy} mice is due to variations in methylation at this locus and is an example of a "disease risk" that is entirely epigenetic. The epigenetic state of A^{vy} is highly unstable in the germline, but there is weak retention of the epigenetic state in the female germline.

A^{vy} serves as a model of epigenetic involvement in fetal programming. When pregnant mice are fed a diet supplemented with methyl donors such as folate, choline, betaine, and vitamin B12, beginning 2 weeks before mating and continuing throughout pregnancy and lactation, their offspring are more likely to carry a silent, methylated A^{vy} allele; mice from these litters are more likely to be brown in color and less prone to obesity and diabetes. The epigenetic effect of the supplemented diet (silencing of the allele) was observed only when the A^{vy} allele was transmitted through the father. To further investigate the heritability of methyl donor supplementation, a pseudoagouti female offspring from this experiment was bred and supplemented with methyl donors only during mid-gestation to target only the primordial germ cells that would give rise to the F2 generation, without affecting the F1 generation. Surprisingly, the F1 generation was affected by mid-gestation supplementation; a shift toward brown or pseudoagouti coat color was observed. Again, the epigenetic effect of supplementation was observed only when the A^{vy} allele was transmitted through the father. This indicates that an environmentally induced effect on epigenotype can survive the epigenetic resetting events that occur between generations and that the mother's diet may influence the phenotype of several succeeding generations.

To determine the cumulative effects of maternal methyl donor supplementation over generations and to determine if the inheritance of the methyl donor effect lasts for more than one generation,

pseudoagouti males were bred to produce subsequent generations, continuing until the pattern of inheritance of the A^{vy} allele stabilizes. Results from the F0 to F4 generations show a trend for decline in the numbers of mice with yellow coats, raising the possibility that stable extinction of the A^{vy} phenotype could occur.

To explore mechanisms by which methyl donors silence the A^{vy} locus, bisulphate allelic sequencing of this locus found that CpG methylation of this region was incomplete, even in mice in which A^{vy} was transcriptionally silent. *In utero* exposure to methyl donors also did not increase the density of CpG methylation in either the F1 or F2 generations. This suggests that methyl donor supplementation does not directly increase methylation of A^{vy}; instead, the locus is more likely to become methylated and may shift to a silent state in early embryogenesis.

Discussion

Dr. Jean-Pierre Issa (MD Anderson Cancer Center) asked whether transmission of the A^{vy} allele had been analyzed in the absence of methyl donor supplementation. Dr. Martin answered that this had not been done, because the paternal effect was not initially recognized. A current goal is to derive an A^{vy} line with an extinguished phenotype, without supplementation. It is possible, although doubtful, that A^{vy} silencing is not mediated by methyl donor supplementation. Dr. Issa asked about the potential effect of strain background on A^{vy} silencing. Dr. Martin answered that strain background does have an effect, because some strains are more likely to have agouti offspring. The strain used for these experiments is well-defined and relevant strain-specific modifiers of the somatic state are known.

Dr. Belinksy asked whether this work supported the idea of methylation changing expression of tumor suppressor genes, but not as a rate-limiting effect. Dr. Martin answered that the methylation patterns observed at the A^{vy} allele are unusual, and he suspects they are a reflection of the underlying epigenetic states that may be transmitted through the germline.

Dr. Waterland described a similar study in which transgenerational exposure was studied via transmission of the A^{vy} allele through the female germline. In this case, there was no evidence of inheritance of effects. Dr. Martin answered that only pseudoagouti mice were bred in this study because these mice likely carried a silenced A^{vy} allele in their germ cells. Dr. Waterland's study involved mainly breeding of yellow mice; because the inheritance and methyl donor effects are weak, increasing the number of mice used in this study likely would reveal a trend toward brown coat color. Dr. Martin said that it is difficult to demonstrate that methylation specifically is the inherited mark, but efforts are underway to create embryonic stem cell lines to find hypermethylated alleles.

A participant commented that offspring receive differential nutrition based on their position in the uterus and that this could affect distribution of the A^{vy} allele. Natural selection also may have a role when pseudoagouti mice are maintained on a high methyl donor diet and then re-bred. Dr. Martin noted that genetic selection does not occur because the strain is isogenic. Additionally, breeding females are drawn from the general mouse colony, not from the experimental groups.

Modulation of Colorectal Carcinogenesis by Dietary Folate: Other One-Carbon Micronutrients and Age as Essential Co-Determinants

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Low folate intake is associated with increased risk for cancer, particularly colorectal cancer. The impact of folate on cancer risk is affected by family history; data from the Nurses' Health Study showed that folate had a robust protective effect primarily on women with primary relatives who had colorectal cancer. Thus, folate inadequacy is not in itself carcinogenic, but rather enhances the likelihood of developing colorectal cancer if there is an underlying predisposition. Folate may modulate carcinogenesis by altering methylation of nucleic acids and/or other macromolecules, resulting in alterations in the stability and integrity of DNA. Folate also is a cofactor for synthesis of purines and thymidines needed for DNA synthesis. Low folate levels can lead to misincorporation of uracil rather than thymidine into DNA, leading to mutagenic effects and DNA strand breaks during DNA repair.

Folate and vitamins B2, B6, and B12 participate in one-carbon metabolism and are biochemically interdependent. A number of epidemiological studies have suggested that these one-carbon nutrients all impact cancer risk. Several surveys also have shown that aging is associated with marginal levels of these nutrients, as well as increased cancer risk. To determine whether multiple, mild one-carbon nutrient deficiencies work synergistically to create a pro-carcinogenic milieu in the colon, C57Bl6 mice were fed diets mildly depleted in folate alone, or depleted in folate and one or all of vitamins B2, B6, and B12. None of the single or double depletion states had an effect on global genomic DNA methylation in colonic mucosa. However, in multiply vitamin deficient mice, a greater than 50 percent decrease in methylation was observed.

The Wnt signaling pathway is involved in colorectal carcinogenesis. Under normal conditions, β-catenin is degraded. In colorectal cancer, defective APC prevents β-catenin degradation and permits translocation of β-catenin to the nucleus, where it promotes transcription of pro-transformation genes such as c-myc and cyclin D1. Folate depletion did not affect APC promoter methylation. However, folate depletion was associated with a trend toward and increases in DNA strand breaks in the APC mutation cluster region (MCR) that also was associated with decreased levels of APC in the mucosa; these trends were more significant in the multiply deficient mice. β-catenin protein expression in colonic mucosa also was increased in the multiply deficient mice, as was nuclear translocation of β-catenin and cyclin D1 expression; several components of the Wnt signaling pathway were increased in a pro-transformation manner. Histologic examination of the colonic epithelium of these mice showed that mild depletion of multiple B vitamins was associated with a decrease in apoptosis.

Age also may be a co-determinant of carcinogenesis associated with one-carbon nutrient status. Uracil incorporation is more likely to occur in colonic DNA of older versus younger rats in the presence of folate deficiency. Given identical levels of dietary folate, older colon has 50 percent less folate than colon of younger animals. Supplementation with folate increases colonic folate

levels to that observed in younger animals, but plasma folate levels remain the same in old and young rats at any level of dietary folate. One key difference is a lack of methyl THF in folate-depleted old rats, but only a modest decrease in methyl THF in depleted younger rats. In mice, increased dietary folate results in higher increases in promoter methylation at the colonic p16 gene promoter in older versus younger animals. Thus, the colonic mucosa of older animals is more vulnerable to qualitative and quantitative changes in folate metabolism compared to younger animals, which translates to an increased susceptibility to DNA base substitution and changes in promoter methylation. In humans, 2 months of folate deprivation resulted in no change in plasma folate, but a 55 percent decrease in colonic folate levels, indicating that a minor degree of folate depletion, as determined by plasma folate levels, is magnified in the colon. Folate depletion is magnified in the colonic mucosa of both animals and humans, regardless of age; this could be attributed to the high proliferation rate of the colonic epithelium.

Discussion

Dr. Issa asked if a 50 percent decrease in genomic DNA methylation in colon was observed in the multiple vitamin B deficient mice. Dr. Mason answered that on an absolute level, a decrease in genomic methylation was observed. The ability to determine absolute genomic methylation is affected by the technology used to detect methylation. Use of liquid chromatography/mass spectrometry (LCMS) for detecting absolute genomic methylation is new; earlier studies relied on the SssI method. Changes in colonic DNA methylation have been observed using this method, but because it is semi-quantitative, it cannot be determined if the drop in absolute methylation was 50 percent. A drop as dramatic as this probably would not be observed in humans.

Dr. Ulrich asked Dr. Mason to comment on the similarities and discrepancies between manipulation of one-carbon metabolites in mice versus humans. Dr. Mason answered that the animals in this study had adequate levels of methionine. Because the human study involved only folate depletion, the effects of multiple one-carbon nutrient depletion in humans is unknown. However, enough similarities exist between rodents and humans that the results of the mouse studies are somewhat translatable to humans; multiple B vitamin deficiencies in humans are likely to have similar effects.

Dr. Milner noted that the colon appears to be the most vulnerable or responsive organ and asked whether other tissues had been examined. Dr. Mason explained that the colon is vulnerable because of the high proliferative rate of mucosal epithelium, which turns over every 72 to 96 hours. This tissue sustains high rates of DNA replication and thus is sensitive to any disruption of DNA synthesis. It is likely that any highly proliferative tissue would be similarly sensitive to B vitamin depletion. Plasma does not reflect changes in dietary B vitamins, and thus is inadequate for a biomarker. Skin scrapings also may not be useful, because surface skin cells are not highly proliferative and thus also would probably not reflect changes in B vitamin levels.

Reversal of Hypermethylation and Reactivation of Genes by Dietary Polyphenolic Compounds

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Polyphenolic compounds are commonly found in the human diet and may affect DNA methylation by inhibiting DNA methyltransferases (DNMT) and by other mechanisms. Promoter hypermethylation and inactivation of a number of genes increases during the progression from normal epithelium to esophageal squamous cell carcinoma. In the esophageal squamous cell carcinoma cell line KYSE 510, these events can be reversed by 2'-deoxy-5-azacytidine (DAC), which is an inhibitor of DNMT. Most DNMT inhibitors are toxic, and less toxic demethylating agents for therapeutic efforts are needed. Epigallocatechin gallate (EGCG) is a major polyphenolic compound in green tea that inhibits DNMT1 activity by docking in its active site. EGCG can reverse CpG island hypermethylation and reactivate methylation-silenced genes, including PAPβ, MGMT, p16, and hMLH1 in KYSE 510; re-expression of both mRNA and protein was observed. The effects of EGCG on p16 are maintained for up to 40 days after completion of a 10-day exposure period; MGMT levels decrease more rapidly. EGCG also synergizes with HDAC inhibitors to increase mRNA expression of RARβ and p16 in esophageal carcinoma cell lines.

Results from other laboratories have shown that EGCG is not a robust inhibitor of DNMT nor is it a strong reactivator of gene expression. EGCG activities also are likely to be cell line-specific. To identify DNA methylation targets, a CpG island array was used that allowed probing of 14,507 unique genes. Using this approach, 22 genes hypomethylated in KYSE 510 cells treated with EGCG were identified and are currently being validated. Some of the hypomethylated genes are expressed in normal esophageal epithelium and others are known to be expressed in Barrett's esophagus. A number of other polyphenols inhibit DNMT1 activity, but more weakly than EGCG. Genistein was a weaker inhibitor of DNMT1 than EGCG, but more effectively reactivated methylation-silenced genes, including p16, RARβ, and MGMT. Demethylation could be enhanced by extending the treatment period with EGCG or genistein or by combination with a HDAC inhibitor. Genistein inhibits DNMT by both competitive and noncompetitive inhibition and has a larger K_I than EGCG. Chromatin immunoprecipitation assays of the promoter regions of RARβ and MGMT genes with anti-acetylated H3 and H4 antibodies showed that inhibition of histone deacetylation may also play a role in reactivation of gene expression by genistein.

Administration of 0.32 percent EGCG in drinking water to mice inhibited S-adenosyl methionine (SAM) levels in the intestine but not in the liver. S-adenosyl homocysteine (SAH) levels were not affected. However, a single high (2000mg/kg) i.g. dose of EGCG elevated plasma levels of homocysteine and decreased levels of methionine at 30 minutes and 3 hours after treatment; liver SAM and SAH levels also were decreased.

Discussion

Dr. Yang explained that the effects of EGCG or genistein on DNMTs other than DNMT1 have not yet been analyzed.

Dr. Dolinoy commented on critical windows of vulnerability and asked if the effects of genistein described here, which were different than her results, could be related to the timing of exposure or to *in vivo* versus *in vitro* conditions. Dr. Yang acknowledged that all of these conditions could cause the different results.

A participant asked if genistein was methylated *in vitro*. Dr. Yang explained that catechols are more readily methylated, but genistein is not methylated. EGCG is methylated *in vivo*. Another participant asked how stabilization of EGCG affected reactivation of gene expression. Dr. Yang explained that stabilization of EGCG resulted in stronger reactivation of p16, but not of MGMT. Dr. Issa noted that 1mM EGCG has had limited effects on promoter methylation, which implies alternative explanations for its effects. Additionally, RARβ does not have CpG islands in its promoter. The effects of EGCG described here could be related to activation of an upstream signaling pathway rather than a direct effect on DNA methylation. Dr. Tim Huang (Ohio State University) commented that treatment of MCF7 cells with genistein results in both hypo- and hypermethylation of genes.

Dr. Milner noted that recent data suggests that Asians and Asian Americans benefit from dietary soy because of the formation of equol in the intestine and asked Dr. Yang if he had analyzed the effects of equol. Dr. Yang answered that he had not performed this analysis.

Panel Discussion

Dr. Dashwood noted that only 5 to 10 genes are expected to be regulated by imprinting and epigenetic means in humans; thus, information from mouse models may not be informative. Dr. Dolinoy countered that this conclusion was drawn using a bioinformatics approach to predict imprinted genes in mice and humans, which found only a 20 percent overlap in predicted genes. Identifying imprinted or epigenetically regulated genes in humans is critical, and the agouti mouse model is valid as an epigenetic biosensor because similar genetic alterations would occur in mice and humans, albeit in different genes.

Dr. Irfan Rahman (University of Rochester) commented that susceptibility appears to impact the effects of dietary supplements. Mice are differently susceptible to environmental influences compared to humans, and differences in the effect of methylation or methylation reversal related to mouse strain have been observed. The impact of background differences in humans should be considered. Dr. Mason answered that Sprague Dawley rats were originally used for studies of methylation; effects on the colon in rats were found to be similar to results in mice. However, although some results may be translatable, all that is observed in animals may not be observed in humans.

Dr. Dwyer asked participants to address how timing of dietary exposure affects modifications, when cells are most susceptible to modification, whether dose matters, and whether effects are

translatable through generations. Dr. Martin answered that the effects may be translatable through generations in humans, but it is unknown whether this is relevant to human biology. These studies do show that it is possible for an environmental agent to affect specific loci in the genome and that changes can be transmitted to subsequent generations. Dr. Mason commented that diet can modify age-related changes in DNA methylation. Within the constraints of animal models, varying dietary folate in older animals affects several aspects of one-carbon metabolism, but this was not observed in younger animals. Dr. Yang observed that the Linxian population was probably marginally deficient in folate and was more susceptible to methylation changes and inflammatory effects.

Dr. Martin stated that there is no evidence that alterations in the methyl donor pool directly methylate DNA. It is more likely that the observed effects on DNA methylation are caused by modifications of histones or other chromatin changes that subsequently cause changes in DNA methylation patterns. In cancer, there is poor evidence of disruption in methylation machinery, but strong evidence of changes in DNA methylation patterns. Dr. Mason agreed that dietary restriction or supplementation may not modify methylation processes directly, but instead may affect other mechanisms that feed back on the methylation process.

Dr. Ulrich noted that differences between global versus site-specific promoter changes in methylation must be distinguished. Global methylation status appears to be linked to deprivation states, whereas site-specific methylation is probably more regulated. Dr. Martin noted that global methylation consists mostly of methylation at retrotransposons, which is directed by histone modifications. Dr. Issa countered that most CpG sites that are methylated in the human genome are not located within retrotransposons.

Dr. Rahman asked how results of experiments on dietary polyphenols in cell lines could be extrapolated for determining safe and effective doses in humans. Dr. Yang answered that *in vivo*, 10-20 uM EGCG is needed to see activity. Timing of treatment and determining the amount of EGCG that enters the cell also must be analyzed.

Session 2: Examining Diet and DNA Methylation Patterns in Human Population and Interventions Studies. What Is Needed To Move Forward?
Moderator: Asad Umar, Ph.D., D.V.M. (DCP, NCI)

Epigenetic Epidemiology of Obesity: Application of Epigenomic Technologies
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Obesity prevalence has increased dramatically in the past 20 years and environmental exposures and epigenetic events occurring at crucial times during development may contribute to this increase. The developmental origins hypothesis suggests that adaptive responses to early nutrition lead to metabolic imprinting, with persistent effects that could be mediated by epigenetic changes. One possible mechanism for metabolic imprinting could involve nutritional

influences on DNA methylation. Methylation requires dietary methyl donors and cofactors and tissue-specific patterns of CpG methylation are established during development, implying a role for nutrition during development. Methylation marks are mitotically heritable and maintained throughout life.

A^{vy} mice are genetically identical, but can have different coat colors based on the degree of methylation at the A^{vy} locus, which can be altered by supplementation with methyl donors during development. Specific transposable elements within the A^{vy} locus induce epigenetic metastability, which allows maternal diet to influence the epigenotype. This system is relevant to humans, because transposable elements such as long interspersed elements (LINEs) and short interspersed elements (SINEs) comprise more than 45 percent of the human genome. Besides shifting coat color, epigenetic dysregulation at this locus can lead to adult onset obesity and type 2 diabetes in these mice. In humans, Prader-Willi syndrome is characterized by obesity in childhood and is caused by an epigenetic mutation.

The theory of transgenerational perpetuation of obesity suggests that differences in the intrauterine environment of obese females can cause developmental alterations in the fetus that may predispose it to obesity. The predisposition to obesity can be perpetuated and amplified transgenerationally. An A^{vy} transgenerational obesity study was performed to test the effects of maternal obesity on offspring obesity and whether the effects could be modified by methyl donor supplementation. The A^{vy} allele was passed through the female germline for three generations and two separate populations of mice were maintained on either a control or methyl-supplemented diet. The adult body weight of these mice increased with successive generations, with 56 percent of the F3 mice weighing over 55 grams. The differences in body weight were due to increased adiposity in the heavier mice. This increase in body weight could be prevented by methyl supplementation. Adult bodyweight was independent of the A^{vy} epigenotype, suggesting involvement of an epigenetic mechanism that does not involve the A^{vy} locus directly. Thus, A^{vy} may be a susceptibility locus, but changes at other loci also are needed.

This work demonstrates that different sources of epigenetic variation, including genetics, epigenetics, developmental stochasticity, and environmental influences likely interact to influence body weight. Maternal obesity during pregnancy and/or lactation may induce epigenetic alterations that perpetuate obesity in offspring. Challenges to understanding this mechanism include defining tissue-specific epigenetic regulation, the importance of specific exposures, the complexity of interacting epigenetic modifications, and the poor characterization of epigenetic regulatory regions. Methods have been developed to perform genome-wide scans for methylation, including methylation-specific amplification (MSA) microarrays, to help identify epigenetic modifications associated with obesity. MSA microarrays have been used to identify genes that undergo methylation changes during early postnatal hepatic development. Approximately 20 genes were identified that had a greater than 2-fold increase in methylation and 50 genes with a greater than 2-fold decrease. For 90 percent of currently tested genes, postnatal methylation changes correlate with expression. MSA microarrays can identify subtle changes in locus-specific CpG methylation and thus provide a tool that permits study of epigenetic alterations associated with obesity and environmental influences on these processes.

Discussion

Dr. Waterland explained that the transgenerational effect on A^{vy} was observed at weaning, but not in the nonagouti mice, suggesting that the transgenerational component requires A^{vy}, but the crucial event does not occur at this locus. Dr. Martin speculated that the agouti locus could sensitize the mice to environmental insults and tumor development.

Dr. Ulrich asked if adipose tissue samples from her randomized controlled trial on exercise and diet in overweight women would be useful and asked if Dr. Waterland had examined mouse adipose tissue. Dr. Waterland answered that analyzing epigenetic effects at the hypothalamus was of more interest. Analysis of adipose tissue is complicated by contamination with stroma; most adipose tissue DNA is actually from the stroma. Dr. Ulrich explained that flow cytometry could enrich the samples for adipocytes.

Dr. Joshua Miller (University of California, Davis) asked if implementation of recommendations for increased folic acid supplementation for pregnant women along with general folic acid supplementation of the food supply could affect the obesity increase, either accelerating it or mitigating it. Dr. Waterland answered that the mice in his studies were supplemented with folic acid, betaine, choline, and vitamin B12, but it is unknown which of these is key to the transgenerational obesity increase. Unpublished data have shown that folic acid alone is insufficient to change coat color. SAM and homocysteine also have been supplemented, but do not appear to augment the effect of folic acid alone.

Epigenetic Epidemiology of Colorectal Cancer

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Global hypomethylation (approximately 10 percent loss of methylation) and promoter hyper-methylation of approximately 300 genes is commonly observed in colon cancer. Age-related methylation changes also are detectable in normal colon mucosa, and these are accentuated in cancer. A CpG methylator phenotype and histone code changes also are observed, and all epigenetic changes are generally inter-related with respect to carcinogenesis. Hypomethylation occurs at LINE-1 elements in colon cancer, although the degree of hypomethylation varies among different types of colon cancer. Cases with the highest degree of promoter hypermethylation generally have the lowest degree of hypomethylation, suggesting that there are shared factors that contribute to methylation changes. Evidence that environmental, dietary, or other exposures affect DNA methylation in colon cancer include different degrees of DNA methylation observed in proximal versus distal colon cancer, association of chronic inflammation with accelerated age-related methylation, and variable association of folate and methylenetetrahydrofolate reductase (MTHFR) genotypes with colon cancer risk.

Spontaneous carcinogenesis in humans could be considered as an epigenetic disease that is influenced by aging. Cells from young, healthy individuals have normal epigenetic patterns, which can change as a result of aging, diet, or other exposures. These exposures cause cells to

acquire epigenetic changes that lead to abnormal epigenetic "fields" or "patches" with faulty gene expression in which cancers arise. The estrogen receptor alpha (ERα) locus becomes increasingly methylated with age. Variation in methylation at this locus could be related to exposure, diet, lifestyle factors, or MTHFR genotype. DNA methylation at ERα was measured in biopsies from the Aspirin/Folate Polyp Prevention Study, which examined the effects of folate supplementation in participants with recent adenomas. Early in the trial, an increase in adenoma and also in prostate cancer was observed in participants receiving folate. MTHFR genotype was not associated with risk for adenomas or colorectal cancer in this study.

Bisulfite-pyrosequencing was used to measure methylation of LINE1 (global methylation surrogate) and ERα and secreted frizzled-related protein 1 (SFRP1) (age-related methylation surrogates) in normal mucosa from more than 300 consenting subjects. Little variation was observed in LINE1 methylation; no correlation was observed between methylation and demographic parameters, folate, or aspirin supplementation; dietary factors; MTHFR genotype; or polyp recurrence. A two-fold variation in ERα and SFRP1 methylation that correlated strongly with age and colon location (proximal vs. distal), but not folate or polyp recurrence, was observed. This work demonstrates that global methylation in colonic mucosa varies little in an adult population at risk for colonic tumors, but that gene-specific hypermethylation (at ERα and SFRP1) is influenced by age and location within the colon. The effects of demographics, lifestyle factors, folate, diet, or MTHFR genotype are likely to be smaller than currently measurable, if they do exist.

Discussion

A participant asked if Dr. Issa had examined the impact of hormone replacement therapy on ERα methylation. Dr. Issa answered that this analysis had not been performed by his group, although there are hints of a relationship.

Dr. Miller asked whether age-related or cancer-related methylation is a random process that occurs throughout the entire genome and then a certain pattern selects for tumor development or if there are regions where changes in methylation are more likely to occur. Dr. Issa answered that a subset of genes seems to be more susceptible to methylation, although what determines this susceptibility is unknown. Conditions during embryogenesis or development could impact susceptibility. Local concentrations of retrotranposons appear to be associated with hypermethylation associated with aging in cancer. Methylation changes may be independent of gene function, but function may in turn modulate methylation because of selective pressure.

Dr. Ulrich commented that because the participants in the Polyp Prevention Study had previous adenomas, the biopsies obtained from "clean colon" may actually have had cancerous precursors not detected by colonoscopy whose growth was promoted by folate. Issa noted that 50 percent of the US population will have an adenoma, whether it is detected or not, so the results of this study are relevant for the general population.

Dr. Waterland asked how the proximal versus distal colon differences in ERα methylation were established and whether this difference could be observed in other genes. Dr. Issa answered that there is some evidence for differences in estrogen or ER concentration in the colon, but the

mechanism for this difference, particularly as it pertains to other genes, is unknown. The data on ERα methylation was an average of results from proximal and distal colon; both sites showed correlation of methylation with age but not with other parameters. The hypermethylation phenotype has a strong proximal-distal relationship which could be due to differences in exposure, differences in cell of origin of the tumor, or related to mucosal differences in proximal versus distal colon. Proximal and distal colon tumors are different. In response to Dr. Belinksy, Dr. Issa answered that participants in the study developed tumors, but mostly small adenomas. Neither global nor gene-specific methylation predicted development of adenomas.

Dr. Milner noted that this population had sufficient folate and supplementation may have resulted in supersaturation of folate; methylation levels may have already plateaued. Dr. Issa agreed that these results would not be relevant to a folate-deficient population.

DNA Methylation Biomarkers to Assess Selenium Chemoprevention for NSCLC
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Hundreds of genes are inactivated by promoter hypomethylation in lung cancer and these changes represent a potential strategy for predicting lung cancer risk. Methylation of specific promoters can be observed in sputum samples and methylation changes were predictive for squamous cell cancer 3 years prior to diagnosis. Methylation changes in sputum and blood thus also could be used to track the effects of chemoprevention. The Lung Cancer Prevention Study, a phase III chemoprevention trial of selenium supplementation in people with resected stage I non-small cell lung cancer, is currently the only lung cancer prevention trial in the United States. Sputum and plasma samples are being collected to determine whether individual gene or methylation changes detected in sputum and/or plasma predict response to selenium and/or cancer recurrence (second primary, local, and extrapulmonary recurrence).

Analysis of promoter hypermethylation of genes involved in lung cancer (p16, MGMT, RASSF1A, GATA5, PAX5α, and PAX5β) showed that the prevalence for methylation was greater in cells from sputum than for cells from plasma; 26 percent of sputum samples showed methylation of three or more genes at baseline. Methylation prevalence for these genes was compared in women at moderate and high risk for lung cancer. Odds for methylation at the examined genes were increased in women with previous cancer, consistent with a greater incidence of field defects and risk. Lung cancer survivors displayed 6-fold higher levels of methylation at three or more genes, indicating that the methylation biomarkers tracked with risk. Methylation index at individual genes was assessed in 283 study participants at study entry and during followup, and demonstrated that the prevalence for methylation of multiple genes increased with lung cancer risk comparing smokers to resected lung cancer patients and incident/stage I patients. Epigenetic changes associated with cancer recurrence suggest that epigenetic therapy could be useful for prevention of lung cancer. Low doses of azacytidine in combination with HDAC inhibitors have achieved 50 percent response rates ranging from high lineage recoveries to complete remissions in myelodysplastic syndrome and acute leukemia. A phase I/II clinical trial to test this therapeutic strategy for lung cancer is underway at Johns Hopkins University and the University of New Mexico.

The positive and negative predictive value for detection of non-small cell lung cancer by analysis of aberrant methylation of a panel of genes in sputum and serum was determined. These genes have been shown to be increasingly methylated in tumors; however, the increase in methylation is not reflected in blood. In sputum, methylation is more readily observed and is independent of histologic cancer type. Sputum had better sensitivity and specificity of approximately 57 percent. Serum had strong specificity, but low sensitivity.

These studies demonstrate that sputum can be used to predict the methylation status of genes in advanced lung cancer when biopsy is not feasible. The positive predictive value of four of the analyzed genes (p16, DAPK, PAX5β, and GATA5) was between 44 and 72 percent. False-positive methylation in sputum is likely due to extensive field cancerization associated with smoking and from which lung cancer arises. Methylation status in small cell and non-small cell lung cancer can be determined by analysis of DNA from sputum and thus can detect both central and peripheral lung tumors. The low sensitivity for detecting methylation in serum likely is due to limited release of free DNA from tumors through apoptosis or fragmentation of the released DNA to a degree that does not allow detection of methylated alleles of the candidate genes.

Discussion

Dr. Milner asked whether selenium supplementation had any effect on methylation. Dr. Belinksy answered that participants with lower levels of selenium intake had increased levels of methylation. A participant cautioned that there is evidence that people with cancer report or recall their diets differently than those without cancer.

Dr. Rahman noted that in sputum, lung macrophages have a half-life of 7 days and asked whether the data reflected methylation in cells from bone marrow and whether any of the subjects in this study had fibrotic disease or chronic obstructive pulmonary disease (COPD). Dr. Belinsky answered that cells in sputum can come from deep within the lung and cytology indicated that sputum contains cells representative of the whole lung. Approximately 40 to 50 percent of lung cancer patients have some degree of obstruction, which leads to increased sputum production and heavy smokers have some degree of fibrosis. Methylation changes in macrophages would not be detected in this study because the genes analyzed are cancer-specific genes. The study used methylation-specific PCR to analyze methylated alleles from exfoliated epithelial cells and is sensitive enough that the presence of macrophages does not interfere.

Modeling Folate, One-Carbon Metabolism, and DNA Methylation
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DNA hypomethylation and CpG hypermethylation are observed in colon cancer. Folate-mediated one-carbon metabolism and pyrimidine synthesis also likely have a role in development of this cancer. Genetic polymorphisms in enzymes participating in one-carbon metabolism, pyrimidine synthesis, and in DNA methylation may have functional effects that

impact colon carcinogenesis. Interactions between DNMT3b genotype and one-carbon intake (folate/methionine) have been observed to affect the risk of developing colorectal adenomas. Folate depletion/repletion studies have shown that moderate folate depletion results in DNA hypomethylation while folate supplementation can increase DNA methylation in the colonic mucosa. The MTHFR 677TT polymorphism is associated with decreased global DNA methylation that also is more readily reduced with folate depletion. However, because methylated CpG islands are "mutational hotspots" for C to T changes, this polymorphism is associated with reduced cancer risk. Decreased MTHFR activity associated with the MTHFR 677 polymorphism leads to increases in homocysteine and SAH, which in turn leads to lowered DNA methyltransferase activity and hypomethylation of CpG islands, reducing risk of mutation of p53 in the colon.

Methionine synthase or MTHFR variants have been suggested to be important for global DNA hypomethylation as an early event in colon carcinogenesis. Some researchers have suggested that low-methyl donor intake is associated with greater promoter methylation, while others have suggested the opposite. A colon cancer case-control study examined associations between CpG island methylator phenotype (CIMP) colon cancer and genetic polymorphisms relevant to one-carbon metabolism and risk of colon cancer. There was limited evidence of a role for one-carbon polymorphisms in risk and little evidence of a role for one-carbon nutrients in CIMP+ etiology.

To more completely understand the role of methylation, one-carbon metabolism, and one-carbon nutrients in colon cancer, the ability of polymorphisms in a folate-metabolizing protein to alter disease risk or drug response must be determined, and whether effects are different under specific conditions, such as in combination with low- or high-nutrient intake or with another polymorphism. A mathematical simulation model of one-carbon metabolism and methylation has been developed based on known enzyme kinetics and biochemical properties. This model can provide mechanistic information and pilot data for targeted studies. The model can be used to analyze interactions between the folate cycle and methionine cycle, including long range interactions. Using the model, activity of a specific enzyme can be perturbed and its effects on intermediates and other metabolites can be determined. The effects on DNA methylation also can be determined.

The model has been used to predict the impact of genetic variation in MTHFR on biomarkers and mechanisms of cancer risk such as SAM, SAH, homocysteine, methylation rate, and thymidine and purine synthesis. The model showed that the DNA methylation rate was insensitive to folate availability. The model predicted that decreases in MTHFR activity would reduce concentrations of SAM, 5-methyltetrahydrofolate and DNA methylation, and would increase SAH and homocysteine concentrations and thymidine or purine synthesis. The model also predicted that as folate concentrations became very high, the reaction velocities of a number of enzymes involved in one-carbon metabolism would decrease. Long-range inhibitions in the folate/methionine cycle helped stabilize DNA methylation rate against fluctuations in methionine input. Low status of folate and methionine was thus predicted to affect DNMT reaction rate; the long range interactions were observed to prevent the decline of the methylation rate as methionine input fell. The model also can help determine the effect of variation in a number of

enzymes or substrates elsewhere in the cycle; changes in activity of some enzymes have far-ranging effects on many components of the cycle, while others have little effect.

This model permits simulation of specific conditions and analysis of their impact on biomarkers and metabolites. Multiple genetic variants and varying states of nutritional status can be analyzed. The information gained from such modeling experiments will help to target experimental studies and integrate information from epidemiologic data analyses. The model has shown that the DNA methylation rate is protected against fluctuations in methionine and folate. The model also permits a greater understanding of the robustness or sensitivity of enzymes in the cycle. This could help identify useful mouse models and point to metabolites or biomarkers that are most likely to be affected and thus should be measured in experimental studies and insure that intake levels of specific nutrients most likely to perturb the cycle are carefully measured.

Discussion

Dr. Ulrich explained that methionine is the most relevant nutrient for methylation capacity, but few in the United States are methionine-deficient. The steady state of SAM and SAH compares well as an index for methylation capacity. Measures of the overall methylation rate are not satisfactory because such measures do not distinguish between types of methylation. Experimental data also has discrepancies that the model has helped to explain.

Dr. Martin commented that DNA methylation placement is directed by chromatin proteins. Alterations in the methylation pattern suggest changes in chromatin proteins, and there is little evidence that disruptions in methyl metabolism directly result in significant changes in methylation patterns by impacting the activity of DNA methyltransferases. Dr. Ulrich explained that the model approximates global DNA methylation, not promoter-specific methylation and is consistent with experimental data on global methylation. Dr. Martin countered that global methylation includes promoter-specific methylation, but is largely composed of methylation at retrotransposons, which is determined by histone modifications. Dr. Issa argued that this was true for yeast and flies, but was not clearly established in mammalian cells. Knockout of histone methyltransferases in mammalian cells has not been observed to have an effect on DNA methylation. Dr. Martin stated that evidence that histone modifications direct DNA methylation is considered to be strong. Dr. Ulrich explained that the DNA methylation link is not the strength of this model, which focuses primarily on one-carbon metabolism and the activity of methyltransferases combined. Dr. Ulrich explained that results from the polyp prevention trials are beginning to be modeled, although folic acid enters the cycle from a different route than those delineated in the model.

Panel Discussion

Dr. Umar asked for feedback on needed next steps, such as analysis of epigenetic marks and tissues on which efforts should be focused.

Dr. Belinsky commented that it has been difficult to find a direct link between one-carbon metabolism and promoter methylation. This link may not exist, or the wrong target is being

analyzed. The process of gene silencing and the importance of histone modifications are known, but not what triggers these events. It will be challenging to understand how nutrition modifies methylation if the trigger is unknown. Possible triggers include DNA damage and repair ability; *in vitro* research has shown recruitment of cytosine methyltransferases and chromatin modifiers to damaged DNA to prevent transcription of a damaged gene. Dr. Ulrich agreed that the understanding of relevant mechanisms is at an early stage. Epidemiological studies could help, but the populations used are generally too limited. A current pilot study involving biomarkers and steroid hormone and insulin metabolites could provide leads for possible mechanisms.

A participant addressed the clear link between cellular SAM and SAH level, in contrast to the lack of clarity linking these levels to methyltransferases activity. Many kinds of methyltransferases exist and most may not respond to changes in SAM or SAH. Low affinity methyltransferases likely would respond to changes more dramatically. Dr. Ulrich agreed that variations in the K_M and K_I of methyltransferases would impact response and said that this would be considered when expanding her one-carbon metabolism model.

Dr. Mason noted that as modeling evolves, events occurring in different tissues will need to be addressed. Not all enzymes are expressed or active in all tissues. Dr. Ulrich explained that her current model primarily used parameters from rat liver and focused on epithelial and hepatic activities because the mechanisms at these sites appear to be similar. Efforts are underway to examine transport of metabolites in and out of cells, but data is needed to validate the model, particularly colon data. The next priority for improving the model is to increase its availability to investigators and then expand it. The goal is to use the model to target methylation, but more information is needed for validation; data from other investigators' experiments would be helpful.

A participant asked how aerobic versus anaerobic mechanisms affect the pathways, given that tumors often develop anaerobic metabolism. Dr. Ulrich answered that this had not yet been examined with the model.

Dr. Umar asked participants to comment on progress in developing biomarkers for outcome and surrogate endpoints. Dr. Belinsky answered that his work on sputum-based biomarkers was encouraging, but the best panel of genes must be identified, and the robustness for positive predictive value in an at-risk population must be determined. It may be less challenging to predict response to therapy than to develop biomarkers for early detection. He currently is testing approximately 60 different genes for use in the early detection of lung cancer, and hopes to validate approximately 10 within 3 years. Concerning prevention studies, selenium is the primary BFC being tested for lung cancer prevention. Other trials focusing on secondary prevention or prevention in people at higher risk should be considered. A benefit to such studies would be a shorter time to outcome.

Dr. Umar asked for opinions on the usefulness of global hypomethylation versus gene-specific hypermethylation for biomarkers. Dr. Belinksy answered that it is difficult to detect global hypomethylation in fluid. Dr. Waterland commented that focus also should be placed on epigenetic changes occurring during development to understand epigenetic mechanisms.

Dr. Umar asked for opinions on the effects of short-term dietary interventions versus long-term dietary intake on epigenetic marks. Dr. Waterland answered that because the processes of epigenetics are not well understood, it is difficult to determine when to intervene and what types of intervention would be most effective.

A participant commented that transcription factor binding and how this modifies chromatin structure and leads to changes in methylation also should be considered. Genistein and resveratrol have receptor binding properties which may be relevant to chromatin structure. Dr. Belinsky agreed that transcription factor binding and regulation of promoter activity likely modulates changes in chromatin structure. Dr. Yang noted that permanent disruption of estrogen signaling silences the progesterone receptor (PR). During the silencing process, epigenetic machinery (DNMT1, polycomb) is recruited and changes in methylation at CpG islands within the promoter region are subsequently observed. Estrogen positively regulates its target gene (PR) and removal results in gene silencing. The estrogen receptor also can repress other target genes.

Dr. Umar asked for comments on high throughput technologies that are needed, particularly for clinical studies. Dr. Yang commented that a hierarchy for methyltransferases activity and CpG site methylation is needed, as well as information on epigenetics in relevant tissues including adipose, sputum, and cells of the oral cavity. Dr. Belinksy said that technologies for methyl-typing tumors are needed. CpG island arrays are available for screening the methylation state of certain genes in tumors. Detection in non-clonal fluids using TaqMan or molecular beacons is not quantitative. Robust multiplex assays and commercialization of these assays are needed. Dr. Huang noted that high throughput technologies for discovery, such as methyl sensitive restriction enzymes, PCR amplification, arrays, immunoprecipitation, and MALDI-TOF, are available. Once candidate genes are discovered, quantitative approaches are crucial for validation.

Session 3: What is the Role of Non-Coding RNA in Transcriptional Gene Silencing? Is There Evidence for Diet and Bioactive Food Components To Modulate This Gene Expression Regulatory Mechanism?
Moderator: Jacob Kagan, Ph.D. (DCP, NCI)

The Role of RNA in the Regulation of Gene Expression in Human Cells
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RNA interference (RNAi) operates in both a post-transcriptional and transcriptional manner to silence gene expression. Post-transcriptional gene silencing (PTGS) involves loading of double-stranded RNA (dsRNA) onto Exportin5 and transport to the cytoplasm, where the dsRNA associates with Dicer and the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), generating small interfering RNA (siRNA). The bottom guide strand recognizes the target mRNA, resulting in degradation of the target mRNA. dsRNAs can be added directly to cells via Transfection to silence specific genes. PTGS is generally transient. In contrast, transcriptional gene silencing (TGS) involves epigenetic mechanisms and is potentially functional for longer periods of time. Transcriptionally active promoters are transcribed by RNA

polymerase II (RNA pol II) and siRNAs targeted to the promoter regions can inhibit transcription through a mechanism that involves histone and DNA methylation and histone deacetylation. DNMT3a co-immunoprecipitates with the targeted loci and siRNA; the protein Argonaute 1 (Ago1) also was part of this complex. Histone 3 methylated at lysine 9 (H3K9me2) or lysine 27 (H3K27me3) also can be found at targeted promoters. Ago1 and RNA pol II are required at the gene targeted for TGS by siRNA.

TGS may involve either shuttling of endogenous siRNAs out of the nucleus and then back to the nucleus to target and silence a particular promoter or the siRNA may remain in the nucleus and regulate gene expression. Research on siRNA has been performed using exogenous siRNA; there currently is no evidence for endogenous siRNA. Two models for siRNA targeting of a specific promoter have been proposed. In the first model, RNA pol II unwinds DNA at the promoter, allowing siRNA to target the promoter and silence it. In the second model, the promoter has commenced transcription and siRNA targets this region, modifies resident nucelosomes, and silences transcription. A recent publication has demonstrated that all promoters transcriptionally silenced by siRNA had a low level of transcription, creating a transcript that included the promoter region. If the upstream region of the transcript is knocked out, transcriptional gene silencing by siRNA does not occur. siRNA binds to the promoter transcript in the presence of the transcriptional complex, which leads to histone and chromatin modification and TGS. DNA methylation might be involved in this process, because DNMTs and histone methylation also are required for silencing.

Ubiquitin ligase (Ubc) promoter targeting was used as a model to analyze the epigenetic silencing complex involved in targeted gene silencing by siRNA. siRNA Ubc167 silences transcription in a TGS manner and is susceptible to the HDAC inhibitor trichostatin A (TSA). siRNA Ubc308 (exon targeting) works in a PTGS manner and is resistant to TSA. Chromatin immunoprecipitation assays demonstrated that silencing by both UbC167 and UbC308 involved H3K9me2, H3K27me3, and Ago-1 at the targeted promoter. The results also suggest that the complex is targeted to the promoter and moves downstream, in addition to causing secondary remodeling of chromatin at the targeted locus; thus therapeutic use of siRNA should target the 5' most region of the gene to prevent off-target effects.

Possible proteins involved in siRNA-mediated TGS include Drosha/DGCR8, Exportin 5, Dicer, and Ago-1 and Ago-2. Epigenetic-related proteins involved in TGS include DNMT3a, HDAC-1, Ezh2, Ago-1, and G9a. If DNMT3a or HDAC-1 are knocked down, TGS does not occur. TGS by siRNA can persist for up to 10 days and histone modifications and DNA methylation are observed at the targeted loci during longterm silencing. siRNA targets the promoter and binds to it, and then recruits protein complexes that promote histone modifications, leading to DNA methylation that increases with time and is required for robust and persistent silencing. Whether siRNA is required for the establishment or maintenance of long-term silencing, or if silencing is maintained once DNA methylation occurs, is currently unknown.

Pyrosequencing has been used to detect operative endogenous siRNAs. Putative siRNAs can be pulled down with antibodies to Ezh2, DNMT3a, and Ago-1, indicating a possible endogenous siRNA pool. Questions remain concerning whether siRNAs are functional in regulating gene expression in human cells. Such an endogenous system could lead to increased DNA

methylation in human cancer cells as a result of uncontrolled siRNA targeting of tumor suppressor genes. A possible therapeutic use of siRNAs would be to design siRNAs to stably repress transcription of a disease gene.

Discussion

Dr. Martin asked Dr. Morris to comment on Piwi-interacting RNA (piRNA). Dr. Morris answered that other investigators have determined that piRNAs can be found in sperm and can regulate gene expression in a transcriptional manner, although their targets are unknown. piRNA may regulate gene expression in sperm as it seeks the egg or may establish an epigenetic profile in the egg.

A participant asked Dr. Morris to speculate on the outcome of silencing if the targeted gene has no promoter CpG island, i.e., would only chromatin modification be sufficient? Dr. Morris answered that the experimental siRNAs used by his group were targeted to CpG-rich regions. Efforts are underway to target phosphoinositide-3 kinase, which has no CpG islands in its promoter and is expressed in a housekeeping manner in every cell. Such genes may not be targeted by siRNAs; however, it is unknown at this time whether endogenous siRNAs regulate transcription in humans. Dr. Morris speculated that siRNAs form a direct complex with the gene, which slows transcription. If this event is positively reinforced, DNA methylation and silencing ensue. A similar effect is seen for the polycomb complex; one member of the complex silences the gene and the other maintains silencing. siRNAs may recruit polycomb proteins. Dr. Richard Eckert (University of Maryland School of Medicine) asked if the polycomb proteins promote or mediate histone modification and then leave the site, followed by recruitment of methyltransferases. Dr. Morris answered that methyltransferases are likely resident at the site because DNMT3a co-immunprecipitates with Ezh2.

Differential Expression of microRNAs During Hepatocarcinogenesis Induced by Methyl-Deficiency in Rats

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Primary hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Development of HCC is a multistage process involving the sequential evolution of distinct histologic states that develop over a period of 20 to 60 years after the initiating defect. Initiation of HCC in normal cells is driven by both genetic and epigenetic modifications; diet and environmental exposures, such as tobacco smoking, also may have a role in risk for and development of HCC. Early detection is the primary goal for reducing death from HCC.

A number of animal models relevant to HCC carcinogenesis in humans exist. The methyl-deficiency model of endogenous hepatocarcinogenesis features chronic deficiency of the methyl donors methionine, choline, folic acid, and vitamin B12 to induce carcinogenesis. Neither exogenous carcinogens nor genetic manipulations are required. HCC develops within 14 to 16

months in male rats and certain mouse strains, and is characterized by a sequence of pathological changes similar to the development of HCC in humans. Recently, the effects of microRNAs (miRNAs) on carcinogenesis have been explored. Such miRNAs can alter gene expression to function as both tumor suppressor genes and oncogenes. Expression of certain miRNAs has been analyzed in cancer cells, but their relevancy to the cancer process currently is unknown.

The miRNA 122a has specific functions in the liver including regulation of cholesterol and fatty acid metabolism, facilitation of hepatitis C virus replication, and regulation of cyclin G1. miRNA 122a also is downregulated in HCC and by methyl deficiency. A number of miRNAs with experimentally confirmed targets have been analyzed for expression changes in HCC induced by methyl deficiency in rats. Most changes were small; the most significant changes were downregulation of the tumor suppressor miRNAs 34a, 16, 181a, and 127. During methyl deficiency, miRNAs 34a and 127 were strongly downregulated, while others showed little change. The protein targets of these miRNAs, E2F3 (34a) and BCL6 (127), were upregulated. E2F3 represses the p53 network. Decreased expression of 34a in cells exposed to radiation rendered the cells more resistant to apoptosis, which could lead to aberrant proliferation of transformed cells. Death associated protein kinase 1 (DAPK1), Fas1g, and cyclins G1, G2, and H also were downregulated.

To determine if alterations in miRNA expression can be used as a biomarker of cancer progression or chemoprevention, rats were placed on a methyl-deficient diet for 9 or 18 weeks and then switched back to a normal diet. Levels of miRNA 34a were reduced and could not be restored in rats fed the methyl-deficient diet for 18 weeks, but could return to nearly normal levels in the rats fed the methyl-deficient diet for only 9 weeks. Early carcinogenic changes also were observed in the rats fed the methyl-deficient diet for 18 weeks. Because dysregulation of miRNA appears to occur early in the process of hepatocellular carcinogenesis but can be restored by restoration of methyl donors, miRNAs are potential biomarkers for early detection of cancer and chemoprevention.

Discussion

Dr. Pogribny explained that these miRNAs had not been analyzed in humans because of a lack of human samples.

Dr. Ross asked whether other epigenetic marks, such as DNA methylation, DNMT activity, or histone modifications, were affected by methyl deficiency. Dr. Pogribny answered that changes in miRNA likely represent a higher level of the epigenetic mechanism. miRNA 34a is regulated by DAPK1, which is downregulated by methylation in a number of cancers. Changes in DNA methylation likely are a consequence of histone modification.

Dr. Dashwood commented on the identification of E2F3 as a target, noting that in the colon, there is a great deal of redundancy in the E2F family members, and asked Dr. Pogribny if he had analyzed other family members. Dr. Pogribny answered that E2F3 is the only confirmed target for miRNA 34a. Changes in E2F1 and E3F2 also were observed, but to a lesser degree.

Dr. Kagan asked whether other targets exist for miRNA 34a, given that most miRNAs do not have perfect targets. Dr. Pogribny answered that there are approximately 100 predicted targets for any miRNA, but he did not know of other miRNA 34a targets. Because of this, confirmed miRNA targets are required to make mechanistic sense of changes in miRNA expression.

A participant asked Dr. Morris about the accuracy of his assay to measure DNA methylation. Dr. Morris explained that the methylation analysis performed by his laboratory is restriction enzyme analysis at the ubiquitin promoter using methylation sensitive and methylation insensitive restriction enzymes followed by RT-PCR; this analysis is qualitative rather than quantitative. Dr. Martin suggested that methylation at sites other than CpG islands also should be analyzed. Restriction enzymes are available that recognize non-CpG sites, but still detect methylation.

A participant asked Dr. Morris to comment on the role of polycomb in siRNA action. Dr. Morris explained that this is not well characterized. Typically, PRC2 modifies DNA and PRC1 is subsequently recruited and ubiquitinates nucleosomes. HK23 appears to be required for sustained silencing and RNA pol II is at the site, but is not active. This suggests that genes retain RNA pol II in an inactive form that can be activated if needed. Inactive RNA pol II has been found as part of the complex that maintains silencing.

THURSDAY, SEPTEMBER 27, 2007

Dietary HDAC Inhibitors: From Cells to Mice to Man
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DNA methylation, histone modification, and nucleosome occupancy comprise the "epigenetic code" that silences (or unsilences) gene expression. Histone modifications such as acetylation, phosphorylation, ubiquitination, and methylation are reversible and can be mediated by dietary factors. Chromatin is dynamically regulated between open and closed configurations. In the closed configuration, the lysine tails of histones can interact with the DNA backbone, constricting the structure and denying transcription factors access to DNA. Closed chromatin that silences tumor suppressor gene transcription is a characteristic of a number of cancers. To maintain an open configuration, acetylation of histone tails masks the positively charged lysines, preventing interaction with the DNA backbone and contribution of the chromatin structure. Because histone acetylation/deacetylation often is disturbed in cancer cells, inhibition of HDACs by chemotherapeutic agents is one strategy for treatment. In promyelocytic leukemia, the PML-RARalpha fusion protein aberrantly recruits histone deacetylases complexes. Treatment with high doses of retinoic acid results in release of the HDAC and recruitment of HATs, resulting in activation of repressed p21 and Bax and subsequent induction of cell cycle arrest or apoptosis.

A number of HDAC inhibitors including butyrate, diallyl disulfide, and sulforaphane (SFN) are found in food. SFN is found in cruciferous vegetables, particularly broccoli, and following

metabolism has a structure similar to known HDAC inhibitors. The two major metabolites of SFN, SFN-cysteine and SFN-acetylcysteine, are effective HDAC inhibitors *in vitro*. SFN metabolites show dose-dependent HDAC inhibitory activity in colon, prostate, and breast cancer cell lines. Known and putative dietary HDAC inhibitors have similar structures, including a 'spacer arm' that permits the functional group of the inhibitor to fit competitively into the HDAC pocket and inhibit its activity. HDAC inhibition is accompanied by an increase in acetylated histones and also in occupancy of the p21 promoter by the acetylated histones, and transcription, and protein expression of p21 in a p53-independent manner. If mice are fed SFN prior to implantation with PC3 prostate cancer cells, tumor growth is retarded and HDAC activity is inhibited by 25 to 35 percent in the xenografts, compared to untreated mice. HDAC inhibition appears to be systemic and is observed in prostate as well as peripheral blood mononuclear cells (PBMCs).

Apc^{min} mice develop polyps spontaneously and serve as a model for familial adenomatous polyposis. Treatment of these mice with 10 μmol SFN per 20 gram body weight results in inhibition of HDAC activity in the gastrointestinal tract and induction of histone acetylation in the colonic crypts. Long-term feeding of SFN results in decreased polyp development and increases in acetylation of histones H3 and H4. The dose used in these experiments is equivalent to 10 servings of broccoli per day; however, broccoli sprouts have 20 to 50 times more SFN and thus represent a more realistic route for diet-based colon cancer prevention. In humans, a single 68 gram serving of broccoli sprouts significantly inhibited HDAC activity in PBMCs 3 and 6 hours after consumption; HDAC activity had recovered by 24 hours. An increase in acetylation of histones H3 and H4 also was observed. HDAC inhibition reaches a plateau within 2-3 days of feeding 68 grams of broccoli sprouts per day. The results of this study suggest that HDAC activity in PBMCs could be used as a biomarker to assess exposure of humans to dietary HDAC inhibitors. It also suggests that dietary agents, as weak ligands for HDAC, might subtly regulate genes such as p21 and BAX to affect how normal cells respond to events such as oxidative stress.

Projects are underway with Dr. Emily Ho to measure changes in histone H3 acetylation in men with a family history of prostate cancer to determine if men who eat one or more servings of cruciferous vegetables per day have increased histone H3 acetylation in prostate compared to men who do not eat cruciferous vegetables. The goal of another project is to analyze histone acetylation and HDAC activity in colon cancer patients and use LCMS to measure levels of SFN metabolites in tissue and in blood to use as possible biomarkers. A project headed by Dr. Williams seeks to clarify the risks and benefits of maternal SFN consumption for the fetus. Using a transplacental exposure model, Dr. David E. Williams has shown that if pregnant mice receive one dose of a carcinogen followed by one week of phytochemical treatment, the pups are protected against development of lymphoma and lung cancer. HDAC activity was inhibited in the lung and colon of newborn pups that had received 1 week of indole-3-carbinol or chlorophyll *in utero*. These studies seek to provide insights into the important question of risk *vs* benefit of exposure to HDAC inhibitors during development, as well as during cancer chemoprevention and/or therapy.

Discussion

A participant commented that epidemiological studies generally do not reveal a strong association between fruit and vegetable consumption and colon cancer risk. Dr. Dashwood answered that people may not eat enough fruit and vegetables, particularly cruciferous vegetables, to benefit with respect to colon cancer. It also can be difficult to determine levels of consumption; cruciferous vegetable food frequency questionnaires that list approximately 50 types of cruciferous vegetables and include portion size questions have been developed and may help in this regard.

Dr. Huang asked how other dietary HDAC inhibitors such as isothiocyantes (ITC) fit into the HDAC pocket. Dr. Dashwood answered that several ITCs were inhibitors and had similar arm structures to sulforaphane; in particular, the ITC cysteine metabolite fits into the HDAC pocket and thus should have inhibitory action. Dr. Huang asked how HDAC activity is decreased after consumption of cruciferous vegetables. Dr. Dashwood answered that the inhibition of HDAC may involve displacement and turnover. There is evidence from colon cancer cell lines plated at low density that HDAC turnover occurs via a mechanism besides competitive inhibition.

A participant asked about the effects of HDAC inhibition during development. Dr. Dashwood answered that the doses of indole-3 carbinol and chlorophyll were equivalent to doses known to protect against lung and colon cancer. The pups were analyzed immediately after birth and did not nurse. HDAC inhibition was not observed in liver. The participant also asked how HDACs are inhibited *in vivo*. Dr. Dashwood answered that HDACs are regulated in the standard manner, involving proteasome-mediated destruction and transcriptional mechanisms. Valproic acid, a clinically-used drug and reported HDAC inhibitor, also targets HDAC2 for destruction.

Dr. Milner noted that wasabi is high in allyl isothiocyantes. He asked whether eating large amounts of broccoli early in life could result in deleterious effects. Dr. Dashwood answered that the compounds he has examined selectively cause apoptosis in cancer cells but not normal cells. In the PREC prostate cell line, which is not a transformed cell line, HDAC inhibition, increased acetylation, and p21 are increased, but G2/M cell cycle arrest is not observed. In response to HDAC inhibition, normal cells upregulate redox-sensitive thiols to protect against apoptosis.

Dr. Barbara Dunn (DCP, NCI) asked whether there were any studies on genetic polymorphisms involved in the pathways and interactions affected by broccoli or its metabolites. Dr. Dashwood answered that polymorphisms in GST certainly do affect bioavailability and retention of ITC's in people, and hence could enhance or diminish HDAC inhibition and histone acetylation status in various tissues.

Diallyl Disulfide Increases Histone Acetylation in Colon Cells In Vitro and In Vivo
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Several epidemiological studies have suggested that garlic consumption can reduce stomach and colorectal cancer risk. A number of organosulfur compounds present in garlic have been found to exhibit anticarcinogenic properties. Diallyl disulfide (DADS) is one of the more abundant organosulfur compounds formed when garlic is cut or crushed. Once absorbed, DADS is metabolized into allyl mercaptan (AM), allyl methyl sulfide (AMS), and allyl methyl sulfone ($AMSO_2$), which has been detected in rat liver, plasma, and urine. DADS exerts various anticarcinogenic effects, including anti-initiating and anti-promoting effects in colon and other organs in animal models, induction of cell differentiation, and cell cycle arrest and/or apoptosis in tumor cells, and modification of gene expression.

In vitro, DADS inhibits HDAC activity of Caco-2 and HT-29 nuclear extracts and increases histone H3 and H4 acetylation in both colon cancer cell lines. Histone hyperacetylation is associated with increases in mRNA and protein levels of $p21^{cip1}$, and cycle arrest in G2/M phase. Chromatin immunoprecipitation assays showed that DADS also increased cyclin-dependent kinase inhibitor 1A promoter-associated histone acetylation.

In vivo, the effects of DADS on histone acteylation and gene expression were assessed in colonocytes isolated from male Wistar rats following intracaecal perfusion with DADS (200mg/kg). Histones H4, lysines 8, 12, 16 of histones H4, and lysines 9 and 14 of histone H3 were hyperacetylated one hour after DADS perfusion. Histone hyperacetylation of total H4 and lysine 8 of histone H4 persisted for up to 21 hours after perfusion, whereas acetylation on other lysines returned to basal levels at this point. Similar results were obtained with administration of a single dose of DADS by gavage. Analysis of gene expression showed that DADS treatment modulated the expression of 3 genes 1 hour after the end of perfusion. Among the 49 genes whose expression was modified 21 hours after the perfusion, 45 were upregulated and four were downregulated. These genes are involved in processes including cell proliferation, inflammation, metabolism/transport, signal transduction, and apoptosis, as well as both pro- and anti-oncogenic genes. Further studies using chromatin immunoprecipitation assays are needed to determine whether histone modification is involved in modulation of gene expression by DADS *in vivo*.

A number of studies have found that pharmacological doses of DADS are associated with histone hyperacetylation and preventive biological effects in tumor cell lines. In contrast, the biological significance of histone modifications deserves to be investigated in non-tumoral cells. Additional investigations are also required to determine whether DADS-induced histone modifications are related to cancer preventive activity *in vivo*.

Discussion

A participant commented that the dose of DADS used in the Caco-2 *in vitro* studies (200 uM) was high and asked whether the effect of DADS could be nonspecific. He also asked whether dose-dependent effects had been observed in either the *in vitro* or *in vivo* models. Dr. Druesne-Pecollo answered that in the Caco-2 cells, DADS had no effect on cell proliferation at lower doses. The dose used *in vivo* was chosen based on available data on gene expression and xenobiotic metabolism. Lower doses need to be tested.

A participant commented that recent work has shown that garlic may not have a long term beneficial effect on cholesterol, and asked for comments on the kind of data that would be reliable and provide valuable information to users. Dr. Milner noted that the benefits of garlic on cholesterol are equivocal for the general population, but garlic can reduce high cholesterol by 10 to 15 percent, equivalent to eating a low-fat diet. He agreed that the doses used in the study were large. Dr. Milner explained that diallyl trisulfide is 10 times more effective than DADS and asked if other DADS metabolites had been tested. Dr. Druesne-Pecollo answered that most of the experiments were performed with DADS. Nevertheless, allyl mercaptan was also tested in HDAC activity experiments and was a more potent HDAC inhibitor *in vitro* than DADS. Dr. Milner commented that higher sulfur content is usually associated with increased potency. He added that older studies have shown that lower dose DADS given orally or by intraperitoneal injection can inhibit tumor growth, and suggested exploring whether these lower doses could modify histone acetylation.

Dr. Dashwood noted that changes in histone acetylation can be demonstrated, but it is more difficult to detect changes in HDAC activity. When tested using a kit to detect HDAC activity, only allyl mercaptan had marked inhibitory activity *in vitro*. The metabolites of certain compounds produce doses with activity, but the effective dose that reaches tissue or cells is unknown.

Dietary Polyphenols Mediated Regulation of Oxidative Stress and Chromatin Remodeling in Inflammation

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A number of chemopreventive phytochemicals are available from diverse dietary sources. Phytochemicals such as turmeric and resveratrol affect HDAC activity and others have antioxidant, anti-inflammatory, and anti-tumor properties. Inflammation is an important factor in a number of smoking-related diseases such as lung cancer and COPD, which is itself related to lung cancer. COPD is characterized by sputum build up and destruction of small airways. Unlike asthma, COPD is unresponsive to steroids, and current treatments for COPD are primarily palliative.

Curcumin is a dietary phenolic antioxidant with anti-inflammatory properties that is present in the spice turmeric. Understanding the mechanism of action of curcumin may allow it to be modified such that it could be used as pharmacologic agent. Regulation of histone acetylation and histone deacetylation by dietary polyphenols is a key event in inflammation. Increased histone deacetylation permits transcription of a number of mediators of inflammation such as TNF-α, IL1β, and IL8. Curcumin inhibits NF-κB transactivation in alveolar epithelial cells and thus can inhibit transcription of these pro-inflammatory cytokines.

Curcumin selectively restores HDAC2 activity and reduces levels of reactive oxygen species in stressed cells. HDAC2 is a transcriptional co-repressor necessary for inhibition of inflammation by steroids. Cigarette smoke extract decreases HDAC2 levels and increases NFκB activity in alveoli. Samples from COPD lung show a significant decrease in HDAC2 activity and the

decrease in activity correlates with the severity of the disease. Alveolar macrophages from smokers and COPD patients show impaired inhibition of inflammatory cytokine release. Through its ability to restore HDAC2 activity, curcumin may be able to restore steroid efficacy and reduce inflammation in the lungs of smokers and COPD patients.

Resveratrol inhibits NFκB activity and also mimics calorie restriction by stimulating sirtuin2 (Sir2), which is a class III protein deacetylase. Sirtuins have roles in cell cycle regulation, apoptosis, metabolism, inflammation, and aging. Cigarette smoke decreases levels of SIRT1, resulting in accelerated aging and bronchitis, in part due to loss of inhibition of NFκB by SIRT1. SIRT1 levels are decreased in lungs and monocyte-macrophage (MonoMac6) cells of both smokers and COPD patients. Resveratrol can restore SIRT1 activity in MonoMac6 cells treated with cigarette smoke extract and also inhibit cigarette smoke extract-induced IL-8 and TNF-α release in these cells.

Dietary polyphenols such as curcumin and resveratrol thus inhibit oxidative stress-induced inflammation, decrease levels of free radicals, and induce endogenous antioxidant defense systems. Curcumin can restore glucocorticoid efficacy by activating HDAC2 and inhibiting NFκB activation and release of pro-inflammatory cytokine release in macrophages and lung epithelial cells. Because of its ability to restore steroid responsiveness, curcumin is a potential therapy for treatment of COPD. Resveratrol also downregulates cigarette smoke-induced pro-inflammatory cytokines by activating SIRT1. Challenges to therapeutic use of these agents include low oral bioavailability and lack of pharmacokinetic information including absorption, distribution, metabolism, and excretion in various tissue and organs.

Discussion

A participant noted that curcumin has only a moderate effect on xenografts in mice and asked if there was any evidence for stronger effects. Dr. Rahman answered that his work had been performed only in primary cells and he did not have data on curcumin effects *in vivo*. He noted that in India, people ingest curcumin daily and smoking rates also are high, but there is no data on the effect of curcumin on lung cancer rates for this population because the life expectancy in India is only approximately 60 years, which is too young for development of many cases of lung cancer.

A participant cited evidence indicating that half the curcumin molecule is as effective as the whole molecule. He asked about the effects of delivery routes, such as emulsion or intravenous injection, on efficacy. Dr. Rahman answered that all polyphenolic compounds have been observed to have beneficial effects on only susceptible populations; little effect probably would be observed on healthy nonsmokers. There is no epidemiological or clinical data on the effects of infusion versus oral ingestion or on bioavailability or tissue distribution, so it is difficult to determine if these compounds will be effective against cancer. Anti-inflammatory effects have been observed, but again only in susceptible groups.

Dr. Huang noted that protective effects of green tea have been observed in epidemiological studies of nonsmokers. The compounds may not be strong enough to exert an effect on smokers.

Dr. Rafman explained that curcumin appears to be ineffective in the presence of tobacco smoke, but a mild effect can be detected when curcumin is administered together with steroids.

Dr. Rahman said that 8.5 grams of curcumin per day probably constitutes an effective dose. Dr. Dashwood noted that valproic acid has been used to treat epilepsy and bipolar disorder and is believed to work by HDAC inhibition through degradation of HDAC2. He asked whether valproic acid could interfere with curcumin and skew data on curcumin effectiveness. Dr. Rahman answered that, in his opinion, HDACs should not be inhibited in normal cells; this could result in an increase in inflammatory cytokines and cell cycle arrest. Dr. Dashwood asked if this meant that Dr. Rahman believed that dietary compounds should not be used as HDAC inhibitors, in spite of everyday consumption of compounds such as curcumin by the general population. Current evidence suggests that normal cells can protect themselves against unwanted effects of BFCs, but the data is limited. Dr. Rahman answered that the specificity of BFCs for particular HDACs would be an issue. He speculated that there could be specific HDACs for cancer and pro-inflammatory conditions.

Epigenetic Regulation of Chromatin Structure and Gene Function by Biotin: Are Biotin Requirements Being Met?

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Biotin modulates a number of signaling cascades and biotinylation of histones is another epigenetic mechanism that regulates chromatin structure and gene function. Biotin is covalently added to histone tails at the exposed surfaces of the nucleosome. Decreased biotinylation of histones has been associated with a number of *Drosophila* phenotypes.

Treatment of histone extracts from human lymphocytes with streptavidin, which binds biotin, and anti-biotin antibodies, demonstrated that the histones are biotinylated. Ten biotinylation sites in human histones have been identified; biotinylation-site specific antibodies have been generated and used to detect biotinylated histones. Two enzymes involved in biotin turnover and biotnylation of histones are biotinidase and holocarboxylase synthetase (HCS), both of which localize to the cell nucleus. HCS has been shown to be associated with polytene chromosomes in *Drosophila*. Nuclear localization of HCS may be mediated by its phosphorylation by tyrosine kinases, and yeast 2-hybrid studies suggest that the interaction of HCS with chromatin is mediated by zinc finger proteins.

Histone modifications have site-specific effects. For example, methylation of H3 on lysine 4 is associated with active chromatin, while methylation at lysine 9 is associated with repressed chromatin. A peptide-based approach was used to identify biotinylation sites on histones. The experiments found that biotinylation targets lysines within the N-terminus of H3 and H4. An antibody specific to H4 biotinylated at lysine 12 has been developed.

Both HCS and biotinidase were knocked down in *Drosophila* using germline transformation with a siRNA-producing vector. Knockdown of biotinidase in females and males did not significantly

affect biotinylation of H3, whereas knockdown of HCS caused a substantial decrease in histone biotinylation in males and females. HCS knockdown resulted in a 50 percent decrease in heat tolerance (survival) in males and females. In contrast, knockdown of biotinidase had less of an effect in males and essentially no effect in females. HCS knockdown also shortened the lifespans of both males and females.

The impact of histone biotinylation on gene repression was analyzed using ChIP assays combined with PCR. Stimulation of Jurkat cells with phorbol 12-myristate 13-acetate stimulated IL2 activation and was accompanied by depletion of biotinylated H4. Because histone biotinylation silences human endogenous retroviruses and maintains genomic stability, the effect of disruption of biotinylation was analyzed by knocking down HCS in Jurkat cells. In wild-type cells, LTR22 could be enriched by pulldown with antibodies to biotinylated H4. By culturing the cells in media containing different concentrations of biotin, it was determined that the abundance of biotinylated histones at the LTR locus was dependent on biotin supply. In the HCS knockdown cells, silencing of the LTRs by histone biotinylation was disrupted; activation of LTRs can lead to genome instability. In addition, spontaneous chromosomal abnormalities are increased in HCS knockdown cells by as much as 200 percent.

Discussion

A participant asked whether biotinylation of histones at different sites has different effects. Dr. Zempleni answered that in the experiments on the LTRs, the biotin supply-dependent biotinylation of H9, K13, H2a, and other biotinylation sites within H3 also were examined. Antibodies against biotinylated species of H3 did not cause enrichment of the LTR. The participant also asked if each specific biotinylation site had a different role in the histone code. Dr. Zempleni answered that he plans to use a ChIP-to-ChIP assay to identify the function of different histone modification sites; high throughput sequencing also may be needed. Mass spectrometry data has shown that biotinylation sites co-exist with other modifications on histones. Biotinylation at K12 may have a different effect on gene regulation, depending on the modification present on neighboring histones.

Dr. Milner asked whether modification of biotinylation on a particular histone had an effect on neighboring histones. Dr. Zempleni explained that if biotinylation of K12b and H4 are increased, a parallel increase in K9 methylation of H3, which is a silencing mark, also is observed, so there does appear to be some crosstalk. Dr. Martin asked how Dr. Zempleni determined that the change in retroviral activity observed with biotin depletion was related to changes at the histone. Dr. Zempleni agreed that there was no direct evidence. Biotinylation at the locus depends on biotin supply, and transcript levels were inversely proportional to biotin supply. In *Drosophila*, the gypsy transposable element jumps more in HCS knockout flies and chromosome instability is increased in biotin-deficient human cells. HCS knockout cells also are impaired in other biotin-dependent cell signaling processes.

Group Discussion

Dr. Mason expressed concern about using high doses of dietary components that are HDAC inhibitors for cancer prevention in healthy people. Since the actions of these compounds are

generalized, de-repression of beneficial tumor suppressor genes or pro-transformation genes could occur. In the fetus, high doses of HDAC inhibitors could de-repress detrimental genes as well. He asked if there were any data from animal models that suggested detrimental effects of dietary HDAC inhibitors. Dr. Dashwood answered that there currently is little information concerning which downstream signaling pathways are regulated by HDACs. His experiments have shown that class I HDACs can be transcriptionally downregulated, but this does not occur for class II HDACs. Dietary HDAC inhibitors may be generalized inhibitors, but do not apparently affect all HDACs the same way. In experiments in which humans were fed broccoli sprouts, marked but transient inhibition of HDACs was observed and no deleterious effects were observed for as long as 2 weeks of eating large amounts of the sprouts. Dr. Williams' transplacental model may help to determine the beneficial and deleterious effects of HDAC inhibitors and whether maternal ingestion of such compounds could interfere with development. Dr. Milner noted that broccoli can constitute up to 5 percent of a rodent diet and the animals do not lose weight.

Dr. Rahman noted that the liver is the primary site for metabolism of polyphenols. Phase 2 genes are upregulated in the livers of animals fed SFN. This raises questions concerning how these compounds induce HDAC in peripheral blood and tumors if they are metabolized in the liver; better data on pharmacokinetics, bioavailability, and other parameters will be needed to address this question.

A participant noted that in an *in vivo* model, dietary HDAC inhibitors go to the gut where gut microbes produce high levels of butyrate, which is a weak HDAC inhibitor. He asked whether there could be competition between butyrate and other HDAC inhibitors or if the butyrate goes directly to gut colonocytes. Dr. Dashwood answered that butyrate is an effective HDAC inhibitor and an inducer of differentiation and cell cycle arrest *in vitro*, but does not inhibit tumor growth efficiently when fed directly to animals. The question is whether adding dietary HDAC inhibitors would act in a synergistic or additive manner. The participant also asked whether a critical point in development at which additional HDAC inhibitor exposure should be avoided had been determined. Dr. Dashwood answered that Dr. Williams will compare DNA methylation patterns at different times during gestation. HDAC inhibitors do appear to markedly inhibit tumorigenesis if delivered during the third trimester through weaning, but are not as effective if delivered only during the third trimester or only through nursing. Dr. Rahman added that HDAC2 knockout mice are embryonic lethals.

Dr. Rahman commented on the need for technology development to better understand the mechanisms involved in biotinylation, acetylation, methylation, and ubiquitination of histones and how HDACs regulate downstream paths. It also is unknown how modifications of histones or HDACs affect DNA methylation.

Dr. Chung Yang (Rutgers University) asked about the site of action of SFN and where it was converted to its cysteine conjugate. Dr. Dashwood answered that the ability of NAC to inhibit HDAC *in vivo* has not yet been proven, nor has binding of the cysteine conjugate been detected in the active pocket of HDACs. LCMS detects low nanomolar concentrations of the parent compound and high nanomolar concentrations of NAC in the plasma of people eating sprouts. Low micromolar concentrations are observed in colon cells.

Dr. Rahman asked Dr. Dashwood if the people in his experimental population were healthy nonsmokers. Dr. Dashwood answered that the data shown at this meeting was based on results from only three people who did not smoke or take dietary supplements. In the future, samples from volunteers obtained during routine colonoscopy will be analyzed; the initial goal of the experiment will not be to prevent tumor formation, but to try to detect histone modifications reliably. Dr. Verma noted that NCI supports many large cohorts and has associated questionnaire data, including for diet; these data could prove useful for answering epigenetic questions.

Dr. Waterland noted that the definitive characteristic of epigenetic mechanism is mitotic inheritance. A recent review asked whether, given that the molecular mechanism of mitotic inheritance is largely unknown and given the dynamic nature of the modifications, could they truly be considered "epigenetic"? Dr. Rahman added that polyphenols have many activities, including anti-oxidant and anti-inflammatory effects and also effects on phase 1 and phase 2 enzymes. It is unclear whether these compounds exert their effects directly through epigenetic mechanisms or through signal transduction pathways. Dr. Milner asked whether prior insult was needed to realize a benefit from changes in histone modifications.

Dr. Dashwood commented that tea polyphenols may be toxic, but generation of NAC and cysteine metabolites requires stimulation of detoxifying pathways. Dr. Rahman said that polyphenols are metabolized in the liver, where they can conjugate with thiols and are thus detoxified. In plasma, the triol concentration is too low to conjugate polyphenols. Dr. Martin asked if pharmaceutical companies had any experience with toxicity of HDAC inhibitors. Dr. Dashwood answered that trichostatin A is a classic HDAC inhibitor and is toxic clinically. Other HDAC inhibitors are less toxic, but have troubling effects on certain blood parameters. Since some of these drugs are used to treat people with serious diseases, the benefits of their use may outweigh the risks. Dr. Rahman noted that rapid inflammatory responses are observed in mice after treatment with some HDAC inhibitors, similar to those seen when VEGF receptor inhibitors are administered. Dr. Dashwood said that drug companies would prefer to target specific HDACs, which could reduce toxicity. Modifying the way compounds fit into the HDAC active site could modulate the specificity or activity of HDAC inhibitors.

Dr. Belinksy noted that most HDAC inhibitors will not be used in healthy people. Determining dosage schedules to minimize toxicity yet maintain a therapeutic effect will be a challenge. To address this issue, animal models and chronic post-initiation models could be used to show if a compound blocks progression of neoplastic disease. Aerosols could be used for systemic delivery; aerosols are expensive and challenging to design and package, but bypass the liver. Dr. Dashwood described a P01 project that calls for examining long-term post-initiation treatment in a colon cancer model. Treatment will occur early and then stop and the effects on histone modification will be determined.

Tools for Epigenetic Analysis in Nutrition

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ChIP allows detection of DNA-protein binding and can be used to analyze epigenetic events accompanying changes in gene regulation and the proteins that are involved. Knockout of the estrogen receptor (ERα) silences progesterone receptor (PR) transcription and is accompanied by loss of marks indicating active chromatin at the PR locus, such as methylation of H3 at K4 and acetylation of H3 at K9. Accumulation of DNA methylation is observed by 36 hours after gene silencing occurs. Reactivation of PR expression requires both ERα and DNA demethylases.

Using a combination of antibodies against ERα and against methylated or acetylated chromatin, the epigenetic states of genes regulated by estrogen were determined. Upregulation of ERα targets was associated with an acetylation to methylation ratio greater than one, while genes with a ratio less than one were more weakly expressed. This work determined that ERα binding alone is not sufficient to predict up- or downregulation of estrogen target genes; instead, binding of co-regulator proteins determine up- or downregulation. Transcriptional regulatory modules of ERα targets include an estrogen receptor DNA binding element (ERE) and binding sites for co-regulator proteins. For example, a c-myc binding site is found near some EREs and c-myc is a positive modulator of ERα-regulated transcription.

To analyze the effect of microenvironment on epigenetic silencing in epithelial cells, a co-culture model was developed in which fibroblasts from healthy breast tissue samples or from breast cancer samples were co-cultured with MCF10A cells (normal breast epithelial cell line). After 4 weeks in culture, the normal fibroblasts form branch-like structures and flow cytometry can be used to isolate MCF10A cells and generate a pure MCF10A culture. Induction of promoter hypermethylation resulted in less methylation around transcriptional start sites in MCF10A cells co-cultured with normal fibroblasts compared with the MCF10A cells co-cultured with fibroblasts from the breast cancer samples. Promoter hypermethylation was not observed in fibroblasts that had not been co-cultured with the MCF10A cells. Comparison of normal breast tissue samples with samples from a primary breast tumor showed that methylation is increased in the tumor cells. This work demonstrates that cell-cell contact and conditioned media were required to regulate methylation patterns.

The methylated DNA immunoprecipitation (MeDIP) assay, which uses an antibody against methylated cytosine, was used to analyze estrogen imprinting and its effects on carcinogenesis. Estrogen imprinting refers to the effects of prolonged exposure to estrogen or related endocrine disruptors early in development that can increase the risk of developing cancer later in life. Diethylstilbestrol (DES) exposure is an example of estrogen imprinting; women exposed to DES *in utero* have a 2.5-fold increased risk of developing breast cancer. Estrogen imprinting is hypothesized to involve estrogen exposure of stem/progenitor cells, which are capable of self-renewal and give rise to amplifying populations of progenitors. Long-lived stem or progenitor cells are susceptible to environmental injury and transmit this "memory" of injury to epithelial progeny through epigenetic mechanisms. Mammospheres, which contain enriched breast

stem/progenitor cells were exposed to high-dose estrogen (70nM) for 2 to 3 weeks. The estrogen was then removed from the medium and the cells were cultured in 2-dimensional culture. MeDIP analysis showed that pre-exposure of the breast stem/progenitor cells to estrogen altered the DNA methylation profiles in their epithelial progeny. Approximately 120 hyper-methylated loci were identified in pre-exposed epithelial cells and 12 were confirmed by MeDIP-PCR. A number of these loci, including CST6 and RUNX3, have been observed to be downregulated in ERα-positive breast cancer. Increased levels of RUNX3 methylation are found in normal tissue adjacent to ERα-positive primary tumors. This work suggests that estrogen-induced epigenetic injury transmitted to epithelial cells may create a large field of cancerization in the human breast.

Discussion

A participant suggested analyzing normal breast tissue from women in the general population to determine if hypermethylation occurs before tumors develop. Dr. Huang explained that his experimental system uses doses of estrogen that are higher than physiological levels. The majority of epithelial cells do not contain ERα, and hypermethylation also occurs through non-estrogenic actions. Upon treatment with estrogen disruptors, methylation changes are observed but these do not necessarily occur through the estrogen signaling pathway.

Dr. Waterland noted the persistent effect of estrogen in the mammospheres and asked whether this occurred through induced epigenetic changes or through clonal selection. Estrogen exposure could select for a sub-population of cells with altered methylation patterns. Dr. Huang agreed that both mechanisms could be involved. Injury associated with exposure could cause an abnormally high proliferation rate. However, ERα can be detected in approximately 80 percent of pre-exposed epithelial cells.

Dr. Belinsky asked for clarification of the mechanism causing increased methylation of target genes in the fibroblast co-culture experiments. Dr. Huang explained that the content of primary fibroblasts was analyzed by gene array and CST6 was found to be downregulated in the co-cultured cells. Genes associated with the Akt pathway also were downregulated. Transfection with AKT resulted in CST6 silencing. Akt signaling may be activated through microenvironmental influences.

Dr. Dunn asked how chemoprevention by nutrients could be assessed in Dr. Huang's co-culture system. Dr. Huang answered that the mammospheres could be used to analyze the effects of phytoestrogens such as genistein to determine if nutritional intervention could reverse the processes and epigenetic effects caused by estrogen exposure. Dr. Dunn suggested that methylation could be used as a surrogate to clarify the pro- and anti-carcinogenic effects of soy and soy products.

A participant commented that analyzing compounds that affect ER and determining subsequent epigenetic events in mixed tissue cultures could be problematic. Dr. Huang agreed that finding the proper host cell and microenvironment to analyze breast cancer progression has been difficult. The MCF10A cell line is ER-negative and no normal cell line that expresses ER could be found.

Another participant noted that 99 percent of women with a mutation in BRCA1 develop breast cancer and that this gene is downregulated in sporadic cases of breast cancer. He asked whether insults could cause epigenetic silencing of tumor suppressor genes, and suggested that nutritional interventions could have an impact by preventing or reversing inappropriate epigenetic silencing. Dr. Huang agreed that a key process to analyze is whether nutritional interventions impact stem progenitor cells.

Folic Acid, Cancer, and Birth Defects: Managing Genome Stability and Expression

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Folic acid fortification represents the first time a population-based intervention has been used to target a specific sub-population, namely women of childbearing age with a susceptibility to neural tube birth defects. The mechanism by which folic acid prevents neural tube defects (NTD) and its possible deleterious effects on other sub-populations, such as people with precancerous lesions, is currently unknown.

One-carbon metabolism produces purines, thymidylate, and activated methyl groups. One-carbon units are partitioned among three pathways of the one-carbon metabolism network. In general, enzymes involved in one-carbon metabolism are present in most cells and many are encoded by housekeeping genes. The total concentration of folate in cells is limiting relative to the concentration of folate dependent enzymes, causing competition among the three pathways for folate-activated carbon. This competition for folate-activated one-carbons occurs between MTHFR and thymidylate synthase, and regulates the portioning of carbons between the methylation pathways and thymidylate biosynthesis. The tissue-specific cytoplasmic serine hydoxymethyl transferase (cSHMT) regulates competition between these two pathways. Under most conditions, carbons are shunted preferentially to MTHFR than to thymidylate synthase. This condition can be reversed by cSHMT, which binds 5-methyl THF with high affinity and sequesters it, inhibiting carbon delivery to the methylation pathway. The enzyme also accelerates the thymidylate synthase pathway by generating carbons from serine, which are preferentially shunted to the thymidylate pathway. During G1 phase, cSHMT is located in the cytoplasm, where it sequesters 5-MTHF. During S phase and G2/M phase, 20 to 40 percent of cSHMT moves to the nucleus, where the nuclear thymidylate biosynthesis pathway is active. This permits carbons to be preferentially delivered to thymidylate synthase. Localization of both cSHMT and TS to the nucleus is modulated by the small ubiquitin-like modifier (SUMO). In this manner, cSHMT serves as a switch by regulating the partitioning of one-carbons between the methylation and thymidylate synthesis pathways.

To determine if cSHMT functions as a regulatory switch in cancer or other pathologic conditions, a comprehensive folate genomic pathway analysis was performed using cSHMT null mice that were generated using the Cre/lox system and determining the effects of cSHMT on metabolism, genome stability, NTDs, and cancer. Mice null for cSHMT are viable and fertile, but an increase in the SAM to SAH ratio is observed, indicating that cSHMT impairs SAM synthesis in the mice. These mice also have decreased thymidylate synthase activity and

increased levels of uracil in the liver, indicating that cSHMT enhances dTMP synthesis and lowers uracil content of DNA.

cSHMT is expressed in the nuclei of actively dividing crypt progenitor cells in the colon, so cSHMT null mice were bred to Apc^{min} mice to determine the effect of cSHMT on colon tumors. When mice with decreased cSHMT levels were fed a control diet, no effects were observed. However, when these mice were fed a folate- and choline-deficient diet, cSHMT null heterozygotes developed an increased number of tumors while cSHMT null homozygotes had a decreased number of tumors. Homozygous cSHMT null mice exhibit a 10-fold increase in thymidylate synthase activity, resulting in an increase in *de novo* thymidylate synthesis, which offers protection against cancer development. Mice overexpressing cSHMT were exposed to azoxymethane, which chemically induces colon carcinogenesis. These mice were protected against colon cancer induction; no effect of diet was observed. Increased cSHMT expression also increases dTMP synthesis, which is inversely correlated with intestinal cancer risk.

A number of dietary components regulate cSHMT, including folate and vitamin B6. cSHMT also is developmentally regulated and zinc-, retinoic acid-, and ferritin-responsive. cSHMT networks also are responsive to lipid and carbohydrate metabolism, cell growth and development, immune response, and cancer. cSHMT knockout mice also have been created and NTDs are observed in both homo- and heterozygotes. This is the first knockout mouse model of NTDs and indicates that low levels of thymidylate contribute to NTDs, rather than increased homocysteine levels.

Discussion

Dr. Yang suggested that depurination is the most common from of DNA damage and may be important in cancer development. He asked whether purine synthesis was affected by cSHMT activity. Dr. Stover answered that purine synthesis was unaffected by cSHMT and that there was no evidence for the presence of purine synthesis enzymes in the nucleus.

Folate, DNA Methylation Machinery, and Breast Tumorigenesis

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Understanding the mechanisms involved in progression from ductal carcinoma *in situ* (DCIS) to breast cancer will aid in prevention of deaths from this cancer. Because aberrant DNA methylation (or de-methylation) can lead to possibly carcinogenic changes in gene expression, understanding the roles of nutrients, such as folate, that affect methylation is important for understanding the transition from DCIS to breast cancer. It is possible that folic acid fortification found in many Western countries promotes progression of pre-existing cancers because colorectal cancer incidence has risen since folic acid fortification began. However, folic acid also is important for normal DNA repair processes and thus may have both beneficial and harmful effects on cancer; the timing of exposure to folic acid in relation to the cancer process may determine benefit or harm.

To analyze the influence of folate (both deficiency and excess) and DNA methylation on the transition from DCIS to malignancy, mice expressing the Polyoma Virus middle T oncogene (PyVmT) under the control of the mouse mammary tumor virus long terminal repeat (MMTV LTR) were developed. MMTV-PyVmT expression is restricted to the mammary epithelium and mice develop multi-focal mammary tumors within 5 weeks of age. The molecular biology of the tumors is analogous to human breast cancer associated with ErbB2/Her2/Neu overexpression. Female mice heterozygous for the MMTV-PyVmT transgene were fed either a methyl-deficient diet or replete control diet for 10 weeks. Examination of whole mount mammary glands revealed that the methyl-deficient mice developed fewer tumors than mice fed the methyl-replete diet. Epigenetic proteins such as DNMT1, MBD2, and MeCP2 were expressed in normal ductal epithelium, in mammary intraepithelial neoplasia (MIN), and in mammary tumors; loss of protein expression was noted toward the center of the tumors. This work showed that dietary methyl deficiency can delay development of MIN and tumors in mice. The role of epigenetic proteins in the development of MIN and tumors remains to be determined.

Because the tumors in the MMTV-PyVmT mice showed diffuse, multi-focal spatial and temporal initiation, isolating the MIN-to-malignancy transition was difficult. To address this, transplantable MIN outgrowths (MIN-O) were developed. In this approach, a single MIN lesion is transplanted from a MMTV-PyVmT female to the gland-cleared fat pad of a nontransgenic mouse of the same background. The MIN-O transplants develop over time distinct proliferation, differentiation, and transformation zones. The hyperproliferative region of the MIN-O can be serially transplanted and latency and tumorigenic properties of the MIN-O transplant are maintained. *In vivo* MicroPET imaging of MIN-O growth also can be performed and used to monitor treatment of the tumors; rapamycin slowed growth of a MIN-O lesion. The MIN-O transplant model mimics human DCIS both biologically and molecularly and the MIN-O lesions and associated tumors are easily accessible for phenotypic and molecular analyses and for assessing the effects of pharmacological and nutritional interventions on the transition to cancer independent of initiation events.

To examine the effects of DNA methylation on breast cancer progression, the MIN-O model was used to determine whether the DNA demethylating agent 5-aza-deoxycytidine (ADC) would decrease MIN-O lesion growth, tumor incidence and size, and increase latency. Surprisingly, ADC caused faster transition of MIN-O lesions to tumors. ADC may be aberrantly upregulating genes that promote tumorigenesis. ADC has been used successfully to treat some forms of leukemia, but these results suggest that it would not be an effective therapy against breast cancer. Ongoing experiments are focusing on the effects of folate deficiency and folate excess on the MIN-O to tumor transition.

Discussion

Dr. Mason suggested that the effects of dietary folate deficiency and ADC on progression of MIN-O lesions might have been other than expected because the MIN-O lesions may have been transplanted at a stage beyond initiation, at which folate cannot modulate progression. Dr. Miller explained that if the initiated stage of the MIN-O lesion is not transplanted, tumors do not develop. These lesions also require the mammary fat pad for growth. The model has limitations for isolating the initiation stage, but is a good model for examining progression. A similar model

for precancerous prostate lesions is in development and models such as this for other tumors also should be pursued.

Making Sense of Skin – Polycomb Genes and Nutritional Chemopreventive Agents

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Polycomb genes (PcG) are epigenetic regulators of stem cell survival and regulate keratinocyte survival and differentiation. PcGs exert their effects primarily through selective silencing of gene expression, although some also activate gene expression. Two PcG product protein complexes have been described. The Eed complex (PRC2) is composed of the Eed, EzH1 and EzH2 proteins and participates in histone deacetylation and methylation. The Bmi complex (PRC1) includes the Bmi-1 polycomb gene product and a number of other proteins. The PRE serves as a binding site for the PRC complexes. PRC2 methylates histones and permits PRC1 to bind the DNA, close the chromatin structure, and silence gene expression. PRCs are regulated by MAP kinase cascades and thus can potentially be therapeutic targets.

Polycomb gene protein products regulate stem cell survival. Adult Bmi-1 knockout mice fail to maintain stem cell survival in the hematopoietic and neuronal systems. Bmi-1 is overexpressed in some epithelial cancers and may enhance "tumor stem cell" survival and tumor expansion. The effects of Bmi-1 overexpression on cancer progression may be mediated through the effects of Bmi-1 on cell cycle regulatory proteins. The Bmi-1 polycomb gene is expressed in the epidermis and may promote keratinocyte survival; Bmi-1 levels are increased in cultured transformed epidermal keratinocytes and epidermal squamous cell carcinoma.

Bmi-1 mRNA and protein are expressed in cultured keratinocytes, where they are localized to the nucleus. Bmi-1 also is expressed in the suprabasal layer of the epidermis, despite the lack of stem cells in this layer. However, survival and differentiation of keratinocytes occurs in this layer, suggesting that Bmi-1 may have a role in cell survival. A Bmi-1 encoding adenovirus was used to deliver Bmi-1 to the nucleus and analyze the effects of Bmi-1 overexpression on keratinocytes. Bmi-1 protected the cells from okadaic acid-induced apoptosis by inhibiting caspase activity. Bmi-1 also affected proliferation to facilitate survival through regulation of cyclins and cyclin-dependent kinases. Elevated levels of Bmi-1 mRNA are found in a number of skin cancer cell lines. Squamous cell tumors display punctate bmi-1 staining throughout the tumor, indicating that Bmi-1 is not limited to tumor stem cells but rather may promote growth and survival of numerous tumor cells.

Nutritionally-derived chemopreventive agents such as EGCG (green tea), curcumin (turmeric), and apigenin (alfalfa sprouts) have proven effective in preventing skin cancer in a variety of model systems. Bmi-1 could be a target for nutrition-based chemoprevention of skin cancer if these agents could suppress Bmi-1 level or activity to reduce cell survival and prevent keratinocyte immortalization, transformation, and cancer progression. EGCG suppresses Bmi-1 expression levels in transformed keratinocytes and also appears to promote Bmi-1 phosphorylation. Phosphorylated Bmi-1 falls out of the PRC1 complex, and exits the nucleus,

and thus can not longer exert its effects on chromatin and gene activity. EGCG also decreases cdk4 levels and reduces cleavage of poly (ADP-ribose) polymerase (PARP). Adding adeno-Bmi-1 to the cells reverses these EGCG-dependent changes and promotes cell survival.

Bmi-1 enhances survival of normal and transformed keratinocytes by enhancing cell proliferation and suppressing apoptosis; this is the first report of regulation of apoptosis by a polycomb gene. Bmi-1 levels are markedly increased in transformed keratinocytes and are likely to enhance survival of these cells. Chemopreventive agents such as EGCG suppress Bmi-1 level and induce Bmi-1 phosphorylation, resulting in displacement of the Bmi-1 polycomb protein complex from chromatin and reduced survival of transformed cells. Interfering with the function of the polycomb stem cell survival genes thus represents a new mechanism of chemopreventive agent action.

Discussion

Dr. Milner asked whether polycomb genes were part of the autophagy response. Dr. Eckert answered that this response is induced in parallel with decreases in Bmi-1. Dr. Milner noted that calorie decreases have been observed to decrease gene activity levels as well.

A participant asked how this system might interact with vitamins A and D to affect epithelial cell growth. Dr. Eckert predicted that there would be interactions with these vitamins since Bmi-1 is a pro-survival factor. Vitamins A and D may regulate Bmi-1 consistent with the need for cell survival or apoptosis.

NIH Epigenomics Roadmap

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Epigenetics is an emerging frontier of science that involves the study of changes in the regulation of gene activity and expression that are not dependent on gene sequence. For purposes of the NIH Epigenomics Roadmap program, epigenetics refers to both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable. While epigenetics refers to the study of single genes or sets of genes, epigenomics refers to more global analyses of epigenetic changes across the entire genome. The overall hypothesis of the NIH Epigenomics Roadmap Program is that the origins of health and susceptibility to disease are, in part, the result of epigenetic regulation of the genetic blueprint. Specifically, epigenetic mechanisms that control stem cell differentiation and organogenesis contribute to the biological response to endogenous and exogenous forms of stimuli that result in disease. The Epigenomics Working Group (EWG) is co-chaired by Samuel Wilson (National Institute of Environmental Health Science [NIEHS]), Nora Volkow (National Institute on Drug Abuse [NIDA]) and James Battey (National Institute on Deafness and Other Communication Disorders [NIDCD]), with Ting-Kai Lee (National Institute on Alcohol Abuse and Alcoholism [NIAAA]) as ex-officio chair. Members of the EWG come from all NIH institutes and the Office of the Director.

The goals of the Epigenomics Roadmap program include establishing an international committee to establish standard practices and platforms; developing new antibody reagents and creating a database; developing epigenomic mapping data and infrastructure to facilitate research in human health and disease; evaluating the epigenome in aging, development, environmental exposure (i.e., physical, chemical, behavioral, and social environments) and modifiers of stress; and developing new technologies for single cell analysis and remote imaging of epigenetic activity in cells, tissues, and whole animals. Interested investigators are encouraged to visit the NIH Roadmap weblink (http://nihroadmap.nih.gov/epigenomics/) for more information about the program and available funding opportunities.

Influence of Genetic Inheritance on Global Epigenetic States and Cancer Risk Prediction with DNA Methylation Signature: Challenges in Data Analysis and Informatics Infrastructure

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Epigenetic changes that are stable through mitosis and sometimes meiosis can be induced by environmental exposures such as diet and disease. Genetic background also may affect the global epigenetic states at the inter-individual and inter-chromosomal levels. Genetic imprinting is a form of epigenetic gene silencing that is determined by the parental origin of the allele. Allele-specific chromatin modifications can be determined using the ChIP-on-ChIP method with a SNP chip. This technique can determine if a particular allele (i.e., of paternal or maternal origin) is preferentially modified. Application of this technique together with principal component analysis (PCA) can cluster epigenetic differences between two families to explore the effects of genetic background on epigenetics and to determine how an epigenetic mark is transmitted through generations.

A population-based epigenomics study of esophageal squamous cell carcinoma is underway. DNA methylation was detected in blood and genotyping and methylation analyses were performed. Analysis of the data found that age does not significantly impact DNA methylation. The possibility of an association between cancer risk and DNA methylation also was explored and 200 SNPs were found at which methylation correlated or associated with cancer. This technique found differences in methylation large enough to discriminate between cases and controls using DNA methylation signature in a test set.

A systems biology approach is needed for analysis of complex diseases. Multivariate analysis will help to distinguish between cases and controls in complex diseases because it permits comparison of multiple genetic loci in a single test, although the effect size must be relatively large. Analysis of allele-specific gene regulation will require integration of large amounts of data. The caIntegrator component of caBIG will help with these analyses.

Discussion

Dr. Martin asked whether complex traits or diseases were the function of many genes or of epigenetic modifications. For example, in agouti mice phenotypic variation occurs in the absence of significant genetic variation. He asked Dr. Lee about a pedigree showing an epigenetic mark segregating with a particular haplotype and asked what was inherited. Dr. Lee answered that the epigenetic mark in agouti mice is the most important contributor to that phenotype, but this is a much cleaner system than what occurs in humans. Dr. Martin noted that attempts to explain complex diseases by multiple interacting genes have not been successful, perhaps because some or all of the phenotype is due to stochastic epigenetic variation. Epigenetic variation could be conditioned by genetic background variation. Some so-called polygenic traits may actually be attributable to epigenetic variation. Dr. Lee answered that a recent review on common diseases promoted a mixed genetic and epigenetic model. Concerning inheritance of the epigenetic mark or the haplotype, the epigenetic mark could have high or low co-segregation with the chromosome, but it is unknown whether the mark was retained or lost and then re-established. Dr. Martin commented that if the mark had a trans-acting effect, analysis would be complicated by changes in background.

Dr. Waterland commented that the PCA analysis shows a clear discrimination between cancer cases and controls. He asked about the methylation signature and whether the cases had received chemotherapy. Dr. Lee answered that he did not know whether the cases had received chemotherapy. The advantage to PCA is that each SNP contributes a small difference, but a large effect is discerned when analysis of individual SNPs is combined. Genome-wide methylation was analyzed using a 500K chip.

Dr. Milner noted that an age relationship for methylation patterns has been observed for the colon, but Dr. Lee's data did not detect a relationship in lymphocytes. Dr. Lee clarified that for analysis of 500 SNP sites, the majority showed no relationship between methylation and age. However, some SNPs were increasingly methylated with age, while others showed decreasing methylation with age. Dr. Milner asked whether blood would reflect events in the esophagus more clearly than events in the colon. Dr. Lee explained that his analyses were performed using normal esophagus tissue. He reiterated that methylation at most loci would show no dependency on age, gender, drinking, smoking, etc., but there would be one group of SNPs with positive correlations and another with negative correlations. Dr. Dunn noted that global hypomethylation increases with age, but this could be due to hypomethylation at repetitive elements that are not found on most SNP chips.

A participant commented on the implications of a 200 allele-specific SNP methylation pattern to discriminate between cancer cases and controls. This work appears analogous to gene expression profiling to discriminate between normal tissue and tumors. He asked Dr. Lee whether he had tested his markers in other samples. Dr. Lee answered that he has information on approximately 500,000 loci, thus even small variations in signal are sufficient for discriminating between cases and controls. There are plans to test this procedure for the ability to discriminate between new samples.

Future Research Directions

During the meeting, a number of research areas and technology needs were identified as important for moving research on epigenetics, diet, and cancer prevention forward. Many of these recommendations are captured in statements below.

Research areas

1. Timing and Exposure – When are bioactive food components (BFCs) beneficial and are there circumstances when they might be harmful?
    a) Identify crucial times for exposure during development and throughout the lifespan
    b) Evaluate if response is related to cancer stage, i.e., during initiation versus progression
    c) Identify circumstances which dictate a beneficial or deleterious response
    d) Assess histone deacetylase (HDAC) inhibitors and other epigenetic modulators in healthy people

2. Improved understanding of the biochemistry and effects of BFCs in epigenetic processes:
    a) Identify relevant BFC compounds and metabolites
    b) Determine effective doses and concentrations
    c) Identify relevant BFC targets
    d) Summarize data on mechanism of action of BFCs
    e) Ascertain pharmacokinetic data
    f) Evaluate effective and safe delivery routes

3. BFCs effects on epigenetic machinery
    a) Identify and characterize triggers of histone modifications and gene silencing
    b) Examine specificity of BFCs for particular HDACs and histone acetylases (HATs)

4. Links between epigenetic events and changes in gene expression
    a) Identify mechanism(s) by which DNA methylation changes are related to changes in gene expression (e.g., direct effects on target DNA or DNA methylation changes as a result of upstream events triggered by a BFC)
    b) Elucidate mechanism(s) by which histone modifications affect gene expression
    c) Expand the understanding of the mechanisms involved in biotinylation, acetylation, methylation, and ubiquitination of histones
    d) Promote a greater understanding of the link between one-carbon metabolism or other pathways relevant to methyl metabolism and promoter methylation
    e) Clarify the hierarchy for methyltransferase activity and CpG site methylation
    f) Evaluate tissue-specific epigenetic effects
    g) Determine importance of genetic polymorphisms in enzymes involved in relevant metabolic pathways (e.g., methylene tetrahydrofolate reductase) that influence epigenetically-controlled gene expression
    h) Identify epigenetic marks to use for early cancer detection and to monitor response to preventive or therapeutic interventions

Technology needs

1. Expand techniques for quantifying DNA methylation and histone modifications

2. Develop methods for genome-wide scans for DNA methylation and histone modifications

3. Create mathematical models to analyze how perturbation of one enzyme in a pathway affects downstream events and data to validate the models. Such models could be used to generate data for pilot studies and identify metabolites or nutrients with important effects that should be carefully monitored in trials.

4. Utilize technology for methyl-typing tumors

5. Develop robust multiplex assays, better high throughput assays, and commercialization of such assays

6. Construct more effective databases, computational approaches, and tools for integrating epigenetic data

7. Characterize reference epigenomes (profiles of DNA methylation and histone modifications)

8. Develop relevant animal and tissue culture models, including models that incorporate the effects of the microenvironment

9. Generate high-throughput assays applicable to screen large populations of diverse ethnicity and races