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The 20th Aspen Cancer Conference:

Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention, and Cancer Therapy 2005†

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INTRODUCTION

The 20th Aspen Cancer Conference on Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention, and Cancer Therapy was held from July 24 to 28, 2005 in Aspen, Colorado. Recognizing the importance of a 20-year history of excellence, the 2005 Aspen Cancer Conference included an extended scientific program as well as several special presentations and events. The scientific program, developed by a Scientific Advisory Committee co-chaired by Benjamin F. Trump (AMC Cancer Center) and Curtis C. Harris (National Cancer Institute, NIH), featured an extra day of seminars by former Aspen Cancer Conference Keynote Speakers. The returning keynote speakers included J. Michael Bishop, Douglas Hanahan, Thea Tlsty, Tony Hunter, and Scott Lowe. In a special public Conference Session held at the Aspen Institute, former NIH director Harold Varmus lectured on *Oncogenes and the Future of Cancer Therapy*, Ms. Karen Allred presented a piano recital, and former Ambassador to the Netherlands K. Terry Dornbush described *A Survivor's Story*, cancer from the perspective of a two-time prostate cancer survivor. Other special programming in acknowledgement of the 20th year of the Aspen Cancer Conference included a whole day devoted to drug discovery with presentations from Cecil Pickett (Schering-Plough Research Institute), James Doroshow (National Cancer Institute), and Carl Barrett (Novartis) a review of the history of the Aspen Cancer Conference in the context of the history of Aspen, Colorado presented by Donald King (National Library of Medicine), and an awards ceremony honoring sustained contribution to the Aspen Cancer Conference by Theodore Puck, Benjamin J. Trump, and Donald King.

The goals of the Aspen Cancer Conference are: to understand the mechanisms of toxicity and carcinogenesis, to understand and minimize cancer risk, to assess the public-health impact of cancer risk, and to develop the scientific basis for improving the diagnosis, prevention, and treatment of toxic and neoplastic disease. The Aspen Cancer Conference fosters intense interaction, discussion, and collaboration among key stakeholders in cancer research. One of the strengths of the Conference is the sustained participation in and support of the Conference

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by leaders in cancer research from government, academic, and industry sectors. Because of its unusual qualities, the Aspen Cancer Conference provides an environment that is uniquely conducive to generating novel insights related to current issues in cancer prevention, intervention, and therapy.

The 20th Aspen Cancer Conference included seven Conference Sessions, a Poster Session by Aspen Cancer Conference Fellows and numerous informal discussions. Session topics included Progress in Drug Development, Metastasis, Inflammation and Cancer, Stress and Cancer, Risk Assessment, Technology Innovations and Apoptosis. At the close of the 2005 Conference, the Scientific Advisory Committee met to select topics for the 21st Aspen Cancer Conference which will held from July 16 to 18, 2006. This meeting summary concisely describes the content of each of the Conference Sessions and the scientific portions of the Special Sessions.

SESSION 1: 20 YEARS OF KEYNOTE SPEAKERS

Mouse Models for Human Liver Tumors

J. Michael Bishop (The University of California, San Francisco)

Numerous oncogenes have been implicated in human hepatocellular carcinoma (HCC), including *myc*, *ras*, *met*, *beta-catenin*, *RB*, *p53*, and *axin*. Recent studies by J. Michael Bishop and colleagues focused on the role of three of these genes: *myc*, *met*, and *beta-catenin*. To study the role of *met*, a conditional transgenic mouse was constructed which allowed doxycycline-regulated overexpression of the human *met* gene at different times during murine development. The endogenous mouse *met* gene is developmentally regulated via posttranslational modification, with a peak of met activity at approximately E13, a pronounced decline in met activity from E13 to birth, and suppressed activity in adult mice with transient reactivation in regenerating liver. Several alternate protocols were tested to determine the effect of inappropriate met expression in developing or adult hepatocytes. When transgene expression was on throughout development and parturition, mice were born with massive hepatomegaly, hyperplastic progenitor cells, no mature hepatocytes, and liver failure. In contrast, if the transgene was turned off just before parturition, normal liver regenerated and mice survived up to 8 mo developing hepatic/biliary carcinomas of mixed lineage. HCC was induced at high incidence when *met* was constitutively overexpressed from birth onward, and both HCC and hepatic adenoma were induced by low level constitutive *met* expression during early embryogenesis and in postnatal animals.

In these mouse models, dysfunctional liver growth, including carcinoma, HCC, and adenoma, was initiated by *met* overexpression. In contrast, *myc* overexpression induces hepatoblastoma. De novo activating beta-catenin mutations were common in *met*-initiated HCC, but not in *met*-initiated adenomas or *myc*-initiated hepatoblastoma. Bishop concluded that these mutations were selected during the progression phase of *met*-initiated tumorigenesis, because the mutations were clonal in individual tumor nodules, and varied from one nodule to another. These conclusions were confirmed with hydrodynamic liver transfection, which gives a 30%-50% efficiency of hepatocyte transformation with the injected DNA constructs. The latter system was also used to demonstrate that an HNF1-alpha dependent pathway is inactivated in *met*-initiated adenomas.

Mouse models of human liver cancer may be useful in developing novel therapeutic approaches to specific cancers. Bishop deduced from previous studies that *myc*-initiated cancers may be sensitive to apoptosis induced by the small molecule cyclin-dependent kinase 1 (CDK1) inhibitor purvanolol. This idea was tested with mice with *myc*-induced hepatoblastomas. Treatment with purvanolol combined with transient suppression of *myc* transgene overexpression produced rapid tumor regression and prolonged survival significantly. This

result may not be specific to *myc*-induced hepatoblastoma, and suggests the possibility that other *myc*-induced or *myc*-dependent cancers may respond to purvanolol or other CDK1 inhibitors.

Regulation and Multi-Targeting of the Angiogenic Switch During Tumorigenesis

Douglas Hanahan (The University of California, San Francisco)

Douglas Hanahan described studies of the angiogenic switch and mechanisms that modulate angiogenesis with a mouse model for pancreatic cancer. In this model, targeted oncogenesis is induced in nodules containing pancreatic islet cells by expressing SV40 T antigen under the control of the rat insulin promoter (RIP-Tag). These genetically engineered mice develop approximately 50% dysplastic/hyperplastic nodules by 5-7 wks, ~10% angiogenic nodules at 7-12 wks and 2%-4% tumors by 12-14 wks. Hanahan examined the requirement for vascular endothelial growth factor (VEGF) signaling in different pancreatic cell types and in different stages of tumorigenesis. The requirement for the VEGF gene was tested by beta cell-specific Cre-lox mediated knockout of the *VEGF-A* gene. This dramatically reduced the number of angiogenic nodules as well as reducing tumor burden and number. Somewhat surprisingly, expression of VEGF-A and VEGF receptor 2 (VEGFR2) in normal pancreatic tissue remained unchanged, despite the impaired angiogenesis in mice with islet cell-specific VEGF knockout. Hanahan explained this result by showing that tumor-infiltrating immune cells secrete matrix metalloproteinase 9 (MMP-9), which mobilizes VEGF in the tumor, while VEGF remains sequestered and inactive in non-tumor tissue. Thus, VEGF and VEGFR colocalize in tumor but not normal cells, and MMP-9 expression is detected in tumor-infiltrating neutrophils and macrophages, but not in pancreatic cells. The role of MMP-9 was confirmed by genetic or pharmacologic inactivation of MMP-9, which reduced angiogenic switching as well as tumor number and size. Similar results for cathepsin proteases and heparanase suggest that they may also play a role in stimulating pancreatic tumor angiogenesis in this model.

The potential of VEGF/VEGFR signaling as a therapeutic target was examined with a small molecule inhibitor of VEGFR, SU5416. Although SU5416 can block angiogenesis in early stages of the pancreatic cancer model, and it slows growth of small tumors, thereby prolonging survival, SU5416 is not effective in later stages of disease and does not cause significant levels of tumor regression. This data resembles the clinical data for Avastin, a human monoclonal antibody to VEGF. However, Hanahan demonstrated that maintenance of tumor-supporting vasculature during late stages of tumorigenesis depends on a PDGFR-dependent interaction between tumor cells and pericytes, and that PDGFR is expressed in pericytes but not in tumor cells. Thus, combination therapy with SU5416 and Gleevec (a PDGFR inhibitor) is an efficacious and tumor-specific method to interfere with maintenance of tumor vasculature during late stage pancreatic cancer, and other combination therapies that inhibit VEGFR and PDGFR have similar potential.

Several lines of evidence show that VEGF signaling blockade loses its efficacy over time. Hanahan explained this result by showing that alternative pro-angiogenic pathways are activated to support tumor growth in the absence of VEGF signaling. These include efrin- and FGF-dependent pathways. Thus, therapeutic agents that suppress these pathways could increase the anti-tumor effect of agents that block VEGF signaling.

In summary, Hanahan emphasized that mechanistic understanding of the process of angiogenesis will facilitate development of effective anti-angiogenic cancer therapies. These therapies will be most effective if they target multiple tumor and tumor-supporting cell types (i.e., tumor cells, infiltrating immune cells, pericytes) as well as multiple proangiogenic signaling pathways (i.e., VEGF, platelet-derived growth factor (PDGF), efrin, fibroblast growth factor (FGF)).

Genetic and Epigenetic Changes in Early Carcinogenesis

Thea Tlsty (The University of California, San Francisco)

Thea Tlsty is investigating genetic and epigenetic changes in the early stages of breast cancer. In previous studies, Tlsty identified discrete foci of preneoplastic variant human mammary epithelial cells (vHMEC) in the breasts of disease-free women. vHMECs have an unusual phenotype, including the ability to evade senescence in culture, genetic instability, p16 hypermethylation, abnormal centrosome number, and telomere dysfunction. Tlsty showed that loss of p16 function via hypermethylation or siRNA-mediated knockdown is sufficient to confer this complex phenotype on normal HMECs, and conversely, wild-type p16 fully complements the phenotype of vHMECs in culture. Comparative genomic hybridization (CGH) analysis of vHMECs suggests that the chromosomal instability of these cells manifests in a stochastic, non-clonal manner. However, in contrast, a non-random signature of hypermethylation is observed in vHMECs. An early target of hypermethylation in these cells appears to be HOXA9, a key regulator of HMEC differentiation, which is inactivated in many human breast cancers. Thus, hypermethylation of HOXA9 stabilizes vHMECs in an undifferentiated state during tumor progression. De-methylation of chromatin with 5-azacytidine reactivates HOXA9 in vHMECs, as indicated by expression of a downstream HOXA9 target. This suggests that the hypermethylation of HOXA9 is functionally related to loss of HOXA9 signaling in vHMECs and that vHMECs retain a wildtype HOXA9 gene. In normal cells, HOXA9 is negatively regulated by the polycomb complex. Tlsty showed that HOXA9 regulation in vHMECs also involves polycomb proteins. In particular, p16 expression appears to be inversely correlated with expression of Polycomb group proteins, and positively correlated with HOXA9 expression.

To facilitate therapeutic approaches to early breast cancer, Tlsty identified a preliminary gene expression signature in vHMECs. These data were used to correlate cyclooxygenase-2 (COX-2) overexpression with tumor progression, suggesting that COX-2 may play a key role in stimulating a tumor-promoting pro-inflammatory process. This idea is strengthened by the fact normal human breast tissue contains foci harboring cells with both COX-2 overexpression and p16 hypermethylation, and these markers are retained in a high percentage of later stage human breast tumor cells.

In summary, Tlsty showed that epigenetic changes play a key role in the preclonal phase of carcinogenesis, and that preclonal variant epithelial cells may be useful for identifying biomarkers for early breast cancer detection as well as novel targets of preventive breast cancer therapy.

Protein Phosphorylation and Intracellular Signaling Networks: Implications for Cancer Therapy

Tony Hunter (The Salk Institute)

Tony Hunter presented a brief overview of the role of protein kinases in human cancer. The human “kinome” includes 518 genes, 150 of which are implicated in disease phenotypes, the majority of which are cancer or cancer-related diseases. Of the 90 human tyrosine kinase genes, at least half have been implicated as oncogenes or tumor suppressor genes in cancer cells. Tyrosine, serine, and lipid phosphatases may play an equally important but as yet less well characterized role in cancer development.

The catalytic domain of protein kinases is highly conserved. Thus, there was early skepticism about the feasibility of finding or designing small molecule inhibitors with sufficient selectivity to avoid undesirable off-target effects. This fear proved groundless, and after several high profile successes with highly selective targeted oncogene inhibitors (i.e., Gleevec™ inhibition

of Bcr-Abl, Iressa™ and Tarceva™ inhibition of mutant epidermal growth factor receptor (EGFR)), it has become evident that agents that inhibit multiple protein kinase targets may be quite useful, if a desirable selectivity spectrum is achieved. While several small molecule tyrosine kinase inhibitors (TKIs) have now been approved for clinical use by the US Food and Drug Administration (FDA), and >30 kinase-targeted drugs are currently in clinical trials or in development, Hunter predicted that novel protein kinase targets for drug therapy and novel kinase-targeted reagents will continue to emerge and be brought to market in the near future. However, to implement such drugs effectively, new methods to customize drug therapies to individual patients will be urgently needed.

The PI-kinase like protein kinases belong to an unusual group of large (>3000 amino acids) multifunctional protein kinases, which includes the Ataxia Telangiectasia Mutated (ATM) protein kinase, ATM-related protein kinase (ATR), and DNA-dependent protein kinase (DNA-PK), all of which are involved in the response of cells to DNA damage. ATM plays a key role in the response to DNA double-strand breaks (DSBs) and loss-of-function mutations cause the human disease Ataxia Telangiectasia (AT). ATM exists as an inactive dimer. In the presence of DSBs, ATM undergoes autophosphorylation at S1981, which stimulates dissociation of the inactive dimer into active monomers. Two other human DNA repair-deficiency syndromes related to AT are Nijmegen Breakage Syndrome and AT-like disorder, which are associated with genetic defects in NBS1 and Mre11, respectively. Previous studies show that Rad50, Nbs1, and MRE11 interact with each other and form a complex called MRN, which also plays an important role in DSB repair. Although Nbs1 is a phosphorylation target of ATM, some evidence suggests that it also acts upstream of ATM in the DSB response pathway.

Hunter used a *Xenopus* egg cell free system to examine the role of ATM and MRN in DSB recognition, and the mechanism by which DSBs activate ATM. When ATM is incubated in the *Xenopus* egg extract with linear plasmid DNA or restriction enzyme-treated chromatin, S1981 is phosphorylated and ATM is activated. In contrast, S1981 phosphorylation does not occur when circular plasmid DNA or intact chromatin is incubated with ATM in the *Xenopus* extract. ATM activation requires a minimum of 200 bp of dsDNA, is blocked by ethidium bromide, and is inhibited in Nbs1-depleted *Xenopus* extracts. However, the C-terminal region of Nbs1 is sufficient to activate ATM in Nbs1-depleted *Xenopus* extracts. Hunter showed that inactive ATM binds linear DNA in the presence but not the absence of Nbs1, and tightly bound terminal DNA-protein complexes, such as Lac I-bound to a tandem repeat of Lac operator sequences prevent ATM activation. These results lead to the following model for ATM activation: MRN binds to and recruits inactive ATM to DNA ends. Upon interaction with the DSB, ATM is activated and then active monomers translocate along nucleosome-assembled DNA, promoting full ATM activation in a cooperative manner, thus greatly amplifying the signal generated by a single DSB.

Escaping p53 Action During Tumor Development and Therapy

Scott Lowe (Cold Spring Harbor Laboratory)

Scott Lowe is interested in identifying novel proteins that participate in the p53 signaling network. To facilitate this goal, Lowe has developed novel short-hairpin RNA (shRNA)-based methods that can be used ex vivo or in vivo in mice to characterize the global p53 signaling network. These methods are intended to expand on and complement existing transgenic and knockout-based approaches in mice. The models described here involve primarily targeted shRNAs, but ultimately, increasingly complex shRNA libraries will be used in similar protocols to screen for novel cancer therapeutic targets.

Initial experiments were carried out in a mouse strain sensitized to B cell lymphomas by expression of a latent *Myc* oncogene. B cells were isolated from these mice, transfected with

targeted shRNAs, and the engineered B cells were then reintroduced into lethally-irradiated animals. Lowe showed that shRNA-mediated knockdown of *p53* or *PUMA* strongly accelerated tumor formation. Similar approaches can be used to test the importance of molecules upstream or downstream in the p53 signaling pathway for lymphoma development or to screen for novel components of p53 signaling.

Lowe used a similar system to modulate and study chemosensitivity of Myc-induced B cell lymphomas. This experiment was carried out as an in vivo tumor cell competition assay, in which genetically-distinct B cell progenitors were labeled with or without green fluorescent protein (GFP), and the relative proportion of “green” and “white” tumor cells was followed in the absence of chemotherapy or in the presence of adriamycin or adriamycin plus rapamycin. When GFP-expressing cells co-expressed shRNA targeting PTEN, green cells outgrew white cells in the absence of adriamycin. In contrast, white cells outgrew green cells in the presence of adriamycin, and the effect of adriamycin was abrogated by addition of rapamycin. Thus, PTEN deficiency increases the rate of tumor growth and also increases the sensitivity of the tumor to chemotherapy with adriamycin. This experiment demonstrates that the effects of PTEN mutation are context dependent in this genetic background.

Previous studies show that many Gleevec-responsive chronic myelogenous leukemia (CML) cells eventually become resistant to Gleevec. One possible mechanism for Gleevec resistance is mutation of the Bcr-Abl oncogene, but some studies suggest that p53 status also plays a role in responsiveness to Gleevec chemotherapy. Lowe tested the latter idea by measuring Gleevec responsiveness in cells with or without p53-targeted shRNA. The results support the idea that wild-type p53 potentiates sensitivity to Gleevec, and that loss of p53 function correlates with resistance to Gleevec in CML cells.

In recent studies, Gregory Hannon (Cold Spring Harbor Laboratory) and Steve Elledge (Harvard University) have collaborated with Lowe and colleagues to develop so-called “second generation” shRNA libraries. These libraries utilize vectors that resemble endogenous microRNAs, but they are engineered to target specific genes with high selectivity and sensitivity. It is thought that these vectors will enter the RNAi pathway more efficiently and result in more effective and more stable gene knock-down than first generation shRNAs. In addition, these microRNAs can be expressed from polymerase II promoters instead of the RNA polymerase III promoters used to synthesize most siRNA constructs. This is important because it will make it possible to express targeted shRNAs in a temporally and spatially-restricted manner, significantly increasing the usefulness of siRNA technology.

SPECIAL SESSION

Oncogenes and the Future of Cancer Therapy

Harold Varmus (Memorial Sloan-Kettering Cancer Center)

Harold Varmus reflected on the status of our country’s “war on cancer,” indicating that there are reasons for both optimism and pessimism towards the prospect of winning this war. Two relatively high profile recent statements reflect these different viewpoints. A relatively pessimistic viewpoint was expressed recently by Clifton Leaf, Executive Editor of *Fortune* magazine, who published an article entitled “Why we are losing the war on cancer,” in which he lamented the lack of success in reducing cancer deaths and the paucity of appropriate models for studying the disease. A contrasting and very optimistic view was expressed by Andrew von Eschenbach, Director, National Cancer Institute (NCI), who announced in 2003 a new goal for NCI to end all cancer-related deaths in the US by the year 2015. Major advances have indeed been made in fighting cancer, as exemplified by the fact that the late Chief Justice Rehnquist remained active on the Supreme Court while undergoing therapy for thyroid cancer. We now

think of *living* with cancer, and even continuing to work while being treated for cancer. Because of advances in cancer research and therapy, we now think of cancer as a chronic disease, instead of thinking of it as a sentence to certain and rapid death.

Varmus also pointed out that great progress has been made in helping patients cope with cancer, for example by mitigating chemotherapy-induced nausea, improved targeting of radiation therapy, improved imaging techniques, and by providing support networks that help resolve psycho-social issues related to surviving cancer and cancer therapy. It is easy to lose sight of the progress made in all of these areas in the last decade. Significant progress has been made in understanding the etiology of liver and lung cancer, and improved and more thoroughly implemented diagnostic methods have been developed for colon, breast, and other cancers. We can also now effectively prevent a few types of cancer.

What has allowed these achievements and this progress to occur? And how can we promote continued or increased progress in the fight against cancer? According to Varmus, two types of information have fueled recent progress in understanding and treating cancer.

First, three types of cancer genes have been identified: proto-oncogenes, tumor suppressor genes, and genetic stability factors. Proto-oncogenes facilitate cell proliferation and gain-of-function mutations in these genes promote cancer; tumor suppressor genes inhibit cell proliferation and loss-of-function mutations in these genes promote cancer; and genetic instability facilitates cancer development and progression, so loss-of-function mutations in genes that promote genetic stability, or gain-of-function mutations in genes that promote genetic instability play a key role in cancer development and progression. Simply identifying these genes has played a large role in supporting advances in cancer research.

Second, the biochemical characteristics of the protein products of cancer genes are being revealed in greater and greater detail, and this has led to greatly increased understanding of the molecular mechanisms of carcinogenesis. However, it remains to be discovered how well we can leverage our increased knowledge and understanding of cancer genes and mechanisms of carcinogenesis towards developing effective cancer therapies, and bringing them to those who suffer from with this disease.

Varmus offered two encouraging reasons why the effort to improve cancer prevention and treatment is likely to succeed. The first encouraging development is that the first “targeted” cancer therapies are now reaching the clinic and improving the quality of life and the prognosis of many cancer patients. The best known example of a targeted cancer drug, Gleevac, is being used to treat a large number of cancer victims in the US and other countries (more than 12 000 new patients per year in the US alone) who suffer from CML, gastrointestinal and stromal tumors (GIST), and several other cancer disease syndromes. This drug is a clear success story, demonstrating that mechanistic understanding of oncogenes, in this case the genes *Bcr-Abl*, *c-kit*, and *PDGF* can be leveraged to generate novel and efficacious cancer therapies.

Second, it is now evident that cancers display “oncogene dependence” or “oncogene addiction.” Because of this fact, it is possible to prevent cancer growth, and induce cancer regression by inhibiting or repressing one or a few genes, a fact that is exploited by the targeted cancer therapeutics referred to above. The clearest demonstration of oncogene dependence comes from engineered mouse models, in which K-ras (or another oncogene) is conditionally expressed under control of an inducible promoter. In some cases, animals that consume food or water containing the inducer doxycycline express the oncogenes, while animals that do not consume doxycycline do not express the oncogene. This and other similar systems have been used to show that tumor growth occurs only when the oncogene is expressed, and conversely, that tumor cells die (through an active process called programmed cell death) when the oncogene is turned off. These models provide the opportunity to perform simple controlled

experiments in which cancer growth can be arrested by inhibiting expression of a single oncogene.

Unfortunately, the real world of cancer treatment is not nearly as simple as the mouse model system described above. Human tumors are genetically heterogeneous, and for drugs such as Iressa, which is used to treat EGFR-dependent non-small cell lung carcinoma (NSCLC), the presence or absence of a single mutation that causes a single amino acid substitution in the active site of the oncoprotein can determine whether a patient will respond to the drug or not. Even in patients that do respond, resistance to the drug of choice often develops after a few months of therapy. Other complications include difficulty in genotyping human tumors, poor clinical trial design, insufficient information to optimize drug dose, and time of treatment for different cancers and individual patients, and the multigenic nature of many human cancers. To solve the latter problem, Varmus suggested that it may be necessary to carry out the recently proposed “Human Cancer Genome Project,” which would systematically define a subset of all mutations in human tumor cells. It has been estimated that such a project would cost \$1.3 billion over 10 years (approximately 0.3% of the total budget of the National Institutes of Health (NIH) over the same time frame), which Varmus felt could turn out to money well spent.

Cultural differences between cancer researchers and clinical oncologists also interfere with progress in winning the war on cancer. Greater emphasis is needed on interdisciplinary collaboration and training, in order to bring these two different parts of our scientific community together. Equally important is better dissemination of new knowledge, including rapid open-access publication of research findings by cutting-edge professional scientific journals. Cancer research data should not only be available to all scientists and clinicians working in the field of cancer research, but they should also be available to cancer survivors and to the taxpayers whose tax dollars are supporting the research.

In closing, Varmus suggested that the “destiny” of oncogenes could be fulfilled if (1) we complete the Human Cancer Genome Project; (2) novel targeted therapeutic strategies against cancer genes are developed; (3) we improve our ability to prevent or treat drug-resistant cancers; and (4) more combination therapies for cancer are developed.

A Survivor's Story

K. Terry Dornbush (Former Ambassador to the Netherlands)

K. Terry Dornbush addressed participants in the 2005 Aspen Cancer Conference with the goal of conveying a sense of what it is like to be a two-time prostate cancer survivor. Dornbush expressed humility in the face of his disease and its recurrence, and honor in having the opportunity to talk about his experience as a cancer patient with researchers who are working at the cutting-edge of the fight against cancer. While Dornbush acknowledged that cancer had brought doubt, fear, anxiety, pain, and emotional stress into his life, he chose not to address this aspect of dealing with cancer in public. Instead, Dornbush emphasized the hopeful side of his story, and the potential benefit that new cancer treatments can bring to other prostate cancer victims *and* himself.

Dornbush demonstrated a large breadth of knowledge about not only current and experimental prostate cancer treatments, but about the historical development of such therapies and their scientific basis and rationale. He chose to undergo radiation therapy as treatment for his recurrent prostate cancer, knowing that the efficacy of this relatively novel treatment for prostate cancer had not yet been established. As if preparing himself for yet another battle with prostate cancer, Dornbush also discussed and encouraged support for “The Prostate Specific Antigen Working Group,” a group of leaders in prostate cancer research who are working to facilitate interim drug approval and to develop treatment guidelines for patients with recurrent

disease. This work has become particularly important to Dornbush, because there are few available treatment options for patients with recurrent prostate cancer, and another indication of Dornbush's ongoing involvement in cancer research is that he and his family have provided direct financial support for development of I-CON-1, a novel prostate cancer therapy based on immuno-stimulatory and anti-angiogenic approaches.

At the end of his address, Dornbush left his audience touched by his story, impressed by his strength of character, hopefulness and knowledge, and inspired to contribute in kind to the fight against prostate and other cancers.

SESSION 2: PROGRESS IN DRUG DEVELOPMENT

Glenn Begley (Amgen, Inc.) chaired and introduced the second Conference Session on Progress in Drug Development. This Session included presentations by Jeffrey Waring (Abbott Laboratories), Tyler Jacks (Massachusetts Institute of Technology), Tona Gilmer (GlaxoSmithKline), Mike Sherman, (Iconic Therapeutics, Inc.), Winald Gerritsen (Vrije Universiteit Medical Center), and Tom Curran (St. Jude Children's Research Hospital). These presentations focused on novel models or novel therapeutic approaches that may resolve ongoing questions or problems in cancer therapy. Issues related to improving the success rate and reducing the attrition rate of oncology therapeutics were addressed throughout this Session.

In introducing this Conference Session, Begley pointed out that the number of oncology products approved per year has been decreasing from the mid-1990s to the present, despite large increases in research and development expenditures in oncology. Approximately 50% of all products in clinical development are oncology drugs, but the success rate of oncology drugs produced by the top pharmaceutical companies over the last 5 years is only 5%. Furthermore, the return rate on venture capital invested in oncology products has remained low and much poorer than competing sectors. This fact, combined with the fact that it typically takes 13 years before an oncology product is brought on the market, has made it more and more difficult to raise the funds needed to support research initiatives in oncology products.

There are many possible reasons for the high failure rate of cancer drugs: for example, patients are often diagnosed with late stage disease, which is more difficult to treat than early stage disease; tumors are heterogeneous and unstable; drugs are often modeled for treating tumors, but tumor metastases are often responsible for cancer mortality; and preclinical data is often a poor predictor of clinical outcome.

Begley argued that new drug development is only one of the factors that limit the success of oncology products. The drugs that we do have are not always used in the optimal manner. For example, prophylactic granulocyte colony stimulating factor (G-CSF) is under prescribed during adjuvant therapy for non-Hodkin's lymphoma and breast cancer, despite clear evidence indicating that significant survival benefit results from its use.

The oncology field must recognize the fact that despite huge investments in financial and other resources to prevent and/or treat cancer, the overall US mortality rate for cancer has remained constant over the past 25 years, while mortality for most other common diseases including heart and cardiovascular disease has declined significantly. Begley ended his introductory comments by urging Conference participants to openly discuss novel approaches that might reduce the rate of attrition for oncology therapeutics.

Shifting Attrition Earlier in Drug Discovery Using Gene Expression Profiling

Jeffrey F. Waring (Abbott Laboratories)

One of the reasons that drug development is so costly, is that drug failures tend to occur late in the drug discovery process. The most common point of attrition is during toxicology testing. According to traditional practice in the pharmaceutical industry, toxicology testing uses the 2-wk bioassay in rodents as well as other cell-based toxicology assays. These animal tests are labor-intensive, drug intensive, and cost intensive, with a price tag of up to \$1 M per drug. They carried out near the end of drug development, in part because large amounts of drug are needed. Because of this situation, there is great interest in and need for assays that can be used earlier in drug discovery to screen for potential toxic effects. There is also currently significant enthusiasm for the potential of toxicogenomics-based assays to help solve this problem. Traditional animal-based bioassays would presumably still be carried out, but the rate of attrition at this step could be lowered significantly, if failure at this step could be predicted accurately with a non-animal-based molecular bioassay. Toxicogenomics-based screens could potentially be employed at the earliest phase of drug discovery to test for target specificity, as well as for lead optimization, preclinical safety testing, or to test putative mechanisms of drug action.

The value of with toxicogenomics in drug development is demonstrated by the results of pilot studies with an Abbott drug called Abbott-001. This drug was initially evaluated at 20, 80, and 200 mg/kg in a repeat dose 2-wk rat bioassay. The drug caused severe myocardial degeneration, necrosis, and death at the high dose; however, initial studies showed no toxic response in rats treated with 20 or 80 mg/kg, suggesting that the drug could might be clinically useful at the lower end of the tested dose range. To explore this possibility, heart tissue from treated rats was subject to gene expression analysis, which identified a distinct gene expression signature associated with drug-induced cardiac toxicity. Furthermore, this study showed that the signature of cardiac toxicity appeared after a single high-dose exposure to Abbott-001 as well as in some, but not all, rats treated for 1 d with 80 mg/kg. Additional studies determined that Abbott-001 kills rats by inducing mitochondrial dysfunction in cardiac tissue. This discovery led Abbott to modify their drug development strategy to include direct assays for cardiac and/or mitochondrial toxicity at an early stage of drug development. Similar application of toxicogenomics is envisioned during development of other types of drugs.

Several in vitro screens are currently available to identify toxic responses in non-animal-based systems. However, it may be possible to identify many toxic responses by gene expression analysis as well. For example, Abbott has used a rat hepatocyte test system to identify gene expression signatures associated with apoptosis, necrosis, phospholipidosis, canalicular cholestasis, microvesicular steatosis, peroxisome proliferation, and exposure to Ah receptor agonists. The ability to accurately classify unknown toxicants based on their gene expression profile has been established, thus laying the groundwork for widespread use of toxicogenomics in toxicity screening.

One possible future application of toxicogenomics may be in anticipating idiosyncratic toxicity in humans. Idiosyncratic toxic reactions are very rare events, thought to be human specific and dose independent, which are not likely to be detected with traditional toxicology studies in animal models. An example is the occurrence of liver toxicity in patients treated with trovafloxacin, a quinalone that inhibits bacterial enzyme DNA gyrase. Liver toxicity was reported at a rate of 150 in 2.5 million humans exposed to the drug. Gene expression studies in human hepatocytes revealed a trovafloxacin-specific signature of oxidative stress, while human hepatocytes exposed to a control quinalone and rat hepatocytes exposed to trovafloxacin did not show evidence of oxidative stress. Even if such an approach is primarily used “after-the-fact,” as described here, if the results help to identify the mechanism of toxicity in humans, they could potentially be used to eliminate the undesirable side effects and improve drug safety and efficacy.

In summary, toxicogenomics-based assays appear to have significant potential to improve drug development by (1) revealing mechanisms of drug toxicity observed in animal models; (2) profiling compounds for toxic effects early in drug development; (3) providing non-animal based methods for toxicity testing; (4) classifying unknown toxicants; and (5) identifying potential mechanisms of idiosyncratic toxicity in humans. Together with increased use of other alternative in vitro toxicity tests, these advances should help shift attrition earlier in the drug discovery process.

Modeling Cancer in the Mouse

Tyler Jacks (Massachusetts Institute of Technology)

Tyler Jacks described a genetically-engineered mouse model for human lung cancer, and its use in screening and development of anti-lung cancer drugs. Human lung cancer is often associated with mutations in K-ras or p53, and the mouse model developed by Jacks is based on oncogenic activation of the endogenous mouse *K-ras* gene with a knock-in strategy. The mutated K-ras allele is driven from the endogenous K-ras promoter, but has a “floxed” stop codon that blocks transcription prior to its removal and gene activation with Cre-lox recombinase. To remove the stop codon, activate oncogenic K-ras transcription, and stimulate lung tumor development, mouse lung tissue is exposed to Cre-lox recombinase via an aerosolized preparation of recombinant adenovirus (Adeno-Cre). Exposure to the virus stimulates K-ras expression in a tissue-specific manner in a nearly synchronous window of time after viral infection. Several cell types in the lung can be infected by adenovirus, but the cells that appear to become tumor progenitor cells are known as broncho-alveolar stem cells (BASC). Adeno-Cre-infected BASC proliferate and progress to adenocarcinoma in approximately 16 wks with little evidence of invasion or metastasis. However, when the K-ras activated mouse also carried a Cre-lox-dependent activating mutation in p53 or a Cre-lox-dependent deficiency in p53, a much more aggressive lung cancer phenotype was observed. The disease phenotype of K-ras p53 double mutant mice more closely resembles late stage human lung cancer in its severity and invasiveness than the phenotype of K-ras mutated mice.

Previous studies suggest that mitogen-activated protein kinase (MAPK) is a key downstream target in the activated K-ras pathway leading to human lung cancer. Surprisingly, MAPK activity was not upregulated in K-ras-dependent tumors in this mouse model. Jacks proposed that the MAPK pathway may be upregulated in the early stages of tumor development in these mice and subsequently turned off (i.e., by upregulation of MAPK phosphatases). In contrast, expression of protein kinase-B alpha (PKBalpha, RAC, or Akt) and *c-jun* N-terminal kinase (JNK) were upregulated, and Jacks showed that inactivation of RAC by Cre-lox-dependent targeted deletion blocked tumor progression.

As described above, conditional K-ras or K-ras p53 mouse models are useful for investigating mechanisms of initiation and progression in lung cancer. However, these models can also be used to study the efficacy of chemotherapy or other therapeutic approaches. Jacks combined this system with novel imaging tools to study the efficacy of traditional cisplatin treatment for lung tumors. Histological analysis showed that many tumors regress after exposure to cisplatin, but other lung tumors in the same animal respond partially or not at all. Small animal imaging with Micro computer tomography (MicroCT) technology confirmed the heterogeneous response of tumors to cisplatin. Furthermore, microarray analysis demonstrated clear differences between the gene expression profile of cisplatin-sensitive and cisplatin-resistant lung tumors. Thus, additional studies may be able to determine the molecular basis of resistance in the tumors that do not respond to cisplatin.

In earlier studies, Jacks demonstrated that expression of cathepsin proteases are upregulated in lung tumors. Jacks exploited this observation to develop a novel “smart” optical imaging

probe which carries quenched fluorochromes that are activated by cathepsin-dependent cleavage. This probe can be used to monitor tumor growth or regression in real time in a non-invasive manner. Combined with genetically-engineered mouse models, use of such probes will facilitate studies of chemoprevention and chemotherapy for lung cancer.

Lapatinib (GW572016): A Novel Approach to Inhibit ErbB1/ErbB2

Tona Gilmer (GlaxoSmithKline)

Tona Gilmer described a dual v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ErbB2)-ErbB1/EGFR TKI known as lapatinib (GW572016) that is being developed as a potential treatment for breast cancer. Lapatinib is a large head group quinazoline that binds with high affinity (3 or 13 nM) and slow off-rate ($t_{1/2} > 2$ h) to ErbB2 and ErbB1, respectively. The crystal structures of inhibitor-EGFR complexes reveal that lapatinib binds deep in the active site and stabilizes an “inactive-like” conformation of the receptor tyrosine kinase, while the EGFR-specific quinazoline inhibitor erlotinib remains solvent exposed and binds to an active conformation of the receptor. Lapatinib is unique among EGFR-targeted TKIs in having a low nM binding affinity for ErbB2.

Preclinical in vitro studies show that lapatinib inhibits proliferation of some but not all human cancer cell lines. Initial analysis suggests that susceptibility to the anti-proliferative effects of lapatinib correlates with overexpression of ErbB2 and with PTEN status in these cells. In cell lines that are responsive to lapatinib, exposure to lapatinib inhibits phosphorylation of Akt1, and downstream targets of Akt1 and stimulates apoptosis. In vitro studies also show synergistic anti-proliferative effects when HER2 positive MDA-MB-361 human breast cancer cells are exposed to lapatinib and trastuzumab. However, lapatinib retains activity against trastuzumab-resistant BT474 human breast cancer cells. Consistent with these results, lapatinib has shown promising results in phase I clinical trials for breast cancer in combination with trastuzumab.

A large phase II randomized trial is underway to test the efficacy of lapatinib as a first-line treatment for advanced or metastatic breast cancer. The inclusion criteria for this study are histologically confirmed invasive breast cancer at primary diagnosis or relapse, no prior treatment except hormonal therapy, and documented amplification of ErbB2 by FISH. Approximately 130 patients have been enrolled and randomized to one of two daily dose regimens. Interim analysis was carried out by an investigator review board and an independent review board after 40 patients had been treated and followed for 12 wks. The investigator review board reported a response rate of 30% and the independent review board reported 35%. There was also a large fraction of patients ($\approx 35\%$) who achieved apparent stable disease over 12 wks. No grade 3 or 4 adverse events (AEs) were observed during interim analysis, and the most common AEs were pruritis, diarrhea, rash, and acne. Initial studies suggest that lapatinib may also have efficacy for treating gastric, renal, and head and neck cancer.

Icon-1™, an Anticancer Therapeutic Candidate Designed to Selectively Destroy Pathological Blood Vessels

Mike Sherman (Iconic Therapeutics, Inc.)

Icon-1™ is an immunoconjugate fusion protein composed of mutated Factor VII fused to immunoglobulin Fc region 2. The mutated Factor VII retains picomolar binding affinity for tissue factor (TF) but lacks the ability to initiate complement coagulation. TF is expressed on the vascular endothelium of pathological blood vessels (PBVs) and on other cell types (including many cancer cells) but not on the surface of normal blood vessels. When bound to PBVs, Icon-1™ triggers immune destruction of target cells via immunoglobulin Fc region 2-mediated activation of Natural Killer Cells. Thus, in contrast to Avastin™, a well-characterized

and FDA-approved anti-angiogenic factor that prevents formation of new PBVs, Icon-1™ can trigger destruction of existing PBV networks.

The efficacy of Icon-1™ was tested in a pig model for wet adult macular degeneration (wet AMD). In this model, wet AMD with robust sustained PBVs is promoted by laser-induced lesions in the pig eye. Both mouse and human versions of Icon-1™ crossreact with high selectivity and sensitivity to PBVs in laser-lesioned pig eyes. Furthermore, PBVs in pig eyes regress within approximately 4 d after treatment. The efficacy of Icon-1™ was also tested in a mouse xenograft model for human melanoma or prostate cancer. Here, the gene encoding mouse Icon-1™ was injected intratumorally with an adenoviral vector and PBV growth was assessed at the site of injection and at distant metastatic sites. PBV and tumor growth was suppressed transiently at both sites for xenografts of human melanoma and prostate cancer. Similar trials are planned with purified human Icon-1™ protein. It is currently hoped that clinical and safety studies in progress or planned in the near future will be successfully completed by early 2006, so that an investigative new drug filing can be made in the first quarter of 2006.

Immunotherapy of Hormone-Refractive Prostate Cancer: Phase I Study with Allogeneic GM-CSF Gene-Transduced Prostate Cancer Cell Line Vaccine in Combination with the Antibody MDX-010 (Anti-CTLA-4)

Winald Gerritsen (Vrije Universiteit Medical Center, Cancer Center Amsterdam)

Novel immunotherapies are being developed and tested as mono- or combination treatments for hormone-refractive prostate cancer (HRPC). One approach uses a vaccine containing GM-CSF-transduced prostate cancer cells (GVAX) and antibody to human CTLA-4. In early studies, isogenic patient-derived human cells were transduced with the GM-CSF gene, and then lethally irradiated prior to intradermal injection. The rationale of this therapy is that the injected cells stimulate a strong inflammatory response, which activates T-cells that destroy both injected and endogenous tumor cells. Better results were obtained with GM-CSF-transduced prostate cancer cells than with prostate cancer cells and soluble GM-CSF, but equivalent results have been obtained with stably transfected LNCaP or PC-3 prostate cancer cells. Relatively mild adverse effects are associated with this therapy, such as rash, short-term flu-like symptoms, or fatigue. Increases in PSA levels are retarded and then stabilized in most patients treated with GVAX. Two phase II clinical trials demonstrated that GVAX has clinical efficacy equivalent to that of Docetaxel in patients with HRPC. Phase II trials are now beginning to examine relative efficacy of GVAX plus docetaxel, GVAX, docetaxel plus prednisone, and docetaxel.

G-VAX is also now being tested in a Phase II clinical trial in combination with antibody to human CTLA-4 (MDX-010). This antibody blocks the interaction between the molecules CTLA-4 and B7, which is present on antigen presenting cells. CTLA-4 is not present on resting T cells but is upregulated for 2-3d after T cell activation. Because the interaction between these molecules antagonizes T cell activation, it was hypothesized that MDX-010 might improve the therapeutic efficacy of GVAX. Preclinical data in a murine model confirmed this idea. In the current human trial for GVAX and MDX-010, patients with HRPC who did not receive prior chemotherapy and had rising PSA levels were treated with a fixed dose of GVAX and a variable dose of MDX-010. Initial results show that GVAX plus MDX-010 may suppress PSA levels effectively at higher MDX-010 dose levels. Some adverse effects were observed including serious immune breakthrough events and severe colitis in approximately 1/5th of treated patients. More data is needed to evaluate the potential of GVAX plus MDX-010 therapy for HRPC.

Use of a Small Molecule Inhibitor of Sonic Hedgehog Signaling to Eradicate Brain Tumors in a Mouse Model

Tom Curran (St. Jude Children's Hospital)

Pediatric medulloblastoma occurs in approximately 600 children in the United States per year, and is lethal in approximately half of these cases. In addition, survivors of the disease who have been treated with radiation and chemotherapy suffer severe loss of cognitive function. Thus, there is great need for improved therapies for pediatric medulloblastoma as well as for other brain tumors. However, development of novel therapies for brain tumors has been hindered by the lack of suitable animal model systems.

Recent studies on the human homolog of the *Drosophila* segment polarity gene, Patched-1 (PTC1) provided the first clues to the molecular basis of pediatric medulloblastoma and potentially other human brain tumors. PTC1 is mutated in a subset of spontaneous medulloblastomas and in hereditary medulloblastomas associated with basal cell nevus syndrome (BCNS). PTC1 encodes a transmembrane protein that plays a critical role in sonic hedgehog (SHH) signaling, by repressing constitutive signaling by Smoothened (SMO) and its downstream effector GLI1. Mutations in SHH are associated with neural tube defects in humans, including spina bifida and holoprosencephaly. In human tumors, inactivation of PTC1 is associated with increased expression of GLI1. Mutations in p53 are also observed in 10%-20% of human sporadic medulloblastomas and patients with Li-Fraumeni syndrome, who carry germline mutations in p53, are predisposed to medulloblastoma. Furthermore, microarray gene expression data suggested that the SHH pathway is activated in human brain tumor cells, as it is in cells carrying a mutation in PTC1.

These data led to the hypothesis that PTC1 or PTC1 p53 mutant mice might provide models for human medulloblastoma. In fact, mice that are heterozygous for PTC1 (PTC +/-) have a 20% incidence of medulloblastoma per year, and this phenotype is strongly enhanced by a homozygous deficiency in p53, confirming the validity of the hypothesis. All PTC1 +/- p53 -/- mutant mice develop brain tumors and most die from the tumors within 12 wks of birth. This high penetrance animal model for medulloblastoma provides a suitable system for testing the efficacy of small molecule inhibitors of SHH signaling to treat brain tumors.

Previous studies identified cyclopamine, a plant-derived alkaloid that binds tightly to SMO, as a potential inhibitor of the SHH signaling pathway. HhAntag, an SHH inhibitor that is approximately tenfold more potent than cyclopamine, was also identified with an in vitro cell culture assay with cells expressing a Gli1-activated luciferase reporter gene. Technical issues prevented detailed characterization of these SHH inhibitors with cultured cells. Nevertheless, it was possible to test the efficacy of HhAntag in vivo by dosing tumor bearing mice twice daily at 20 or 100 mg/kg. Three endpoints were examined: target inhibition by measuring Gli-1 expression; pathology reduction by histological assays of brain tissue; and long-term survival of tumor-bearing mice. The results showed that Gli1 inhibition by HhAntag was dose dependent in vivo. Furthermore, tumor volume was dramatically reduced in mice treated twice daily with 20 mg/kg of HhAntag for 2 wks and tumors were completely eliminated in mice treated twice daily with 100 mg/kg. In long term studies, survival increased significantly in animals dosed daily at 100 mg/kg; however, the amount of drug available was insufficient to fully characterize the impact of HhAntag on long term survival of mice with late stage brain tumors. These data suggest that HhAntag has significant promise as a treatment for human brain tumors. Additional studies are needed to determine optimal treatment regimens and the relative efficacy of monotherapy or combination therapy with HhAntag.

SPECIAL SESSION

Update on Cancer Therapy and Research Advances

Cecil Pickett (Schering-Plough) chaired a special Session of the 20th Aspen Cancer Conference that featured presentations concerning the strengths and weaknesses of the drug development process in academia, government, and pharmaceutical companies by James Doroshow (National Cancer Institute) and J. Carl Barrett (Novartis Institutes for Biomedical Research, Inc.). This Session also included an extensive open discussion session on ways to facilitate development of oncology drugs by promoting greater information exchange as well as collaboration and cooperation among research groups working on similar and/or complementary drug development issues.

The oncology field is currently moving heavily towards developing targeted oncology products. However, it may sometimes be overlooked that this is a more challenging task than the problem of developing non-targeted cytotoxic oncology drugs. Five essential criteria must be met in order to develop a targeted oncology drug: (1) the drug target must be validated; (2) the agent must interact with the target with sufficient selectivity and sensitivity; (3) the agent must have demonstrated therapeutic effects; and (4) assays must exist to quantify the agent and its effects in vivo; and (5) the agent must be efficacious in a clinical setting.

The most successful examples of targeted oncology therapeutics are the following: EGFR family inhibitors, Bcr-Abl inhibitors, anti-angiogenesis factors, proteasome targeted agents, CD20 inhibitors, targeted cytotoxic drugs.

To improve development of targeted oncology therapeutics in the future, the following challenges must be met: better approaches are needed to identify drug-responsive patient populations; pharmacodynamic markers of biochemical activity are needed to facilitate dose selection; surrogate markers of efficacy must be developed and applied; imaging technology should be developed for studies of drug efficacy; and the level of enrollment in clinical studies should be improved.

Reinventing Drug Development: A View from the National Cancer Institute

James Doroshow (National Cancer Institute)

James Doroshow proposed a novel approach by which the National Cancer Institute (NCI) might promote a new paradigm of clinical trials with increased emphasis on molecular markers of therapeutic outcome. In contrast to the existing process, which involves phase I, II, and III clinical trials, the new paradigm would include a pilot phase (phase 0) followed by broad phase I/II trials. Phase 0 trials would be “first-in-man” studies with fewer patients per trial (6-10 vs. 15-25 patients) and a shorter time frame than the typical phase I trial (3-6 vs. 6-12 mo). Phase 0 trials would also focus on development of molecular endpoints that assess biological effects (pharmacokinetic/pharmacodynamic, PK/PD) as well as preliminary toxicity evaluation including doselimiting toxicity and maximum-tolerated dose. Functional imaging would be used routinely. The ultimate goal of phase 0 pilot studies would be to develop a database of “in-man” studies to guide further agent development. In contrast, current phase I “first-in-man” studies are often based solely on in vitro or animal model studies. The broad phase I/II studies in the proposed new paradigm would be designed to test efficacy of a single agent on multiple tumor types or in multiple tissues. Inclusion criteria could be relaxed with regard to prior patient treatment, and assays would be designed to evaluate dose, biomarker endpoints and to obtain useful PK/PD data.

The overall goal of this proposal is to increase the number of patients entered in early agent trials and to efficiently produce critical pharmacodynamic data in humans, which is needed for

definitive agent development. To implement this plan, significant resources would need to be developed and/or refocused at NCI and greater integration, and collaboration would be needed in drug development and clinical therapeutics. Management infrastructure as well as widespread support in the research and clinical communities would also be essential to the viability of this plan.

A Tale of Two Targets

J. Carl Barrett (Novartis Institutes for Biomedical Research, Inc.)

J. Carl Barrett contrasted two distinct oncology drug targets; one of these targets is an unconditional success story, while the other has so far achieved less success as a therapeutic target in cancer patients. The success story is Bcr-Abl, the critical enzyme in pathological changes associated with CML. More than 95% of newly diagnosed CML patients respond to the Bcr-Abl-targeted TKI Gleevac (imatinib), and responsive patients demonstrate a low disease relapse rate. When resistance to Gleevac develops, it is usually associated with mutation in the Bcr-Abl kinase active site or amplification of the Bcr-Abl gene. Recently, a novel compound, AMN107, was developed that targets Gleevac-resistant Bcr-Abl. AMN107 binds to the same target site in Bcr-Abl as Gleevac, but it binds with higher affinity (IC₅₀ of 25 nM vs. 649 nM). Like Gleevac, AMN107 also binds to and inhibits the TK activity of PDGFR and c-Kit, but the affinity of AMN107 for these three RTKs covers a narrower range (25-160 nM) than Gleevac (39-649 nM). AMN107 inhibits 32 of 33 previously characterized Gleevac-resistant mutants of Bcr-Abl in vitro. Furthermore, AMN107 induces apoptosis and inhibits proliferation of Bcr-Abl transfected or primary leukemia cells and increases survival in a mouse model for CML. One rationale for the success of Bcr-Abl as an oncology target is that CML cells lack compensatory pathways to promote tumor growth. Thus, inhibition of Bcr-Abl is sufficient to kill the tumor. However, it is not yet known whether Gleevac or AMN107 effectively kills tumor stem cells, which is essential to prevent disease relapse.

The second target for oncology therapeutics discussed by Barrett is the receptor for vascular endothelial growth factor (VEGFR). VEGF, another oncology drug target, binds to six different VEGFRs encoded by three VEGFR genes. VEGF and VEGFR comprise a key node in normal and pathological angiogenesis. PTK/ZK has broad specificity for all six isoforms of VEGFR. Early proof of concept studies for PTK were encouraging, including anti-tumor and anti-metastatic effects in rodent tumor models. Furthermore, Avastin, a monoclonal antibody that inhibits VEGF-A, has shown clinical benefit for several cancers. Thus, it seemed possible that an additional block to pathological angiogenesis might enhance or be equal in efficacy to Avastin. Unfortunately, the results of an ongoing Phase III clinical trial for PTK in metastatic colorectal cancer patients have been less encouraging than anticipated. The trial is a multinational, randomized phase III trial with 1168 previously untreated metastatic colorectal cancer (CRC) patients. Patients were randomized into two groups receiving 5-fluorouracil (5-FU) plus placebo or 5-FU plus 1250 mg/day oral PTK. Patients were stratified at the outset of the trial based on performance status and serum lactate dehydrogenase (LDH) with an LDH cut-off value of $1.5 \times$ the high normal LDH value. High LDH has been identified previously as a negative prognostic indicator for survival from mCRC. The primary early endpoint in this trial (as of March, 2005) was early progression-free survival assessed by radiologic review by both an unblinded investigator-based lab and a blinded central lab. The late primary endpoint is overall survival, but these data are not yet available. The results show an overall hazard ratio of 0.88 ($P = 0.118$) for central lab assessment and 0.83 ($P = 0.026$) for local lab assessment. However, it appears that the high LDH subpopulation may be more responsive to PTK, showing hazard ratios/ P -values of 0.68/ $P = 0.012$ and 0.60/ $P = 0.001$ for central and local lab assessment, respectively. It is not yet known whether the high LDH subgroup is biologically significant.

Additional studies are needed to assess the efficacy of PTK for mCRC and the suitability of VEGFR as a therapeutic target. It is possible that VEGFR is a poor oncology target because there are compensatory pathways to support tumor vascularization in the presence of agents that inhibit VEGF or VEGFR. It is also possible that specific patient subpopulations will respond to VEGFR-targeted therapeutics, but the criteria for identifying such subpopulations are not yet known.

Key Discussion Points

- Novel molecular endpoints may greatly enhance the value of future clinical trials. However, extensive histological studies are needed to support and or complement novel molecular approaches, and a dedicated surgeon may be needed to perform such studies in a timely manner.
- Quantitative linear pharmacokinetic assays should be developed to assess drug efficacy in preclinical and clinical studies.
- The project proposed by Doroshow represents would involve the NCI in new aspects of the drug development process. Some discussion participants questioned this proposal, suggesting that greater specialization among companies and institutions developing oncology drugs may be appropriate at the present time. According to this view, pharmaceutical companies should perform high throughput assays for validated targets, and academic labs should identify and validate targets and study drug mechanisms. This suggestion appeared to be controversial; however, because some participants expressed support for a less stratified approach to drug development.
- One of the bottlenecks in drug development is identifying susceptible patient populations and markers that are predictive of drug efficacy. There is little understanding of how to do this at present. Cross-sector collaborative efforts in this area were encouraged.
- Metabolic profiling was suggested as a means to elucidate the important biological differences between mice and humans. It was also suggested that an NCI initiative in this area might be appropriate investment for NCI in the future.
- In cases where antibody-based and small molecule-based therapeutics have been developed for the same target, a comparison of the advantages and disadvantages or each approach would be useful. A systematic study of this kind might reveal principles that could be used to optimize therapeutic approaches for different cancers or patient subpopulations.
- Drug concentrations are too infrequently measured in the target tissue of treated animals and patients. Whenever possible, these data should be obtained and used to improve understanding of drug efficacy.
- Additional cross-sector and intersector collaboration is needed for developing combination oncology therapeutics. Intellectual property issues and material transfer agreement issues currently represent large barriers to these collaborations. New approaches are needed to overcome these barriers.
- Additional mechanisms are needed to promote resource and information sharing among cancer researchers in a non-competitive environment. These mechanisms might include a biomarker bank or an open access toxicogenomics database.

SESSION 3: METASTASIS

David Kaufman (The University of North Carolina, Chapel Hill) introduced and chaired the Conference Session on Metastasis, which included presentations by J. Carl Barrett on

metastasis suppressor gene KAI1 and by William Dougall (Amgen, Inc.) on the RANK-RANKL signaling pathway in osteoclastic and osteoblastic bone metastases. Many solid tumors can now be eliminated by surgery, radiation, or chemotherapy, but metastatic cancer has proved less tractable to therapeutic approaches, and it is metastatic disease that is usually responsible for cancer deaths. Different tumors tend to metastasize to preferred sites and some sites of metastasis such as spleen are uncommon for all tumors. These patterns are poorly understood, but may in part be determined by preferred routes of metastatic spread and the pattern of blood flow from the primary tumor site. The four classical steps of metastasis are migration, invasion, dissemination, and growth. Metastasis only occurs if cells are capable of dissociating from the primary tumor site, migrating to distant sites through tissue, lymphatics and/or blood vessels, surviving during migration, exiting the circulation into a target tissue, and growing at the distant metastatic site. Most of these steps remain poorly understood, which limits the ability to inhibit or treat metastatic disease. Thus, greater understanding of the metastatic process is essential to progress in developing effective tools to inhibit or eliminate metastatic cancer.

Molecular Mechanisms of Metastasis

J. Carl Barrett (Novartis Institute of Biomedical Research, Inc.)

The metastatic process is exceptionally complex and not well understood. One important approach that could improve our understanding of metastasis is to identify genetic mechanisms that regulate or control metastatic growth. Previous studies indicate that metastasis is both positively and negatively regulated. Some of the positive regulators of metastasis are ARM1, ATX, CD44, Cox2, Cyr6a, Ezrin, HMG-I, Laminin-5, c-Met, MTA1, oncostatin M, Twist, RAGE, S100A4, S100A9, Semaphorins, Thymosin- β 15, Wnt-5a, and IGF1. Negative factors regulating metastasis include Annexin7, BRMS1, CC3, CEACAM1-4S, CRSP3, DAP-kinase, E-cadherin, HEPSIN, HPIHSa, KAI-1, KiSS1, Maspin, Melastatin, MKK4, NESH, NM23-H1, PTEN, RhoGD12, SFRP1, SHPS-1, Syk, TSP-1, tropomyosins, and VDUP1.

KAI1/CD82 is a transmembrane glycoprotein with four membrane-spanning segments. KAI1 belongs to the tetraspanin protein superfamily, and it is thought to play roles in cell motility, invasiveness, and cell-cell interactions. There is evidence for interactions between KAI1 and several cellular signaling pathways including integrin-, e-cadherin-, and EGFR-mediated signaling. KAI1 was first identified during cell fusion experiments as a gene on human chromosome 11 that suppresses the metastatic potential of cells. Several types of solid tumors appear to be susceptible to KAI1-mediated suppression of metastasis including melanoma, prostate, and breast. In addition, loss of KAI1 is often associated with progression in prostate and other cancers. However, no mutations in the *KAI1* gene have been identified, despite extensive sequencing efforts, and the mechanism by which KAI1 is inactivated during cancer progression is not yet known. Some possible mechanisms include chromatin remodeling, interaction with dominant-negative binding partners, or posttranslational modification.

In summary, metastasis is regulated by both positive and negative factors that act through multiple pathways and mechanisms. The expression of these genes may be further modulated by chromatin remodeling, and the plasticity and flexibility of the metastatic cascade may have implications for therapeutic approaches to prevent or inhibit metastatic disease.

The RANK/RANKL/OPG Axis in Multiple Myeloma and Bone Metastases

William Dougall (Amgen, Inc.)

Bone metastasis occurs in the majority of patients with advanced breast and prostate cancer, and in a significant number of patients with cancers of the kidney, lung, thyroid, or bladder.

When tumors metastasize to bone, the normal bone remodeling process is disturbed and the balance between bone deposition and bone resorption is altered. Bone remodeling is driven by osteoclasts and osteoblasts, which resorb and deposit bone, respectively, and by the RANK/RANKL/OPG signaling pathway, which regulates osteoclast differentiation, survival, and activation. RANK (Receptor Activator of NF- κ B), and its ligand RANKL, are members of the tumor necrosis factor (TNF) receptor and the TNF superfamilies, respectively. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that downregulates RANK-dependent signaling. In the case of osteoblastic metastases, factors such as endothelin-1, insulin-like growth factors (IGFs), and transforming growth factor-beta (TGF-beta) may act to increase osteoblast function, resulting in increased bone formation. In osteolytic metastatic lesions, the focal lesions are due to the tumor-dependent release of PTHrP, eicosanoids, growth factors (Transforming growth factor-alpha, M-CSF), or cytokines (IL-1, IL-6, IL-8, TNF-alpha), which stimulate osteoclast differentiation or activity. However, osteolysis is linked to increased osteoblast activity by a process known as "coupling," and osteolysis also releases growth factors that stimulate tumor growth, thus generating a "vicious" cycle of increased tumor growth and increased osteolysis.

The mechanism by which tumors stimulate osteolytic or osteoblastic metastases was examined with pharmacological and genetic approaches that alter RANK/RANKL signaling. A mouse model for osteolytic disease was developed in which MDA-MB-231 human breast cancer cells are injected into the left ventricle of the heart, and circulating cancer cells generate osteolytic lesions in the long bones of the mouse within a few weeks. This process is completely blocked by co-injection of recombinant OPG, indicating that it requires RANK/RANKL signaling. Osteoclasts and RANK/RANKL signaling also play a role in osteoblastic metastases, as shown in a mouse model for LuCaP prostate cancer cell-induced bone metastasis. In this model, cancer cell-induced increases in bone mineral density were eliminated by injection of soluble RANK:Fc, a pharmacological analogue of OPG that includes the extracellular RANKL binding domain of RANK linked to immunoglobulin region Fc. Additional markers of bone turnover (serum osteocalcin, serum alkaline phosphatase (BAP), urinary cross-linked N-telopeptides of type I collagen) as well as markers of tumor growth (prostate specific antigen, PSA) were also suppressed by RANK-Fc. Thus, RANK/RANKL signaling and osteoclastic activity play a role in both osteolytic and osteoblastic metastatic disease.

AMG 162 is a fully human monoclonal antibody to RANKL that blocks the activity of soluble and cell surface endogenous RANKL. AMG 162 does not bind to other TNF superfamily members and binds to RANKL with high selectivity and high affinity. In phase I clinical trials, AMG 162 has been well tolerated at doses of ≤ 3 mg/kg. Initial results suggest that a single dose of AMG 162 produces sustained reduction in bone turnover in breast cancer or multiple myeloma patients with no significant adverse effects. Additional studies are needed to determine the efficacy of AMG 162 for treating or preventing bone metastatic disease.

SESSION 4: INFLAMMATION AND CANCER

Thomas J. Slaga (The University of Texas, San Antonio) introduced and chaired the Conference Session on Inflammation and Cancer, which included presentations by Gerald Wogan (Massachusetts Institute of Technology), Curtis Harris (National Cancer Institute), and Raymond DuBois (Vanderbilt-Ingram Cancer Center). Slaga indicated that the evidence for a link between chronic inflammation and cancer is increasing. The inflammatory agents that play a role in cancer can be infectious or non-infectious; both types of agents activate stromal inflammatory cells and stimulate hyperproliferation and mutagenesis in affected tissues, which increases the risk of subsequent dysplasia and cancer. Many host factors enhance the inflammatory response, including cytokines, chemokines, interleukins, and reactive oxygen/nitrogen species; in contrast, glucocorticoids and the glucocorticoid receptor (GR) play a

primarily negatively role in regulating the inflammatory response. For example, GR negatively regulates genes encoding proinflammatory factors such as ICAM1, ELAM-1, IL-2, IL-6, IL-8, IL-1b, iNOS, and COX-2. Glucocorticoids have been used extensively for their anti-inflammatory properties; however, other types of anti-inflammatory agents are often preferred, because pharmacological glucocorticoids tend to lose efficacy during prolonged use and because they can have undesirable side effects. It may be possible to overcome the disadvantages of common glucocorticoids, through the discovery of novel natural and synthetic glucocorticoids with improved pharmacological properties.

Reactive Nitrogen and Oxygen Species in Elevated Cancer Risk Associated with Chronic Inflammation

Gerald Wogan (Massachusetts Institute of Technology)

A number of human diseases, including ulcerative colitis, hemochromatosis, viral hepatitis, and Barrett's Esophagus are linked to chronic inflammation and elevated cancer risk. Extensive studies have shown that reaction oxygen species (ROS) and reactive nitrogen species (RNS) play a central role in the pathology of these diseases, and for this reason they have been called "oxyradical overload diseases." Two main types of inflammatory cells, the macrophage and the neutrophil, generate significant amounts of ROS and NOS in vivo. Neutrophils produce nitric oxide (NO) and hydrogen peroxide (H_2O_2) via myeloperoxidase and macrophages produce nitrous anhydride (N_2O_3), peroxynitrite ($ONOO^-$), nitrosoperoxy carbonate ($ONOOCO_2^-$), superoxide, and hydroxyl radicals. These species promote deleterious deamination, oxidation, and nitration of biological macromolecules including DNA, protein, and lipid with cellular endpoints including mutagenesis, cytotoxicity, cell-cycle arrest, and/or apoptosis.

The role of ROS in chronic inflammation and carcinogenesis has been studied more extensively than the role of RNS. This is in part because of the lack of suitable model systems to study the biological effects of RNS. To address this need, Gerald Wogan and colleagues developed an in vitro NO delivery system that allows analysis of the biological impact of RNS on cells in culture. In this system, the culture chamber is infused continuously with 50% O_2 , 5% CO_2 , and 45% N_2 through one port and with a variable concentration of NO gas through a second port. The NO is delivered via permeable 150 mm of polydimethylsiloxane tubing, and the amount of NO in the culture medium is a function of the length of the tubing in contact with the medium and the concentration of NO. Using 1 to 10% NO, the steady-state NO concentration in the culture medium varied from 0.4 to 2 μM , while the physiological range of NO in cells is thought to be 0.01-1.0 μM .

In initial studies with human lymphoblastoid TK6 cells, Wogan and colleagues observed two thresholds for growth and viability in the presence of exogenous RNS. The first threshold is for steady-state concentration and the second is for cumulative dose (i.e., steady-state concentration $\times$ duration). TK6 cells showed significant loss of viability above but not at or below a steady-state concentration of 0.5 μM and the maximum cumulative dose that did not cause loss of viability was 150 μM min. In contrast, in p53-deficient NH32 cells, these thresholds were shifted to significantly higher values, indicating that p53 plays a role in the response to NO. The number of apoptotic cells in the TK6 culture increases with increasing dose of NO; however, when exposed to the same NO dose, fewer p53-deficient NH32 cells enter apoptosis than TK6 cells. Thus, p53 likely plays an important role in triggering DNA-damage induced apoptosis in cells exposed to RNS.

The *HPRT* mutation spectrum in TK6 cells exposed to NO includes mostly AT to GC and AT to TA base substitution mutations, implicating etheno adducts as the primary mutagenic lesion generated by NO. The NO-induced mutation spectrum at *HPRT* resembles the spontaneous

mutation spectrum at *HPRT*, suggesting that NO might contribute to spontaneous mutagenesis. p53-deficient NH32 cells are hypermutable at *TK1* in response to NO or 4-nitroquinolineoxide, and NO induces sister chromatid exchange in NH32 but not in TK6 cells.

In summary, with a controlled NO delivery system, these studies show that exposure to NO increases mutagenesis and stimulates apoptosis in p53-proficient cells, while in p53-deficient cells, mutagenesis increases to a greater extent but cells evade apoptosis and demonstrate increased viability. Based on the Hanahan-Weinberg molecular hallmarks of cancer, these data are consistent with the hypothesis that RNS play a significant role in carcinogenesis in eukaryotic cells.

Chronic Inflammation and Cancer: Radical Causes of Cancer

Curtis Harris (National Cancer Institute)

The relationship between chronic inflammation and cancer is illustrated by the human disease ulcerative colitis, in which chronic inflammation of the colon is tightly linked to a highly elevated risk of developing colon cancer at an early age. One approach to studying the cellular interactions involved in this phenomenon is to grow co-cultures of target cells and macrophages, an inflammatory cell that generates ROS and RNS including significant amounts of nitric oxide (NO) after cytokine activation. In cultures containing cytokine-activated macrophages and MCF-7 breast cancer cells, p53 is upregulated and activated by phosphorylation at many sites including serine residues 15, 20, 33, 46, and 315 and lysine 382. These events are at least in part due to NO exposure, because they are partially suppressed by an inhibitor of the NO metabolic pathway. This is consistent with the observation that inducible nitric oxide synthetase (iNOS), ser15-phosphorylated p53, p21, and Mdm2 are coordinately upregulated in a subpopulation of patients with ulcerative colitis, while they are expressed at very low levels in normal colon.

Gene expression profiling is another approach that can help explain the impact of chronic inflammation on cancer risk. This approach could potentially identify common or distinct pathways that are activated by different components of inflammatory/oxidative stress. Surprisingly, only 14 of approximately 1400 differentially regulated genes were activated in common by NO, H₂O₂, hydroxyurea, and hypoxia. In contrast, exposure to NO, H₂O₂, hydroxyurea, and hypoxia specifically induced 842, 272, 283, and 315 genes, respectively. p53-dependent genes were generally downregulated at early time points after exposure and upregulated at later time points. This may indicate that p53-dependent transrepression plays a role in the early response to inflammatory stress. The importance of the p53 pathway in the response to inflammatory stress is underscored by the fact that 8 of the 14 common response genes to inflammatory stress components are p53 target genes with p53 response elements. Furthermore, p53 mutations occur at an elevated frequency in cells from ulcerative colitis patients and patients with other diseases linked to chronic inflammation.

Molecular epidemiological studies also provide evidence of a link between chronic inflammation and cancer. Approximately 60 common human single nucleotide polymorphisms (SNPs) have been mapped to genes that participate in the inflammatory response. SNPs in one of these genes, *mannose binding lectin2* (*MBL2*), are associated with predisposition to bacterial, viral, and fungal infections and lower levels of *MBL2* expression. Recent studies of colon cancer risk in approximately 300 patients and 500 controls suggests relatively strong positive correlation between polymorphism at *MBL2* and risk of colon cancer ($P = 0.001$ and odds ratios of 3.3-4.2).

Molecular Targets for Cancer Prevention

Raymond DuBois (Vanderbilt-Ingram Cancer Center)

A significant body of epidemiological evidence indicates that chronic use of low to moderate doses of non-steroidal anti-inflammatory drugs (NSAIDs) reduces mortality from colorectal and other cancers. These drugs inhibit the first step of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and/or cyclooxygenase-2 (COX-2). However, very recent studies indicate that COX-2 specific inhibitors of the “coxib” class (celecoxib and rofecoxib) are associated with elevated risk of adverse cardiovascular effects, which has raised significant controversy over their use. In light of these data, it is worth considering directly whether COX-2 is a rational anti-cancer target, or in other words, “is there a biologically-plausible explanation for the observation that chronic use of COX-2 selective NSAIDs reduces cancer risk?”

Data that support this idea includes the following. (1) COX-2 but not COX-1 expression increases in colorectal adenomas and in colorectal cancer cells. (2) Elevated expression of COX-1 correlates with poor prognosis in colorectal, non-small cell lung, breast, cervical, and esophageal cancer. (3) A promoter variant that reduces expression of COX-2 is associated with reduced risk of colorectal adenoma in individuals not exposed to chronic NSAIDs. (4) Tissue-specific overexpression of COX-2 in transgenic mice correlates with higher tumor frequency in the tissue with elevated COX-2. (5) Genetic inactivation of the prostaglandin synthesis pathway downstream of COX-2 reduces colon cancer risk in mouse models of colon cancer. (6) Exogenous prostaglandin E2 (PGE2) stimulates colon adenoma growth and colon carcinoma metastasis in the mouse.

Clinical studies in humans also suggest that COX-2 may play a role in promoting cancer growth in patients with familial adenomatous polyposis (FAP). In a recent phase II trial, polyp number and size was decreased by 15% or 31% in patients dosed with 100 or 400 mg of celebrex twice daily, respectively. Based on these and other data, coxib NSAIDs are currently approved by the US FDA for use in patients with FAP or other high-risk forms of cancer. However, based on evidence showing an increase in risk for thrombotic cardiovascular effects when coxib NSAIDs are used chronically at high doses, coxib NSAIDs are no longer approved by the US FDA for use at any dose in patients at low risk for cancer.

The mechanism by which coxib NSAIDs increase risk for adverse cardiovascular events is currently not known. However, studies have been initiated in the zebrafish model system to address this question. Preliminary results show that COX-1, mPGES, and EP4 are expressed abundantly in early embryogenesis through gastrulation, but COX-2 is not expressed during most of embryogenesis in the zebrafish. Furthermore, inhibition of COX-1 causes a defect in vascular development with no effect on arterial development. This indicates that the prostaglandin synthesis pathway is essential for posterior vascular development in the zebrafish. Experiments are underway to determine if similar results are observed in other species.

SPECIAL LECTURE: ASPEN CANCER CONFERENCE FELLOW 2001

Genetic and Epigenetic Events and Colorectal Cancer

William Grady (Fred Hutchinson Cancer Research Center)

The current paradigm for colorectal carcinogenesis (CRC) involves discrete histological stages in which normal epithelium progresses through early and late adenoma stages to fully metastatic cancer. Many specific genetic changes have been linked to the adenoma to carcinoma progression sequence including mutations in APC, CTNNB1, BRAF, KRAS, TGFBR2, and others. Epigenetic changes have also been observed during these discrete steps

of the polyp→cancer progression sequence, with the most notable epigenetic studied to date being aberrant DNA hypermethylation. Less is known about the specific timing and frequency of these epigenetic events; however, a large number of genes have been shown to be methylated during colon carcinogenesis including MINT31, MGMT, MINT2, CDKN2A/p16, TIMP3, THBS1, HIC-1, APC, p14, and hMLH1. Recent studies by William Grady and colleagues suggest that methylation occurs on a stochastic basis during the adenoma to carcinoma progression in CRC. Consistent with this model, a significantly higher fraction of methylated genes are observed in later adenocarcinoma stages than in early or late adenoma stages. In addition, some genes, such as MLH1, tend to be methylated later in CRC than others.

Microsatellite instability is a common feature of a subset of CRC tumors. These tumors are deficient in mismatch repair (MR) due to mutation and/or hypermethylation of *MR* genes, which include MLH1, MSH2, MSH3, MSH6, PMS2, and PMS1. Grady proposed and tested whether hypermethylation of MLH1 might drive cancer progression in MSI-positive CRC tumors by examining the status of an MSI-target gene at different cancer stages. The results are consistent with the possibility that hypermethylation of MLH1 occurs at the early to late adenoma transition and precedes microsatellite-induced mutation of TGFβ receptor 2 (TGFB2), which occurs in later CRC stages. Grady also used TGFB2-deficient mice to demonstrate that loss-of-function mutations in TGFB2 increase the frequency of carcinogen-induced tumors. These data suggest that epigenetic and genetic events both contribute to CRC progression, and in some cases, epigenetic events may specifically promote subsequent procarcinogenic mutational events.

The above results suggest that aberrant DNA hypermethylation could potentially be used as a molecular marker of CRC progression and/or susceptibility. However, a suitable assay is needed to test and/or implement this idea. Initial tests showed that conventional methylation-specific PCR assays run on serum samples from CRC patients could detect methylated MLH1 in the serum of 30% of people with CRC that also carried methylated MLH1. These results demonstrated the feasibility of using methylation specific PCR (MSP) on remote media (i.e., blood, sputum, urine, stool, etc.) but also showed that the conventional assays are not sensitive enough to be useful in a clinical setting. Therefore, an alternative assay was developed for use with fecal DNA. This was a significant challenge, because a very small fraction (approximately < 0.5%) of the DNA in fecal sample derives from CRC cells. Preliminary results with a novel ultrasensitive MSP assay developed in the Grady lab were encouraging, showing strong correspondence between results in tissue and fecal samples, and a higher proportion of methylated target genes in colon adenoma patients than in normal controls. However, a significant background of target gene methylation was detected in the controls. This could indicate the presence of precancerous colon tissue (also known as a field effect), that the gold standard assay, colonoscopy, has a significant misdiagnosis rate for colon polyps (it is estimated to be 10%-20%), or that methylation occurs at a significant frequency in normal colon tissue and is not relevant to cancer or cancer predisposition. Additional experiments will be carried out to explore these possibilities.

SESSION 5: RISK ASSESSMENT

Ray Tennant introduced and chaired the Conference Session on Risk Assessment, which included presentations by Cheryl Walker (The University of Texas, MD Anderson Cancer Center) and Jim Swenberg (The University of North Carolina, Chapel Hill). Tennant discussed limitations on the process of risk assessment and prospects for moving beyond these limitations with genomics-based technologies such as gene expression profiling. Risk assessment, which attempts to define the probability of adverse health effects caused by exposure to environmental agents, is compromised by the use of assumptions and extrapolations to account for missing information or knowledge. Risk assessment is also a prototype application of systems biology,

because it attempts to simultaneously integrate the impact of multiple factors on the function of a biological system.

Toxicogenomics is a tool being used to conduct systems-level analysis of the impact of environmental exposures on biological systems. Toxicogenomics has successfully identified gene expression signatures associated with specific chemical exposures or the responses to such exposures. As such, the potential of toxicogenomics to facilitate risk assessment has been demonstrated. However, the greater potential of toxicogenomics is that it can not only identify annotated genes and gene processes that contribute to specific biological responses, but it can identify novel genes and gene processes that play a role a biological response or pathway. In general, this is done through text and/or literature mining, and thus the effectiveness of this approach is limited by the available information and the quality of the tools/algorithms used for text and/or data mining.

The NIEHS will be releasing in the fall of 2005 an open access version of The Chemical Effects on Biological Systems Knowledgebase (CEBS). It is hoped that the release of CEBS will encourage exploration of toxicogenomics-based approaches to risk assessment. Tennant solicited feedback on the usefulness of CEBS as well as input on how to make CEBS better and/or easier to use.

Developmental Programming of Tumor Suppressor Gene Penetrance

Cheryl Walker (The University of Texas, MD Anderson Cancer Center)

TSC2 is a tumor suppressor gene encoding tuber in that negatively regulates Akt1 signaling. TSC2 is frequently lost or decreased in the human disease uterine leiomyoma, which predisposes to severe uterine fibroids. Studies with a rodent model for uterine leiomyoma (the Eker rat) show that tumor incidence in TSC2-deficient rats is approximately 65%. However, recent studies by Cheryl Walker and colleagues demonstrated that exposure to environmental estrogens can increase the penetrance of the TSC2 mutation, and increase tumor incidence in TSC2-deficient rats to nearly 100%. In these studies, TSC2 and wild-type rats were exposed to the synthetic estrogen diethylstilbesterol (DES) during three different early developmental periods: postnatal days 3-5, 10-12, or 17-19. In all cases, estrogen-dependent gene expression was induced in DES-exposed TSC2-deficient rats. TSC2-deficient rats exposed during postnatal days 3-5 or 10-12, but not rats exposed during postnatal days 17-19 or wild-type controls, also showed altered transcription of estrogen-dependent genes at 5 mo of age. Furthermore, the incidence of uterine tumors in 16-month-old rats increased to nearly 100% in TSC2-deficient rats exposed to DES on postnatal days 3-5 or 10-12. Although tumor incidence increased significantly in DES-treated TSC2-deficient animals, no change was observed in tumor latency, the rate of loss of heterozygosity at TSC2, or the rate of apoptosis or extent of vascularization in the reproductive tract.

DES-treatment induced estrogen-responsive gene expression and increased tumor incidence in intact females, but these effects were blocked when rats were ovariectomized prior to puberty. This result suggested an explanation for these data as follows: exposure to DES during a critical developmental window between postnatal days 3 and 16 causes “developmental reprogramming” in the female reproductive tract. This causes hyper-sensitivity to subsequent estrogen exposure. Because endogenous estrogen is removed by ovariectomy, the ovariectomized rats were protected from the deleterious effects of early developmental exposure and developmental reprogramming.

These studies indicate that environmental exposure to an estrogenic compound during a critical developmental period (i.e., a window of susceptibility) alters cancer susceptibility in adult mice. Similar effects have previously been observed for other human diseases including

cardiovascular disease and diabetes, but this is one of the first demonstrations of latent effects on adult cancer susceptibility caused by early environmental exposure. If humans are also susceptible to estrogen exposure during a developmentally-defined window of susceptibility, it is possible that the window is quite large, potentially lasting for several years.

Biomarkers

James Swenberg (The University of North Carolina, Chapel Hill)

James Swenberg presented recent studies of 1,3-butadiene (BD) carcinogenesis with a novel biomarker assay for the cyclic N-terminal valine adduct of hemoglobin, pyr-Val. Earlier studies demonstrated that BD is metabolized into three reactive intermediates, 1,2-Epoxy-3 butene (EB), 1,2:3,4-Diepoxy-ybutane (DEB), and 3,4-Epoxy-1,2-butanediol (EBD). These species form DNA and protein adducts, the major adducts being trihydroxybutylguanine and valine adducts generated by DEB.

To improve detection of BD adducts in humans and other species, Swenberg developed an immunoaffinity capillary LC-MS/MS assay for the pyr-Val adduct of hemoglobin. Synthetic peptides were generated that correspond to the N-terminal 11 residues of the adducted mouse, rat and human hemoglobin molecules. Stable isotope labeled peptides were also synthesized to be used as internal standards and to facilitate quantification of biological samples. A polyclonal antibody was raised against the synthetic adducted human 11mer, and this antibody was used to prepare an immunoaffinity column. To analyze BD adducts in animal or human samples, hemoglobin (HB) is purified, the appropriate species-specific internal standard is added, the sample is digested with trypsin and applied to the immunoaffinity column. Subsequent analysis is by capillary LC-MS/MS.

The LC-MS/MS antibody affinity assay for Pyr-Val HB was used to quantify adducts in rats and mice exposed to BD at 1-60 ppm. The results show a linear dose-dependent increase in the pyr-Val HB adduct in exposed male and female mice, but no adduct was detected in exposed rats. Adduct formation was also assessed in humans occupationally exposed to 1 ppm BD. For this experiment, 50 mg of HB was analyzed from mice, rats, and humans exposed to the 1 ppm of BD. The adduct level in male and female mice was 31 and 23 pmol/g, respectively; in contrast, 0.7 or 0.9 pmol/g of adduct was detected in male and female rats, but adduct was not detected in humans. These results confirm previous reports of strong species-specific differences in BD metabolism, and BD-induced DNA and protein adducts.

Vinyl Chloride (VC) is a known animal and human carcinogen, which is associated with high incidence of hepatic angiosarcoma in occupationally-exposed humans. Controversial data also suggest that VC exposure may be linked to brain tumors in occupationally-exposed humans. VC is metabolized to the active carcinogen chloroethylene oxide by Cyp2E1 that forms mutagenic etheno DNA adducts including N2,3-ethenoguanine (EG), 1,N6-ethenodeoxyadenosine, and 3,N4-ethenocytosine. EG also forms spontaneously in vivo, most likely due to lipid peroxidation, and DNA from unexposed control humans and animals contain detectable amounts of EG. Because EG is formed at a low basal level in untreated cells, it is possible to assess the impact of low-dose VC exposure with reasonable accuracy and confidence. If animals are exposed to C13-labeled VC, the endogenous adduct can also be distinguished from VC-induced adduct. This approach was used to demonstrate that rats exposed to VC at a dose comparable to current exposure limits experience a 0.5%-5% increase in total EG burden. However, even this small increase in adduct burden does not occur in the brain of exposed rats, most likely because the amount of Cyp2E1 in brain is insufficient to drive adduct formation. This result is consistent with the observation that brain tumors have not been associated with occupational VC exposure because that maximum allowable VC dose was lowered to 1 ppm.

Swenberg also presented a novel quantitative assay for pyrimido[1,2-a]purin-10(3H)one (M1G). This assay detects and traps the ring-opened aldehyde form of M1G with aldehyde reactive probe (ARP). The ARP-M1G-deoxyribose (ARP-M1G-dR) conjugate is stable and can be readily isolated with standard solid phase extraction technology. Using this assay, M1G appears to be a reliable biomarker of oxidative stress, that is, less subject to artifacts during DNA isolation than other biomarkers of oxidative DNA stress such as apurinic (AP) sites.

Swenberg used this assay to show that M1G accumulates in a linear dose-dependent manner in calf thymus DNA treated with bleomycin, hydrogen peroxide, or hydroxyl radical. However, hydroxyl radical induces 8-oxoguanine approximately 100-fold more efficiently than it induces M1G (10^{-6} vs. 10^{-8} lesions per bp, respectively). Estrogen metabolites also induce low levels of M1G in DNA in vitro. Endogenous M1G is present at a higher level in rat heart than in lung, brain, kidney, pancreas, or liver, and at higher level in rat liver mitochondrial DNA than in nuclear DNA. Metabolic labeling was used to show that endogenous M1G is induced by hydroxyl attack on DNA and not as a result of lipid peroxidation, as has been suggested previously. Lastly, Swenberg indicated that XPA-deficient mice are proficient in repair of M1G, suggesting that nucleotide excision repair is not likely to be the only pathway for repair of M1G DNA adducts.

SESSION 6: TECHNOLOGY INNOVATIONS

John Lechner chaired and introduced the Conference Session on Technology Innovations, which included presentations by Neal Rosen (Memorial Sloan-Kettering Cancer Center), Jack Griffith (The University of North Carolina, Chapel Hill), and Linda Griffith (Massachusetts Institute of Technology). Technology innovations continue to bring improvements to both cancer diagnostics and cancer therapeutics. Novel imaging tools are currently one of the most important novel areas of technology development, bringing new capabilities to analyze protein-protein or protein-DNA interactions and new assays to monitor cancer drug efficacy. Smart imaging probes are being developed, which couple tumor-specific binding moieties and imaging tools. For example, HER2-positive breast tumors can be imaged with biotinylated anti-HER2 and avidin-labeled gadolinium complexes as a MR contrast-enhancing agent. Another smart imaging system called “luciferase complementation imaging” uses the N- and C-terminal halves of luciferase each fused separately to protein-interacting partners. Luciferase signaling is only turned on when the two interacting proteins are bound to each other. This allows dynamic assessment of protein-protein interactions and protein trafficking in vivo. In Jack Griffith’s talk presented later in the Session, a nanoscale biopointer is introduced. The nanoscale biopointer is a short streptavidin end-labeled linear DNA molecule that “points” to biotinylated subunits within macromolecular complexes when the complexes are visualized by electron microscopy. Technology innovations related to progress in stem cell research and tissue engineering are also anticipated in the near future.

Imaging Pharmacodynamics and Biologic Response

David Solit (presented by Neal Rosen) (Memorial Sloan-Kettering Cancer Center)

There are many good pharmacological targets for cancer prevention, and there are many good pharmacological agents that lack toxic side effects and show promising preclinical results. Despite these facts, success in preventing and/or treating cancer or significantly reducing overall mortality from cancer has been very limited. Why is this so? Neal Rosen offered a few possible reasons for this lack of success. First, while drugs are developed “rationally” based on available evidence, often that evidence is inadequate to determine if the selected targets and pathways are truly relevant to cancer development and/or progression in vivo. In other words, “we do not really know which targets are physiologically important” for cancer progression. Second, our model systems are inadequate. To improve use of model systems, they must both

inform and be informed by clinical investigations in humans. Third, in many cases, it is not known whether the target is adequately inhibited in vivo, in part because researchers rarely measure the biologically effective target tissue dose and rarely assess pharmacokinetic and pharmacodynamic properties of anti-cancer drugs. In other words, dose and schedule are very important determinants of treatment outcome.

The latter point is illustrated by the importance of schedule when treating with Taxol plus HSP90 inhibitor 17-AAG (which degrades Her-2) to inhibit tumor growth in a mouse model system. The maximum effect was achieved with a single simultaneous dose of both drugs. With this dosing schedule, tumor growth was almost completely inhibited for up to 60 d. In contrast, much weaker and less sustained suppression of tumor growth occurred when other dose schedules were used, including 17-AAG prior to Taxol, Taxol prior to 17-AAG, or either drug alone. One possible explanation for this effect is that 17-AAG causes tumor cells to arrest in G₁, when Taxol is expected to have little efficacy. Another drug combination whose efficacy is highly dependent on schedule is Iressa plus Taxol. In a xenograft mouse model for non-small cell lung carcinoma, a pulse of Iressa followed by continuous Taxol strongly suppressed tumor growth, while continuous dosing with Iressa and Taxol did not.

These examples underscore the need to directly assess the impact of pharmacological agents on their target. Novel imaging methods are currently being developed to provide this capacity. For example, it is possible to monitor inhibition of HSP90 in vivo by imaging expression of HSP90 client proteins such as Her-2. However, initial studies with a herceptin derivative tagged with a radiotracer for PET imaging were not successful because herceptin has a relatively long half-life. In contrast, the half-life of a labeled F(ab')₂ fragment of herceptin is approximately 2 h, which is suitable for in vivo imaging studies. This reagent can be used for microPET analysis of xenograft tumor suppression in 17-AAG treated mice. Uptake of the radiotracer was constant in normal mouse tissues with or without 17-AAG, but significantly lower in tumors in 17-AAG-treated mice. The quantitative accuracy of the PET data was confirmed by direct measurement of the radiotracer in tumor samples. When adapted for human use, this method could be used for patient selection and dose guidance in therapeutic cancer trials. In addition, the approach could be generalized for use with other drugs that degrade/destabilize their target or to monitor drug-induced changes in expression of cell surface proteins.

Use of Electron Microscopy to Visualize Single DNA and DNA-Protein Complexes

Jack Griffith (The University of North Carolina, Chapel Hill)

Electron microscopy (EM) is a highly specialized but particularly valuable tool in the arsenal of the molecular biologist studying DNA metabolism in eukaryotes. EM has facilitated particularly important advances in the study of telomeres, p53, and mechanisms of DNA replication.

In the area of telomere structure and biology, EM provided the first structural evidence for telomeric T-loops, strand-invaded structures in which a 3'-terminal single-stranded DNA tail forms a loop, invades and pairs with one strand of a homologous dsDNA telomeric repeat. EM was used to visualize T-loops in telomeric DNA from humans, peas, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. EM was also used to show that the telomere binding protein TRF2 promotes T-looping in telomeric DNA.

The mechanism by which telomeres are maintained in normal eukaryotic cells is understood in some detail. In normal cells, the enzyme telomerase is required for telomere maintenance and continued growth of cells in culture; however, many immortal cell lines bypass the requirement for telomerase and maintain their telomeres by a process known as alternative lengthening of telomeres (ALT). ALT cells do not express functional telomerase, have very

long heterogeneous telomeres and require a functional *rad52* pathway in yeast. This suggests that telomeres are maintained by a pathway involving homologous recombination. EM analysis provided support for this idea by revealing the presence of abundant extrachromosomal “T-circles” in telomere-enriched DNA from ALT cells. T-circles are small dsDNA circles composed of telomeric DNA repeats. T-circles are approximately the same size as T-loops that have been detached from the chromosome arm on which they form. Two-dimensional pulsed field gel electrophoresis provided evidence that T-circles replicate by rolling circle replication. Therefore, it has been proposed that T-circles replicate autonomously and that homologous recombination between T-circles and telomeric chromosome ends stochastically lengthens chromosomes, thereby avoiding cell senescence. These data are important for cancer therapy, because telomerase has been considered as a therapeutic target for anti-cancer drugs. However, ALT cells are expected to be resistant to telomerase-targeted drugs. Thus, it is important to understand the mechanism of ALT and to develop anti-cancer therapeutics targeting essential components of ALT-mediated cell immortalization.

p53 is a well characterized tumor suppressor protein that has structure-specific DNA binding properties. This property of p53 was first discovered in EM images of p53 bound specifically to bulged or mismatched DNA or other non-canonical B DNA structures. In an in vitro eukaryotic DNA replication assay, p53 also specifically binds to “chicken feet” structures, in which the replication fork has stalled and partly regressed. p53 appears to halt fork regression, promote lesion removal, and then facilitate replication restart at the stalled replication forks. p53 may play a similar role during replication of telomeric DNA, which is predicted to be particularly prone to replication slippage.

A long-standing question in eukaryotic replication has been whether one or two DNA polymerases are active at a bi-directional replication fork. The eukaryotic bi-directional replication fork has been described as a “trombone-like” structure, in which the lagging strand DNA loops back towards the junction between nascent leading strand DNA and unreplicated leading strand DNA. An usual EM-based method was developed to reveal structural information about the replication machinery at the replication fork. First, specific replication proteins were N- or C-terminally tagged with biotin. Then, short linear DNA molecules were three-end-labeled with streptavidin. When the streptavidin-tagged DNA molecules were added to replicating DNA and the replication intermediates were visualized by EM, the labeled DNA appeared as a visual “biopointer” that marked the position of the biotinylated protein in the reaction. The reaction appeared to be stoichiometric, such that two biopointers corresponded to two biotinylated polymerase subunits and three biopointers corresponded to three biotinylated polymerase subunits. After many EM fields and many DNA molecules were scored, it was concluded that two DNA polymerases participate in DNA replication at the eukaryotic replication fork. A similar approach could be applied to many different biotinylated replication factors as well as to other protein complexes and other biological processes. Alternatively, it may be possible to use antibody- or hexa-histidine-tagged DNA to generate biopointers with alternative specificities.

Microscale 3D Models of Vascularized Tissue (Liver)

Linda Griffith (Massachusetts Institute of Technology)

When hepatocytes are removed from the liver and grown in 2-dimensional (2D) collagen sandwich culture, they rapidly lose many of their unique characteristics, including hepatocyte-specific patterns of gene expression. This has hampered studies of hepatocyte biology, including attempts study hepatocellular toxicity in vitro. However, Linda Griffith and colleagues have recently begun to coculture rat hepatocytes and other rat liver cell types in a prototype 3-dimensional (3D) microscale bioreactor. In this system, hepatocytes retain their

in vivo properties for many weeks in culture. This system holds promise for studies of drug metabolism and toxicity in hepatocytes as well as to advance progress in tissue engineering.

The 3D microscale bioreactor system is constructed on a silicon or plastic substrate, which has an array of 300×200 micron collagen-coated wells. A permeable filter is placed at the bottom of each well and the wells are seeded with hepatocytes. The hepatocytes adhere to the surface and in the presence of other liver cell types, the hepatocytes organize into tissue-like structures. Endothelial cells migrate to the tissue-fluid interface. Fluid is continuously perfused from bottom to top and horizontally across the top surface of each well. Both perfusion flow and horizontal flow are required for cell viability. The bioreactor is scaleable, with the most recent prototypes having 8 or 12 wells.

RT-PCR was used to compare expression of liver specific transcription factors and phase I/II detoxification genes in 2D and 3D hepatocellular cultures. The results showed that hepatocytes in 3D culture expressed HNF1a, HNF1b, HNF3a, and HNF4a at near physiological levels, but hepatocytes in 2D culture had up to 6 log changes in expression of these genes. Similar results were observed for many phase I/II detoxification genes including Cyp2B1, Cyp2C11, Cyp1A2, GST-Ya, and others. The level of testosterone hydroxylase activity in 3D cultures was also close to the in vivo activity level; in contrast, 2D hepatocellular cultures showed 8- to 20-fold lower testosterone hydroxylase activity.

When hepatocytes were co-cultured with EGFP-labeled endothelial cells, macrophages, and stellate cells from liver, tissue-like structures, including large vessel endothelium, formed readily, and specific cell subpopulations were non-randomly distributed in the bioreactor wells. Three dimensional co-cultures of hepatocytes with other liver cell types, and 3D hepatocyte monocultures had significantly different global patterns of gene expression; in contrast, patterns of gene expression were more similar for 2D hepatocyte co-cultures and 2D monocultures. The significance of the gene expression changes in 3D hepatocyte co-cultures are not currently understood. Surprisingly, only subtle changes were observed in the expression of phase I/II enzymes.

In future experiments, the 3D bioreactor culture system may be adapted for studies of liver carcinogenesis. Other important research goals are increased automation, higher scalability, and increased throughput. Eventually, 3D bioreactors may be useful for tissue engineering. However, 3D bioreactor hepatocyte cultures now offer an improved system for study of drug metabolism in hepatocytes.

SESSION 7: STRESS AND CANCER

Stuart Yuspa (National Cancer Institute) chaired and introduced the Conference Session on Stress and Cancer, which included presentations by Neal Rosen (Memorial Sloan Kettering Cancer Center), Michael Karin (The University of California, San Diego), and James Fox (Massachusetts Institute of Technology). A substantial body of evidence suggests that stress can have a significant impact on cancer susceptibility. This could be due to both direct and indirect effects of stress-inducing agents on cancer-prone cells and tissues (i.e., cytotoxicity, inflammation, ROS, and immune responses). Examples of stress-accelerated carcinogenesis include an increased rate of mouse mammary tumor virus-induced carcinogenesis or higher incidence of UV-induced skin tumors in stressed versus non-stressed mice. This Conference Session considered the role of three cellular mechanisms that modulate the response to stress and its influence on carcinogenesis: molecular chaperones, anti-apoptotic NF- κ B signaling, and chronic inflammation. The best characterized molecular chaperones are the heat shock proteins, which play an important role in stabilizing cellular components that are adversely impacted by cellular stress. Similarly, the NF- κ B signaling pathway is a strong anti-apoptotic pathway that counteracts the proapoptotic effects of stress and modulates immune and

inflammatory processes. The role of chronic inflammation in cancer was discussed in an earlier Conference Session. In this Session, the role of chronic infectious agents and cytokines in gastric cancers was considered, with an emphasis on rodent model systems for human diseases such as inflammatory bowel disease and gastric cancer. The role of specific cytokines in specific cancers has been demonstrated. For example, TNF α is required for skin carcinogenesis in mice and mice deficient in IL-1 β or IL-6 show altered cancer susceptibility.

Heat Shock Proteins as a Molecular Target of Cancer Therapy

Neal Rosen (Memorial Sloan Kettering Cancer Center)

HSP90 is an abundant protein chaperone that plays important roles in stabilizing protease-sensitive client proteins, conformational maturation of gain-of-function mutant proteins, and refolding denatured proteins in stressed cells. When HSP90 is inhibited in normal eukaryotic cells, many HSP90 client proteins, including Her2, Met, Raf, and AKT are destabilized and efficiently degraded. Normal cells exposed to the geldanamycin derivative 17-AAG also undergo cell-cycle arrest and subsequent apoptosis. HSP90 is often expressed at a high level in tumor cells, and is thought to be required for folding mutant oncoproteins such as mutant v-src, Bcr-Abl, and b-Raf.

The potential of HSP90 as a target for anti-cancer therapeutics first became apparent when it was discovered that the naturally occurring antibiotic and anti-tumor drug geldanamycin binds to and inactivates HSP90. However, because the specificity of HSP90 was poorly understood, it was initially thought that a clinically-effective dose of an HSP90-targeted drug would be associated with unacceptable levels of toxicity. In contrast, recent experiments by Neal Rosen and colleagues demonstrated that growth of xenograft tumors in mice can be almost completely blocked by non-toxic doses of the HSP90 inhibitor 17-AAG. There are three plausible (nonmutually exclusive) explanations for this result: (1) Tumor cells may require stable expression of an HSP90 client protein for growth and proliferation; (2) Tumor cells may require HSP90 for refolding mutant oncoproteins on which they are dependent for growth and proliferation; or (3) HSP90 may be limiting in tumor cells because of a heavy burden of unfolded proteins, which may reflect tumor-associated hypoxia and/or other types of tumor-associated cellular stress.

There is evidence to support at least the first two mechanisms proposed above. For example, 17-AAG causes specific loss of Her2 expression in Her2-dependent breast cancer cells. In addition, 17-AAG promotes degradation of tumor specific mutant b-Raf in human melanoma cells, but it does not alter the stability or expression of wild-type b-Raf in normal human melanocytes. Furthermore, the volume of mutant b-Raf-dependent tumors was inversely proportional to the dose of 17-AAG and the extent of b-Raf degradation in tumor cells from animals treated with 17-AAG.

Initial phase I clinical studies are in progress with 17-AAG and taxotere as treatment for metastatic breast cancer; preliminary data from this trial suggest that moderate doses of 17-AAG lack severe toxic side effects in humans. However, more complete assessment of the therapeutic potential of 17-AAG await completion of this and other clinical studies. Nevertheless, the absence of hepatotoxicity in patients treated with 17-AAG to date is encouraging. In addition, alternative HSP90 inhibitors that interact with the ATP/ADP binding pocket that binds to 17-AAG are being developed. Future studies may reveal that one of these compounds has higher efficacy and lower toxicity than 17-AAG.

The NF- κ B Signaling Pathway in Cancer Development and Therapy

Michael Karin (The University of California, San Diego)

NF- κ B plays a central role in propagation of immune responses and NF- κ B signaling is induced by many pro-inflammatory stimuli such as tumor necrosis factor α (TNF α), IL-1, lipopolysaccharide, and dsRNA. The downstream effects of NF- κ B are complex but are known to include pro-inflammatory, immunostimulatory, and anti-apoptotic effects. I κ B is the main upstream regulator of NF- κ B, and because I κ B-deficient cells are also completely deficient in NF- κ B signaling, tissue specific knockouts of I κ B have been very useful for analysis of the biological roles of the NF- κ B signaling pathway.

Given the relatively strong association between certain cancers and chronic infection-associated inflammation (i.e., gastric cancer and *H. pylori*, liver cancer, and hepatitis B/C viruses), it is reasonable to propose that NF- κ B signaling might play a role in inflammation-associated carcinogenesis. This idea was explored by Michael Karin and colleagues with primarily mouse models of inflammation-associated colon cancer and tissue- or cell type-specific deletion mutants of mouse I κ B. When I κ B-deficiency was introduced in epithelial cells, the number and size of colitis-associated tumors decreased and pro-apoptotic pathways were stimulated. Similar effects were observed when I κ B-deficiency was introduced in epithelial and myeloid cells, but proliferation of transformed epithelial cells was suppressed. This suggests that activation of I κ B in myeloid cells promotes the proliferation of transformed epithelial cells through a paracrine mechanism (i.e., secretion of IL-6 and TNF α).

The importance of NF- κ B signaling in chemical carcinogenesis was also examined with a mouse model for HCC and liver cell type-specific deletion of I κ B. Wild-type and I κ B-deficient mice were given a single dose of diethylnitrosamine (DEN) at 15 d and sacrificed at eight mo to score HCC incidence. Surprisingly, when I κ B deletion was induced in hepatocytes, HCC tumor number and size increased relative to wild-type controls, and % survival decreased significantly. A increase in DEN-induced apoptosis, necrosis, and cell proliferation was associated with the higher rate of tumor growth in mice with hepatocellular deficiency in I κ B. This suggested that DEN may induce a higher level of compensatory proliferation in I κ B-deficient than in I κ B-proficient hepatocytes. Additional evidence supporting this idea was obtained by feeding mice anti-oxidants before and immediately after dosing with DEN, and by performing the same experiment in mice lacking I κ B in both hepatocytes and Kupffer cells. Under the latter two conditions, tumor number and volume was not stimulated by the I κ B-deficiency. These data suggest that compensatory proliferation plays a critical role in chemical carcinogenesis in liver, providing an opportunity for oncogenic mutations to be fixed in initiated hepatocytes. Kupffer cells likely play an essential role in this process, because they secrete pro-inflammatory factors TNF α and IL-6 in response to carcinogen-induced cellular necrosis.

In summary, these studies support a critical role for inflammation in chemical and non-chemical carcinogenesis. Inflammation associated with cellular necrosis may drive growth and progression of many solid tumors. Furthermore, these relationships may be relevant to the efficacy of cancer therapeutic agents, which are likely to produce both apoptotic and necrotic cell death in target tumor cells. Thus, it may be possible to improve the efficacy of cancer therapeutic agents by inhibiting necrotic cell death or by targeting tumor-associated inflammatory cells during anti-cancer treatment.

Chronic Inflammation is the Culprit: Rodent Models of Infectious Gastrointestinal and Liver Cancer

James Fox (Massachusetts Institute of Technology)

Infectious agents play key roles in many types of cancer, most notably in gastric, cervical, and hepatocellular cancer, which are tightly linked to infection with *Helicobacter pylori*, human papilloma virus, and hepatitis B/C viruses, respectively, The World Health Organization

estimates that approximately 18% of the total world cancer burden is related to chronic infectious processes.

To prove that *H. pylori* is a class I carcinogen, it was necessary to develop an appropriate in vivo model system in which the impact of infection on cancer incidence could be studied. This was achieved with an *H. pylori*-related virus, *H. felis*, which infects cats, dogs, and rodents. Experiments performed with this model system demonstrated definitively that infection of C57BL/6 mice with *H. felis* strongly increases the incidence of gastric cancer at 12 mo postinfection.

Although the link between *H. pylori* and gastric cancer in humans is unequivocal, exceptional human populations exist in Africa and South America that have a very high incidence of *H. pylori* infection, but a low incidence of gastric cancer. The explanation for this observation remained obscure, until it was discovered that the cancer-resistant human populations also carry a high burden of helminthic parasites. While *H. pylori* infection stimulates a Th1-mediated pro-inflammatory immune response, helminthic parasites stimulate a Th2-mediated non-inflammatory immune response. Furthermore, in the case of dual infection, a Th2-dominated non-inflammatory response is observed, suggesting that helminthic parasites are protective against cancer because they reduce pro-inflammatory Th1-mediated signaling. When this explanation was tested in mice infected with *H. polygurus*, *H. felis*, or both, the dual-infected mice had much lower incidence of gastric cancer than mice infected with *H. felis*. Data from human epidemiological studies in coastal and Andean populations in Columbia are consistent with the idea that intestinal parasites are protective against *H. pylori*-induced gastric cancer.

H. hepaticus colonizes the liver and colon in mice and infection with *H. hepaticus* is associated with colitis as well as with hepatitis and hepatocellular neoplasms in mice. Significant mouse strain dependence has been noted in susceptibility to *H. hepaticus*-induced disease and this strain-dependence correlates with strain-dependent differences in the extent of virus-induced inflammation, suggesting that inflammatory responses modulate disease susceptibility in a mouse-strain specific manner. This is consistent with the observation that *H. hepaticus*-induced disease can be suppressed in susceptible mouse strains by injecting the mice with CD4+CD25 + regulatory lymphocytes (T regulatory cells). T regulatory cells are known to downregulate inflammatory responses in an IL-10 and TGF β -dependent manner, and disease suppression was not observed when mice were injected with IL-10-deficient T regulatory cells.

Gall bladder disease is linked to gall bladder cancer in humans. Although previous studies did not suggest that infectious disease played a role in gall bladder disease, recent studies in mice do suggest such an involvement. In particular, a mouse strain was identified that is highly susceptible to gall stones when fed a lithogenic diet, but studies with this strain were inconsistent, showing loss of susceptibility under specific pathogen-free conditions. This suggested a role for infection, which was confirmed when gall bladder disease was induced in infected but not in uninfected isogenic animals.

In summary, these studies underscore the important ways in which inflammation and innate immune responses modulate pathological processes including cancer in animal models. Where available, human data are consistent with experimental observations in animal model systems.

SESSION 8: APOPTOSIS

Scott Lowe (Cold Spring Harbor Laboratory) chaired and introduced the Conference Session on Apoptosis, which included presentations by Craig Thompson (The University of Pennsylvania), Maureen Murphy (Fox Chase Cancer Center), and Stuart Yuspa (NCI). The importance of apoptosis in carcinogenesis is well known and this fact was recognized when

Hanahan and Weinberg, in their seminal paper on the phenotype of cancer cells, named “disruption of apoptosis” as one of the six “hallmarks of cancer” (Hanahan D, Weinberg RA, 2000, The hallmarks of cancer. Cell 100: 57-70.). Apoptosis has been extensively studied in recent years, and these studies have revealed that apoptosis is mediated by exceedingly complex interrelated signaling networks. Several anti-apoptotic proteins are oncogenic, while other regulators of apoptosis, such as p53, are tumor suppressor proteins.

There is significant interest in the role played by apoptotic signaling in anti-cancer therapy. Importantly, it is clear that the outcome of both conventional and targeted chemotherapy is highly dependent on the status of apoptotic pathways in targeted cells, a fact which explains some of the variability or “context-dependence” in cancer treatment outcome. The fact that drug efficacy is difficult to predict with a high degree of accuracy is consistent with the fact that apoptosis can be dysregulated by different mechanisms in different cancers or in different specimens of the same cancer. Greater understanding is needed of how distinct apoptotic signaling pathways are coordinated, and of the role of thresholds in triggering apoptosis versus cellular survival. More information on these aspects of apoptotic signaling may facilitate future development of improved anti-cancer therapies.

Mechanisms of Apoptosis

Craig Thompson (The University of Pennsylvania)

In eukaryotic multicellular organisms, individual cells have lost the capacity for cell autonomous survival and proliferation. Instead of surviving or proliferating, isolated cells undergo apoptosis when deprived of exogenous sources of essential growth factors. The pro-apoptotic proteins Bax and Bak are required for the intrinsic pathway of apoptosis. However, some Bax $-/-$ Bak $-/-$ mice survive to adulthood, which challenges the idea that apoptosis is absolutely essential for development and homeostasis in metazoan organisms. When grown in culture in the absence of essential growth factors, Bax $-/-$ Bak $-/-$ splenocytes survive for more than 96 h, while wild-type cells uniformly undergo apoptotic cell death within this time period. Over-expression of anti-apoptotic Bcl2 suppresses apoptotic cell death in cultured cells, but these cells survive for a limited period of time (approximately 4 wks) by autophagy-dependent catabolism of intracellular components, after which they succumb to necrosis. Autophagy also plays an essential role in avoiding starvation in Bax $-/-$ Bak $-/-$ cells, and autophagy inhibitors such as chloroquine or 3-methyladenine causes rapid loss of viability in these cells.

In a subset of human and animal tumor cells, oncogene stress and the rapid cell growth associated with oncogene stress is associated with overexpression of members of the anti-apoptotic Bcl2 family. Thus, it was of interest to ask if tumorigenesis could be suppressed by inhibiting Bcl2. Recent studies identified a small molecule inhibitor of Bcl2, ABT-737, which binds with high affinity to Bcl2 and blocks Bcl2-mediated suppression of Bax- Bak-dependent apoptosis. When tumor cells were screened for susceptibility to ABT-737, the susceptible cell lines included several Myc-dependent tumors, as expected. Many of these cell lines also inactivate p53. When p53 is reactivated in these cells, tumor growth is transiently suppressed, presumably by its ability to induced apoptotic cell death. However, tumor suppression can be potentiated by reactivating p53 (to stimulate apoptosis) and simultaneously treating with the chloroquine to inhibit autophagy-dependent survival.

In developing novel anti-tumor treatments, it is important to understand the role of bioenergetic stress in cancer development and progression. Cancer cells typically have high bioenergetic requirements and are characterized by a glucose-dependent metabolism, which is essential to support high ATP utilization and to maintain the concentration of intracellular ATP. This fact lies behind the sensitivity of cancer cells to DNA alkylating drugs, which induce poly(ADP-ribose) polymerase (PARP)-dependent depletion of cytoplasmic and nuclear NAD. Depletion

of NAD selectively inhibits glucose-dependent ATP production resulting in necrotic cell death in cancer cells, but it does not kill non-cancer cells. The fact that alkylating agents induce necrotic but not apoptotic cell death is clearly demonstrated by the fact that cyclophosphamide is equally effective at regressing solid tumors in wt and Bax ^{-/-} Bak ^{-/-} mice.

In summary, inhibition of Bcl2 may be an effective therapeutic approach in tumors subject to oncogene-induced bioenergetic stress. Inhibitors of autophagy, such as chloroquine, may potentiate the efficacy of pro-apoptotic anti-tumor agents. And in tumor cells that display extreme glucose-dependence, it may be possible to induce necrotic tumor cell death by inactivating the pathways that support glucose-dependent ATP production.

The Role of p53 in Programmed Cell Death

Maureen Murphy (Fox Chase Cancer Center)

The human *p53* gene has two common polymorphisms that may alter p53 function in normal and cancer cells. One of these polymorphic sites is Pro72/Arg72 and the other is Pro47/Ser47. The Pro72 allele of p53 was cloned first and is considered “wild-type” although the Arg72 allele is more prevalent in some populations. Pro72 is homozygous in 10% of Caucasians and 40% of African Americans. Pro47 is more common than Ser47, with Ser47 occurring at a frequency of 1% in African Americans, always in cis with Pro72.

The codon 72 polymorphism lies in the proline-rich domain of p53, which is required for p53-dependent transcription-independent induction of apoptosis after DNA damage. The functional importance of the codon 72 polymorphism was examined by overexpressing temperature sensitive forms of the codon 72 variants in cultured cells in vitro. The results showed that p53Arg72 induces apoptosis approximately 15-fold more efficiently than p53Pro72. Initial studies revealed no differences in transactivation, transrepression, or protein interaction capabilities of the two codon 72 variants of p53. However, the Arg72 variant was transported to the mitochondria more efficiently than the Pro72 variant, due to more efficient binding of Mdm2. In addition, p53Arg72 induced oligomerization of BAK more efficiently than p53Pro72. These two factors explain enhanced apoptotic signaling by p53Arg72. The proline at codon 47 of p53 is required for phosphorylation of Ser46 by proline-directed kinase p38. In addition, p53Ser47 induces apoptosis less well than p53Pro47 and has decreased ability to transactivate the p53-response gene PUMA.

Because the mouse and human *p53* genes are not conserved at codon 72, it is necessary to “humanize” the mouse p53 gene before functional studies of the codon 72 polymorphism can be carried out in vivo in the mouse. Future studies will examine the impact of codon 72 and codon 47 p53 SNPs on cancer susceptibility in vivo with humanized mouse models.

CLIC 4, a p53, and TNF α Regulated Chloride Channel Protein, Is Essential for Cell Viability and a Potential Molecular Target for Cancer Therapy

Stuart Yuspa (National Cancer Institute)

Chloride intracellular channel 4 (CLIC4) is a p53-inducible transmembrane protein that undergoes subcellular redistribution in stressed cells. CLIC4 was first identified as a p53-regulated protein in keratinocytes, whose expression is also regulated by differentiation state and TNF α . CLIC4 belongs to a large family of well-conserved membrane proteins including the prototypical chloride channel protein p64. CLIC family members are localized in several membrane compartments including nuclear, microsomal, and plasma membranes. The promoter of the human *CLIC4* gene has three p53 binding sites, which activate expression of an alkaline phosphatase reporter gene in the presence of p53. The CLIC4 promoter also has two Myc consensus binding sites, which are functional based on responsiveness to Myc protein

in reporter gene and chromatin immunoprecipitation assays. Overexpression of Myc induces CLIC4 and stimulates apoptosis in cultured cells. However, Myc-induced apoptosis is blocked by expression of CLIC-targeted siRNA, suggesting that CLIC4 is required for Myc-induced apoptosis.

The intracellular localization of CLIC4 changes in response to cellular stress, with rapid redistribution of CLIC4 from the cytoplasm to the nucleus. This appears to play an important role in CLIC4-stimulated apoptosis, because apoptosis is accelerated by targeted expression of CLIC4 in the nucleus or mitochondria. Furthermore, when CLIC4 is targeted to the nucleus, it induces apoptosis even in Apafdefective cells. Surprisingly, CLIC4 is excluded from the nucleus in many tumor cell types, including skin, kidney, and prostate, although nuclear-targeting of CLIC4 induces apoptosis in cancer cells as it does in non-cancer cells. Preliminary experiments suggest that adenovirus-mediated expression of nuclear CLIC4 can suppress the growth of solid tumors in vivo. Additional studies are needed to define the mechanism by which nuclear CLIC4 regulates apoptosis. Nevertheless, these data suggest that CLIC4 may be a useful target for anti-cancer therapy.