National Cancer Institute

Think Tanks in Cancer Biology

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Cancer Susceptibility and Resistance Think Tank

Rosters

Think Tanks in Cancer Biology
Summary Report

I. What we sought to accomplish

A year ago, Dr. von Eschenbach charged the Division of Cancer Biology (DCB) with conducting a series of Think Tanks to assess the state of cancer biology research and to recommend to the NCI a research agenda that would accelerate progress in cancer research. The process of fulfilling this charge began with an internal identification of scientific areas of unusual promise for rapid progress. Eight areas were chosen as the topics of Think Tanks. The areas were quite different in scope and in the maturity of the scientific disciplines involved. As a result, each Think Tank had to be structured differently, to deal with the unique questions and opportunities in each area. The eight Think Tanks are listed below, each with a brief description of its goal:

1) **Tumor Immunology** - This area of basic cancer biology has gone from relative obscurity to intellectual vitality over the past several years, as a result of critical basic science and translational advances, coupled with new concepts and methods in basic immunology. Immunotherapy of cancer had disputed translational potential for many years, but is now an established and expanding therapeutic modality. The field has untapped promise, but basic science advances have made clear how much still needs to be known about how the immune system and tumors affect one another. In addition, clinical advances depend on improving the rate at which immunological and other biological therapies are brought to the point where they can be used in clinical studies, and on devising clinical trials structures that accurately and efficiently allow evaluation of efficacy. These basic and translational issues were the focus of discussion.

2) **The Tumor Microenvironment** - The microenvironment in which a tumor arises has a profound influence on its progression. Tumor cells and stromal elements such as endothelial cells, fibroblasts, lymphoid and myeloid cells, extracellular matrix and cytokines interact dynamically and depend on one another for growth and survival. The tumor microenvironment has been the focus of intense interest within NCI and the scientific community for some time because it is the key to understanding tumor biology and strongly affects the likelihood of successful cancer therapy. While a consensus has been reached that this area deserves substantial additional support, this Think Tank had the goal of specifying the type of initiative(s) that would best facilitate rapid advances in knowledge about the microenvironment.

3) **Tumor Stem Cell & Self-Renewal Genes** - Recent results suggesting that at least some tumors contain only a small number of cells with tumor-initiating potential (loosely termed tumor stem cells), have profound implications for carcinogenesis, cancer biology and cancer therapy. Think Tank participants discussed current evidence for the existence of tumor stem cells in different tumor types, how they might arise, and the genetic (and possibly epigenetic) pathways important in maintaining the tumor stem cell state.

4) **Cell Decisions in Response to DNA Damage: Survival vs. Programmed Cell Death** - When cells encounter DNA damage, they can repair the damage or decide to try to live with it, either of which leads to survival, or they can choose to undergo programmed cell death. This life or death decision involves a complex interplay between DNA damage sensing and repair pathways, the pathways controlling the cell cycle, and cell death mechanisms. With an understanding of how these decisions are made, the response to radiation and/or chemotherapy could be improved by increasing the likelihood that cancer cells would die in response to therapy-induced damage while decreasing the chance that normal cells would die. Experts in DNA damage, cell cycle control, and apoptosis, who do not meet regularly, met at this Think Tank to consider prospects for fruitful interaction.

5) **Cancer Etiology: Role of Exogenous and Endogenous Chemicals** - Classical studies in chemical carcinogenesis have definitively established an important role for exogenous and endogenous chemicals in cancer development and defined some of the mechanisms involved, particularly those related to DNA adduct formation. Other areas, such as the role of damage to proteins and lipids, and identifying markers of exposure, have received less emphasis. The challenge in the field is to build on what has been done by integrating with other fields of biology (particularly integrative cancer biology and cancer susceptibility) to build a system-wide understanding of the complex process of carcinogenesis. The participants discussed the connections that exist between chemical carcinogenesis and areas such as inflammation, biological carcinogenesis, reactive oxygen species and systems biology, and how to strengthen them.

6) **Epigenetic Mechanisms in Cancer** - Epigenetic mechanisms are those that lead to heritable changes in cell behavior without changes in DNA sequence. The dramatic changes in gene expression that characterize tumor cells come about through both genetic and epigenetic mechanisms. Dramatic advances in genetics and genomics, and smaller advances in epigenetics, have sharpened our appreciation of the critical role that epigenetics plays in development and cancer, through DNA methylation, histone modification and changes in higher order chromatin structure. The Think Tank was organized to discuss evidence for epigenetic phenomena important in cancer and to prioritize NCI activities to advance the field.

7) **Inflammation and Cancer** - Chronic inflammatory disease has been known for many years to predispose to cancer development, and most tumor sites show evidence of ongoing inflammation. Inflammation can benefit a tumor by leading to production of mutagens, such as reactive oxygen and nitrogen species, and growth-stimulatory cytokines. At the same time, the proper inflammatory signals are necessary to allow the development of an effective immune response against the tumor. The participants in the Think Tank discussed how to sort out the positive and negative influences of inflammation in cancer, including that due to infectious agents, and how this understanding is expected to contribute to more effective strategies for cancer prevention and treatment.

8) **Cancer Susceptibility and Resistance** - The consensus of two prior meetings of mouse geneticists, population scientists, and statisticians is that the ability to understand human cancers as complex traits will require new statistical and computational methods for modeling gene/environment interactions, and assembling data for high-level gene network analysis. This Think Tank was set up to explore novel developments in mathematics and engineering design and their application to cancer

susceptibility research, both in modeling human populations and hypothesis testing/candidate gene validation in rodent models.

9) **Integrative Cancer Biology** - The first Think Tank area to be addressed with a major initiative was systems biology or, in this specific context, integrative cancer biology. Cancer is sufficiently complex that it will never be adequately understood based on the expression of a few genes or the regulation of a few signal transduction pathways in the transformed cell. It is an emergent property of derangements in complex intracellular systems in a context set by equally complex interactions of the tumor cell with intercellular environment. No report is included here for the Integrative Cancer Biology Think Tank because a solicitation has already been done for Integrative Cancer Biology Programs, and these have now been funded. This was the key recommendation of the Think Tank. Recommendations from several of the other Think Tanks dealt with the need to foster this area (see below for discussion), so this is likely to remain a topic of great interest for NCI for the foreseeable future.

II. How the Think Tanks were organized and run

The general goal of each Think Tank was to assess a specific area of cancer biology, determining where the science is and where it is going, and asking what NCI could or should do to facilitate progress. In areas that had been assessed previously in workshops and where NCI had made a commitment to do initiatives, such as the tumor microenvironment, the emphasis was entirely on what NCI should do. In other areas, where less had been done and no funding commitment existed, the emphasis was more on assessing the state of science in the field. A general charge was developed for each Think Tank, as follows:

Questions to Consider:

- Where does the field stand today?
- What would we have to know to move the field forward dramatically?
- What are the gaps and/or roadblocks to progress?
- Are there areas of expertise that need to be, but have not been, brought to bear on the problems in this field? If so, how can this be remedied?
- What cross-cutting tool, enabling technology and/or infrastructure needs to be developed?
- What can NCI do to move the field forward?

Each Think Tank was unique in scope and maturity of the field, so each had subtly different goals and had to be organized somewhat differently. Accordingly, the organizers of each Think Tank were given guidelines or "points to consider," rather than firm rules. These organizational guidelines are included as Appendix A. A critical element of every Think Tank was the selection of outside co-chairs. These individuals are distinguished scientists with unusual breadth of vision in the area to be discussed and strong organizational ability. They were recruited very early in the planning process, and played a leading role in identifying other participants, defining the critical questions to be discussed and setting the agenda. They were also involved in assembling the reports included here, and generally took the

lead if an account of the Think Tank was prepared for publication in an appropriate scientific journal. In every case, the recommendations included in the Think Tank reports are those of the co-chairs and the other participants.

Each Think Tank was organized independently, but it was obvious from the outset that some of the Think Tanks overlapped. For example, tumor immune responses are influenced by the tumor microenvironment and cancer etiology involves a strong element of inflammation, at least as a cofactor. This was seen as an asset because it made it possible to build in a level of continuity in these areas by inviting key participants to attend two or more Think Tanks. In some cases, formal participation in two Think Tanks was not possible, but advice was always sought where overlaps were anticipated to ensure that similar issues were addressed.

III. Overall recommendations

Each Think Tank was unique in focus and participants, and each resulted in a series of recommendations that will be addressed individually. All of the reports are appended, and for easy access the recommendations have been collected at the end of each report. This section will deal with a number of scientific and support-mechanism issues that emerged independently in multiple Think Tanks, with details on each given below. The scientific issues of the tumor microenvironment and integrative cancer biology (systems biology) were mentioned in virtually every Think Tank. Both of these subjects have been identified as high-priority areas for NCI support. The Think Tank reports provide a wealth of details on what needs to be done in these areas and reinforces their importance for cancer biology in the future. It was emphasized in these areas and others that studying cancer is impossible without also studying normal biology. The derangements in cancer can only be understood in terms of their deviations from normal behavior. This increases the scope of what must be done, and challenges us to coordinate with agencies supporting studies of normal biological development and other diseases.

Among the issues dealing with support mechanisms, it was not a surprise that the participants uniformly identified a need for additional and more flexible mechanisms to support multi-disciplinary, generally multi-institutional efforts to address complex issues like integrative cancer biology and the tumor microenvironment. A related concern was the difficulty of establishing training programs that prepare students and investigators for research in a multi-disciplinary environment. To balance this concern, each Think Tank also emphasized the indispensable contribution by, and the continuing importance of, research supported by investigator-initiated R01 grants.

Some of the issues were unexpected. Inflammation was recognized as important enough to deserve its own Think Tank, but it was surprising how prominent a role it occupied in other Think Tanks. This suggests that it needs an even more prominent role than had been envisioned in initiatives to study the tumor microenvironment, where inflammation is a nearly constant finding. A related common theme was the role of microbial flora as a cofactor in tumor development. While biological carcinogenesis, with an emphasis on cancer caused by viruses, has always been supported within NCI, examination of a cofactor role for microbes that are not directly transforming has lagged behind. This is one of several

areas that bridge cancer biology and etiology. Support for technology development, in general, appears to remain a challenge despite the addition of many new programs in recent years. Think Tank participants consistently reported limitations in funds for reagent preparation (e.g., monoclonal antibodies), model development (both genetically engineered animals and complex, three-dimensional tissue cultures), and state-of-the-art imaging. Funding for critical resources needs to be factored into plans in many areas, but it was also surprising that in some instances Think Tank participants recommended that NCI make available resources, including reagents, databases, animals and facilities, that already exist. This indicates that more effort needs to be put into ensuring that all members of the cancer research community are aware of the resources NCI currently provides. If there are problems of quality or access with existing resources, these need to be evaluated as well.

The Tumor Microenvironment

Evidence continues to accumulate that growth and migration of normal epithelial cells are subject to many levels of regulation by neighboring cells, extracellular matrix, and local levels of soluble signaling molecules. Cancer cells lose critical aspects of these controls, but they lose them gradually and rarely lose them all. Thus, one way of looking at cancer initiation and progression is as an iterative and progressive renegotiation of constraints carried out between a developing clone of epithelial cancer cells and its stromal microenvironment. This perspective suggests two principal lessons. First, attempts to understand tumor behavior or to treat cancers must take into account far more than the intrinsic properties of the malignant cells to be successful. And second, attempts to model tumor behavior must go beyond using tumor cell lines cultured on plastic surfaces, to three-dimensional culture systems and *in vivo* studies. The Tumor Microenvironment Think Tank provided a detailed blueprint for integrated studies, and several of the other Think Tanks emphasized specific aspects of the microenvironment that are often overlooked in overviews of the subject.

The tumor microenvironment has become an active area of investigation in the NCI R01 grant portfolio, a recent development underscored by the creation of a new CSR Study Section called Tumor Microenvironment. The Think Tank participants emphasized the importance of continuing this support, but also recommended the formation of a network or alliance to encourage cooperative, interdisciplinary studies beyond the scope of R01 grants. The Tumor Microenvironment Network would bring investigators experienced in this area together with scientists with complementary expertises. It would leverage existing grant support, but provide incremental funding for cooperative projects and for the creation of freely accessible, common resources that would benefit the entire research community. The goals of the Network would be to:

1) Characterize all of the cellular and non-cellular components of the normal, wounded, and tumor tissue microenvironments. The microenvironment that sustains and shapes tumor stem cells would also be of interest. Characterization would involve probing the genomics and proteomics of stromal and tumor cells, and developing antibodies and other reagents useful for visualizing, quantitating and comparing different microenvironments, and storing the data in public databases. Static characterization would rapidly be extended to studies of dynamic interactions, using real-time imaging methods.

2) Facilitate communication and data exchange through caBIG.

3) Create and make available to the research community three-dimensional tissue culture and animal models in which microenvironmental influences can be studied.

4) Delineate the role of the microenvironment in tumor progression and metastasis, and in response to radiation and/or chemotherapy, including characterization of the metastatic tumor microenvironment.

5) Determine the role of inflammation in shaping the tumor microenvironment in the earliest phases of tumor initiation and during progression. Delineate how inflammatory processes facilitate or inhibit the development of an effective antitumor immune response. It will be critical to determine the role of the microbial flora in establishing the nature and degree of inflammation.

6) Understand the effects of androgens and/or estrogens on inflammation and other aspects of the microenvironment.

7) Explore the role of host genetics in influencing stromal elements, and determine whether mutations or epigenetic changes in the stroma influence tumor growth and capacity to metastasize.

8) Encourage the use of state-of-the-art imaging technologies in microenvironment studies and the development of techniques to bypass current limits on imaging. This is critical because only imaging has the capacity to capture the dynamic and contact-mediated aspects of this complex system.

9) Provide an environment conducive to interdisciplinary training programs.

10) Translate the basic knowledge obtained to improve diagnosis and early detection of cancer, and to discover and validate therapeutic targets derived from the tumor microenvironment.

Think Tanks other than the one focused specifically on the tumor microenvironment have influenced our sense of priorities in this area. Tumor Immunology emphasized the critical roles of the tumor and lymph node microenvironments in determining the effectiveness of antitumor immune responses. The Tumor Stem Cell Think Tank noted that the long-term proliferation potential of a tumor can controlled by a small number of tumor stem cells. Hence, the niche that supports those stem cells must be characterized and stem cell markers developed so that the most important subset of interactions can be studied. The Inflammation Think Tank highlighted evidence that inflammation is strongly associated with tumor initiation and progression, suggesting that the common inflammatory aspects of the microenvironment facilitate tumor development. At the same time, there is the paradox that inflammation, at least in infectious diseases, is associated with brisk immune responses, but tumors generally exhibit evidence of ongoing inflammation while suppressing immune responses. It is clearly insufficient to say that inflammation is present or absent; the phenomenon must be dissected to its

molecular roots if interventions are to be developed. In Cancer Etiology, the macroenvironmental influences of both chemicals and microbes were discussed. Chemical exposures have impact far beyond mutagenesis, extending into a range of effects on the microenvironment. Striking evidence also exists in some tumor types that the normal microbial flora strongly influences the microenvironment. Viruses and bacteria must be assessed as important components of, or cofactors for a permissive tumor microenvironment. Finally, it was emphasized in Cancer Susceptibility that some of the complexity that has made it difficult to identify the genes responsible for individual variation in susceptibility comes from the context dependence of gene expression. Individuals may have a series of genetic polymorphisms that would predispose to cancer development, but it may not make a difference unless the microenvironment in which the tumor must develop supports the expression of the susceptibility gene(s).

Integrative Cancer Biology

As Fiscal Year 2004 is ending, NCI is funding a series of Integrative Cancer Biology Programs, the first organized foray into systems biology in the context of cancer. The Think Tanks provided strong evidence that this is an important direction for the Institute to pursue and that its influence will be felt throughout cancer research. Integrative Cancer Biology is complementary to the reductionist studies that comprise the majority of the grant portfolio. It attempts to address the complexity of many interactive and interdependent biological processes, starting by making high-throughput measurements of critical parameters. Biological lessons will be extracted from the masses of data through the use of advanced bioinformatics tools and the construction of predictive computational models of the cancer process. Although integrative biology is most often identified with the analysis of signal transduction pathways and other regulatory circuits within a single cell, it is equally applicable to complex processes involving multiple cells and extracellular molecules. Thus, the tumor microenvironment can be fully characterized only through the use of high-throughput analytical methods, and participants in the Think Tank acknowledged that a predictive model of the interactions that drive the microenvironment can be obtained only through the methods of integrative biology. The same is true of the related fields of tumor immunology and inflammation.

Other areas explored in the Think Tank process are similarly dependent on the emerging field of integrative cancer biology. Epigenetic influences on gene expression are mediated through DNA methylation, covalent modifications of histones, and higher order chromatin structure effects. Progress in the field is absolutely dependent on being able to measure these molecular changes on a genome-wide scale and knowing how to extract meaningful patterns from this mass of data. The DNA damage response and the cell cycle and cell death machinery are complex, interacting systems each made up of many quasi-stable molecular complexes. The dynamics and interactions of these systems must be understood in detail if they are to be manipulated for patient benefit in cancer. Determining how tumor stem cells function and how they come about involves achieving a molecular understanding of the cell regulatory pathways that underlie "stemness," or the ability of some cells to maintain unlimited replication potential and to divide asymmetrically into another stem cell plus a daughter cell committed to differentiation. The stem-cell genetic program is in turn heavily influenced by the cell and molecular milieu of the stem-cell niche. Etiology and susceptibility are two different views of the earliest stages of tumor development. They have been recognized for some time to involve numerous

genetic and environmental influences impinging on complex cellular homeostatic mechanisms. These disciplines all need high-throughput data, bioinformatics support, and computational modeling to address critical issues.

The Challenge of Comparing the Normal and the Tumor State

The National Cancer Institute has finite resources and cancer biology presents an enormous array of promising areas of investigation directly relevant to the NCI mission. To maximize the impact of our efforts, it is tempting to focus exclusively on the cancer state, leaving studies of the normal state to other funding agencies. In six of the Think Tanks, participants explicitly recommended against this course of action, pointing out a need to understand cancer in the context of normal biology. In the Tumor Microenvironment, normal constraints on cell growth and mobility are gradually loosened as the tumor develops. We need to know much more about the normal constraints individually, and about how they are coordinated at a systems level, before the tumor microenvironment can be fully characterized. The Cell Decisions in Response to DNA Damage are similarly complex and also must be better described in the normal case before they can be manipulated for therapeutic benefit in cancer. In Tumor Immunology, the major advances in understanding that have occurred in the last ten years have come from conceptual advances in immunology as a whole. The critical questions that remain are the same for basic immunology, autoimmunity, chronic infectious diseases and cancer, although the perspectives on the questions differ slightly among these fields. Inflammation in cancer has a marked stimulatory effect on cancer growth not because of its intensity, but because it fails to resolve the way acute, physiological inflammation does. It shares this characteristic with autoimmunity and certain chronic infections, and it is from a comparison of all these states that further understanding of the process, and the ability to modulate it selectively, will come. Cancer appears to use the stem-cell program of several tissues to further its own causes, but so little is known about the regulatory program within the normal tissue stem cell and the cell-cell interactions of the stem-cell niche that it is difficult to characterize cancer stem cells or to determine the path by which they became transformed. Epigenetics is similarly a young field, in which a great deal of basic knowledge must be accumulated before its role in cancer can be clarified.

The challenge is to identify those elements of these fields that the NCI should attack with its own resources and those where it should work in coordination with other NIH Institutes and other funding agencies. Leveraging of resources is difficult, but necessary. Some of the NIH Roadmap areas are relevant to scientific issues listed above, but a great deal more remains undone. There is no large-scale project on epigenetics on the horizon, despite its documented importance in many human diseases. Similarly, while the NIH has some coordinated activities related to human embryonic stem cells, tissue-specific stem cells (with the exception perhaps of hematopoietic stem cells) have received scant attention. The Think Tank recommendations make it clear that catalyzing larger-scale studies of some critical cross-cutting biological issues must be a high priority for NCI to provide the necessary context for progress against cancer.

Mechanisms to Foster Collaborative, Interdisciplinary Research

NIH grants, built around the R01 traditional research grant, have been the engine of creativity that has brought us to the current exciting point in cancer research. The Think Tank participants uniformly acknowledged the past and continuing importance of these individual grants. During the Think Tanks, however, they focused on needs that are difficult or impossible to meet through this mechanism. These were generally large-scale efforts, especially those that required input from scientists in diverse disciplines. The Tumor Microenvironment Network, described above, is an example of the recommendations, but similar networks were suggested in immunotherapy, stem-cell research, epigenetics, etiology and susceptibility. In other cases, less formal (and smaller scale) resources for collaboration were recommended. The Inflammation Think Tank recommendations included one to "sponsor interactive fora that interface experts drawn from different disciplines to address the multi-faceted topic of inflammation and cancer at a deeper level." The Cancer Etiology Think Tank made several recommendations for collaborative undertakings, including one for instrument development and use. The Epigenetics Think Tank recommended formation of a working group to discuss and begin outlining a Human Epigenome Project. In this case, the smaller initiative could lead to the development of a large-scale effort.

For very large projects, such as a Tumor Microenvironment Network, the size and expense of the proposal makes it appropriate for funding through the RFA process. The NCI has available a wide variety of funding mechanisms and governance models for large projects of this type. It is noteworthy that in recent years RFAs at NCI and elsewhere at NIH have increasingly emphasized collaboration and interdisciplinary teamwork, part of a well publicized trend toward "team science." The large projects included in the Think Tank recommendations, including the Tumor Microenvironment Network, exemplify that trend. These recommendations came from the outside Think Tank participants, rather than NCI staff, indicating that the trends in RFAs reflect the current thinking of the research community.

Many of the recommendations were modest in cost and involved more coordination than direct research support. These recommendations were made because there are very few investigator-initiated NIH funding mechanisms that can support any of these varied activities. Critical problems in cancer research and other areas of biomedicine increasingly require a variety of expertise and/or the sharing of data or reagents in a manner that is not facilitated or sometimes even possible when support comes exclusively from grants to individual principal investigators. Constraints on collaborative and interdisciplinary research also exist at research institutions. Rigid departmental structure, intellectual property policies and concerns about indirect costs can make some types of research more difficult.

With sufficient resources, NCI could address all of these recommendations through available mechanisms such as contracts, supplements and workshops. DCB fully intends to address as many of the high-priority recommendations as possible through such efforts. What this will not do, however, is alter the fact that few such efforts can be initiated directly by the research community through investigator-initiated mechanisms. Some collaborative research can be carried out through Program Projects, but these offer a limited range of flexibility. They typically have a small number of projects, all of which normally extend for the entire project period. There is also a perception that multi-institutional Program Projects are difficult to get funded. SPORE-like grants were recommended in a couple of cases, based on the added flexibility of a changing cast of projects and integral training, but

these are possible only in response to an initiative. The DCB Activities to Promote Research Collaborations (APRC) program is relatively flexible, but it is relatively small and short-term in nature. What is needed is a highly flexible, permanent program open to investigator-initiated applications to support modest-scale, collaborative, interdisciplinary research efforts. DCB will work toward the design of such a program.

Collaborative and Interdisciplinary Training

Each recommendation for a collaborative and/or interdisciplinary research program was accompanied by a recommendation for a program that would train students, postdoctoral fellows and established investigators to take optimum advantage of the opportunities such a program would create. There are differences in opinion as to the ideal way to attract and train scientists comfortable with both wet-lab biology and computational modeling, for example, or with both chemistry and biology, but there is universal agreement that any barriers that exist between disciplines need to be removed. Some barriers exist at the level of research institutions, but NCI must strive to create incentives for flexible training programs that will meet the needs for the next generation of research scientists. Three types of suggestions about training were made during the Think Tanks. One was to incorporate training into large-scale interdisciplinary initiatives. This was done in the Integrative Cancer Biology Programs, and is a feature of some other NCI programs. The second was to place some leverage back in the hands of graduate students by inaugurating individual pre-doctoral fellowships in which the range of subdisciplines and the mentor(s) could be determined by the graduate fellow and not the institution. The third was to reserve a portion of NCI postdoctoral training grants for explicitly interdisciplinary programs. While institutions are moving to respond to the need to change training paradigms, the Think Tank process made it clear that NCI must work to facilitate and accelerate such change.

IV. Concluding Remarks

The Think Tank process provided an opportunity for both NCI funded investigators and DCB scientific staff to think broadly and deeply about the directions cancer biology must take in support of the goals of the NCI.

The individual Think Tank reports are attached. Each of the individual recommendations deserves, and will receive, consideration by DCB staff. The resulting initiatives, both large and small, will reflect a unified agenda for cancer biology over the next several years.

The cross-cutting themes, summarized here, all derive from the need and emerging ability to study cancer biology as a complex system. Integrative cancer biology will be the necessary new fundamental discipline driving this transformation. We believe that understanding the tumor microenvironment is an initiative that can focus the new tools and approaches and provide results with dramatic translational impact. To support this endeavor and others, the emerging trend toward team science as a component of cancer biology research needs to be reinforced with a reengineering of NCI funding mechanisms and approaches to encourage more collaborative and interdisciplinary interactions, accompanied by continued support of an investigator-initiated research portfolio.

Appendix A

Guidelines for Organization of Think Tanks

1. Think Tank Goals - Concrete goals should be set early in the planning process, and modified as necessary. The goals should be communicated to the participants before the Think Tank.

2. General Structure of Agenda - Think Tanks need to be distinguished from scientific workshops. Presentations, if any, should be short and not involve primary data. Slide talks should be banned or held to a strict minimum. The bulk of the time should be devoted to brainstorming and structured group discussion.

3. Number and Type of Participants - To optimize discussion, the number of outside participants should generally be limited to fifteen. The participants should cover the critical subdisciplines and points of view. It is desirable to have a mix of established authorities and rising stars. It may also be useful to have a few key individuals who have useful perspectives but are not identified with the field in question. Achieving all of this with such a small number of participants requires judgment, and the mix will be different for each Think Tank.

4. Think Tank Summary - Each Think Tank must produce a summary of discussion and recommendations for internal use. The summary should be done in a timely fashion, and should be done by, or have substantial input from, the outside Chair(s). Depending on the goals of the Think Tank, it may be useful to produce a meeting report or review article for publication in an appropriate journal. This should be decided in advance, in consultation with the outside Chair(s).

5. Schedule/Location - Barring unusual circumstances, Think Tanks should be held locally. The length is expected to be in the range of 1.5-2 days. The schedule of an evening session, followed by a full day, followed by a morning has become common, but should be modified as appropriate. Breakout sessions are fine, as needed.

Executive Summary of the Tumor Immunology Think Tank

Three major themes emerged from the presentations and discussions at the Workshop:

1) Unequivocal evidence has emerged from a number of sources of the capacity of the immune system, alone and in combination with other modalities, to effect clinically meaningful antitumor immune responses. Specific examples include: a) the growing success of monoclonal antibody therapy (i.e., rituxan and herceptin), b) the understanding that cure of leukemias and some lymphomas by allogeneic BMT derives in large part from the antitumor response of donor T cells transferred to the patient (the so-called graft-vs.-tumor effect). In fact, GvT from donor lymphocytes is the only way to cure CML, c) dramatic antitumor effects after adoptive transfer of melanoma-specific T cells expanded *ex vivo*, d) antitumor effects of IL-2 in melanoma and renal cell carcinoma.

2) Recent advances in basic cellular and molecular immunology have been truly revolutionary, and have given us an unprecedented framework for understanding how the immune response is initiated and regulated, from specific cell types (i.e., dendritic cells and T regulatory cells) to specific molecules and signaling pathways. An understanding of how these pathways function and intersect, as well as how the immune system naturally interacts with developing cancers, will provide unprecedented insights and tools to effectively manipulate antitumor immunity. Already, these insights are leading to the conclusion that the most effective immunotherapies will employ combinatorial approaches that impact the antitumor immune response at multiple points.

3) Infrastructure limitation with respect to preclinical models of cancer, production of immune cells for adoptive therapy in patients, vaccine generation and availability of clinical grade recombinant molecules (i.e., cytokines, antibodies, etc.) for early phase clinical testing are severely limiting progress in the translation of the most promising immunotherapeutic combination strategies. Additionally, the growing regulatory burden for biologic therapies threatens to destroy even the current ongoing progress toward clinical translation.

Facilitation of the development and translation of rationally designed combination immunotherapy strategies should be the major NCI mandate in this area. This will require the dual approaches of empowering academically based groups for independent early stage translation as well as proactive promotion of effective public-private partnerships in this area.

Introduction

1) Enhanced understanding of immune regulation.

It is becoming clear that the immune response is finely tuned via a set of activation and inhibitory signals expressed by critical cellular subsets. In addition to the continuously expanding knowledge base of T cell and B cell biology, a new explosion of knowledge over the past decade has occurred related to dendritic cells, NK cells, NKT cells and T regulatory cells. Each of these cell types has been shown to be central to regulation of both innate and adaptive immunity.

The myriad of cellular interactions that ultimately regulate immune responses are mediated by specific ligand-receptor interactions that in turn trigger intracellular signaling pathways. In addition to the antigen receptors on T and B cells (TCR and BCR), over 100 cytokines and cell surface molecules regulate the amplitude and quality of the output response. These ligands and receptors, many of which have been molecularly identified, appear to be roughly evenly divided between activating (e.g., IFN-α,β,γ, B7-1/2, CD28, CD40) and inhibitory (e.g., IL-10, TGF-β, CTLA-4, PD-1). Likewise, intracellular signaling pathways triggered by receptors are becoming defined in terms of how they activate or inhibit important immune effector functions as well as cellular lifespan. Indeed, more than any other system in the body, apoptosis control is a major mechanism of regulation in the immune system.

a) While much is known about individual molecules and pathways in isolation, there is much more to be learned about how these pathways interact in a coordinated fashion. Understanding the physiology of these interactions will require integration of classical molecular biology and biochemistry approaches with newer approaches in 4 areas: genomics, proteomics, systems biology and *in vivo* imaging.

b) Enhancement of specific activation pathways or blockade of specific inhibitory pathways has been shown to induce or exacerbate autoimmunity. Conversely, early studies using antibodies and recombinant fusion molecules has demonstrated that combinations of activating signals (such as vaccines that enhance dendritic cell function) and blockade of inhibitory signals (such as anti-CTLA-4) can dramatically enhance antitumor immunity. The development of combination approaches that simultaneously activate tumor-specific or tumor-selective immunity and block immunologic checkpoints is the most important translational mission in the cancer immunology field. Maximizing the window between antitumor efficacy and intolerable autoimmunity will require a significant investment in understanding the mechanisms of immune regulatory pathways.

2) Understanding the interaction between the immune system and the tumor microenvironment.

It is now absolutely clear that tumors express tumor-specific (from mutations and rearrangements), tumor-selective (gene expression changes due to epigenetics), and tissue-specific antigens (relevant targets for tumors derived from dispensable tissues) that the immune system can potentially recognize. If the tumor were simply an inert bag of antigens, the immune system would have no trouble eliminating all cancers. However, tumors interact actively with their environment, including the immune system. It is now emerging that an integral element of tumor biology is the immunologic

effects of oncogenic changes. Examples include the inhibition of dendritic cell maturation by tumor derived factors such as VEGF and the finding that activation or inactivation of various Stat signaling pathways not only affect tumorigenesis but also have profound effects on how the immune system senses invading cancer cells. These interactions dramatically affect the balance between immune surveillance and tolerance induction. At the effector stage, it is clear that features of the tumor microenvironment, such as stromal structure and hypoxia, dramatically affect the traffic and function of immune effector cells at the metastatic site, even when appropriately activated. The mechanisms of immune interactions with the tumor microenvironment is a critical and understudied area of cancer immunology that will impact significantly on the success of immunotherapy strategies. This is a specific area that the NCI should encourage.

3) Infrastructure and regulatory barriers relevant to translation of promising immunotherapy combinations.

The diversity of immune regulatory pathways amenable to manipulation with vaccines, antibodies, and small molecule reagents offers both unprecedented opportunities and challenges for effective translation. Cell-based therapeutic opportunities, including adoptive T cell approaches, dendritic cell vaccines and bone marrow transplant-related immunotherapies, likewise offer tremendous opportunities and challenges. It is a general consensus that barriers to effective translation are mounting rather than coming down. The realization of successful cancer immunotherapy will live or die depending on whether translation is facilitated or blocked. There were 4 areas that were identified as critical to address:

a) Paucity of good preclinical mouse cancer models useful in immunological studies. Cancer immunology was largely ignored in the animal models consortium efforts despite the fact that some of the most important innovations in mouse genetics were pioneered to study the immune system *in vivo*. A specific effort to make the opportunities and resources of the animal model consortium directly available to the cancer immunology field is important. This will require a proactive effort on the part of the NCI.

b) Measurements of human immune responses. Although anti-tumor responses are the final arbiters in the evaluation of tumor immunotherapies, development of these therapies will only proceed in an efficient and rational manner if better means of measuring human immune responses are developed. Current methods are largely *ex vivo* and do not necessarily inform us about the behavior of the cells or agents in the patient. Substantial opportunities exist for NCI to actively promote the development of novel methods for the detection and measurement of the activity of the human immune system. Specific attention should be given to non-invasive imaging methods, including but not limited to PET and MRI based methods. These approaches can be used not only with labeled cells to evaluate homing to tumor sites, but also potentially for high-resolution determination of *in situ* lymphocyte function such as cytokine secretion or cytotoxic granule release.

c) Lack of availability of clinical grade biologic reagents to the immunotherapy community. As described above, there is a wealth of exciting biologic reagents that, if applied in proper combinations, can dramatically enhance immunotherapy potency. These range from antibodies (i.e., anti-CTLA-4), soluble ligands (i.e., soluble CD40L), and cytokines (i.e., IL7, flt-3L) to more

complex recombinant viral and bacterial vaccines and finally engineered cells. Most of these are virtually unavailable to the immunotherapy community.

The three current sources for production of these reagents are invaluable, but at present not adequate to meet the increasing need.

i) RAID – while BRB/RAID is a critical mechanism to produce biologic reagents for investigators and has an extremely dedicated and expert development staff, its ability to supply these reagents is far too slow. Only a tiny fraction of RAID-approved reagents have been delivered and most that have been delivered take >3 yrs to produce. These delays are due to understaffing (staff is <10% required to complete the project portfolio relative to industry standards), tremendous bureaucratic inefficiency, lack of appropriate expertise among the review groups that select projects into the pipeline (gumming the system with flawed projects), and ineffective outsourcing and backsourcing.

ii) Institutional Processing Facilities – These facilities are becoming an important resource for institutions with highly active translational missions. However, they are extremely expensive to maintain. None of the NCI funding sources come close to adequately supporting these facilities.

iii) Companies – Biotechnology and pharmaceutical companies own a tremendous number of valuable molecules and more complex reagents at the level of patents, production expertise and actual clinical grade stocks. Most of these reagents are not available to the immunotherapy community to use in novel and promising combinations and many are not being developed at all. Much of this problem comes from the corporate culture of favoring complete control over the reagent over release of the reagent to groups that wish to utilize it in a fashion other than what the company is interested in or in combination with agents not owned by the company.

d) Regulatory barriers. FDA barriers continue to mount, driving the cost and administrative burden of doing the most innovative trials to virtually unbearable levels. The burdens are typically borne by the translational clinical investigator, who has minimal resources to meet the requirements. Some of the problem is that communication between FDA and investigators is inadequate. Much of the problem is that the NCI and the immunotherapy leadership are not appropriately educating the FDA on which safety regulations are necessary vs. frivolous, relative to the severity of the disease being treated.

Specific Recommendations for the NCI

1) Continue to promote basic research on mechanisms of immune regulation with special emphasis on interactions between immunology and tumor biology/microenvironment. This could involve an RFA to bring together tumor biologists and immunologists to specifically address these questions. In addition, promote the development of animal models of cancer useful for the testing of immunotherapies.
2) Promote the development and application of new imaging technologies to study immune function *in vivo* in both animals and patients.
3) Create a mechanism for supporting collaborative, interdisciplinary consortia that is not organ site focused but rather modality focused – i.e. immunotherapy. This would greatly facilitate interactions among immunologists, cancer biologists, and clinical investigators interested in translating the most innovative and promising combination immunotherapy approaches. Inter-institutional collaborations should be emphasized with this mechanism. To provide optimal flexibility as well as emphasis on translational work, this mechanism could be based in part on the SPORE model.
4) Develop a strategic plan to effectively identify, acquire, and make available to the community the most promising immunomodulatory reagents such that their creative clinical development is most efficiently facilitated. This will involve a paradigm for interaction with the corporate world as part of the "public-private partnership."
5) Improve the availability of new biologics for immunotherapy by:
    a) Convening a blue ribbon panel to review BRB/RAID that will be charged with developing specific recommendations on how to enhance the efficiency, quality and speed of reagent production.
    b) Developing a mechanism to support infrastructure for the most active institutionally based facilities committed to cellular and biologic reagent production for biologic therapy of cancer.
6) Develop a strategic plan to proactively interface with the FDA so that regulations are applied intelligently and flexibly and communicated in an effective and consistent fashion to clinical investigators. This should involve the recommendation of a separate review process for academically based pilot trials of combination immunotherapy approaches for patients with advanced cancer or those prognostically defined as a high probability of relapse. Also, because of the unique regulatory issues associated with biologic reagents as opposed to small molecules, consideration should be given to creating an NCI advisory/liaison group to the FDA biologics branch.

Executive Summary of the Tumor Microenvironment Think Tank

The microenvironment in which a tumor originates plays a critical role in tumor initiation and progression, and may be an important factor in developing therapeutic approaches. The tumor microenvironment, or stroma, influences the growth of the tumor and its ability to progress and metastasize. It also can limit the access of therapeutics to the tumor, alter drug metabolism and contribute to the development of drug resistance. Because of their role in all the stages of tumor development, stromal elements represent attractive therapeutic targets. Manipulating host-tumor interactions may be important in preventing or reverting malignant conversion, and re-establishing normal control mechanisms.

Despite the importance of tumor-stromal interactions, there is a limited understanding of the stromal composition, and of the complex relationship between the tumor cells and the surrounding host cells. It is now acknowledged that tumor cells and their stroma co-evolve during tumorigenesis and progression. Stroma consists of cells, extracellular matrix and extracellular molecules. Among the identified cells are fibroblasts, glial cells, epithelial cells, adipocytes, inflammatory cells, immunocytes, and vascular cells. However, the precise nature of the cells that comprise normal stroma, how these cells or newly recruited cells are altered during tumor progression, and how they reciprocally influence tumor initiation and progression are poorly understood.

As these salient and outstanding questions are addressed, it will be possible to begin to develop complementary therapeutic strategies targeted at both the microenvironment and the tumor. Among the approaches envisioned to target the tumor microenvironment are the development of drugs that induce apoptosis or inhibit the function of the stromal cells, or the factors secreted by stroma that are required for tumor progression and metastasis. It is expected that understanding the tumor microenvironment will lead to the development of better diagnostic tests and/or improved therapeutic strategies. Finally, it may be possible to develop strategies to prevent the development of tumors based on our understanding of alterations in the microenvironment that enable tumor development.

The research priorities listed by the think tank include: a better understanding of tumor microenvironment, and the identification and characterization of the signatures of seemingly normal cells within the tumor microenvironment and signatures that reflect changes that occur as cancer cells interact with the host microenvironment. Achieving these goals can be expedited by encouraging interdisciplinary research teams and multi-institutional collaborations. Similarly, advances in technologies will be critical for progress. Among the technologies that have been identified as critical are: 1) novel *in vitro* 3D matrix reconstitution and organotypic models, and animal models; 2) techniques, such as laser capture microscopy, for the isolation and characterization of stromal cells; 3) the discovery of novel stromal markers through molecular profiling and their application for the development of reagents for *in vivo* imaging to visualize tumor-host interactions. These technologies will provide the tools for a better understanding of the tumor microenvironment and for the development of tissue- or cell-specific targeting agents. Successful approaches to respond these needs can have a dramatic effect on making the tumor microenvironment "hostile to the tumor," thereby transforming cancer into a "chronic, but benign" disease.

Introduction

The intrinsic and extrinsic influences that transform a normal epithelial cell into a malignant cell are very complex. Enormous advances have been made over the last several decades in identifying the molecular and genetic changes of cancer cells and the pathogenesis of neoplasia. This has led to the identification of oncogenes and tumor suppressor genes and associated signaling mechanisms by which they modulate growth, survival and proliferation. These studies have generated novel therapeutic reagents such as Tamoxifen, Herceptin and Gleevec.

Stromal influence on epithelia cells begins at fertilization and continues during adulthood, wherein the microenvironment controls normal development and homeostasis. For example, macrophage association with the developing mammary gland is critical during development and CSF-1 or CSF-1R null mice (devoid of macrophage) have defective mammary glands.

Research on tumor-host interactions collectively suggests that (a) tumors are not autonomous masses of cells but function as organs composed of many interdependent cell types that contribute to tumor development and metastasis, and (b) the interactions between the tumors, the extracellular matrix (ECM) and stromal cells is bidirectional and dynamic; stromal cells include fibroblasts, adipocytes, glial cells, smooth muscle cells, and resident and recruited vascular and immune cells.

The tumor microenvironment and the malignant cells themselves constitute the tumor entity that clinicians confront when treating cancer patients. The cell-cell and cell-matrix interactions that influence the behavior of cancer cells are targets with as much potential for the development of effective therapies as the tumor cells themselves. The microenvironment can exert both positive and negative influences on tumor cells. Stromal cells can also impart stimulatory and growth inhibitory effects on tumor cells, e.g., the malignant potential of teratocarcinoma cells can be restrained during embryonic development resulting in cancer-free adult mice. Similarly, attenuation of β1 integrin (laminin receptor), EGFR or MAPK activation in highly aggressive human breast cancer cells results in a reversion of the aggressive phenotype.

The cancer cell is absolutely dependent on the stroma for its proliferation, progression, and metastasis; examples include the role of inflammatory cells (via cytokine and protease secretion) in tumor cell proliferation, angiogenesis, invasion and metastasis; the interaction of host immune cells with the vasculature; the interaction of tumor cells with the angiogenic endothelial cells, and the role of lymphangiogenesis during metastasis to the regional lymph nodes. Stromal cells can also influence organ-specific metastasis as evidenced by the role of stromal-derived cytokines and growth factors (e.g., PTHrP, CXCR4, SDF1, TGF B and RANKL) in breast and prostate cancer and multiple myeloma metastasis to bone. Finally, interaction of bone marrow stromal cells with multiple myeloma cells has been shown to contribute to the development of drug resistance.

Stroma can be targeted for therapy. Recent successes (a) in patients with multiple myeloma, where bone marrow stroma was targeted using proteasome inhibitor to attenuate bone metastasis, and (b) the development of anti-angiogenic drugs (Avastin and Thalidomide) which target the endothelial cells illustrate progress towards this goal.

The two major goals are: to obtain information about the microenvironment that would facilitate the diagnosis, prevention or treatment of cancer, and translating this information into useful clinical applications. These broad goals can be achieved through the following specific objectives: (1) identify the key components of the tumor microenvironment and define how these are altered during tumor development. Identify the stromal compartment of normal tissues and compare how these are altered in carcinogenesis. (2) Determine which alterations in the tumor microenvironment that are critical for the development, progression and metastasis; elucidate the mechanism responsible for induction of these changes. (3) Identify tumor cell stem cells and characterize the interactions between stromal cells and tumor stem cells, as well as the role of tumor stem cells in metastasis. (4) Develop therapeutic strategies to target the tumor microenvironment and interfere with site-specific metastasis by the (a) development of drugs that induce death or inhibit the function of the stromal cells that are required for progression; (b) development of reagents to target specific factors produced by stromal cells that are responsible for progression, and (c) blocking the induction of stromal factors responsible for tumor cell survival and proliferation. (5) Develop diagnostic tests to predict outcome and/or design treatment. (6) Develop strategies to prevent the development of tumors based on an understanding of the alterations in the microenvironment essential for tumor development.

These issues can be best addressed with the availability of novel technologies and model systems. Thus the development of novel *in vitro* 3-dimensional matrix reconstitution and organotypic models or animal models, isolation of stromal cells from normal and tumor cells using techniques such as Laser Capture Microdissection will aid in the identification of stromal markers through molecular profiling technologies. The availability of stromal markers will facilitate better reagent development for *in vivo* imaging to visualize tumor-host interactions as tumor cells invade and metastasize. The availability of stromal markers will also expedite the generation of reagents for tissue- or cell-specific targeting.

Significance

Critical stromal elements of the tumor are attractive targets for prevention, because they have maximal influence over tumor cells in the early stages of tumor development. As targets for therapy, they are less likely to be genetically unstable than tumor cells and thus less likely to develop drug resistance. Manipulating host-tumor interactions has the potential of reverting the malignant phenotype and establishing normal control mechanisms. Eventually, the desired goal is to reduce or eliminate metastasis-associated morbidity and transform cancer metastasis in to a chronic but benign disease.

Specific Recommendations for the NCI

1. **Establish an Interdisciplinary Tumor Microenvironment Network** that includes pathologists, cancer biologists, cell biologists, oncologists, engineers, physicists, bioinformatics experts and industry representatives. Such a group will facilitate the study of normal and malignant tissue microenvironments. This can be accomplished by both centralized resources and widespread, varied funding opportunities.

2. **Encourage study of the normal tissue microenvironment as a prerequisite for understanding the microenvironments of wounded tissues and tumor tissues**

Establish and support a repository of normal stromal cells and matrix molecules to facilitate this research

Develop additional and improved 3-dimensional reconstitution and organotypic models that will permit the *in vitro* study of microenvironmental elements

Key issues in understanding normal stroma are

- What % of tumor is stroma?
- What are the various cells in the stroma?
- What are the matrix molecules?
- How does it change over time and with tumor progression?
- Genomic and proteomic analysis of tumor stroma and involvement of bioinformaticists

3. **Develop a better understanding of tumor dependence on stroma**

- Since experimental manipulation of stromal components *in vivo* has profound effects on tumor growth, progression to metastasis and immune response, there is a need for selective agents that target specific cellular and molecular stromal elements, and inhibit tumor progression.

4**. Delineate the role of the microenvironment in tumor progression – key issues**

- Do tumor promoters affect the stroma and facilitate metastasis?
- Are there differences between the microenvironment of a primary tumor as compared to secondary sites?
- Does the stromal compartment contribute to the "metastatic signature" of tumors?
- Are some 'stromal' cells really tumor cells in disguise?
- Can the microenvironment of tumors be effectively neutralized to inhibit progression?
- Is organ metastasis achieved solely via hematogenous spread, or do transiting cells initially exit via lymphatics and subsequently spread hematogenously?
- What is the role of inflammatory cells during invasion, migration, tumor growth and metastasis? To achieve this it will be necessary to study inflammation *in vivo*, in relevant immune-competent (mouse) models of progression (transgenic or syngeneic xenograft).

- Do stromal elements interact or modulate the tumor stem cell 'niche' and do these interactions change as tumor progress and metastasize?

5. **Stromal Genetics – key issues**
   - What is the role of host genetics in influencing the stromal formation?
   - Does stromal composition differ in different inbred mouse strains?
   - Do epigenetic changes influence stromal composition?

6. **Encourage the use of existing technologies for visualizing the components of the stroma at the level of individual cells and molecules**
   - Expand access to multiphoton microscopy and deconvolution microscopy to study the microenvironment.
   - Develop novel imaging technologies to study tumor microenvironment; recruit/collaborate with engineers and physicists who can apply the most current imaging technologies to the study of the normal and tumor microenvironment.

7. **Translation of basic knowledge to human disease**
   - Discover and validate therapeutic targets derived from the tumor microenvironment
   - Encourage mouse and human research to improve our chances of discovering useful targets.

8. **Role of NCI**
   - Establish a network of interconnected, multidisciplinary investigators and collaborative groups to work together on understanding the tumor microenvironment, facilitated by both centralized resources and widespread, varied funding opportunities.

Such an infrastructure should
- Provide centralized administrative support
- Provide and maintain repositories
- Produce and distribute reagents
- Provide core facilities
- Facilitate interdisciplinary collaborations
- Train scientists

What kind of technologies should NCI support?
- Animal models / fluorescent markers
- Imaging (multiphoton) at one micron resolution
- Spectral deconvolution microscopy
- Selection of live cells from tumors
- 3D matrix reconstitution
- Organotypic models

Where would such facilities reside?
- In individual labs

- In “Centers of Excellence”
- In central or decentralized cores
- At meetings and conferences
- At courses on the tumor microenvironment

9. **Establish and encourage a Systems approach** to the study of the tumor microenvironment

10**. Encourage interactions with other NCI-supported multidisciplinary groups** such as EDRN, MMHCC, SPOREs, and Cooperative Groups

11. **Encourage interactions with other ICs** with similar interests (NIEHS, NIDDK, NHLBI, NIAMD, and NIDCR)

Executive Summary of the Tumor Stem Cell & Self-Renewal Genes Think Tank

The Tumor Stem Cell and Self-Renewal Genes Think Tank was convened to identify the major scientific issues in the field and provide recommendations to the NCI to help advance research in this area. The participants came to the following scientific conclusions:

- Efforts are required prospectively to isolate and purify the tumor initiating cells (T-IC) from a larger variety of hematological and solid tumors. Since many tissues do not have as rich a source of cell surface markers as the blood system, effort will need to be expended to develop the means for T-IC purification.

- The normal tissue stem cell is a likely target for the initial carcinogenic insult, because it has the long life necessary for accumulating the multiple genetic or epigenetic changes required for malignant transformation.

- Although the events required for malignant transformation may all accumulate in the normal tissue stem cell, this cell does not necessarily become the tumor stem cell.

- It appears that the genetic and biochemical pathways regulating the "stem" phenotype in normal stem cells are subverted later to maintain the tumor stem cell phenotype.

- There is evidence to suggest that epigenetic mechanisms can produce an inheritable tumor stem cell phenotype. Epigenetic control also underlies a great amount of stem cell regulation, as exemplified by the polycomb gene Bmi-1. Thus, there is a link between normal stem cell regulation and the control of cancer stem cells.

Finally, it was recommended that the National Cancer Institute:

- Continue support for opportunistic basic research into tumor stem cell biology.

- Develop a Research Consortium to encourage transdisciplinary approaches and to provide specialized research reagents.

Introduction

The clonal nature of most malignant tumors is well established. Experiments spanning several decades have shown, however, that as many as 10^6 murine or human tumor cells are required to transplant a new tumor from an existing one. Two theories have been developed to account for the observation that apparently not every tumor cell is a tumor initiating cell (T-IC). The stochastic theory predicts that every tumor cell can form an entirely new tumor; however, entry into the cell cycle is a stochastic event with low probability. Alternatively, tumor cells may exist in a hierarchical state in which only a small number of cells possess tumor initiating potential. If the stochastic model is correct then tumor cells are biologically homogeneous and genetic or epigenetic programs that allow for tumorigenesis are operative in the majority of cells that comprise a tumor. The hierarchical model, however, predicts the tumor cells possess a functional heterogeneity and that quantitatively the cells capable of tumorigenesis are a relatively minor population among the bulk of tumor cells.

Recent data from both hematologic malignancies and solid tumors have suggested that there are only minor populations of cells in each malignancy that are capable of tumor initiation. These tumor initiating cells have the functional properties of a tumor stem cell. They appear to be capable of asymmetric division and self renewal, and are only a minor faction among the bulk of more differentiated cells in the tumor. These observations have profound implications for tumor biology research as well as successful tumor therapy. The Tumor Stem Cell Think Tank addressed several of the outstanding scientific questions that will define research in this area, as well as the needs of the research community to promote progress in this research.

(1) <u>What is the current evidence for tumor initiating stem cells among tumors arising from a variety of tissues?</u>

Currently, tumor stem cells have been isolated and characterized in several hematologic malignancies and two solid tumors. The critical experimental design that underlies all these studies is the development and use of a functional assay for tumor establishment and the prospective isolation of the T-IC. Often normal tissue stem cell markers are used to identify these populations, but a functional assay such as transplantation of human leukemic stem cells into immunodeficient murine models such as the NOD/SCID mouse is most important in identifying tumor initiating cells. One of the first tumors in which a stem cell was identified was acute myeloid leukemia (AML). In this disease, the frequency of the leukemic stem cell (LSC) was approximately 1 per million AML blasts, establishing that not every AML cell had LSC capacity. A $CD34^+$, $CD38^-$ cell fraction representing 0.1-1% of the tumor cells possessed all the leukemia initiating activity in the NOD/SCID model. By contrast, the $CD34^+$, $CD38^+$ cells and the $CD34^-$ cells, which comprise most of the cells in the tumor, could not initiate leukemia. A multiple myeloma stem cell has also been characterized. Multiple myeloma cell lines and primary patient derived cells express the cell surface marker syndecan -1 (CD138). Expression appears during the course of B-cell differentiation. A population of cells representing <5% of the cells in the bulk population of multiple myeloma cells were found to be $CD138^-$ and possessed *in vitro* clonogenic potential. These cells also engrafted successfully into NOD/SCID mice, whereas $CD138^+$ cells did not engraft. $CD138^-$ cells were also $CD19^+$ and $CD20^+$, and they expressed higher

levels of KI67 (a cell proliferation antigen) than CD138+ cells. Recently a mammary carcinoma stem cell has been isolated primarily using three cell surface markers (CD44, CD24, and epithelial specific antigen). The tumor initiating capacity of the cells was verified in a NOD/SCID engraftment assay, and the T-ICs represented only 2% of the unfractionated cells.

Finally, a putative brain tumor stem cell has also been isolated. These cells appear to be between 0.3 - 25 % of the cells in the brain tumors examined. They are positive for the neural stem cell marker CD133 and have a marked capacity for self renewal and differentiation. Transplantation of these putative neural tumor stem cells into the forebrains of NOD/SCID mice yields tumors phenotypically resembling the tumors from which the stem cells were isolated.

The think tank participants arrived at a consensus that:

A. Isolation of stem cells from more hematological malignancies and solid tumors is required to validate the general nature of the presence of stem cells in tumors.
B. Functional assays, such as NOD/SCID mouse engraftment and single cell tumor initiating capacity assays, are necessary to establish the true stem nature of isolated cells. Cell surface morphological markers alone are insufficient to accurately characterize stem cells.
C. Efforts are needed to isolate prospectively and purify the T-IC. Since many tissues do not have as rich a source of cell surface markers as the blood system, effort should be expended to develop the means for T-IC purification.

(2) Is the tumor stem cell a derivative of a normal tumor stem cell or a later more differentiated progenitor cell?

It is unclear whether tumor stem cells arise exclusively from normal tissue stem cells, or from progenitors that have differentiated from the stem cell itself. From a theoretical standpoint, it has been well established that neoplasia arises as a consequence of the acquisition of multiple oncogenic events. Thus, the initial events must occur in cells that persist. Hierarchical differentiation of blood cells from a stem and progenitor population has been well studied and characterized in the hematopoietic system. It is thus not surprising that a great deal of evidence on the nature of the tumor stem cells is available from studies of hematopoietic malignancies. In AML, the stem cell population appears to share many of the markers of the normal hematopoietic cell (e.g. $CD34^+$, $CD38^-$, $HLA\text{-}DR^-$); however the leukemic stem cell appears to overexpress the IL-3 Rα subunit (CD123) relative to normal hematopoietic stem cells (HSC). The similar cell surface properties, self-renewal properties, and complexity of stem cell hierarchies, as determined by clonal stem cell tracking of both LSC and normal stem cells, suggest that they are closely related and have been interpreted to suggest that LSC are derived from HSC. In the blood system, only the stem cells have the long-life required to accumulate all the initiating mutations and these could be passed to the progeny that are continuously produced from such stem cells.

In experimental murine systems, it has been found that the more committed myeloid progenitor cells, especially those employing the MLL oncogene, are also capable of becoming tumor stem cells. This gene codes for a transcription factor that regulates Hox gene expression. The Hox genes have been

implicated in stem/progenitor cell expansion and self renewal. MLL fusion proteins created by chromosomal translocations are frequently associated with acute lymphoid and myeloid leukemias. Transduction of an MLL fusion protein into purified populations of either hematopoietic stem cells or more committed granulocyte macrophage progenitors gives rise to cells that produce a rapid AML. Although the disease is similar with both populations, the stem cell fraction is much more potent. Thus, a more differentiated cell can also act as a tumor initiating stem cell if the genetic alteration endows this cell with self-renewal capacity. In chronic myelogenous leukemia (CML), the t(9;22) translocation joining the BCR and ABL genes is found in hematopoietic stem cells, but the mRNA and protein for the fusion gene are found only in later progenitors. In AML patients carrying the AML-1 Eto translocation in hematopoietic stem cells, the stem cell fraction is still capable of normal differentiation, but the more committed cells possess clonogenic leukemia potential only, suggesting that the AML/ETO translocation created a pre-leukemic stem cell. Additional alterations occurred in the more committed cells to create a frank leukemia. Thus, the tumor stem cell can arise in hematologic malignancies from multiple alterations occurring in normal stem cells, or the initial alteration could occur in the stem cell, with additional hits occurring in a more differentiated progenitor cell. Finally, it is also possible that under some rare circumstances, the initiating event could occur in the committed progenitor to convert it into a self renewing stem cell. It will be important to determine if the leukemogenic pathways obtained in experimental murine systems are recapitulated in the human disease.

Mammary tumor stem cells are $CD44^+$, $CD24^-$, and epithelial stem antigen positive, and this phenotype overlaps with that of epithelial stem cells. Definitive evidence, however, that these cells arise from the normal mammary tumor cells is thus far lacking.

The tumor stem cell isolated from a variety of brain tumors, including slowly proliferating astrocytomas and highly malignant medulloblastomas and glioblastomas, contain tumor stem cells that express the CD133 antigen and nestin found on normal neural tumor stem cells. The neural tumor stem cell does not contain any of the markers characteristic of more differentiated neural cells. Again, these data suggest that the normal neural stem cell is the precursor of the brain tumor stem cell. This conclusion about neural tumors is supported by molecular genetic studies performed in neural cells. Simultaneous knockout of p53 and NF-1 in neuronal precursor cells results in the development of brain tumors. Imaging studies demonstrate that the tumors arise in two areas: the subventricular zone of the lateral ventricle and the hippocampus. These areas of the brain have been previously shown to be reservoirs of normal neural stem cells. Studies of the generation of neurofibromas from Schwann cells have also shown that primitive neural crest cells can become tumor stem cells. The transcription factor Krox20 participates in the differentiation of these Schwann cells, but has recently also been shown to become expressed in the primitive neural crest. Homozygous deletion of the Krox20 gene using a Krox20-Cre system causes some of the deleted cells to become neurofibromas. It is postulated that the cells must accumulate other stochastic events (e.g., loss of NF-1) to become fibromas, because all the cells do not become transformed.

The participants concluded the following:

A. Among the solid tumor stem cells identified there is an overlap between the phenotype of the normal tissue stem cell and the tumor stem cell, but definitive evidence as to whether tumor stem cells are actually transformed normal stem cells is not yet available.
B. In the hematologic and neural malignancies there is evidence that tumor stem cells may be heterogeneous. Some may arise from the normal stem cells population, but others may arise from more differentiated progeny cells that have acquired self renewal capacity.
C. The normal tissue stem cell is a likely target for the initial carcinogenic insult, because it has the long life required to accumulate the multiple genetic or epigenetic changes required for malignant transformation.
D. Although the events required for malignant transformation may all accumulate in the normal tissue stem cell, this cell does not necessarily become the tumor stem cell.

(3) <u>What genetic pathways may be important in maintaining the tumor stem cell state?</u>

The proteins involved in self renewal in normal tissue stem cells appear to be subverted in tumorigenesis to allow the tumor initiating cells to maintain self renewal capacity. Two families of proteins related to self renewal were considered in detail: the poycomb gene Bmi-1 and the Wnt signaling pathway proteins. The polycomb genes have an essential role in embryogenesis, regulation of the cell cycle and lymphopoiesis. These genes are essential for the silencing of other families of genes. It has been shown by RT-PCR analysis that knockout of the polycomb gene Bmi-1 in mice results in a progressive loss of all hematopoietic lineages. This loss results from the inability of the Bmi-1 $^{(-/-)}$ stem cells to self renew. Bmi-1 $^{(-/-)}$ cells displayed altered expression of the cell cycle inhibitor genes p16 ^{INK4a} and p19^{ARF}, and down regulation of a gene coding for an inhibitor of apoptosis. The p16 and p19 proteins interact with the p53/Rb regulated cell cycle pathways. Introducing genes known to produce acute myeloid leukemia (AML) into Bmi-1$^{(-/-)}$ hematopoietic stem cells (fetal liver cells) induced AML with normal kinetics. However, the Bmi-1$^{(-/-)}$ leukemic stem cells from primary recipients were unable to produce AML in secondary recipients. These results demonstrate that Bmi-1 is also required for self renewal of leukemic stem cells in AML.

Another group of genes involved in self renewal are those involved in the Wnt signal transduction cascade. The Wnt protein binds to a receptor called Frizzled and activates cell fate decisions during tissue development. It has been shown that deletion of the TCF-4 gene, a transcription factors at the end of the Wnt signal transduction cascade, causes early neonatal death in mice. The mice lacking the gene have a single histological defect - the intestinal stem cell lining is absent. It has also been shown that inhibitors of Wnt signaling leads to inhibition of hematopoietic stem cell growth *in vitro* and reduced hematopoietic reconstitution *in vivo*. Activation of Wnt signaling in hematopoietic stem cells leads to increased expression of Hox B4 and Notch-1 genes previously replicated in self renewal of hematopoietic stem cells. The Wnt signaling pathways has been shown to be involved in both hematopoietic malignancy and colon carcinoma.

Although the Wnt ligands themselves are only rarely involved in tumorigenesis, mutations mimicking Wnt receptor (Frizzled) activation induce a set of genes associated with repression of differentiation and potentiation of self renewal. In general, these mutations involve Wnt signal transduction proteins: activation of ß-catenin and inactivation of the (APC) adenomatosis polyposis coli protein. In myeloid

leukemia, non-phosphorylated ß-catenin accumulates in granulocyte macrophage progenitors as they progress toward leukemia. These normally more committed progenitors can thus acquire self renewal properties. A similar accumulation of non-phosphorylated ß-catenin has also been observed in multiple myeloma cells. In colon cancer, the APC gene is mutated early in the development of 90% of colon carcinomas. Similarity in gene expression patterns between populations of colon cancer cells and colon epithelial stem cells has also been observed by DNA microarray analysis. It is possible that mutations in the Wnt signaling pathway maintain the program of stem cell genes in the "on" position.

Two other proteins that may play a role in tumor stem cell biology are nucleostemin and the tumor suppressor PTEN. Nucleostemin is abundant in self renewing cells such as mouse embryonic and neural stem cells as well as several human cancer cells. Although the exact function of nucleostemin is not yet known, it behaves like a molecular switch to control cell division, perhaps through binding to p53. Knockout of the PTEN phosphatase in prostate cancer cells allows the expression of genes associated with metastasis. As metastatic cells are likely prostate tumor stem cells, it is likely this gene may regulate expression of stem cell related genes.

In conclusion, it appears that the genetic and biochemical pathways regulating the "stem" phenotype in normal stem cells are subverted to maintain the tumor stem cell phenotype.

(4) Can the tumor stem cell phenotype be epigenetically programmed or reprogrammed?

Human tumor cells often demonstrate abnormal patterns of DNA methylation. DNA methylation provides an epigenetic mechanism for altering gene expression by silencing genes. Hypermethylation frequently underlies the silencing of tumor suppressor genes. The opposite condition, in which DNA is hypomethylated, often in concert with regional hypermethylation, has been observed in a spectrum of human tumors. That such hypomethylation can have a fundamental effect on tumor cell development has recently become clear. Transgenic mice that are heterozygously deleted for a DNA methyltransferase show a substantial decrease in total genomic methylation in all tissues. At 4 to 8 months of age, these mice develop an aggressive T-cell lymphoma with a high frequency of trisomy in Chromosome 15. There appears to be a link between DNA hypomethylation and chromosomal stability. Chromosome 15 is frequently duplicated in T-cell lymphomas and the c-myc oncogene is located on this chromosome. c-Myc is overexpressed in many of the hypomethylated tumors and in T-cell lymphomas as well.

Further evidence that hypomethylation may have a causal role in carcinogenesis was obtained by crossing the DNA methylase heterozygote mice with mice prone to develop soft tissue sarcoma with simultaneous loss of heterozygosity (LOH) of the NF-1 and p53 genes. The resulting transgenic mice develop sarcomas at an earlier age and demonstrate an increase in LOH in the hypomethylated versus the normally methylated cells. The increase in the rate of LOH is the result of a specific effect of hypomethylation on the stability of pericentric and centromeric chromosomal regions.

Other experiments with nuclear cloning provide evidence that the tumorigenic phenotype of tumor stem cells can be reprogrammed epigenetically. Introducing the nucleus of a murine melanoma cell into an enucleated murine ovum with subsequent transfer into a surrogate female resulted in a normal

offspring. The neural crest derived cells including melanocytes were all normal in the resulting mouse. Thus, the micro-environment induced a genetic reprogramming. However, the genetic mechanisms that existed in the tumor nuclei were maintained as these mice had a high incidence of neoplasia. Thus, both genetic and epigenetic mechanisms may be active in neoplastic development. This result with melanoma was a rare experimental success among a number of tumor nuclei tested, and the reasons for the lack of repeated success with nuclei from other tumors is under investigation.

That the microenvironmental niche that a cell resides in can have profound effects on the cellular phenotype is also demonstrated by studies on the fate of embryonic stem cells derived from the blastocyst inner cell mass. When they are transplanted back into another embryo, they maintain their normal phenotype. If, however, they are transplanted into a differentiated microenvironment such as kidney or liver, the cells form a teratoma.

In conclusion, there is evidence to suggest that epigenetic mechanisms can produce an inheritable tumor stem cell phenotype. Epigenetic control also underlies a great amount of stem cell regulation as exemplified by the polycomb gene Bmi-1, thus providing a link to the control of cancer stem cells.

Future Directions:

At the conclusion of the Think Tank, a number of important future basic research questions were synthesized from the scientific discussion. They are summarized below:

1) What governs the rate of proliferation of stem cells and in particular tumor stem cells? A corollary question would be whether the size or quality of the stem cell microenvironment (niche) acts as a constraint on stem cell growth?
2) What creates the stem cell niche for a tumor stem cell (i.e. do tumor stromal cells constitute the tumor stem cell niche?)
3) Does the transition from semi-linear to exponential cell growth occur when the tumor cells become independent of the stromal niche?
4) Can oncogenes and their associated mutations affect asymmetric versus symmetric divisions in stem cells?
5) Is the object of Darwinian selection in the tumor the T-IC, rather than the more differentiated tumor cells? It is likely that alleles for malignancy spread in a tissue because they arise in stem cells and provide advantages to the tumor stem cells that carry them.
6) Are the phenotypes of invasion and metastasis uniquely connected to the tumor stem phenotype?
7) Stem cell quiescence versus growth and differentiation must ultimately be understood in terms of progression through the cell cycle. It will be important to determine whether the retinoblastoma (Rb) gene product is as critical in this process as it appears. Rb plays a key role in cell cycle progression and differentiation in a number of tissues. Hypophosphorylation of Rb forces cells to leave the cell cycle and enter G_0, therefore regulation of this protein is likely to play a role in regulating true stem cell state. This is substantiated by studies on the Bmi-1 gene and the genes it regulate that interact with the Rb/p53 cell cycle pathway.
8) Most human carcinogens are strong tumor promoters and weak initiators of carcinogenesis. If tumor promoters work by increasing the size of the target population, then they must work by

increasing the population of already initiated cells. Thus, it is important to understand whether tumor promoters work on initiated epithelial stem cells or on stromal cells, as the stromal cells may control the size of the stem cell niche.

9) Can the roles of mutation and epigenetic mechanisms be distinguished in the generation of the tumor stem cell phenotype?

In addition a number of questions related to the future of cancer therapy were also considered.

1) Can the current benchmark for measuring the success of cancer therapy, tumor shrinkage, be changed to something more biologically relevant? The success of therapy can only really be measured by understanding the effect of the therapy on the tumor stem cell. Unfortunately this is difficult to obtain because of the lack of tumor stem cell markers.
2) Can we develop xenograft models that recapitulate the stem cell transition to more differentiated progenitors in a tumor? Such models might be useful to begin understanding the effects of therapy on tumor stem cells.
3) Are there methods for treating tumors that might cause a collapse of the stem cell niche? It is likely that most human tumors depend on stromal cells that define the niche and can control the size of the stem cell niche.
4) Are the cells that form the tumor stem cell niche different from those forming the normal tissue stem cell niche? Might any differences present an opportunity for directing selective therapy to tumor stromal cells?
5) Finally, might tumor stem cells be more or less sensitive to apoptosis inducing stimuli? Such information is critical in designing therapies that destroy the tumor stem cells responsible for continued expansion of the tumor and subsequent metastasis.

Specific Recommendations for the NCI

Technical Recommendations:

1) Develop methods to encourage surgeons involved in clinical research to collect tumor specimens directly into trypsin disaggregation solutions and provide viable freezing of cells in large banks to allow scientists to isolate unselected populations of tumor stem cells.

2) Encourage the development of real time PCR technology to examine gene expression in tumor stem cells. This technique appears to give more valuable information than DNA microarray technology for analysis of stem cells.

3) Improve *in vivo* and *in vitro* functional assays for tumor stem cells to allow for more accurate identification of these cells in concert with cell surface phenotype identification.

4) Improve *in vivo* organotypic assays to understand symmetric versus asymmetric cell division.

Administrative Recommendations:

1) Continue National Cancer Institute support for opportunistic basic research into tumor stem cell biology.

2) Develop a Research Consortium to facilitate transdisciplinary approaches and to provide specialized research reagents that will advance research in this area.

Executive Summary of the Cell Decisions in Response to DNA Damage: Survival vs. Programmed Cell Death Think Tank

Current cancer therapy relies heavily on DNA damaging agents (radiation, DNA alkylating agents, etc.) to induce programmed cell death in cancer cells. The proven success of this therapy, albeit only in some patients, implies that cancer cells are more sensitive to killing by DNA damaging agents than normal cells. This increased sensitivity is believed to be due largely to defects in DNA damage response pathways within the cancer cell. Compelling experimental evidence suggests that it is possible to dramatically modulate the sensitivity of cells to DNA damage. Similarly it may be possible to modulate the cell-specific and stimulus-specific responses to DNA damage leading to cell death. These responses vary dramatically between cell and tissue type, metabolic state and genetic background. If the sensitivity of cancer cells to DNA damaging agents and the cell death response to them could be specifically increased (or the sensitivity of normal cells to these agents be specifically decreased) by just one order of magnitude this would lead to a significant increase in cancer cure rates.

The obvious benefits of such an outcome are:

- reduced collateral tissue damage from the use of lower doses of radiation or chemical agents that would be required to kill cancer cells,
- reduced risk of second cancers from cells irradiated at the edges of the therapeutic radiation field,
- improved tumor targeting by combining the focusing power of radiation therapy with pharmacologic enhancement of tumor susceptibility to DNA damage.

A major goal of the workshop was to identify the knowledge and resources needed to optimize the DNA damage response leading to programmed cell death in human cancer. The Think Tank participants put forth a number of recommendations that can be summarized into 4 broad areas:

- Support systems biology analyses of the human DNA Damage Response (DDR) networks including the complete mapping of the biochemical and regulatory circuitries that link the cell-cycle checkpoints, apoptotic, and DNA repair pathways with the DDR.
- Support further identification of molecular targets for enhancing programmed cell death in response to DNA damage, particularly by investigating p53-independent pathways of DDR-induced cell death and by investigating strategies to modulate activity of DDR sensor and mediator proteins to amplify cell death signals.
- Develop reagents to study the DDR *in vivo*, particularly a resource library of phosphospecific antibodies and DDR read-out reagents for quantitative and dynamic measurements.
- Convene a conference focused on enhancing molecular pathology approaches for the analysis of DDR in human tissues and undertaking comparative studies of the DDR in normal and tumor cells/tissues to elucidate novel anti-apoptotic components altered in cancer cells.

Introduction

The introduction of DNA damage is a major therapeutic strategy for killing cancer cells. However, cell death is not the sole option for cells in response to DNA damage. In brief, the damaged cancer cell has two options: To die by regulated programmed cell death (PCD), or to survive by preventing cell division until DNA repair can be completed. In their initial stages, both PCD and survival/DNA repair processes initiated by DNA damage share the same signaling cascades based on high-level protein kinases (e.g., ATM, ATR) and secondary kinases (e.g., Chk1 and 2) along with a number of other proteins involved in signal detection and "mediation" of signaling and repair. What is less clear, however, is how, at the molecular/mechanistic level, human cancer (and also normal) cells that have sustained DNA damage assess its severity and make the ultimate cellular decision between death and survival. The aim of this Think Tank was to advance understanding of the mechanisms involved, with the ultimate goal of finding strategies that will select or enhance cell death.

Think Tank Program
Session I: DNA Damage and Repair

Discussion topics included:

- How do human cells (normal and cancer) detect primary DNA damage?
- How are signals of primary DNA damage amplified by the cell?
- What are common and distinct responses to different types of DNA lesions?

The most lethal type of DNA damage is DNA double strand breaks (DSBs). DSBs can be caused directly by radiation or indirectly after DNA modified by chemotherapeutic drugs is processed by cellular enzymes. Both radiation and DNA-modifying chemotherapeutic drugs are currently used to treat human cancer.

The response to a DNA DSB includes DNA repair and DNA damage signaling components. DNA repair pathways are clearly important; however, DNA repair genes are not frequently mutated in human cancer and it is not clear that inhibiting these pathways will lead to cancer-specific killing. DNA damage signaling pathways (also known as DNA damage checkpoint pathways) include sensors, which detect the presence of DNA DSBs, transducers, which produce a DNA damage signal, and effectors, which induce cell death or cell cycle arrest (transient or permanent). Mutations that inactivate DNA damage checkpoint genes are extremely prevalent in human cancer, resulting in rewiring of the DNA damage signaling pathways and differences in the response of cells to DNA damage.

In the last ten years there has been tremendous progress in our understanding of DNA damage checkpoint pathways. Most of the known DNA damage checkpoint genes were cloned within the last ten years. Their function is just beginning to be understood and key research discoveries, especially with regard to the DNA DSB sensors, were published in the last year or are still in the process of being published. The emerging theme is that there is a small number of DNA DSB sensors and the response of the cell to DNA damage (cell cycle arrest or cell death) depends on which sensor is being used to

recognize the damage. Crystallography analysis suggests that recruitment of at least one sensor (53BP1) to DNA DSBs is amenable to pharmacologic intervention. Inhibition of 53BP1 recruitment to sites of DNA DSBs would result in increased recruitment of other sensors (specifically, the NFBD1/MDC1-Mre11 complex), which is thought to be more potent in promoting cell death in response to DNA DSBs.

Cells also have at least two transducers of the DNA damage signal in the form of the protein kinases ATM and ATR. How these kinases are activated by the DNA DSB sensors is not yet clear. The pathway downstream of ATM and ATR is somewhat better understood, although there are still gaps in our knowledge there as well.

In conclusion, how cells detect the presence of DNA DSBs and transduce the DNA damage signal represents a major gap in our knowledge. The recent progress in this field suggests that we are at the verge of making considerable progress. Characterization of the DNA DSB sensors and transducers is expected to identify promising and pharmacologically amenable targets for development of cancer therapeutics.

Session II: Checkpoints and Apoptosis

Discussion topics included:

- How do cells assess DNA damage information to decide between survival and death?
- Is DNA repair linked to either checkpoint or apoptosis induction?
- Are DNA lesions sufficient to trigger apoptosis?

The p53-dependent pathway which induces apoptotic cell death in response to DNA damage is clearly relevant in terms of cancer therapy toxicity, since normal tissues exposed to DNA damage can undergo apoptosis. Stabilization of p53 following DNA damage results in its accumulation in both nuclear and cytosolic compartments. Cytosolic p53 can directly activate the proapoptotic activity of Bax to permeabilize the mitochondrial outer membrane. It remains to be determined to what extent this cytosolic effect, independent of transcription, contributes to p53-mediated death. If cytosolic p53 is apoptotic, the transcriptional upregulation of PUMA may provide another block to the anti-apoptotic functions of Bcl-2/Bcl-xl. However, in most solid tumors these pathways are unlikely to contribute to DNA damage-induced death, because in most tumors p53 is inactivated by mutations. Therefore it is anticipated that inhibiting p53-dependent apoptosis may enhance the therapeutic index of cancer therapy, although there are still reservations regarding whether such an approach would be beneficial in humans.

p53-independent cell death pathways are likely to be of greater relevance to killing of most solid tumors in response to therapy. Cell death in p53-mutant cancers may be due to apoptosis or perhaps more likely to progress through the cell cycle with unrepaired DNA DSBs, which occurs when DNA damage checkpoint genes are mutated. While in yeast and humans it is well established that defects in DNA damage checkpoint genes lead to extreme radiosensitivity (for example, in ataxia-telangiectasia patients in which the ATM transducer is mutant), a link between mutations in DNA damage

checkpoint genes in human cancers and their radiosensitivity has not been systematically investigated (with the exception of the effect of p53 mutations). Recent studies have identified a p53-independent apoptotic pathway, involving p53-related p63 and p73, in mediating DNA damage induced apoptosis. Activation of p73 by genotoxic agents has been shown to involve the nuclear c-Abl tyrosine kinase and contributes to the apoptotic response to p53-negative cells. But in general, it is fair to say that we do not understand well how DNA DSBs kill cancer cells that bear mutant p53.

In addition, apoptosis is not the only way for damaged cells to die. Excessive or persistent damage can trigger other modes of cell death, including necrosis and mitotic catastrophe. It is also possible that these modes of cell death could offer opportunities for new therapeutic strategies.

Session III: DNA Damage and Cancer Therapy

Discussion topics included:

- How can our knowledge about DNA damage responses be best exploited to therapeutic benefit?
- What are the current roadblocks to translating our knowledge about DNA-damage responses to therapeutic strategies?

Tumor cells start out as intrinsically more sensitive to apoptosis by virtue of their obligate oncogenic lesions, which push cells closer to their apoptotic activation threshold. This is thought to explain the innate sensitivity of most tumors (at least initially) to the crude and blunt types of classical therapies we currently use. Clearly, such innate apoptotic sensitivity is eroded during tumor evolution, and especially in response to the strong selective pressure of classical cancer therapies. However, such erosion is an evolutionary process and arises through a specific and restricted repertoire of anti-apoptotic mutations that the tumor acquires. Because of this, tumor cells remain chronically dependent on their restricted repertoire of anti-apoptotic mutations for their survival. In contrast, normal cells have no pro-apoptotic oncogenic lesions and are supported by a variety of trophic signals by virtue of their residing in their correct orthotopic somatic environments. By definition, tumor cells are outside their orthotopic environment and should therefore be acutely sensitive to therapies that negate their anti-apoptotic mutations.

Unfortunately, we know too little as yet of the range or repertoire of anti-apoptotic mutations that operate in different types of cancer cell. Since such mutations are acquired through natural selection, and since different selective pressures operate on different tissue types as they evolve into tumors, anti-apoptotic mechanism are likely to vary between different tumor types. This provides an unparalleled wealth of possible therapeutic targets, if we could but identify them. This must remain a priority.

Conclusions:

Pathways

A substantial body of data exists on the pathways of responses to DNA damage in bacteria and yeast, and studies of pathways generally follow two main strategies: mutations in pathways that trigger cell death, and responses of cells (i.e., responses at the cellular level) to damage. Despite extensive information on some pathways in certain organisms, many key gaps in knowledge exist in how cells detect and repair DNA lesions and orchestrate these cellular responses. Further investigation of DNA repair mechanisms in mitochondria and of the mechanisms of target resistance and sensitivity is also warranted. We also need to increase our knowledge of how DNA damage kills cells that lack a functional p53, and the role of metabolism and microenvironment on cell death decisions in response to DNA damage. We have accumulated clustered knowledge in areas such as apoptosis and DNA repair, but we lack the knowledge needed to link these clusters. The basic mechanisms of DNA repair and molecular interactions in signal transduction pathways are probably best studied in cellular models, including yeast and mammalian cells, and advances in these areas may assist in the identification of novel therapeutic interventions (e.g., IR and targets of NHEJ repair).

It is also critical in this area of investigation to understand the pathophysiology of normal versus tumor tissue and the progression of normal tissue to tumor tissue. It is assumed that mutations in mechanisms that alter DNA repair or damage response pathways can potentiate tumor development. However, it is not clear whether this process is (1) a stochastic phenomenon that "opens the door" to a cell progressing to transformation or to cell death; or (2) an ordered, repeated process that continues until reaching a specific threshold beyond which repair no longer occurs, ultimately generating a cancer cell or causing a cell to die. Tracing the effect of a DNA repair defect from its initial stage to the final outcome in a highly advanced tumor cell would likely be highly informative in distinguishing between these two possibilities and in identifying different death suppression mechanisms that lead to variable sensitivity to DNA damage. Comparisons of immediate responses to DNA damage, which appear to involve posttranslational changes, and longer term adaptive responses, would likely be informative.

Tumors may be considered as dysfunctional tissue. For example, tumor cells are attracted to a type of vasculature to which normal cells are not. In addition, specific lesions in tumors prevent terminal differentiation. A more sophisticated analysis of signaling pathways within currently curable human tumors would be enormously valuable. While these tumors (mostly pediatric leukemias, sarcoma, germ cell tumors, certain lymphomas) have been studied in the past, a more sophisticated approach, which compares and contrasts their signaling responses to chemotherapy (particularly DNA damaging agents) relative to incurable tumors, is likely to shed important light on key variables in treatment success.

Tools

Considering that the NCI Developmental Therapeutics Program is being "reshaped," the NCI could now play a direct role in providing infrastructure and support for the development of new tools. The NCI should be looking hard at data already in hand with an eye to making it more accessible (e.g.,

cross-indices of chemical compounds vs. the 'NCI60' cell lines) or simply funding development to make needed reagents available (e.g., screening to identify panels of phospho-specific antibodies that work on tissue). A model is found with the development of the PubMed database, where the previous MedLine was rolled over into something that is web-accessible, readily searchable and easily elaborated to add additional features at the local level.

In addition, improved, advanced computer and web-based systems modeling programs relevant to DNA damage signaling also are needed. Participants noted a two-dimensional computer model developed by Kurt Kohn that incorporates the underlying biochemical mechanisms involved in the cell cycle (a similar model for apoptosis also has been developed). Although these models can be helpful and serve as a platform for further investigation, participants generally agreed that a research model should be dynamic and should allow for contingency pathways and multiple decisions by the cell. It was noted that other more quantitative computer models are under development. However, because of the limited amount of data available, applications of these models currently are, in turn, also limited.

Sophisticated mouse models that accurately recapitulate human neoplastic processes are also needed. Most existing transgenic and knockout models merely "mimic" cancer. Efforts, like that of the MMHCC, to more faithfully recapitulate relevant neoplastic processes should be strongly supported. Faithful mouse tumor models are essential since, as discussed countless times in the Think Tank, the response of somatic cells (normal and neoplastic) to DNA damage and/or other therapeutic insults is, in great part, dictated by interaction between the tumor cell and its dynamic somatic environment.

A particularly attractive suggestion regarding animal models was to engineer "reporter transgenics/knockins." Such mice would contain germline-encoded reporters (such as GFP, LacZ, Luciferase) that would respond to a variety of important signaling or transcriptional pathways (eg p53 responsive, NF-κB responsive, HIF, hypoxia, and many more). Until molecular imaging becomes possible via PET (not likely for a while still), such animals could prove enormously valuable.

Human Tissues

Much of the emphasis in the past two decades had been on defining genetic differences between tumor and normal cells. This has come, quite naturally, from the emphasis on cancer as a somatic genetic disease process. The most likely way to identify truly useful differences between normal and tumor cells (i.e., those that when targeted would have the offer the largest possible therapeutic indices) is to determine which normal pathways in tumors are still functional and can be exploited in the background of the tumor to force specific endpoints, such as cell death. This analysis will be facilitated by the ability to assay the activity of damage response networks by protein expression levels.

Few tools are available to study responses to DNA repair in tissues. However, such tools are necessary to map processes in normal and tumor tissues. To accomplish this goal 'modern molecular pathology' needs to be better defined in this context. For example, what functional imaging or histopathology markers are useful to examine endpoints such as apoptosis, energy state and viability *in vivo* or in tissue/tumor specimens from humans or mouse models? Methods that cut across different types of specimens: e.g., functional imaging, biopsy samples and archival tumor specimens (currently the

world's largest untapped human tumor biology resource) would be particularly valuable. The suggestion of phosphorylation site-specific antibodies that work on tissue is a good example, as is simple staining for p53 abundance. This would be the best place to fund some descriptive normal biology, which we desperately need to provide the 'numerator' for all of the tumor studies and begin to understand the tissue correlates of therapeutic index. Use of imaging as a noninvasive tool to examine molecular pathways in carcinogenesis and in response to DNA damage is still in the early stages of development but holds great promise. Expanded use of skin (or biopsies) was suggested as a model for imaging studies and assessment of the pathology of normal versus carcinogenic cellular processes.

At least two types of effort are focused on mechanisms and markers of DNA repair in human tumor tissue. They are academic-commercial laboratory collaborations developing reagents (e.g., phosphoepitope-specific antibodies) and researchers conducting tumor specimen analyses (e.g., chromatin status or modification in relation to tumor type or stage). Forging better connections between the two would facilitate movement of new analytical reagents and techniques into the clinical setting and yield more information from human specimens.

Broader issues that need further investigation include gaining a better understanding of the persistence of DNA damage versus transient DNA damage systems; determining the relative rates of proliferation and apoptosis in conjunction with DNA damage signals *in situ* (e.g., in oncogenic mouse models); assessing processes in real time; conducting longitudinal studies in humans; and understanding the different mechanisms underlying chemotherapy and radiation. One notable gap that warrants further investigation involves the distinct differences in the *in vitro* versus *in vivo* responses to DNA damage and therapeutic interventions (e.g., radiation). An approach to gaining a better understanding of these differences and bridging the gap between these systems may be through the study of pathway reporters inside tumors (see Tools). Another general challenge to the field is the standardization of technologies, assays, definitions, interventions, and outcomes across research to improve the power of between-study comparisons.

Targets, Strategies

The emerging science indicates that it will be possible to differentially modulate sensitivity to DNA damaging agents in cancer and normal cells. Practically all cancer cells have mutations targeting DNA damage response genes. Mutations in these genes are essential for cancer development and result in rewiring of the DNA damage response pathways in cancer cells. Because of this difference in rewiring, inhibiting the same molecule is expected to have different effects in normal and cancer cells. We need to support research to identify and characterize DNA double strand break sensors and transducers. These proteins are promising targets for development of drugs that could specifically enhance the efficacy of current cancer therapeutics. Research to validate which of these sensors and transducers would be suitable targets for development of cancer therapeutics should be supported, including development of prototypical small chemical inhibitors to test proof of principle. It is expected that the proposed research will utilize a multitude of experimental systems, including human cells, human cancer tissues, mouse models and lower eukaryotes, as each experimental system has its own advantages and disadvantages and knowledge gained in one system can be easily applied to the others. In addition, tumor cells have different sensitivities to DNA damage and other apoptotic stimuli.

Sensitization models that focus on unique mutations and/or unique clinical situations with high sensitivity may help determine the role of DNA damage and repair in the carcinogenic process.

It is critically important to explore ways in which sub-lethal pro-apoptotic signals can be combined to overcome the apoptotic-buffering threshold in cells. Merely refining, exacerbating or increasing the persistence of DNA damage alone may not accomplish selective tumor cell death. No amount of damage signaling is going to provide a therapeutically discriminate pro-apoptotic signal if, as in many tumors, DNA damage-induced apoptosis has become compromised by the mutation of p53. Exacerbating DNA damage also carries with it the future problem of inducing further oncogenic mutations. While it is reasonable to argue that therapy-induced mutagenic/carcinogenic risk is secondary to curing the immediate neoplastic disease in patients, it would be better to avoid the problem completely if a better way of triggering apoptosis in tumor cells can be found. No tumor cell has ever been identified that lacks the apoptotic machinery if hit hard enough. This reflects our understanding that the programmed cell death (PCD) machinery is highly redundant and mechanistically defocused to employ several overlapping processes, including mitochondrial dysfunction, pro-phagocytic signaling, and caspase activation, each of which is alone sufficient to ensure death of the affected cell. Therefore, it appears that PCD cannot be lost by progressive mutation. Rather, tumors acquire refractoriness to apoptosis through corruption of upstream signaling pathways and through lesions that raise the threshold at which apoptosis is triggered. Thus, adroit stimulation of multiple disparate apoptotic pathways, each at a sub-lethal level, might together trigger activation of the apoptotic program.

Other research questions of interest focus on the role of the cell cycle in DNA repair and cell responses to DNA damage. We must find out whether blocking the cell cycle is a good clinical strategy.

Specific Recommendations for the NCI

- ***Analyses of the Human DNA-Damage Response Networks (DDR)***

    - Complete the mapping of biochemical and regulatory circuitries that link the cell-cycle checkpoints, apoptotic and DNA-repair pathways into the hDDR with special emphasis on putative regulatory nodes.
    - Develop a quantitative, computer-driven, systems-biology model for the hDDR network in human cells.
    - Complete (or initiate) the integration of hDDR proteomics, gene-regulatory and gene expression databases with more dynamic cellular metabolite and energy profiles as functions of DNA damage.

- ***Identification of Molecular Targets & Translational Strategies for Enhancing Programmed Cell Death over Cell Survival in Response to DNA Damage***

    - Support more research on ways to modulate the activity/expression of early-time ("upstream") mediator and sensor proteins to activate the pathways of programmed cell death.
    - Support more research on the underlying mechanisms of p53-*independent* programmed cell death, because in most tumors p53 is inactivated by mutations.
    - Validate, as targets, mechanisms other than apoptosis that can induce cell death in human tissue, such as those linked to metabolic pathways that are altered in specific tumors.
    - Place greater research emphasis on mechanisms that determine whether dual-function ("downstream") signaling proteins (e.g., p53, E2F1) signal survival or death responses.

- ***Normal versus Cancer Tissues***

    - Conduct comparative proteomic studies of tumor cells/tissues, progenitor cells, and normal cells/tissues to elucidate additional anti-apoptotic components altered in cancer.
    - Characterize and catalogue molecules from the signaling, checkpoint and apoptotic parts of the hDDR network as possible therapeutic targets.
    - Develop predictive profiles for radiation sensitive/resistant individuals; tumors with or without functional p53, ATM or other key signaling proteins, in order to tailor DNA-damage-based therapy to the patient.

- ***Reagents to Study and Technology to Study the DDR* In Vivo**

    - Develop a resource library of phospho-specific antibodies or other "readout" reagents that can provide quantitative measures of DDR signaling and output pathways (e.g., caspase induction and apoptosis; focus formation and DNA repair). This should include phospho-specific antibodies that can examine the activities of the DNA-damage signaling pathways in cultured cells and primary human tumors, before and after treatment and at different stages in tumor progression, etc.

- Develop small molecule inhibitors of specific targets implicated in the response of cells to radiation, particularly where the target protein three-dimensional structure is known.
- Promote dynamic imaging in the study of mechanisms of DNA repair in *in vivo* and *in vitro* studies.
- Develop programs or incentives to allow for research access to early-phase clinical drugs belonging to pharmaceutical companies and studies of pharmaceutical reagents in mouse models of human cancer.
- Support application of molecular imaging capabilities to monitor the dynamics and cellular compartmentalization of the hDDR.
- Convene a conference or think tank focused on new tissue markers and modern pathology to improve connections between investigators in basic science and clinical research.

Executive Summary of the Cancer Etiology: Role of Exogenous and Endogenous Chemicals Think Tank

For many years, the focus in carcinogenesis has been on initiation, and particularly on the direct induction of DNA damage by chemical carcinogens. While this is an important aspect of carcinogenesis, further progress depends on the use of a more balanced approach, emphasizing that the carcinogenic process is continuous and dynamic. Chemicals (both exogenous and endogenous) can contribute to tumor formation at any part of the process, and do so by interacting with a broad range of molecules including proteins, lipids and RNA, as well as DNA. For example, studies investigating the pro-carcinogenic effects of reactive oxygen and nitrogen species (ROS) (RNS) on cancer initiation and progression identified a large number of cellular damage effects along the progression continuum, including evasion of apoptosis, insensitivity to antigrowth signals, self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, and tissue invasion.

If a new, more comprehensive approach to carcinogenesis is to be maximally productive, a systems biology approach will be needed to deal with complexities head-on, focusing not on individual components but rather on networks that can be measured, modeled, and manipulated. It will be necessary to generate very large, highly accurate datasets describing the behavior of all components in the system. This will require highly sensitive, newly available technology that can identify a range of biomarkers measuring exposure. Benefits that will flow from these broadened investigations include:

- The identification of biomarkers useful for assessing risk, for earlier diagnosis and for measuring therapeutic efficacy using newly developed technologies with extremely high sensitivity.
- An understanding of how endogenous and exogenous chemical exposure impacts repair pathways, epigenetic changes, and protein and lipid function, and how they alter cancer susceptibility, which will lead to novel targets for prevention and therapy.
- Development of better strategies for chemoprevention, exposure avoidance, and healthy lifestyles.

The Think Tank identified knowledge gaps and resources needed to improve prevention strategies and identify at-risk populations that can be summarized into 5 broad areas:

- **Chemical processes and pathways:** Expand studies to include a broader spectrum of the effects of endogenous/exogenous chemicals and their reactions in the carcinogenesis continuum. Use a systems approach to interacting signaling networks at the cellular and microenvironment levels.
- **Biomarkers:** Design approaches for the development, validation and application of chemical biomarkers of exogenous and endogenous carcinogen exposure. Use damage products measurements (e.g., DNA, protein, and lipid changes; urinary and plasma metabolites) for early detection, risk assessment, and monitoring therapeutic efficacy.
- **Models:** Develop models with sufficient dynamic range to study combined chemical exposures and enable modulation of endogenous chemical products via knockdown, pharmacologic, chemopreventive, or dietary manipulation. A range models are needed, from microbes through vertebrates to three-dimensional organ culture systems.
- **Technology:** Enable collaborative access to newly developed, high sensitivity, high resolution, expensive instrumentation for high-throughput data collections.
- **Recruitment, collaboration and resources:** Train the biologists, chemists and modelers who must work collaboratively on chemical carcinogenesis in an interdisciplinary environment.

Introduction

The classic Berenblum paradigm of multistage carcinogenesis, conceptually dividing the process into discrete stages of initiation, promotion and progression, requires extension and modification to take into account current advances in cancer research. It is clear that carcinogenesis is a continuous, dynamic process. Human cancers are caused or modified by exogenous and endogenous chemicals, and the same chemical can have multiple effects along the initiation to progression continuum. To eliminate the burden of cancer, research priorities must reflect the continuity and enormous complexity of the carcinogenesis process. Although the identification of exogenous carcinogens is nearly complete, the identification of endogenous carcinogens is not. In many cases, exogenous exposure and endogenous processes predispose to cancer through the same ultimate effectors, such as ROS and RNS. Similarly, much is understood about metabolic activation and detoxification, about DNA adducts, and DNA repair, but knowledge of the biological consequences of DNA adducts is incomplete, and the effects of carcinogens on other molecules, such as proteins and lipids, are largely unknown. Critical to further advances, both animal and human studies highlight the need to consider the timing and duration of chemical exposure. To understand the contributions and interactions of all these factors, a systems biology approach is essential.

Animal models suitable for studying chemical carcinogenesis are very limited. While genetic loss-of-function mutations (largely knockout mice) have provided extremely valuable insights into genetic factors in tumor development, these ablative models often do not replicate the effects of chemicals. For example, modulation (vs. ablation) of DNA repair pathways results from exposure to endogenous and exogenous chemicals. As a result, while most cancers do not exhibit mutations in DNA repair pathways, damage continues to accumulate.

A theme that permeated the Think Tank and can be seen in much that follows is the importance of having biologists and chemists work in close collaboration to maximize progress in the field. Biologists are familiar with the complex changes in cell biology and physiology that occur during cancer development and progression, and have expertise with *in vivo* experimentation. Chemists understand the reactive potential of carcinogens, have the ability to synthesize proposed intermediates in carcinogen activation, and have access to technology that can identify carcinogens and their metabolites. Ensuring collaborations between these groups is complicated by differences in scientific approach and, often, their location in different schools within a university.

The Think Tank recommendations can be summarized under five topic areas: I. Chemical processes and pathways; II. Biomarkers; III. Models; IV. Technology; and V. Recruitment, Collaboration, and Resources. Recommendations in each area are summarized below, followed by discussion points from the presentations and the literature.

I. Chemical processes and pathways: The field's research focus must expand to include a broader spectrum of effects resulting from exposure to endogenous and exogenous chemicals throughout the carcinogenesis continuum. Such investigations should include the consequences of exposure on RNA, lipids, and proteins, and epigenetic modifications to DNA and protein. To accomplish this, it will be important to investigate the consequences of infection and tumor promoter exposure on cancer

development and to identify signaling pathways that are either dependent or independent of reactive oxygen/nitrogen species (ROS)/(RNS) and other effectors of inflammation. Interdisciplinary teams will use a systems approach to determine how cells and signaling pathways interact within target cells and in their tissue microenvironment in response to carcinogen exposure. To learn how committed stem cells in target tissues respond to chemical exposures and how such exposures modulate their function may be a key to understanding their resistance to therapy and their persistence following treatment-induced remission.

A) Carcinogen effects on RNA, lipids, proteins and DNA: DNA damage and mutation occur along the continuum of carcinogenesis, not just during "the initiation phase." The field has focused on DNA, but carcinogens also damage proteins, RNA, and lipids. DNA, protein and lipid damage recovery pathways intersect, and responses to protein and lipid damage are just as important as DNA damage in mediating cell recovery from exposure. Although protein damage has been understudied, recent work identified protein adducts as biomarkers. For example, a mouse skin carcinogenesis study detected 95% of the labeled carcinogen bound in damaged protein and most of the remainder in RNA; the smallest amount of the label was associated with DNA. Even less is known about the consequences of carcinogens on lipid metabolism, or the pro- and anti-apoptotic effects of peroxidized lipids. The role of bioactive lipids in signal transduction needs attention and recent technologies have now enabled an investigation of these processes. Other factors that should be considered are:

***DNA Adducts*:** DNA adducts can lead to p53 gene mutations in specific tissues like bronchial epithelium. The p16 gene is only methylated among smokers. Neither the tissue specific responses to exposures nor their underlying mechanisms are understood and they require investigation.

***Pathways*:** When epigenetics, protein pathways, and apoptotic pathways, are considered, it seems it is not specific genes, but pathways that are consistently altered in carcinogenesis. To identify pathways that influence cell death and mutation, genomic phenotyping for damage sensitivity could be useful. It is important to keep in mind that more than 1000 yeast proteins are involved in recovery from some carcinogenic agents. Because several interacting pathways are involved in recovery from a carcinogenic insult, it is important to identify synergism among these recovery pathways.

***Timing of exposure*:** The timing of exposure over the life course can be critical with respect to an individual's risk of developing cancer. Further, huge threshold differences in metabolism and detoxification may relate to differential gene expression at specific developmental stages; these differences are not well understood.

B) Role of diet, hormones and carcinogenic processes: Effective prevention requires the identification of exogenous and endogenous carcinogens and their interactions. Among other environmental agents, dietary substances may be a key factor. Dietary effects operate in a background of genetic differences and circulating endogenous hormones, both or either of which can alter an individual's exposure risk.

C) The roles of inflammation, ROS and RNS and microbial flora: Inflammation is "the perfect storm" for carcinogenesis, causing DNA damage and activating production of growth-stimulatory cytokines. Anti-inflammatory compounds have chemopreventive effects in animal models and humans.

Recently, attention has turned to the role of inflammation, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), throughout cancer initiation and progression. ROS and RNS cause a variety of different types of cellular damage; in addition to increased mutation rates, cellular damage results in evasion of apoptosis, insensitivity to anti-growth signals, self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, and tissue invasion—all hallmarks of tumorigenesis. Declining defense mechanisms during aging may increase sensitivity to inflammation. A number of human cancers result from the combination of infection and carcinogen interactions. Infection contributes to inflammation, but pathogenic processes specific to certain infectious agents also play important roles in carcinogenesis. The synergy of the hepatitis B virus (HBV) with aflatoxin is striking--the occurrence of liver cancer increases dramatically in infected people. The appropriate analysis of these complex interactions requires a systems approach.

D) The microenvironment in carcinogenesis: Although only one cell type may be capable of forming a given tumor, chemical exposure of the microenvironment is likely to have direct and/or indirect consequences. Exposure of animals to a carcinogen that directly affects one cell type may indirectly influence other cells in the tissue environment. In terms of prevention, inhibition of IKKβ in target cells is more effective than its inhibition in the surrounding inflammatory cells. An analysis of the different cell types' sensitivity may reveal chemical interactions in specific genetic pathways.

E) Tumor promotion: If it is possible to distinguish between factors affecting tumor initiation (genetic changes) and those that give the target cell its proliferative advantage, chemoprevention strategies might focus on inactivating the latter. Carcinogens not detected by the Ames test are likely to be tumor promoters, and are likely to involve ROS. Dioxin is an example of a substance that causes no DNA damage, but is a potent promoter in human skin and liver, acting through a single receptor that alters gene products influencing apoptosis, ROS, and cytokines. Better assays to detect tumor promoters should be developed. Age and gender also influence tumor promotion and are relevant to exposure assessment. For example, the ability to remove oxidative damage from the prostate may diminish with age and the commonly observed loss of COX2 activity in prostate cancer. Age and gender effects are clearly illustrated in diethylnitrosamine induction of hepatocarcinoma.

F) The analysis of complex carcinogenic mixtures: The assessment of complex mixtures of compounds, such as tobacco smoke, remains a particularly challenging area that requires considerable attention and resources. There is a scientific consensus that mixtures need to be investigated, but methods do not exist to address the complexities inherent in such studies at budget levels that will survive the NIH peer review process. Until now, most bioassays have used single, large dose exposures to a single chemical. Findings from the analysis of a single adduct species in a clean system cannot be extrapolated to real life exposures. People are much more likely to experience combinations of low dose exposures. A consensus is needed on scientifically acceptable methods to study mixtures in cells and animals that take into account differences in susceptibility due to developmental stage, genetic polymorphisms, and gender.

II. Biomarkers: Although biomarkers were not initially a part of the Carcinogenesis Think Tank agenda, chemists and carcinogenesis specialists are especially well equipped to identify them since biomarkers represent a spectrum of chemically complex substances such as chiral lipids, and

endogenous protein and DNA adducts. Biomarkers can be used to monitor exposure and treatment, and as tools for early diagnosis. The chemists' focus can now be expanded to detecting protein, lipid and DNA damage. Epidemiologists need biomarkers of past exposure history. A key issue in animal studies is to identify methods to serially sample the same animal using urine or plasma rather than having to serially sacrifice animals. This would dramatically reduce the number of animals needed in carcinogenesis experiments.

A) Assessment of past exposures: Samples collected during epidemiology studies can be used to determine past exposure history of the subjects once carcinogen biomarkers are identified. Although hair is regularly used for chemical analysis, its use for protein assessment is questionable. Tools with sufficiently high sensitivity to study biomarkers reflective of low exposure levels are needed.

B) Detection of present disease: Since tumors have different patterns of protein expression than normal tissues, it should be possible to identify altered protein patterns in blood or urine as cancer biomarkers via proteomics. Biomarker development and validation require both animal and human studies. Markers that track disease processes or predict cancer progression would be especially useful. The study of liver cancer in China illustrates that a successful biomarker study requires a high risk population so that sufficient numbers are available for statistically meaningful results. The study revealed a non-linear interaction of HBV and aflatoxin in determining liver cancer risk.

C) Prediction of future disease: *Fortune* magazine suggested that "The NCI should commit itself to finding biomarkers that are predictive of cancer development." The development of DNA, protein and/or lipid biomarkers that indicate cancer potential is a major goal. They could provide targets for chemoprevention strategies and guide patient counseling on lifestyle choices. Biomarker identification teams should also identify mutations so that adducts can be correlated with key mutations.

III. Models: Discussion focused on animal models that have the potential to provide a mechanistic understanding of carcinogenesis. It is important to determine the appropriate model to use, since a given model may be useful for a particular organ system, but not for all. Two models illustrate available insights:

***Hepatocarcinogenesis*:** Rat liver hepatocarcinogenesis was induced by genotoxic hepatocarcinogens and the initiated preneoplastic cells were isolated. In wild-type animals treated with a single *in vivo* dose of the hepatocarcinogen diethylnitrosamine (DEN), superoxide production by the Kupffer cells increased and enhanced DNA damage and nitrotyrosine in liver proteins. Phox-/- knockout mice treated with DEN showed less DNA damage and almost no nitrotyrosine production. It thus appears that the cell injury, DNA damage, apoptosis/necrosis, and proliferation result principally from increased superoxide release by Kupffer cells. This model illustrates the importance of interactions between different cell types during the first steps of carcinogenesis. Infection, intestinal problems and ethanol can also stimulate Kupffer cells, and thus influence carcinogenesis.

***Genetically Modified Animals*:** When induction of NF-κB in mice is accompanied by deletion of IKKβ in intestinal epithelial cells, tumor incidence decreases. NF-κB, a transcription factor that regulates the expression of anti-apoptotic genes, decreases the susceptibility of cells to apoptosis,

and may have a role in tumor promotion. In this model, IKKβ and NF-κB provide a molecular link between inflammation and cancer. However, in models of hepatocellular carcinoma, IKKβ disruption increases tumor number and size, as well as increasing apoptosis. In organs that can regenerate, increased apoptotic cell death pushes more cells into the proliferation cycle. Hence, a complex relationship exists between apoptosis and tumor formation, and promotion.

A) Animal models—strengths and weaknesses: Animal studies, particularly using rodents, have been the backbone of chemical carcinogenesis research. New methods of genetic manipulation in these species, and particularly in mice, offer promising new opportunities for research. The NCI Mouse Models Consortium is a useful resource to the carcinogenesis community. Some newer mouse mammary tumor models are metastatic, resembling the human situation. Mouse models may prove useful in identifying the carcinogens that cause breast, bone, and prostate cancer. Interestingly, a mouse model that develops lung cancers in response to cigarette smoke involves many of the same genes associated with human lung cancers. Do these genes play a role in initiation and/or progression? However, studies in rodents also have limitations. For example, tests have revealed that only 40% of chemical carcinogens harmful to animals are also harmful to humans, and at lower doses, only half of these were toxic in humans. Most mouse tumors are rarely metastatic or invasive, and mice do not develop gastric cancer. Additionally, mice have higher glutathione transferase levels than do humans, which limits their usefulness for some types of research. Gene knock-out mice are widely used, but there is some concern that the complete absence of a gene is not always a good mimic of a drastically reduced level of expression of the gene, a situation more commonly seen in carcinogenesis. Aflatoxin studies illustrate another limitation using selected animal models. Fisher 344 rats metabolize aflatoxin much as people do, but cannot be infected with hepatitis B virus (HBV). Thus, there is no animal model that truly replicates the interaction of HBV and aflatoxin observed in human studies, but one should be developed. In looking for models beyond rats or mice, what are the options? Lower eukaryotes can be useful, particularly where the specific mechanisms involved are known. In DNA repair, the choices are yeast, mammals, or *C. elegans*; little is known about DNA repair in *Drosophila*. The growing appreciation of the importance of stem cells in carcinogenesis suggests that *in vitro* studies of embryonic and adult stem cell cultures may be very useful. Currently, methods to maintain and manipulate such cultures are limited, but rapid progress is anticipated.

B) Inflammation is a confounding factor in animal studies: Even brief inflammatory episodes during the course of a carcinogenesis study can affect the outcome. The flora in animal facilities differ, so the occurrence of tumors may be high in one facility and low in another. The existence of at least 20 types of *H. pylori*, makes it possible to miss their presence in supposedly *H. pylori*-free animals. If the inflammatory agents (pathogens) are removed, the ability to generate the phenotype (tumors) may be lost. Despite its importance, few animal models are available to study inflammation.

IV. Technology: Think Tank discussions of technology focused on four broad areas: A) new technologies, B) shortcomings of techniques, C) sensitivity and other challenges, and D) fiscal constraints.

A) New technologies: Mass spectrometry has been the most widely used analytical technique in chemical carcinogenesis, and spectacular strides have been made in this area in recent years. Real-

time mass spectrometry with blood flow detection is a reality. Various types of mass spectrometry have improved to the point that they are approaching their limit of sensitivity. New types of mass spectrometers include the exquisitely sensitive Fourier transform ion cyclotron resonance (FTICR); MALDI-TOF/TOF, which can characterize proteomes, lipidomes, and DNA adducts; and the high resolution triple quadrupole, which can quantitate lipids, DNA adducts, proteins and protein adducts. Current work involves increasing their specificity still further.

Proteomics, lipidomics, and related genomic-level, high-throughput analyses are needed in carcinogenesis, as they are in many other areas of cancer research. Mass spectrometry is a very useful technique in these areas, but more technologies are needed. Further techniques of protein analysis utilize radioactive labels to detect and pinpoint changes in protein patterns after a challenge. Stable isotope proteomes can be used as standards to run in 2-D gels. The proteomics laboratory at the Medical University of South Carolina is using chromatography columns on a chip, which are more sensitive than existing separation techniques. These chips should be generally available in two to three years.

The importance of non-invasive analytical procedures has been emphasized above, and intravital microscopy offers substantial promise in this area. Two-photon microscopy can penetrate tissue, at least to millimeter levels. It is possible with this technology to measure activities spectroscopically, an ability which could be applied to look for a ROS spectral signature.

B) Shortcomings of techniques: There are many areas in which technological improvements are still needed. Inferring sequential changes in animals from serial sacrifice has serious pitfalls. The development of non-invasive techniques to track changes in a single organism would substantially improve the quality of such data. In the case of an inflammatory response, for example, an adequate imaging methodology to obtain real-time records of organ-by-organ changes would be invaluable. 2-D gel analysis and protease digest proteomics are not quantitative. Even using antibody array information to detect up-regulation of proteins may fail, if it is applied at the wrong time, and information on labile protein modifications is very easy to lose during analysis.

C) Sensitivity and other challenges: Analytical and instrumental sensitivity was a recurring topic of concern. More sensitive mutagenesis assays are needed--current assays cannot detect mutations less frequent than one part in 10^6. For detection of many endogenous substances (such as endogenous vinyl chloride at the DNA and gene level), more sensitive and artifact-free instrumentation is required. Assessing the carcinogenic potential of low-dose exposures is difficult, as is interpreting the "U"-shaped curves which may result. Ongoing challenges for technology include methods to separate modified from unmodified adducts; the tools to do a mass balance experiment, looking at many pathways in a system such as chlorination and bromination; the chemical means to detect changes in real time, perhaps using *in vivo* probes; improvements in the chemistry of detection so that not only end products of a reaction are detected; and the means to link carcinogens clearly to disease or disease subtypes.

D) Fiscal constraints: Some sensitive instruments are costly to obtain and maintain. Researchers are slow to invest in technology not available within a P01 or R01 budget structure, until its suitability to

their research challenges has been well-established. As a result, MRI or PET scans are rarely done in animal facilities due to their expense, although MRI resolution can show oxidative stress levels, changes which might have regressed by the time animals were euthanized.

V. Recruitment, Collaboration, and Resources: Although not among the designated discussion topics, personnel and resources were regularly mentioned as factors impacting the success of research programs and the future development of the field.

A) Recruitment: Students perceive the field of chemical carcinogenesis as doing the same thing for the past 30 years--testing chemicals and not asking mechanistic questions. Academic structures can be blocks to cross training, although some institutions have integrated chemistry with biology or instituted interdisciplinary programs that expose students to research in carcinogenesis. T32 grants to support young people entering the field and multi-disciplinary training grants funded by NCI have been effective in training and recruiting new people, but more effort is needed in this area. Think Tank participants agreed that a combination of improved marketing approaches and funding can attract promising graduate and post-doctoral students for future leadership in the field of chemical carcinogenesis.

B) Consortia: Consortium grants, including those within a single institution, were attractive to some Think Tank participants. Sharing biological specimens, tissue arrays, databases, and other resources benefits the research community and should be required. NCI can assist by coordinating efforts to acquire and divide up tissue (especially human samples), and provide material to investigators as needed. Collaboration between chemists and biologists is also productive, and is relevant to the recruiting concerns voiced above. In the context of the roadmap initiative, some participants had done an exercise regarding inter- and trans-disciplinary research and how to form a team. The essential factor seemed to be that everyone must address the same, specific question. Other Think Tank participants expressed considerable skepticism about the benefits of consortia. Some advised caution in forming large groups across the country, which can be inefficient in getting information and publications together. In one opinion, the worst thing NCI could do is to have a big urine bank or blood bank--researchers should be closely associated with the collection process in order to know the source and storage conditions.

C) The Grant System: A major impediment to consortium formation is the lack of convenient funding mechanisms. NCI's strict rules for funding a program projects were viewed as counterproductive. One solution is for NCI to develop the needed infrastructure by funding individuals to develop these resources. Some believe there is a lack of knowledgeable study sections members. People with seniority and collaborative experience aren't on these sections, and conflict of interest requirements preclude participation by the most informed and up-to-date people because of their previous associations or collaborations.

Specific Recommendations for the NCI

The Think Tank identified knowledge gaps and needed resources to improve prevention strategies and identify at risk populations. Technologies that expand discovery opportunities are now available.

Chemical processes and pathways

- Expand research focus from DNA adducts and mutation analysis to include a broader spectrum damage resulting from exposure to carcinogens throughout the continuum of tumorigenesis.
- Develop a systems approach to interacting signaling networks at the cellular, microenvironmental and macro-environmental levels.
- Expand studies of reactive oxygen species and other mediators of inflammation to identify the variety of cellular and microenvironment damage they cause.
- Enable studies of low levels of exposure, in which perhaps 1 cell in 1000 is altered. DOE support of studies to look at the effects of low levels of radiation that cause no change in cell cultures could serve as a model.
- Identify carcinogens and other etiologic factors in breast, colon, and prostate cancer.

Biomarkers

- Provide resources for the development, validation and application of chemical biomarkers resulting from exogenous and endogenous carcinogen exposure and their damage products (e.g., DNA, protein, and lipid changes; urinary and plasma metabolites).
- Identify biomarkers for early detection, risk assessment, and monitoring therapeutic efficacy; identify markers that track past exposure to carcinogens.
- Develop instrumentation for high-throughput sample processing, to permit multiplexing analytical procedures for biomarkers.

Models

- Develop models with sufficient dynamic range to permit analysis of combined chemical exposures; enable modulation of endogenous chemical products via knockdown, pharmacologic, chemopreventive, or dietary manipulation.
- Provide more realistic funding for studies using genetically modified mice. In many cases, investigators must produce 3-4 times the required number of animals to obtain the desired genotypes. When modifier genes are found, follow-up studies are often not done because they can take three years and 1000 cages of mice.
- Make effort to standardize animal nutrition, health status and knowledge of endogenous bacterial flora. Encourage studies in which all investigators use animals with the same microbial flora.
- Use models of cancer progression, as well as initiation and promotion, for studies of combined etiological agents including mixtures. Inflammatory processes must be considered when designing cancer biology models and in implementing human translational studies.
- Develop a useful spectrum of biological systems, including single cell-microbes, invertebrates such as *C. elegans* and *Drosophila*, vertebrates including rodents, and three-dimensional organ culture systems, which can be used to look at cell/cell interactions and to study tissue interactions.

- Pay more attention to timing of exposure and the influence of developmental stages on carcinogenesis.
- Develop a controlled carcinogen-induced tumor model in which proteomics and genomics can be used to look for chemical/biological interactions.

Technology

- Using available, newly developed high sensitivity, high resolution instrumentation, develop high throughput technology in **cooperative arrangements**. Due to their expense, requirement for high level expertise, and the need for quality control, such instrumentation must be shared.
- Establish and support instrumentation centers within major institutions to make costly technology widely available.
- Develop non-invasive technology (e.g. imaging and serum sampling) to track changes over time, (avoiding serial sacrifice); develop methods to do mass balance experiments, improve the chemistry to detect changes in real time (*in vivo* probes). Develop technology to mark tumor stem cells and initiated cells for visualization and isolation.
- Design approaches to make stronger *in vivo* correlations between adducts, biomarkers, mutations, initiated and pre-neoplastic cells and cancer—a process limited, at least in part, by our lack of high-sensitivity mutational assays to extend dose-response curves for biomarker-mutation correlations.

Recruitment, collaboration and resources

- Support fundamental training in chemistry.
- Support database development (an area currently buried in the depths of grant proposals) and mathematical modeling predictions of how biological systems respond to carcinogen exposure.
- Increase support for developmental projects. These are currently ignored by NIH.
- Support cross-training to emphasize the special role of chemists at all levels, including training grants and PI visits to collaborating laboratories.
- Maintain NIH sponsored synthesis of high quality standards (labeled and unlabeled, small molecule and macromolecular).

Executive Summary of the Epigenetic Mechanisms in Cancer Think Tank

The purpose of the Epigenetics Think Tank was to bring together experts from basic and translational sciences to discuss the potential of epigenetic research to inform cancer biology, diagnostics and therapeutics, and to make specific recommendations to NCI for action in this field. The think tank began with brief presentations by each participant on the first evening. On the second day, three discussion sessions addressed cancer epigenetics, basic mechanisms in chromatin structure, and epigenetic technology and epigenomics. A special presentation was included on the European Epigenomic Network of Excellence. On the final morning, the panel summarized the discussions and prioritized recommendations.

The Think Tank highlighted a clear link between epigenetic alterations and cancer. Epigenetic changes such as DNA methylation and imprinting are targets of environmental carcinogens and diet, and probably are responsible for much of the population variation relevant to cancer risk. The role of hypomethylation in gene activation, aging and chromosomal instability is an emerging significant issue. The panel was particularly excited by the apparent role of epigenetic changes in the earliest stages of carcinogenesis, because these are potentially reversible with treatment. This provides an epigenetics-based strategy for cancer prevention. Epigenetic changes also clearly play an important role in tumor progression, tumor cell heterogeneity and possibly metastasis. A key need is to map these early changes through the analysis of methylation and other epigenetic marks in a well-defined set of reference human normal and tumor tissues. In addition, it is important to determine the temporal relationship of epigenetic alterations, as well as their relationship to underlying DNA sequence variation in the population.

This is an exciting time in the understanding of the mechanisms of epigenetic transcriptional regulation and chromatin structure. Protein complexes that manipulate nucleosomes, organize larger chromatin domains and set boundaries of chromatin structure have been discovered in recent years; now research must focus on discovering how they work in such cellular functions as replication, transcription and differentiation. Crucial next steps are to understand the role of key histone modifications, cis-acting elements, and regulatory proteins in setting, maintaining, and reprogramming epigenetic memory. Other key unknowns are the determinants of higher-order chromatin structure, the function of micro RNAs or siRNA in mammalian gene regulation and genome stability, and the mechanism of transmission of epigenetic marks through cell replication.

The Think Tank concluded that the highest priority should be development of a U.S. Human Epigenome Project of analogous scope to the Genome Project. The immediate establishment of a Working Group to define the criteria and plan the approach to the Epigenome Project is timely and highly recommended. There was strong consensus that we are now at the stage at which we can “genomicize” epigenetic information. Most of the necessary technologies are already available, although some tools for analysis of epigenetic alterations at the genome-wide level are still needed. New quantitative and computational methods may be required, including integrating epigenetic information with existing databases. Mapping epigenetic information to the human genome will maximize its usefulness and allow us to determine the relationship between genetic and epigenetic variation in the population, and its relationship to cancer. Such a project would be immediately worthwhile for its basic science insights as well as for enabling the use of epigenetic information in prevention or therapeutic strategies.

Introduction

The think tank began on the first evening with brief presentations of scientific advances, questions and barriers by each participant. The following day, three discussion sessions addressed Human Cancer Epigenetics; Basic Mechanisms in Chromatin Structure and Epigenomics; and Technology and Translation. There was also a presentation on the European Epigenome Program. On the final morning, the discussions were summarized, and the participants created a prioritized list of major and minor recommendations to NCI which will best advance the science.

In each session, the discussion was organized around a set of major questions. The panel began with these questions, and proceeded to discuss the issues and most critical next steps, and concluded each discussion with the next crucial questions to be answered and needs for the field to progress.

Human Cancer Epigenetics

Mechanisms of cancer initiation and progression by epigenetic events
Epigenetic changes are modifications to the genome that are heritable during cell division but do not involve change in DNA sequence. Expression of individual genes, of gene regions or a whole transcriptional program are regulated not by the DNA sequence, which is the same in every cell, but by the epigenetic marking and packaging that regulates chromatin structure. DNA methylation, histone variants and post-translational modifications, nucleosome positioning factors, boundary setting elements, and chromatin loop and domain organizer complexes are all elements of the epigenome.

Alterations in methylation, imprinting and chromatin are ubiquitous in cancer. A clear link between epigenetic changes and cancer, as well as other diseases, has in many case been established. And beyond these specific instances, epigenetics has the potential to explain many aspects of cancer: these marks can be modified by environmental factors; the age dependence of cancer corresponds to epigenetic changes in aging; and the quantitative nature of epigenetic changes corresponds to changes seen in cancer. The frequency of epigenetic alterations is orders of magnitude greater than that of genetic mutation, but unlike mutation, they are reversible.

The most frequently identified epigenetic alterations in cancer are changes in DNA methylation, although others have been identified as well. DNA methylation at CpG sequences (frequently in stretches called “islands”) is a basic marker of the genome that appears to have several functions. Methylation of gene regulatory regions has long been associated with transcriptional inactivation, and differences in methylation pattern in the promoter regions of tumor suppressor genes are documented in some cancers (e.g., p16/INK4a in pancreatic cancer). Hypomethylation is thought to either permit gene expression where it should not occur, or to encourage chromosome instability, especially when it occurs in the centrosome region. In general, global hypomethylation with local hypermethylation of CpG sequences is associated with cancer, although this concept may eventually be revised.

Two well described instances of epigenetic changes involved in cancer were used to illustrate different epigenetic mechanisms of cancer initiation throughout the meeting. Both involve loss of imprinting (LOI), which is DNA methylation that silences specific genes on either the maternal or paternal allele lineage. In Wilms' tumor, approximately 95% of cases do not follow the two-hit model as it has been understood, with an inherited mutation in one allele of a tumor suppressor followed by a mutation in the other allele; instead, the second hit usually is LOI that activates the IGF2 gene. In colon cancer, IGF2 is also frequently activated, but another mechanism is involved, in which genetic and epigenetic mechanisms appear to be complementary. The LOI seen in these tumors also exists in the surrounding normal tissue and appears to predate the tumor; the theory is that this "field effect" renders the mucosa highly susceptible to subsequent insults, such that the overexpression of IGF2 (or other growth factors) due to LOI renders mutations such as APC mitogenic rather than apoptotic.

While the link between epigenetic changes and cancer is clear, the mechanisms behind the abnormal changes – indeed, the mechanisms by which normal patterns are established and maintained – at this time are largely unknown. The relationship between cause and effect is also controversial; while histone acetylation and DNA methylation are permissive for transcription, there is conflicting evidence as to whether these marks are the signals for gene expression, or if they follow the passage of the polymerase and serve to reinforce the "active" gene structure. DNA methylation differences at specific loci exist in Wilms' tumor and Beckwith-Wiedemann syndrome, but a mosaic of methylation states is observed in the population of tumor cells. This raises the question of the degree to which the methylation pattern is a cause of the malignant growth. Because normal methylation patterns are not well defined, it is difficult to determine what is abnormal in cancer. In spite of the widely documented link between methylation changes and cancer, none of the known methyltransferase genes are known oncogenes or tumor suppressors. Thus, several crucial questions remain to be answered for methylation to be optimally used in cancer diagnosis or treatment.

A fascinating current issue is whether there are sequence variations in the human genome that create susceptibility differences by predisposing the site to epigenetic changes. Noncoding DNA such as repetitive elements and transposons may play a role in genome-wide methylation patterns. These elements are the main sites of CpG methylation in the genome. The example discussed by one think tank participant was that of a transposed element that altered the methylation pattern at a gene locus in a mouse model in a manner that responded to dietary factors. In the offspring, the transposon could be differently methylated depending on the maternal diet, with a higher methylation state leading to an obesity phenotype, demonstrating a means by which disease states could have an underlying genetic/epigenetic basis that responds to environmental factors. In this example, a genetic event led to the possibility of stochastic epigenetic changes. There is currently no known human locus where methylation variation leads to trait or susceptibility variation, or where a transposable element creates a site of potential expression variation; however, the likelihood was thought to be high that such examples will be found.

Histone modification, especially acetylation, has long been recognized to influence transcriptional capacity, but we must now begin to understand how DNA methylation and histone modifications

have coordinated effects. A self-reinforcing cycle between these two epigenetic marks appears to exist, such that transcriptionally active or inactive regions of chromatin (i.e., euchromatin or heterochromatin) are self perpetuating through replication. The most important next step will be to understand the degree to which changes in these epigenetic marks are the causative regulatory factor in chromatin function changes, or whether other complexes play the regulatory role and these marks play a mainly reinforcing or maintenance role. This issue was the focus of some debate, since different results have shown these marks to play both an active and a passive role. Histone acetylation, for example, has been shown to cause transcriptional activity and also to result from the passage of the polymerase complex, thus being self-reinforcing, while loss of single specific histone acetylations can cause the spread of heterochromatin over a gene region. The recently proposed histone "code" of modifications was challenged by some panel members because such questions of cause and effect cannot yet be answered.

While DNA methylation of specific genes or general pattern changes are the most widely reported epigenetic signature in cancer, a cancer connection with chromatin organization is also well documented. SNF5, a subunit of the SWI/SNF chromatin remodeling complex family, is deleted in the aggressive pediatric cancer rhabdosarcoma. It acts as a tumor suppressor, activating expression of p16/INK4a and repressing several cyclins and CD44, a cell surface glycoprotein implicated in metastasis. Another example is that of the tissue-specific BUR (base unwinding region)-binding protein SATB1, which appears to organize chromatin loop domains and act as a positioning factor that attaches genes to Matrix Attachment Regions (MARs), giving them the proper nuclear localization for transcriptional activation. SATB1 specifically binds to the major break region in the characteristic chromosomal translocation of some lymphomas, creating the MAR which is thought to be a susceptible site. It will be interesting to search for similar proteins specific to other cell types and determine their expression or activity in cancer cells, since SATB1 has the ability to cause global changes in gene expression pattern.

Several specific needs were identified to advance our understanding of the epigenetic mechanisms functioning in cancer. It will be important to define genetic-epigenetic relationships; e.g., to identify mutations that lead to epigenetic changes. It will also be necessary to determine the relationships among such transacting modifiers of the epigenome as methyltransferases, transcription factors and genome organizers, as well as identify the factors and/or pathways that are activated in conjunction with these relationships. Another significant area in need of investigation is the effects and dynamics of epigenetic modifications of repetitive elements, transposons, pseudogenes, and novel transcripts. New insight into the organization and functioning of transcriptional networks and pathways may be gained from understanding principles of chromatin organization. New experimental model systems (e.g., to study RNAi; global CpG methylation and hypomethylation in cancer; pseudogenes in heterochromatin origins; and histone acetylation/deacetylation) are needed to study these features. Finally, it will be important to identify and develop applications for reagents for site-specific modifications of histones, such as acetylation-site-specific antibodies, and identify ways to decrease cost and increase availability of these reagents.

Interplay of epigenetics and cancer – where does epigenetics fit in the big picture of initiation and progression?
Despite the cancer associations highlighted above, the question remains of how frequently epigenetic gene activation or silencing is a "first cause" event that initiates dysplasia or creates susceptibility. Depending on the cancer, the first hit may be a mutation followed by epigenetic changes leading to a functional second hit, or abnormal or age-related epigenetic changes may create conditions that enhance environmental assaults or release suppression of tumor progression. Once the primary tumor is under way, the many steps by which a motile, metastatic phenotype emerges are likely to include epigenetic changes in gene regulation. Tumor cells generally must obtain a motile, spindle phenotype while traveling to the new site, then change phenotype again as they become established as a metastatic lesion. Regarded in this light, the reversibility and changeability of epigenetic states, as opposed to mutations, is a thought provoking idea. The reexpression of genes in metastatic cells, and the regulation of some genes involved in the angiogenic switch, are thought potentially to have an epigenetic etiology. In all of these questions, the lack of experimental models is a major barrier. Specifically lacking are models of very early epigenetic changes or very early stages of disease.

One promising focus for research is the question of epigenetic methylation changes as a timing event in the path from stem cells to differentiated cells to tumor cells (i.e., DNA methylation as a developmental timer). Another area of investigation might involve examination of epigenetic changes in "normal" cells and cells predisposed to cancer, to distinguish between normal epigenetic variation or changes with age, and potentially dysregulating epigenetic changes leading to disease. The epigenetic status of normal cells surrounding tumors, including angiogenic, immune, and stromal cells, may also play a significant role, and is virtually unknown. It will also be necessary to identify the relationship of epigenetic changes to chromosome instability and recombination in cancer.

Comprehensiveness of epigenetic studies of cancer – how much information do we need?
It will be important to conduct a genome-scale analysis of domains of epigenetic change at the local level, in gene groups or clusters, and globally. A genome-wide screen for DNA methylation changes would avoid the bias from candidate gene studies; on the other hand, the data set from a single region would allow us to study all epigenetic features, and determine how they relate to each other. There were conflicting opinions on which approach would be most valuable at this time. It will be necessary to investigate the functional significance of specific epigenetic marks in order to better understand how to resolve this question. It is important to determine the relationship between hypomethylation and gene activation, as there are several examples of loss of methylation linked to new gene expression and cancer. Before demethylating agents become widely used to treat cancers, this question must be addressed. The relationship of epigenetic changes to histone modifications is also important, and may involve the results of clinical applications, such as the effects of drugs on gene expression.

It will be highly informative to add epigenetic information to existing databases of genetic information. Gene regions could be layered with information on methylation and other marks that relate to transcriptional activity. However, as discussed in the final section on Epigenomics, a

judicious approach to selecting experimental material and identifying the regions of chromatin to analyze must be used. To date, drugs that target DNA methylation and histone acetylation have been used with some degree of success in the clinic, albeit the more that is learned about the function of these epigenetic marks the less clear it becomes how the drugs work.

Basic Mechanisms in Chromatin Structure

Key players in chromatin organization
A number of molecules with critical roles in chromatin organization, remodeling and epigenetic modification have been identified in recent years. However, our understanding of which ones are the key regulators and how they accomplish their roles in development, in the identity of different tissues, or during cellular functions such as replication is far from complete. Further basic research to better understand the mechanisms of action and determine the regulatory pathways of the known enzymes and complexes is crucial and timely. At the same time, it is important to try to broaden the spectrum of known chromatin modifying enzymes, if we wish to form a comprehensive picture. To understand how chromatin structure marked by specific epigenetic modification is established, it is necessary to identify additional players at the levels of DNA, RNA and proteins, and then go on to identify the dynamic relationships between them. The role of non-coding RNAs is addressed in a later section.

There is evidence that DNA methylation/demethylation affects gene transcription by signaling chromatin structure changes, rather than simply "blocking" access by polymerases or transcription factors. Thus, since DNA methylation status is the most widely and readily measured epigenetic mark, it will be important to determine its role in chromatin organization. At the same time, it is important to search for long-term epigenetic marks other than DNA methylation and histone modification.

A major challenge in the near future will be to identify key determinants of locus-specific chromatin structures; that is, key histone modification sites and key cis-acting DNA elements (e.g., BURs, which are recognized by global gene regulators) which have dominant roles in the formation of specific chromatin structure, as well as the protein complexes which interact with these sites. To understand how epigenetic states are established, it will be necessary to identify new interacting proteins that are involved in chromatin targeting and remodeling. These partner proteins currently include PCNA, RNA polymerase, nucleosome assembly factors, CTCF, and loop organizers. Other factors must be presumed to exist which have yet to be identified; however, it is not too soon to begin to ask which part of the genome is regulated by which of the known factors.

The known molecules that regulate accessibility of the DNA to regulatory factors include DNA methyltransferases, histone variants, and enzymes that covalently modify histones through acetylation, phosphorylation, methylation and other chemical moieties. Another class of chromatin-modifying complex includes those that move nucleosome positions to reveal or occlude DNA sequence. Three families of these complexes have been identified – SWI/SNF, ISW1, and

NuRD – all of which consist of a core subunit with an ATPase domain, plus associated subunits presumed to be regulatory. Other chromatin modifiers include the Methyl-CpG-Binding Domain (MBD) protein family, Position-effect variegation (PEV)-related factors, and chromatin loop organizers. Although presumed to exist based on the observed dynamics, neither DNA demethylases nor histone demethylases have yet been identified. Discovery of such crucial enzymes will have high impact on understanding of chromatin structure maintenance and remodeling. Additionally, any of these enzymes, loop organizers or boundary element-binding proteins may be subject to post-translational modifications that regulate their functions, although very little is known at all about such regulation of these molecules. Many of these proteins can be studied in yeast; for example, yeast studies have demonstrated that Sir2 histone deacetylase induces the spread of heterochromatin, while Sas2 acetyltransferase disrupts heterochromatin, and together, they appear to establish chromatin boundaries. Efforts are now being directed at understanding the roles of known higher eukaryote analogs of these enzymes.

Mechanisms for higher-order folding of chromatin

To understand chromatin structure and function, it is essential to expand our knowledge of how chromatin is packaged beyond the nucleosome structure. Which proteins are responsible for higher-order packaging (e.g., loop domain organization) and the biological significance of such chromatin folding are fundamental questions that have only recently been formally addressed. It will be an important step to identify more loop organizers in addition to SATB1, in different tissues and at different developmental stages, and to distinguish between general and specific proteins. This will require improvement in the highly variable loop capture assay, and the development of new technologies to analyze higher-order chromatin structures (e.g., in mega-base domains).

It is necessary to determine the role of loop structures in delineating functional chromatin domains that are marked by specific epigenetic modifications. Such an approach, which introduces the third dimension into epigenetic analysis, will reveal the contribution of higher-order folding to epigenetic modification and overall genome organization. It will be important to identify the mechanisms that regulate functions of loop organizers such as SATB1, and to discover additional analogous protein complexes. Finally, it will also be important to analyze the roles of loop structure and loop organizers in cancer and explore their diagnostic utility. Some observations suggest that the organizer protein BORIS competes with the related protein CTCF to disrupt boundary function, and may alter transcription pattern on a large scale when abnormally expressed in cancer. Additionally, the global organizer SATB1 is aberrantly expressed in some cancers. It will be significant to explore whether these proteins could be effectively targeted in cancer therapy.

The role of RNA in gene regulation and genomic organization

Non-coding RNA appears to play several roles in chromatin regulation. Emerging evidence suggests that micro RNAs (miRNA) or siRNAs can regulate over very large chromatin territories. These small RNAs, produced by defined processing pathways, are known to silence genes at the translational and perhaps also the transcriptional level; however, a role in maintaining heterochromatic regions is currently only postulated. It will be important to determine the

generality of RNAi control in the genome beyond repetitive elements, and the function of RNAi in genome organization. Currently it is unknown how many miRNAs are present in our genome. It is still to be determined how they can be identified, and how their targets can be validated. Then, moving beyond quantification of miRNAs, we will have to determine the role of miRNA in gene expression, where it acts, and what link if any it has to cancer. Another intriguing question is whether viruses and other pathogens modify the epigenetic profile of the genome in ways that involve siRNAs and/or miRNA, leading to dysregulation and potentially diseases such as cancer.

Intergenic transcription is a novel mechanism by which heterochromatin may be excluded from transcriptionally active regions. It will be necessary to determine the link between intergenic transcription and RNAi. An important question is whether siRNA-based regulatory networks exist, and what the role and mechanisms of large noncoding RNA transcripts (e.g., Xist) are in chromatin function.

Epigenetic inheritance in somatic cells

This area of chromatin epigenetics research in particular is still in its early stages and warrants further, extensive investigation. DNA methylation is initiated de novo by DNA methyltransferases and maintained by Dnmt1, and this provides a mechanism of epigenetic inheritance. However, there are epigenetic phenomena in organisms such as *Drosophila* that lack any significant DNA methylation. Epigenetic states may be transmitted through specific chromatin structures, such as histone modifications, but how this is achieved is largely an enigma. Lysine methylation on histones 3 and 4 provides relatively stable marks on histones. But, again, how such marks are maintained through the cell cycle or through development is not known, given that there are mechanisms for active loss of histone methylation, in addition to passive loss by dilution at each round of replication. Replication timing may be a crucial mechanism in setting or preserving the epigenetic marks, since euchromatin replicates early in the cell cycle, while heterochromatin replication is delayed. The histone modification "code" has not yet been demonstrated to be heritable. The true function of histone modification patterns remains to be determined, and there was disagreement as to whether an active role in determining chromatin functions has been convincingly demonstrated, as opposed to a more passive role as a result of replication or transcription.

The question of what epigenetic information is preserved through replication, as well as the related question of what modifications are causative of chromatin organization as opposed to simply the more passive result of replication, were the topics of extensive discussion. The roles of DNA methylation and histone modifications were discussed in this light.

A critical next step is to determine the mechanisms of the replication of specific chromatin structure, identifying the DNA sequences and the targeting of chromatin remodeling factors involved in setting up specific chromatin structures during or after DNA replication. Part of the same effort will be to test the role of histone modifications in epigenetic inheritance through the cell cycle, and to determine the dynamics of histone modifications during DNA replication and the cell cycle. We must deepen our understanding of how DNA methylation relates to gene expression and chromatin structure.

Epigenetic remodeling and genome stability
It will be important to determine the mechanisms of regulating or reversing the more stable epigenetic marks. For example, DNA methylation can be either an active or a passive process, as discussed above, and once methylated DNA demethylation may be via an active or passive mechanism. Also, when histones are replaced with variants that have different functions or modification sites, are these mechanisms active regulatory mechanisms or secondary results of other functions? In any case, it remains unknown if they have relevance to cancer.

Chromosomal rearrangements are a common feature of a wide variety of neoplastic lesions and are thought to have a causal role in tumorigenesis. Genome instability, leading to carcinogenesis, may occur due to the genome-wide DNA hypomethylation observed in aging. Global hypomethylation is frequently observed in tumor cells, and has been associated with abnormal chromosomal structures, as observed in cells from patients with ICF (Immunodeficiency, Centromeric instability and Facial abnormalities) syndrome. However, the mechanistic link between genomic instability and hypomethylated genomic DNA remains to be discovered.

It will be very important to determine the mechanism for the creation of neocentromeres. Centromeres are key players in genome integrity, and defects in their function results in mis-segregation of chromosomes and aneuploidy. Neocentromeres are ectopic centromeres that originate occasionally despite the complete absence of normal centromeric alpha-satellite DNA in the regions. No alteration of DNA sequence occurs, and it remains unknown how they are created, but they are still able to form a primary constriction and assemble a functional kinetochore. Neocentromeres have been detected in certain cancers, and are thought to be generally detrimental. However, no systematic screening for these structures has been conducted.

Finally, it will be important to investigate the role of chromosome territories, and factors that organize these territories, in recombination and genome instability. Chromatin must be viewed in the context of whole chromosome territories, organized into specific nuclear zones. These territories occupy non-random positions in the interphase nucleus. The mechanisms confining each chromosome in a defined territory remains unknown, however, as well as the question of whether changes in the position of the territories occur during differentiation or carcinogenesis. The specific location of genes within nuclei is important for proper gene expression, and nuclear architecture contributes to the localization of genes. Thus, improper positioning of genes may contribute to their improper regulation, potentially leading to cancer. Cancer is associated with disruption of gene expression patterns and global disorganization of chromosome organization within the nucleus. The roles of nuclear architecture, including chromosome territories, in genome stability and proper gene regulation must be studied in depth.

Epigenomics, Technology, and Translational Opportunities

We are presently approaching the stage where we can begin to "genomicize" epigenetic information. At the think tank, much of the discussion of needed technology and translational opportunities took place in the context of developing a Human Epigenome Project, analogous to the Genome Project. The science is clearly developed to a point that it is timely to plan a strategy for such an effort. The key question is what information the efforts should focus on obtaining.

There are two decisions that need to be made before launching into a large-scale effort. First, what epigenetic information should be gathered and incorporated into the Epigenome? DNA methylation is the first consideration, and most of the investigators want to make genome-wide DNA methylation profiling using microarrays the top priority. After that, other important elements that should and could be incorporated include unequal allelic gene expression (including imprinting); histone variants and modifications; chromatin organizer proteins; and cis-acting marks for epigenetic signals. It must be remembered that different types of epigenetic information present different challenges. For example, the two fundamental epigenetic marks, histone modifications and DNA methylation, differ in stability; certain histone modifications can change quickly (acetylation reversed by deacetylases, methylation reversed by histone replacement, etc.), while CpG methylation of DNA is more stable.

Second, what tissue samples should be used? Unlike DNA sequence, epigenetic marks and states certainly differ between tissues and change with the age of the donor. A discussion is needed about what we know and what we need to know, to permit these choices to be made. It would be useful to ask what relationship exists between diversity in the population and diversity in epigenetic marks, and what new epigenetic population-based and family-based algorithms need to be developed.

Key Issues and Potential Pitfalls

New technologies are needed to study some questions of higher order chromatin organization and function, and broader training is needed in current state-of-the-art procedures such as the loop capture assay or the ChIP-on-chip assay. Nonetheless, current methodology is sufficient for conducting genome-wide screens of much of the epigenetic information. Integration of epigenetic information into existing genome databases would be optimal. A computer screen that incorporates all levels of epigenetic information for each stretch of DNA sequence in the genome was envisioned by some of the panel. In principle, data from genome-wide or chromosome-wide bisulfite conversion/sequencing, histone modifications, and mRNA expression profiling, can be overlaid on the existing human genome sequence, to provide an integrated picture of the genome and epigenome. This information, with associated annotations, could be displayed at a resolution ranging from the whole chromosome to the individual nucleotide in a clickable format, simply by adding a series of new "information fields" to available, widely used genomic databases. With this coordination of data, the project would begin to provide answers about the potential for epigenetic therapy and how the epigenome can be targeted for chemotherapy or chemoprevention.

Discussion of precisely what epigenetic marks and what tissues would be included first did not produce a consensus at this meeting. All agreed that future organizational meetings would be required to plan an Epigenome Project. This project should take an approach somewhat like the Human Genome Project, with a sequencing effort that provided baseline information on which further genetic variation could be identified. A comprehensive analysis of DNA methylation is needed, including quantitative variation in the density and breadth (i.e. numbers of modified residues) of methylation marks. In addition, novel bioinformatic approaches must be developed.

In contrast to the DNA sequence, the epigenome varies among different normal cell types and, perhaps more dramatically, between normal and cancer cells. So, selection of the "reference cells" and "reference tissues" for analysis in the Epigenome must be done with extreme care. To deal with the problem of cell heterogeneity, microdissection will be essential in preparing the samples. An even more fundamental issue concerns the identification of the closest normal counterpart for any give human cancer type, i.e. intelligent and meaningful selection of the "control" samples for comparing to the cancer samples. Significant progress is being made in this area, largely through gene expression profiling and immunophenotyping, and this information needs to be taken into account in making this critical decision on sample selection. It would be a disastrous waste of resources to analyze the epigenome in tissue samples that subsequently prove to be poorly defined and heterogeneous.

Feasibility of a Human Epigenome Project

Despite these issues to be worked out, it was the consensus of the panel that beginning an Epigenome Project is feasible now. Methods for determining the pattern of CpG methylation of DNA and corresponding patterns of histone modifications across the human genome are well established. Chromatin immunoprecipitation/ChIP-on-chip assays can rapidly score histone modification, potentially tiled across an entire genome, and bisulfite conversion/DNA sequencing, as well as several complementary approaches including microarray-based methods and genome fractionation using McrBC endonuclease, can achieve the same objective for DNA methylation. Furthermore, such a Project will be highly leveraged by the completed Human Genome Project.

The most useful next steps towards beginning a Human Epigenome Project were discussed. It will be crucial to identify epigenetic markers other than methylation, and develop high-throughput tools to target and characterize these markers fully. Also, it will be necessary to identify new compounds and reagents, and compare these agents against standardized reference sets. The tissue bank needed for the project must be designed carefully. It must contain adequate amounts of material across appropriate populations, and have samples varying in age, tissue type, and disease state. A significant consideration is how banked tissue samples should be processed and stored.

A hypothetical organization of a Human Epigenome Project would include several Core Facilities, as well as a number of Pilot Projects that would be expected to leverage the epigenetic data in specific research areas:

Core Facilities
DNA Methylation Core
Histone Modification Core
Gene Expression Core
Bioinformatics Core (interfaced with the caBIG consortium http://cabig.nci.nih.gov/caBIG/)
Tissue/Pathology Core

Pilot Projects including but not restricted to
Genome Imprinting and Allelic Asymmetry
Interindividual Variation in Epigenetic Marks
Mechanisms of Epigenetic Marking
Environmental Effects on the Epigenome
Cancer Detection using Epigenomic Targets
Epigenomic Drug Targets in Cancer Treatment
Bioinformatic Approaches to Epigenome Analysis

Benefits of a Human Epigenome Project
Epigenetic marks, defined as modifications of the genome that can be faithfully propagated through cell divisions, and that affect central biological processes like gene expression and genome stability, have been amply shown to influence both cancer predisposition and tumor progression. But in contrast to the DNA sequence, our understanding of these epigenetic marks remains fragmentary, with most if not all of the available data coming from highly selected regions of the genome, examined by non-exhaustive methods. It is likely that a comprehensive analysis of the epigenome, carried out in carefully chosen and well defined normal and cancerous cells/tissues, will produce some truly surprising and paradigm-shifting results, and may well yield fundamental insights into cancer pathogenesis, detection and treatment. This integrated epigenomic information will allow us to begin answering the question of what is the basis for epigenetic therapy and how can the epigenome be targeted for chemotherapy or chemoprevention.

Following some discussion of the European Epigenomic Network of Excellence (see below), Think Tank participants discussed pursuing a similar effort in the United States that would comprise a significant, dedicated investment whose management and direction would be formulated by an established network or committee. The justification for pursuing such a project included:

- The series of discoveries in the last 10 years showing that epigenetics is central to understanding of the development and genetics of cancer.
- Moving forward with an Epigenome Project is timely in light of the completion and findings of the Human Genome Project.
- To gain a better understanding of human disease, including cancer, investigators need to take a genome-wide perspective of epigenetics.
- It will facilitate the standardization and dissemination of information across the research community.
- It will increase visibility of the research community as a coordinated group.

- Improved cost effectiveness, decreased redundancy, and better comparisons are expected outcomes with standardization across research teams, labs, and groups.
- Keeping the project in the public domain would help not only to maintain but to encourage openness of communication, development, and discoveries, including technological developments and advances.
- This strategy would provide an infrastructure for reviewers to improve genome study sections through increased awareness, expertise, and innovation.
- It will provide a much-needed, coordinated opportunity for quantitative/statistical epigenetics and the development of new tests, tools, and measures.
- It will provide a missing component of integrative biology.

Special Presentation: European Union 6th Framework Programme – The Epigenome

Dr. Patrick Varga-Weisz summarized the EU's Epigenomic Research Program, which provides a platform and structure to facilitate the coordination of epigenetics research beyond local efforts. The program takes advantage of established research groups and facilitates the development of new research talent. A "core consortium" of 25 leading scientists in epigenetic research receives funding through a Joint Program of Activities (JPA) for the first phase of the Epigenomic Network of Excellence (NoE), which will run from 2004 through 2009 with an annual budget of approximately 12.5 million Euros.

The purpose of the Epigenome Program is to integrate European epigenetics and epigenome research; to support new researchers; and to facilitate dialogue among investigators. More specifically, the three key goals are to coordinate and support:

- European epigenetic research: To unravel epigenetic mechanisms in the post-genomic era, the NoE will prioritize research into molecular mechanisms of epigenetic control. To achieve this goal, the NoE will mount an internationally competitive research program through a Joint Program of Activities (JPA).
- The next generation of European scientists: A funding program for 22 independent investigators will distribute monies in two rounds over 5 years to integrate the most promising newly established teams (NET program) in Europe.
- An interactive Website: Scientists and the public will be provided with up-to-date knowledge of the field through a Web site maintained by the NoE. This Web site [for the preliminary Web site, go to http://www.epigenome-noe.net/] will develop into a major resource for the scientific community, and it will seek to establish a dialogue with the public by providing first hand knowledge in an appropriate form for a non-specialist audience.

The NOE research program is organized into eight distinct subprograms focusing on different aspects of epigenetic control:

- Chromatin modification

- Nucleosome dynamics
- Non-coding RNA and gene silencing
- Xi and imprinting
- Transcriptional memory
- Assembly and nuclear organization
- Cell fate and disease
- Epigenomic maps

Specific Recommendations for the NCI

Specific major recommendations
1. Establish a Human Epigenome Project of analogous scope to the Genome Project. This was the highest priority recommendation. The Project could include:

- Developing a baseline of epigenetic information and profiles that includes reference samples, and expanding exploration of integrative, comprehensive epigenetic profiles of normal and tumor tissues.

- Developing detection tools to genomicize epigenetics; i.e., to lead to robust, cost-effective, high-throughput, genome-level analysis.

- Developing novel bioinformatic approaches to epigenetics, including computational and statistical tools and comparative epigenomics. A computer screen which incorporates all levels of genetic/epigenetic and chromatin knowledge was envisioned.

2. Organize a planning meeting, and establish a Working Group, to define the needs for a Human Epigenome Project. This was unanimously agreed upon. The questions to be determined include: tissues to be used; how many individuals; relationship to Genome Map; what will be measured – DNA methylation, histone modifications, other chromatin proteins

General recommendations
3. Support technology development for epigenetic profiling as well as for studies of basic chromatin organization is needed. This may be addressed as part of Epigenome Project; or may relate to Recommendation 4 below; or may require an R21 initiative to develop technology.

4. Encourage and foster R01-based epigenetic research, particularly for basic research in mechanisms of chromatin structure and gene regulation.

5. Encourage and support translational research with a focus on early epigenetic variations and translational consequences, e.g., how do these variations alter adult susceptibility to disease?

6. Expand training opportunities, including cross-platform training for post-docs, junior and senior faculty, and institute U.S.-European collaborations or cross-training agreements.

Executive Summary of the Inflammation and Cancer Think Tank

Inflammation is a response to acute tissue damage, whether resulting from physical injury, ischemic injury, infection, exposure to toxins, or other types of trauma. It can play a role in tumor suppression by stimulating an antitumor immune response, but more often it appears to stimulate tumor development. Epidemiologic and clinical research indicates an increased risk of certain cancers in the setting of chronic inflammation. For example, two inflammatory bowel diseases, ulcerative colitis and Crohn's disease, predispose to cancers of the intestinal tract. Basic research, in turn, has shown that many of the processes involved in inflammation (e.g., leukocyte migration, dilatation of local vasculature with increased permeability and blood flow, angiogenesis), when found in association with tumors, are more likely to contribute to tumor growth, progression, and metastasis than to elicit an effective host anti-tumor response.

Interestingly, inflammation functions at all three stages of tumor development: initiation, progression and metastasis. Inflammation contributes to initiation by inducing the release of a variety of cytokines and chemokines that alert the vasculature to release inflammatory cells and factors into the tissue milieu, thereby causing oxidative damage, DNA mutations, and other changes in the microenvironment, making it more conducive to cell transformation, increased survival and proliferation.

Chronic inflammation appears to contribute to tumor progression by establishing a milieu conducive to development of different cancers. However the precise mechanism by which it does so remains to be determined. Infection is a common cause of inflammation, and evidence indicates that the presence of microbes can be a cofactor in the tumor promoting effects of inflammation. Tumor cells produce various substances that attract inflammatory cells, which then secrete an array of soluble mediators. These further stimulate proliferation of the initiated cell, tissue disruption in the stroma, and tumor growth. Leukocyte infiltration, and particularly macrophages, can lead to enhanced angiogenesis, which is associated with a poor prognosis in some tissues.

The role of inflammation in metastasis is less well defined than its roles in cancer initiation and progression. The soluble mediators secreted by tumor-associated leukocytes promote cell motility, and induce angiogenesis, vascular dilation and extravasation of tumor cells. Particularly interesting is the recent finding that metastatic cells leave the tumor as microcolonies, containing lymphocytes and platelets as well as the tumor cell. Inflammation continues to play a role at metastatic sites by creating a cytokine milieu conducive to tumor growth.

Although there is a strong association between chronic inflammation and cancer, investigators have not yet uncovered all the molecules, pathways, and mechanisms involved, and numerous questions remain to be resolved about the mechanisms and targets of pro-inflammatory mediators of tumor development. These are articulated in the body of the report and the recommendations that follow. Furthermore, to understand the role of inflammation in tumor formation and progression, we also need to understand its role in maintaining homeostasis and responding to damage in normal tissue. An appreciation of the importance of inflammation has already led to clinical trials of anti-inflammatory drugs (e.g., COX-2 inhibitors) for cancer prophylaxis and treatment. The results obtained will provide clues to the dominant mechanisms at work, and will help in the design of a new generation of interventions.

Introduction

Inflammation involves a complex set of interactions between soluble mediators and immunocytes, triggered in response to tissue injuries that include trauma, infection, toxic agents and autoimmune responses. Such injuries trigger a cascade of cellular infiltrations and cytokine releases that result in local cellular proliferation and repair of tissue damaged. While sustained proliferation alone is insufficient to initiate cancer, a functional relationship between inflammation and cancer has been recognized for a long time. The current discussions centered on our current understanding of the role of inflammation in cancer initiation, progression and metastasis and highlighted areas in which there are major, unresolved questions.

Discussion Themes

I. Inflammation and Cancer Initiation

Although inflammation is a necessary response to clear viral infections, to repair tissue insults - either chemical exposure or injury - and suppress tumor initiation/progression, chronic inflammation is also clearly correlated with increased risk of developing cancer. Inflammation may become chronic either because an inflammatory stimulus persists or because of dysregulation in the control mechanisms that normally turn the process off. Many of the cells, cytokines and systems (e.g., leukocyte migration, dilatation of the local vasculature and angiogenesis) involved in inflammation are also found in a variety of tumors. Chronic inflammation caused by intestinal flora leading to the inflammatory bowel diseases, ulcerative colitis and Crohn's disease, is clearly linked with a higher incidence of colon cancer. The use of mouse models has furthered our understanding of the contributing cellular and molecular factors in colon cancer. Similarly, dietary intake of proinflammatory carcinogens has been associated with prostate cancer. Chronic inflammation resulting from esophageal reflux gives rise to gastroesophageal reflux disease (GERD) and Barrett's esophagus, also linked with a higher incidence of cancer. In the case of Barrett's, chronic inflammation leads to the production of TNFα. This, in turn, induces the nuclear translocation of β-catenin and transcriptional activation of proliferative signals.

The molecular basis for the increased risk is thought to be two-fold: 1) generation by inflammatory macrophages of reactive oxygen (ROS) and nitrogen (RNS) species leads to DNA damage in the surrounding epithelial cells and 2) enhanced proliferative signals mediated by cytokines released by inflammatory cells increase the number of cells at risk for mutations. In combination, DNA damage and proliferative signals create a circumstance conducive to the development of cancer. ROS and RNS can cause extensive damage to essential proteins (e.g., DNA repair enzymes), to DNA and to the mitochondria through a series or cascade of reactions. Among the many possible mutations that may result from oxidative DNA damage are the formation of single- and/or double-stranded breaks and the stimulation of recombination events. Free-radical damage can be caused by the pro-inflammatory prostaglandin enzyme, cyclooxygenase 2 (COX-2), which leads to the production of highly reactive peroxide intermediates at high

levels in a local tissue environment. Drugs that selectively inhibit the COX-2 enzyme, including NSAIDs, are being studied to determine their impact on local tumor biology and development, and in clinical trials. Recent studies have suggested protective effects of COX-2 inhibitors in colorectal cancer and breast cancer. Several small studies of colorectal, non-small cell lung cancer, breast, cervical and esophageal tumors have shown that increased COX-2 levels are associated with poor clinical prognosis. Animal models for colorectal cancer show similar patterns of COX-2 expression and response to COX-2 inhibitors as human neoplasias.

Inflammation results in the recruitment of leukocytes secreting a variety of proliferative cytokines and angiogenic factors to the site of tissue insult. These cytokines, necessary for proper wound healing, stimulate epithelial proliferation, which if unchecked could lead to dysplasias and ultimately cancer. Paradoxically, cytokine deficiency (e.g., GM-CSF, IL-2 and IFNγ) can also lead to tumor development. Immune homeostasis consists of a succession of pro- and anti-inflammatory signals. Loss of the anti-inflammatory signals leads to chronic inflammation and proliferative signaling. The mechanisms involved in the interplay of microbes and defective immune homeostasis is an area that requires further delineation. Future steps will involve clinical studies to determine whether individuals have polymorphisms or genetic variations that affect specific cytokine pathways.

The discussion highlighted the duality of inflammation in controlling and promoting tumor development. While chronic inflammation can establish conditions conducive to tumor initiation and progression, compelling data also suggest that the presence of lymphocytic infiltrates in a variety of tumors is associated with a good clinical outcome. The major challenge in this area is to understand the balance between inflammatory tumor suppression and promotion and how to control it.

II. Inflammation and Cancer Progression

It is generally accepted that chronic inflammation – triggered by toxins, microbes or autoimmune reactions – plays a major role as a tumor promoter. However, the precise function of inflammation in tumor progression remains to be elucidated. Tumor cells produce various cytokines and chemokines that attract leukocytes, which in turn produce cytokines and chemokines that stimulate further tumor cell proliferation; the inflammatory tumor microenvironment is characterized by the presence of host leukocytes both in the stroma and around the tumor. A developing neoplasm can contain a diverse leukocyte population, including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes. These inflammatory cells secrete an array of cytokines, interleukins, interferons and other soluble mediators and further induce secretion of cytokines by resident stromal cells.

Interestingly, both cytokines that promote and suppress proliferation of the tumor cells are produced. As in the case of cancer initiation, it is the imbalance between the effects of these two classes of activity that results in tumor promotion. For example, in the presence of GM-CSF and IL-4, monocytes differentiate into immature dendritic cells,

which migrate into inflamed peripheral tissue, capture antigens and then migrate to lymph nodes to stimulate T lymphocyte activation. Deletion of GM-CSF from Polyoma T transgenic mice reduces cancer progression. This is correlated with reduced macrophage infiltrates, which play a major role in the transition from adenoma to carcinoma. GM-CSF vaccines that stimulate dendritic cell and T cell responses are being combined with anti-CTLA4 treatment in clinical trials to potentiate the anti-tumor response.

In contrast, IL-6 and CSF-1 secreted by tumor cells can skew monocyte differentiation towards the macrophage lineage. Although tumor associated macrophages can kill tumor cells when activated by IL-2, IL-12 or interferon, they also produce a host of compounds – angiogenic factors, growth factors, proteases and cytokines – that either contribute to cancer progression or blunt the anti-tumor response. Macrophages generate a variety of proteases, including cathepsin B, which contribute to tumor growth. Stromal fibroblasts and monocytes enhance this proteolysis. During tumor progression, the degradation of the matrix and stromal fibroblasts appears to be focal, suggesting that widespread degradation may not be necessary for tumor growth. In mammary cancer models, a variety of leukocytes are found at the tumor-stroma interface. A complex interaction between tumor, stroma and inflammatory cells results in the secretion of protease and matrix degradation and the entrapment and degradation of fibroblasts by the tumor.

Other studies have indicated that hypoxia signaling pathways are engaged very early in cancer development. Hypoxia stabilizes HIF-1α which in turn induces VEGF secretion by epithelial cells that stimulates microvascularization and angiogenesis.

The spatial relationship between inflammatory cells and tumors is now being investigated by imaging of a range of live human tumor and associated cells. Examination of the interaction reveals that although T cells will home to the tumor, they stay on the periphery and do not enter. Real-time images of mammary tumors in mice show that the tumor regions are metabolically active, compared with surrounding stroma and fat cells. These regions also have a much greater inflammatory response; associated immune cells, particularly T cells, are very active and mobile. In mammary cancer models, imaging reveals that tumor associated macrophages preferentially line up along the lumenal side of the tumor-associated vessels. Macrophages in this region are relatively static, whereas those at the stromal interface are very active and motile, suggesting differential behavior within the tumor. The functional significance of this remains to be determined.

Although the role of inflammatory cells and soluble mediators in tumor progression is now well documented, the details of the cellular and molecular interplay between stroma and tumor progression remain to be elucidated.

III. Inflammation and Metastasis

Unlike tumor progression, where the role of inflammation in promoting cancer cell proliferation and stromal/matrix degradation is reasonably well understood, the role of inflammation in metastasis is less well defined, although appears to be important. The

cytokines and chemokines secreted by tumor associated macrophages and leukocytes promote cell motility and induce angiogenesis and the growth of tumor-associated vessels, providing an egress route for metastatic tumor cells. The leukocytes also promote vessel dilation and extravasation of tumor cells. Particularly intriguing is the observation that metastatic cells leave the tumor as microcolonies, containing lymphocytes and platelets, the latter allowing attachment to distal organ sites. Tumors that are unable to form such microcolonies are not malignant.

At the distal, metastatic sites, evidence suggests that inflammation continues to play a role in the establishment of metastases. At the sites of prostate cancer metastases in the bone, inflammation triggers the secretion of TGF-β by osteoclasts. TGF-β in turn induces the cancer cells to secrete PDGF which further stimulates the osteoclasts, leading to bone degradation and stimulation of cancer cell growth. PDGFR on tumor-associated endothelial cells increases their levels of bcl-2 and bcl-xl, rendering them resistant to apoptosis and chemotherapy. Similarly, in brain metastases of melanoma, astrocytic infiltrates upregulate MDR (multiple drug resistance) in the tumor cells, making them more resistant to chemotherapy.

Next Steps and Important Questions

- Despite the evidence for the role of inflammation in cancer initiation, progression and metastases, other evidence suggests that cancer proceeds through inflammation-dependent and independent stages. These stages need to be defined and characterized.
- To better understand the critical role of inflammation in initiating and modulating tumor behavior, host-pathogen interactions need to be defined at a molecular level, the phenotypes of hematopoietic cells (leukocytes, monocytes, platelets, etc.) involved in wound repair and tumor initiation need to be characterized and the roles of endocrinological mediators on inflammation need to be examined.
- To better understand the role of inflammation in tumor progression, tumor stage should be correlated with intensity and repertoire of hematopoietic infiltrates and with the levels of cytokines and proteases present; biomarkers for pre-malignant and malignant lesions need to be identified and validated.
- To better understand the role of inflammation in metastasis, better pre-clinical models need to be developed, the molecular relationship between primary and metastatic tumor cells needs to be resolved, the nature of the inflammatory responses that influence primary versus metastatic tumor need to be determined, and the role of the hematopoietic network in tumor extravasation and migration needs to be elucidated.
- Real-time imaging models need to be refined and extended to allow better definition of the relationship between inflammatory and tumor cells that influence the cancer initiation, progression and metastasis process.
- Tumor immunotherapy approaches should include targeted intervention of inflammation-mediated growth, pairing molecular information of inflammatory infiltrates with that of specific tumors. Therapy should be specifically directed to both the organ microenvironment and the tumor.

Specific Recommendations for the NCI

- Continue to support basic research aimed at characterizing the role of inflammation at all stages of cancer and specifically at determining the role of hematopoietic cells in both metastasis and normal development.
- Encourage collaborative studies between basic and clinical investigators. Support studies that characterize inflammatory cells in the tumor microenvironment and correlate these with clinical outcomes and prognosis.
- Consider mechanisms to stimulate multi-agency, multi-institutional and transdisciplinary collaborations to more rigorously define critical interactions that occur between tumor cells and their inflammatory microenvironment.
- Sponsor a series of workshops and/or interactive fora on inflammation and cancer that interface experts from different disciplines (i.e., toxicologists, cellular and tumor immunologists, systems biologists, cancer biologists).
- Develop and standardize reagents and protocols for analysis of archived tissues. Establish a uniform database of existing reagents and well-defined and catalogued tumor types that are or are not associated with inflammation.
- Establish and make available conditional tissue- or cell-specific pre-clinical models to study the biology of inflammation-dependent cancers and to stimulate novel prevention, diagnostic and therapeutic strategies.
- Consider mechanisms to provide investigators with
    - access to imaging tools for *in vitro* and *in vivo* analysis to characterize immunocyte/tumor interactions
    - more sophisticated and cheaper imaging modalities, perhaps by encouraging development in the private sector through the SBIR mechanism
    - training in the use and application of imaging techniques

Executive Summary of the Cancer Susceptibility and Resistance Think Tank

At present, we assume that cancer develops in a particular person because of her/his unique genetic composition and exposures, and the complex interplay among them. Intensive studies of strongly cancer-prone families inheriting the same mutant allele of a cancer gene illustrate that more than just pre-disposing genes confer and modify risk of developing cancer. Many parameters of the malignancy – severity, timing, therapeutic response, and even manifestation of the cancer itself – may differ markedly among cancer-prone siblings and between generations. Technological advances enable us to identify many of the familial and non-familial genes and aberrant processes that confer some of the susceptibility to cancer, and to assess the probability that certain environmental exposures contribute to the likelihood of disease. However, we require a fuller exposition of the genetic architecture of cancers as complex traits if we are to define population and individual cancer risk more accurately. Genetic architecture is the fusion of the effects of interactions among genes and environmental perturbants; it is thus more than the sum of those individual factors. To achieve significant advances in insight about cancer risk, we must propose and test new theoretical models of genetic architecture and use experimental model systems to test the resulting hypotheses about individual susceptibility and resistance factors, their interactions, and their manifestations. This will require that we redirect many of our on-going epidemiologic and cancer modeling efforts toward coordinate activities that play to the strengths of each research community and its unique contribution to the collective effort.

In addition to epidemiologists and cancer modelers, the scientific redirection includes augmenting the intellectual inputs to include computational biologists, mathematical modelers, and evolutionary biologists, among others. To encourage and sustain the new interactions requires access to human specimens and data, development of new analytical strategies to tease apart the contributors to genetic architecture and new algorithms to model genetic and non-genetic interactions, interdisciplinary training in computational analysis and modeling, and computer systems capable of rapid high-level data analyses.

Vision and Overall Goal: To understand the complex interactions of genetic and environmental influences; to predict the risks of cancers for individuals and populations; to enhance prevention, perfect diagnosis, target treatment, improve prognosis prediction, and greatly reduce the burden of cancers.

To achieve these goals, the scientific community must:

- Integrate studies of experimental model systems, human populations, and computational models of molecular signatures in normal and dysregulated states relevant to particular features of cancer susceptibility.
- Improve cancer-related phenotyping (i.e., the analysis of molecular and systemic changes in cancer progression) by applying emerging sensor, imaging, nano- and molecular technologies to individual humans and animals, biological specimens, cells, extracellular matrix components, and cell-cell interactions.
- Identify sources of individual variation in cancer-related biological processes, both genetic and non-genetic, and model the genetic-environmental and stochastic interactions.
- Develop and apply technologies to investigate genetic variation broadly, including alleles, SNPs, haplotypes, and epigenetic alterations of nuclear and mitochondrial genomes, miRNAs, highly repetitive sequences, transposons, gene copy number polymorphisms and other evolutionary molecular features.

Roadblocks and Challenges that currently hamper progress in understanding cancer susceptibility were identified. ***Roadblocks include*** the cost and limited scope of current high-throughput technologies for the molecular characterization of tissues and tumors and the genotyping of individuals; the inadequate scope of systemic clinical measures of cancer initiation and progression; the difficulty of assessing environmental exposures relevant to cancer development; the lack of an adequately large human cohort for prospective studies of susceptibility, along with the necessary infrastructure and biological repositories; the need for long-standing informed consent valid for multiple studies; and the serious lack of infrastructure and requirements for data sharing and standardization. ***The major challenges are*** the development of new statistical and computational approaches adequate to deal with the level of complexity believed to exist in cancer susceptibility, involving multiple genes and environmental factors, interacting in complex, non-linear ways; the integration of human studies with those in experimental systems to optimize understanding of carcinogenic mechanisms; and developing interdisciplinary training and research environments that incorporate the perspectives of biologists and computational scientists.

Introduction

The overall goal of defining cancer susceptibility in terms of the interaction of genetic variation and environmental factors is an ambitious one that cannot be achieved with a single line of investigation. It will require integrating studies in experimental model systems, human populations, and computational models. Studies are required not only on frank malignancies, but on normal physiology and dysregulated states along the continuum from the earliest phase of initiation through metastasis. Several specific requirements were identified:

1. Improve cancer-related phenotyping (i.e., the analysis of molecular and systemic changes in cancer progression) by applying emerging sensor, imaging, nano- and molecular technologies to individual humans and animals, biological specimens, cells, extracellular matrix components, and cell-cell interactions.

Cancers are a very heterogeneous collection of diseases; correlating phenotype with genotype depends on a detailed knowledge of the clinical manifestations of each cancer type. Susceptibility studies are designed to find and test associations between genotype and a specific cancer phenotype. Early studies of tumor classification through the use of DNA microarrays, as in NCI's Director's Challenge program, suggest that molecular phenotyping is a powerful approach to stratify cancers in ways not possible using histopathology and the few previously available molecular markers of cancer behavior. Such disease stratification will sharpen the ability to study susceptibility.

Although microarray studies are useful, and allow researchers to ascertain the expression level of thousands of genes simultaneously, expression analysis alone does not meet the need for a complete phenotyping program. Techniques to expose epigenetic changes and

to define the serum and cellular proteome are the next frontiers. Studies of epigenetic change are important because DNA sequence is not the only heritable change in cells and individuals. Proteomics is important because mRNA levels correlate imperfectly with protein levels and proteins are subject to many post-translational modifications that strongly influence their functions. Metabolomics will become increasingly important as the field of cancer phenotyping matures. Moreover, there is a need to evaluate not only absolute levels of genes and gene products, but their rates of change.

Studies of normal tissues and the tumor microenvironment further demonstrate that it is insufficient to determine the phenotype of the cancer cell; the properties of tissues and alterations of those properties as tumors emerge are what is most relevant. The behavior of a cancer cell is influenced by the cells and molecules with which it has contact; fibroblasts, infiltrating lymphoid and myeloid cells, endothelial cells, the extracellular matrix, and soluble molecules, such as growth factors, cytokines, chemokines, nutrients, and oxygen, all influence tumor phenotypes.

Characterizing tumor phenotypes at this level will benefit many areas of cancer research, and such studies are proceeding. However, it is worthwhile emphasizing how critical these studies are for susceptibility research.

2. Examine all cancer-related biological processes for sources of individual variation, both genetic and non-genetic, and model the genetic-environmental and stochastic interactions of genetic networks and cancer phenotypes.

Susceptibility to cancer results from both genetic and non-genetic individual variation within a human population. Genetic variation is commonly equated with polymorphisms in germline DNA sequence, but this is an over-simplification. Among other potentially relevant genetic complexities are: Imprinting; X-chromosome inactivation; mitochondrial DNA; segmental inversions; deletion or alteration of regulatory elements; jumping elements; tri-nucleotide repeats; and others. Although susceptibility to many cancers is influenced by multiple genes, a recent study suggests that genetic variation is a minor component in cancer susceptibility, contributing perhaps 25% to the risk. The rest is attributable to a wide range of environmental factors, including smoking, diet, hormonal status, chemical exposures, microbial flora, and history of inflammation.

Environmental factors add unsolved methodological complexities to the evaluation of cancer susceptibility. Genotypes can be determined with high precision, but current ways of quantitating environmental exposures generally do not capture all the information needed for a large study. Dietary histories, for example, are notoriously inaccurate. Biomarkers of most chemical exposures are lacking. Microbial flora and inflammation are increasingly recognized as factors in cancer development, but no consensus exists on which factors to measure or how to evaluate their impact.

With so many challenging variables to deal with in susceptibility studies, it is tempting to make the simplifying assumption that the contribution of each susceptibility factor is independent. However, the available evidence indicates that this would be a mistake. In

Epistasis and the Evolutionary Process (2000), Templeton states that, using proper computational methods and algorithms, one finds complex interactions (epistasis) more often than additive, single-factor effects. In studying susceptibility, it is necessary to ask complex questions and develop complex models. A shift away from single gene or single environmental factor analysis to multi-gene, multi-factor analysis is required, but current analytical and statistical tools are inadequate to support such a shift.

3. Develop and apply technologies to investigate genetic variation broadly, including alleles, SNPs, haplotypes, epigenetic alterations of nuclear and mitochondrial genomes, miRNA, and other factors as they are identified.

A few relatively high-throughput technologies exist to evaluate some types of genetic variation, but improvements in existing technologies and the development of new ones are required. Genome-scale sequencing efforts mean that complete genome sequences for quite a number of people will soon be available. One spin-off of this sequencing work has been the development of methods for whole-genome scanning using microsatellites, and now single-nucleotide polymorphisms (SNPs). SNP typing is rapidly becoming the technology of choice for genotyping as the cost is reduced, and the value of the technique will rise rapidly with further cost reductions and the development of analysis techniques that can handle the masses of data generated. The HapMap project leads the way in identifying and validating human SNPs, and in organizing them into haplotypes to maximize the information content of SNP mapping. Similar efforts are underway for model organisms.

However, more progress is needed in other areas of genetic analysis. High-throughput assessment of epigenetic variation is at least a decade behind that of DNA sequence variation, and new techniques are required to interrogate the genomic architecture for regulatory features. With the discovery of potential new sources of genetic variation, such as regulatory micro RNAs, the need for technology development continues to increase.

Specific Recommendations

1. Given the scale of data sets and complex biological systems, create new computational, mathematical, and statistical models and analytical tools that embrace, rather than simplify or ignore, the complexity of variation in susceptibility and resistance to cancers. Support large-scale coordination of studies with calibration, interoperability, data federation, and standardization of data formats, natural language processing tools, and dataset query tools.

Current analytical tools and approaches are inadequate to deal with the complexities inherent in assessing susceptibility (see Requirement 2, above). Orders of magnitude more data and types of data will need to be acquired, assembled, and analyzed into information, and knowledge extracted. This will require development of new approaches to statistical analysis, integration of modeling efforts into the field, and changes in how data are collected, annotated, and stored.

Analytical Approaches

The currently accepted picture of cancer susceptibility, involving multiple genetic and environmental factors acting together in a non-additive fashion, cannot be adequately tested experimentally because of limitations in statistical methods and computational resources. These limitations necessitate the introduction of simplifying assumptions that are likely to be invalid. As a result, potentially critical variables are ignored or oversimplified. The types of analytical methods that need to be developed involve combinatorial problems. Epidemiological analysis is generally restricted to use of a linear modeling paradigm, but such statistical methods are unsuitable for the amount and complexity of data that will be needed to test realistic hypotheses. It is necessary to shift from parametric methods to those that deal more successfully with complex interactions. Significant computational resources are required to deal with high-dimensional data sets; although computing power is increasing, new algorithms and strategies are needed to solve the problems and model complex systems. The methods depend on non-linearity and non-additivity and involve applied biostatistics, a field that moves forward slowly. To anticipate the requirements of research five to ten years in the future, research is needed now in the way statistical methods and design algorithms are applied.

Modeling

While simple problems can be analyzed directly, problems as complex as cancer susceptibility must be computationally modeled to understand what data needs to be collected and what analytical approaches are likely to be successful. Modeling a biological process at early stages of a project can provide information about the amount of data needed to represent the process accurately, and ensure that enough data is collected to make analyses feasible. Modeling can also help to determine what types of interactions among variables are most likely to affect the outcome, and focus computing power on the most productive problems.

Models and experiments are refined in an iterative process that requires regular interaction among model developers, model simulators, and biologists. The proper integration of modeling into laboratory studies or epidemiologic projects requires building a mutually beneficial working relationship with experts in the quantitative discipline in which modelers are typically trained. Because the empirical approach relies heavily on statistics, empirical scientists and statisticians have learned to communicate well. Similarly productive communication between biologists and computational scientists is less common, limiting the development of modeling.

Data Issues: Standardization, Calibration, Test Validation, and Integration

Standardized nomenclature, controlled vocabularies, and natural language processing are needed. Many data sets cannot be pooled because they are not standardized. Data collection must be standardized – especially in the large-scale cohort (see Recommendation 3, below) – and data sets integrated so that comparisons can be made. One approach to standardization and facile data sharing is to aggregate the necessary technologies in one place. This might involve a consortium of many academic institutions using the same standards, so that collection could be correlated with sample

analysis. Even modeling should be standardized to enable investigators access to several well-defined pathways to work with.

Calibration is critical for pooling of some data types, but it is currently difficult because groups do not use the same controls, even where controls are possible. In addition, in some fields such as structural and functional MRI, no calibration exists to permit cross-comparisons among instruments, so no valid comparisons are possible.

Test validation is needed, especially for the most fundamental data types. If thousands of SNPs will be typed on DNA samples, the procedure must be validated. The Think Tank participants recommend obtaining a uniform set of data using lymphoblastoid cells from a control population for this process.

Finally, mechanisms are needed to integrate new data into what already exists. Scientific communities need to pool information in ways that maximize the use of the resources. If a large-scale cohort were to be established, it will pose particular challenges of data integration because the database is likely to be a federated system, assembled by linking datasets at many separate locations. The community's computer development effort needs to build integrated models that can maintain security.

2. Create a common and widely available repository with existing resources of well-selected, maintained, and annotated human biological specimens (immortalized lymphoblastoid cell lines, serum, plasma, urine, etc.) for genetic, epigenetic, proteomic, and metabolomic analyses of early-onset cancer cases, including those with positive family histories. Create common databases. Develop a corresponding resource from a special elderly subpopulation to study resistance to cancer development in individuals with high lifetime exposure to carcinogens. Require data deposition in freely accessible databases, with resource sharing of repository materials.

Developments in analytical techniques, modeling, and data collection and storage, all described above, will become especially useful when applied to large, highly informative population studies. The most ambitious such proposal is described in the next section, but there was general agreement at the Think Tank that at least two studies hold special promise in the short term. The first is a study of early onset cancer patients and their families, to look for particularly influential susceptibility genes.

The second study would focus on cancer resistance, the opposite side of susceptibility. The carcinogenic potential of smoking is very high, but some heavy smokers live to be 100 years of age, and eighty-five percent of smokers do *not* develop lung cancer. Family studies should be developed based on unusual resistance to cancer. For example, Peter Shields and others have cohorts of 90-year old smokers from Veterans' Hospitals; these cohorts would be a valuable addition to a large cohort study. Some individuals develop multiple cancers, but live a long life because their tumors do not metastasize. The interactive genes that confer apparent resistance to metastasis should be identified.

Susceptibility or resistance to progression or metastasis should receive the same attention as susceptibility or resistance to initiation.

These two studies are important on their scientific merits, but they also provide an opportunity to test developments that need to be applied to all studies in cancer susceptibility. In addition, they can serve to pilot high-quality repositories for biological samples, strategies for dealing with privacy issues, and ways to mandate data and resource sharing.

Biological Resources/Repositories

For these studies and the large cohort study described below, a repository for human specimens is a necessity. A common resource of biological specimens should be developed. This should include a standard set for iterative studies, with enough samples for test training sets of data. Repositories containing material useful in the study of cancer susceptibility exist throughout the world, but their optimal use by the scientific and clinical communities is hampered by impediments. Beyond the existing repositories of samples from "common" types of cancer and populations, susceptibility studies require cell lines from families to investigate familial gene variation. The success of ongoing efforts by the NCI to optimize the large-scale and efficient acquisition, storage, annotation, and distribution of biological samples is critical to progress in cancer susceptibility.

Resource Sharing

It is important to capitalize on existing resources, including large datasets and large specimen stores, many of which have not yet been analyzed. Investigators could contribute these samples for analysis, and retain access to the data for a reasonable period of time (e.g., six months) before the data enters the public domain. NCI could facilitate such resource sharing by paying for processing the material.

Privacy, Sample identification, and Patient Concerns

New mandates for patient privacy provide challenges for the study of cancer susceptibility. It is critical to retain some identification with genetic information and to keep family data together, yet current requirements to de-identify data impede progress in assessing gene-environmental interactions. Informed consent is also a potential problem. Durable informed consent must be obtained so that samples are approved for varied, long-term uses. These issues generated considerable discussion at the Think Tank, but the Think Tank participants were optimistic that, given sufficient attention, these problems could be dealt with.

Data Sharing

Little, if any, epidemiological data relevant to cancer susceptibility is freely available for computer downloading. In addition, there are hundreds of linkage studies in human genetics from which conclusions have been published, but the underlying raw data remain unavailable. As a result, experimentalists have no way to compare their data to that of other labs, and computer scientists developing algorithms useful for cancer research have no way to test them, short of entering into formal collaborations. The NIH

or NCI could, and should, exert pressure for release into the public domain of raw data underlying published analyses. In a number of large-scale research projects funded by NCI or elsewhere at NIH, data release is required, and sometimes even facilitated. This practice needs to be much more widespread, and reasonable schedules for data release need to be negotiated with input from the scientific community. In coordination with funding agencies and professional societies, journals can facilitate data release by requiring release of underlying data as a condition of publishing the conclusions.

Datasets are unavailable because they are difficult and expensive to produce. They can be reused by the original investigator in new studies, and no incentives exist for sharing them. No credit is given in university promotion and tenure decisions for releasing data helpful to others in the field. In fact, even collaborating with others to publish analyses of aggregated datasets results in multi-author publications for which most participants receive little credit. The physics research community had no choice but to work together when experiments became exorbitantly expensive, and they have succeeded in changing the culture so that collaborations have intrinsic value. This must happen in the cancer susceptibility research community if important problems are to be addressed.

3. Develop a trans-NIH multiple-disease-oriented framework for initiation of a very large-scale human population study, with multiple ethnic groups, environmental exposures, and family histories. Ensure widespread access to specimens, cell lines, and data.

A central discussion point of the Cancer Susceptibility Think Tank was the need to establish a million-person cohort from which standardized samples and data could be obtained and shared among research groups interested in complex diseases like cancer. The rationale and logistics underlying such a large project, and its relationship to existing cohorts, elicited extensive discussion. Among the considerations:

Why a million-person cohort?
Statisticians working on the proposal for the American Gene and Environment Study concluded that a cohort of this size should be adequate for analysis of gene-gene interactions of the type anticipated to play a role in cancer susceptibility. An article by Francis Collins in S*cience* mentioned the need for a large-scale longitudinal human cohort, taking tissue samples pre- and post-disease, to make best use of the technology – not just after the disease has spread. Case control studies typically yield biased information because they are based on the surviving subset of people with that disease. Few studies have collected long-term human tissue samples.

To design such a large population study is obviously challenging – deciding what to analyze for and how to obtain a large enough dataset that the signal-to-noise ratio is robust in the face of real human variation. The difficulties are compounded by the high cost of the undertaking, which necessitates that the cohort be applicable to research on a broad spectrum of diseases. The studies must be very long-term; thus the information and biological samples obtained and the way in which they are stored must be carefully chosen to preclude their obsolescence. Population characteristics can change

dramatically in a short time, as illustrated by variations in colorectal cancer rates with environment and time.

Building the cohort: Recruitment and Time-frame
A trans-NIH multiple-disease-oriented cohort should build on existing cohort studies, and pilot novel strategies for database construction and recruitment. It is critical to define the minimum data set needed, and to make a *centralized data base* attractive to the cohort consortium. Specimen resources should also be centralized, or at least federated, to ensure access to them. It is also critical to put in place a mechanism for obtaining available tissue when a participant develops cancer and has a biopsy or surgery.

A million-person cohort will reach maximal utility within 5-10 years, but recruitment should start as soon as possible. It is reasonable to anticipate that technology will advance, and sequencing and genotyping will become much easier to do. The 5-year goal should be based on optimizing currently available technologies, while the 10-year goal should assume the availability of high-throughput techniques applicable to large populations.

Specimen Collection, Standardization, Data Analysis, and Accessibility
When investing in such a large study, one important step is to determine whether the current methods of defining diet and other environmental exposures are adequate to capture all the information that may be of value. Exposure measurements must be refined. It will also be critical to know the genetics of cohort participants, from the germline to somatic genetics and disease.

Specimen collection should include fresh tissue, benign as well as malignant, associated with a minimum data set containing residency history and ethnicity. A federated (virtual) facility would store and distribute tissue and data. The "million cohort" database would be accessible to all. Collection of tissue would be enabled, as would international interactions. A grid system should be set up at NIH in which all data from the "million cohort" would be included, with enough computer power to do the modeling and analysis. The level of quality control must give assurance of standardization and comparability of the data.

4. Exploit animal models for identification and validation of cancer phenotypes and susceptibility and resistance genes. Foster comparative genomic and phenotypic studies of human and rodent (mouse and rat) cancer susceptibility and resistance.

Many laboratories use inbred and outbred mouse and rat strains to disclose the genomic loci that are associated with complex traits, such as tobacco carcinogenesis, addiction to alcohol, tobacco and other drugs, diet-induced obesity, and hypertension, among others. There are usually several dozen or more implicated loci, none of which has a major effect by itself. Comparative genomics reveals that the majority of the implicated loci are conserved across three species – human, rat, and mouse – inspiring confidence that murine species can be informative for the genetic and environmental determinants of

human disease susceptibility. The genomes for other species have also recently been sequenced. In particular, championship bloodlines of dogs display inherent susceptibility to a variety of cancers, and the exceptional documentation of these bloodlines will enable cross-comparisons between human populations and animals that share the same environment. An expanding list of publications on the bases of susceptibility and resistance to cancer and other diseases attests to the fact that cross-species comparisons are not only useful, but also more facile, approaches to defining complex traits.

Researchers are more optimistic about understanding genotype to phenotype mapping using animal models rather than human models. It is possible to generate animals in which the activity of several loci are diminished or increased at the same time, and have a strain with a very acute phenotype within a few months. An unbiased set of phenotyping strategies can be applied to the strains to expose novel disease manifestations that may be overlooked in human population studies. In addition to unaltered inbred or outbred mice, mice that are genetically altered to be sensitive or resistant to carcinogens are available. These strains can be bred to others which differ in sensitivity to identify which of the many contributing genes is dominant. Most wild mouse strains have a low incidence of cancer, and studies of them indicate that they have a number of dominant resistance genes. These are only a few examples of existing mouse resources and approaches that can be applied to the problem of understanding cancer susceptibility.

Several years ago, mouse models were designed to map simple, single-gene-based traits; new mouse models are designed specifically for analysis of complex traits and the contributions of environmental effects. An integrative approach using newer mouse models to illuminate results obtained with the million person cohort and to experimentally model the resulting hypotheses would be particularly powerful. Even with the best methods to model gene-gene or genetic-environment interactions, statistical patterns that emerge from the mathematical models require experimental validation in animals. This may require knockout mice for evaluating multi-genotype variations. Not all the experimental techniques are in place yet, nor have mice been engineered to examine all the genotype interactions, but increased efforts to employ integrative human/animal/computational strategies are likely to pay substantial dividends in the study of human cancer susceptibility.

5. Establish a trans-NIH initiative for the development of common mouse resources for the analysis of genetic and environmental effects on development of cancer and other complex trait diseases. Facilitate multi-level, multi-lab studies of specimens after environmental exposures for gene-environment interactions for the development of computational models representing the complex genetic and non-genetic networks associated with disease susceptibility and progression.

Using wild-derived strains and out-bred populations, it is possible to expand genetic variation far beyond that available in standard inbred strains. Recombinant inbred (RI) lines are a powerful tool in the search for susceptibility genes, and, even more importantly, for the conduct of a systems biology approach to the correlation of genotype and phenotype. A number of Think Tank participants expressed considerable enthusiasm

for the creation of a large, trans-NIH resource of RI strains to facilitate study of cancer susceptibility and other similarly complex genetic traits, such as diet-induced obesity. Several trans-NIH groups are evaluating common mouse resources to understand complex human diseases, and there are international efforts underway to coordinate the development and deployment of these resources. The precise nature of common mouse resources for the analysis of complex gene-environment interactions in human diseases still requires consensus-building within the relevant research community. These efforts are the subjects of a series of workshops currently in development.

6. Radically modify training programs to provide immersive interdisciplinary learning environments for biologists, mathematicians, computer scientists, statisticians, epidemiologists, and clinical investigators.

The field of cancer susceptibility needs investigators who can understand and apply high-level computational and modeling techniques in analyzing multiple genetic and environmental interactions. Think Tank participants identified several approaches for overcoming this problem.

Develop cross-training, interdisciplinary programs
Cross-training in biology and computational science is challenging. Most of the participants expressed a preference for educating scientists with programming or statistical genetics or mathematical modeling backgrounds about biology, rather than teaching biologists the math and computational skills. Thus, it is important to identify students with good quantitative skills at the undergraduate level who are comfortable with statistical concepts so that mathematics is a fundamental component of their broad education. It is also important to educate biologists and epidemiologists how to generate and apply models. Too often, data that are analyzed are not translated into a model, or the models that are created only verify the original assumptions and add nothing new.

A major change is needed in graduate training to prepare scientists in quantitative and biological areas, but successful prototypes exist. For example, an innovative Ph.D. program exists for mathematicians, physicists and computer scientists to learn biology as well as use their technical skills, and for biologists and chemists to spend most of their time learning how to model. The field of Neuroscience is unusual in that it allows researchers to return at any stage of their career for cross-disciplinary training. Similar opportunities would be of value in cancer research.

Provide grant support for interdisciplinary education
Most NIH training grant programs limit the opportunities for students to pursue interdisciplinary opportunities or learn computational methods, yet students should be rewarded for seeking training in other areas. Competitive grants and awards designated for that purpose would encourage pursuit of interdisciplinary education. A few NIH grants, awarded to an institution or an individual, do require interdisciplinary training, with two mentors and work in two labs in different areas. Expansion of these programs is strongly encouraged, could be done immediately, and would have an impact within ten years. Anything that increases cross-disciplinary training is an opportunity with a good

payoff. Such training could be encouraged by NCI/NIH and implemented without additional funds – just reconfiguration of the present priorities.

Support summer programs and/or workshops

Although interdisciplinary training is hard to find within an institution, some institutions convene special summer programs. NCI or NIH could support a program of intensive lectures to introduce young people to concepts such as model building. A program might involve 50-75 students, staff support, and facilities that can deal with animals, cells, human material, and computational materials. Students could be familiarized with enough mathematics to feel comfortable, and high-end lecturers could be brought in, as is the practice at courses at Woods Hole and Cold Spring Harbor. Courses of this type have not been implemented to provide interdisciplinary training for mathematicians and cancer biologists. Another possibility for productive interaction would be a genetic analysis workshop, in which the participants could work with a shared dataset and apply their own methods to analyze the data.

Increase access to hardware and large-scale resources

Students occasionally have problems with access to computers, but their main problem is accessing higher-level analyses. Students who are unable to parallelize their data may take months to learn the algorithms, and are unable to perform analyses on the higher power machines. In another three years, there will be more computers with unusual architectures; there is an immediate need to train biomedical scientists to use these new computing technologies and to provide more parallelization training so the next generation of scientists will be able to move into the new systems. Grants to purchase instrumentation to be used for training would encourage institutions to provide training.

Encourage collaborative career development

At many universities, junior faculty members who publish with other people receive less credit than for a solo publication; this strongly discourages collaborations and interactions across disciplines. Yet within academia, there are a few models like Biostatistics, in which people build careers with collaborative studies and still advance the core of their own discipline. The work-style of NIH-supported individual investigator communities is generally counter to the kind of collaborations needed to make real progress. NCI should investigate and implement incentives of recognition as well as financial rewards to encourage necessary culture change.

Biomedical scientists tend to utilize computer scientists as technicians, so computational scientists hesitate to get involved in collaborations in this area. When they are recognized as a critical intellectual partner, computer scientists contribute novel ideas to the design and execution of the project, and analysis of the outcome. It also helps to have a critical mass of computational people involved in biological research, so that they have a community to interact with on theoretical and development issues.

Specific Recommendations for the NCI

Realizing that the goal for cancer susceptibility research set out in the Executive Summary will be possible only with advances in basic knowledge about cancer and genetic variation, and the introduction of new tools and approaches, the following are recommended.

Requirements to achieve the goal

- Integrate studies of experimental model systems, human populations, and computational models of molecular signatures in normal and dysregulated states relevant to particular features of cancer susceptibility.
- Improve cancer-related phenotyping (i.e., the analysis of molecular and systemic changes in cancer progression) by applying emerging sensor, imaging, nano- and molecular technologies to individual humans and animals, biological specimens, cells, extracellular matrix components, and cell-cell interactions.
- Identify sources of individual variation in cancer-related biological processes, both genetic and non-genetic, and model the genetic-environmental and stochastic interactions.
- Develop and apply technologies to investigate genetic variation broadly, including alleles, SNPs, haplotypes, and epigenetic alterations of nuclear and mitochondrial genomes, miRNA, highly repetitive sequences, transposons, gene copy number polymorphisms and other evolutionary molecular features.

Specific recommendations

- Given the scale of datasets and complex biological systems, foster the creation of new computational, mathematical, and statistical models and analytical tools that embrace, rather than simplify or ignore the complexity of variation in susceptibility and resistance to cancers. Support large-scale coordination of studies with calibration, interoperability, data federation, and standardization of data formats, natural language processing tools, and dataset query tools.
- Create a common and widely available repository with existing resources of well-selected, maintained, and annotated human biological specimens (immortalized lymphoblastoid cell lines, serum, plasma) for genetic, epigenetic, proteomic, and metabolomic analyses of early-onset cancer cases, including those with positive family histories. Create common databases. Develop a corresponding resource from a special elderly subpopulation to study resistance to cancer development in individuals with high lifetime exposure to carcinogens. Require data deposition in freely accessible databases, and resource sharing of repository materials.
- Develop a trans-NIH multiple-disease-oriented framework for initiation of a very large-scale human population study, with multiple ethnic groups, environmental exposures, and family histories. Ensure widespread access to specimens, cell lines, and data.
- Exploit existing animal models for identification and validation of cancer phenotypes and susceptibility and resistance genes. Foster comparative genomic and phenotypic studies of human and rodent (mouse and rat) and other mammal cancer susceptibility and resistance.

- Establish a trans-NIH initiative for the development of common mouse resources for the analysis of genetic and environmental effects on development of cancer and other complex trait diseases. Facilitate multi-level, multi-laboratory studies of specimens after environmental exposures for gene-environment interactions for the development of computational models representing the complex genetic and non-genetic networks associated with disease susceptibility and progression.
- Radically modify training programs to provide immersive interdisciplinary learning environments for biologists, mathematicians, computer scientists, statisticians, epidemiologists, and clinical investigators.

Tumor Immunology Think Tank

January 22-24, 2003

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Tumor Microenvironment Think Tank

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Tumor Stem Cell & Self-Renewal Genes Think Tank

March 24-26, 2004

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Cell Decisions in Response to DNA Damage: Survival vs. Programmed Cell Death Think Tank

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Epigenetic Mechanisms in Cancer Think Tank

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Cancer Susceptibility and Resistance Think Tank

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