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Meeting Report

Modern Criteria to Establish Human Cancer Etiology

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Abstract

The Cancer Etiology Branch of the National Cancer Institute hosted a workshop, "Validation of a causal relationship: criteria to establish etiology," to determine whether recent technological advances now make it possible to delineate improved or novel criteria for the rapid establishment for cancer causation. The workshop was held in Washington, D.C., December 11–12, 2003, and participants were among the international leaders in the fields of epidemiology, chemistry, biochemistry, microbiology, virology, environmental and chemical carcinogenesis, immunology, pathology, molecular pathology, genetics, oncology, and surgical oncology. There was a general consensus that the rapid identification of human carcinogens and their removal (when possible) or the establishment of specific preventive and therapeutic measures was the most desirable and effective way to have a rapid and positive impact in the fight against cancer. From a clinical perspective, it may be as important to target initiators, cocarcinogens and promoters, if by removing any one of them tumor growth can be prevented. Future studies should focus on interactions among and between different biological, chemical, and physical agents. Analyses of single agents can at times miss their carcinogenic potential when such agents are carcinogenic only in subgroups of individuals because of their genetic background, diet, exposure to other carcinogens, or microbial infection. Epidemiology, molecular pathology (including chemistry, biochemistry, molecular biology, molecular virology, molecular genetics, epigenetics, genomics, proteomics, and other molecular-based approaches), and animal and tissue culture experiments should all be seen as important integrating evidence in the determination of human carcinogenicity. Concerning the respective roles of epidemiology and molecular pathology, it was noted that epidemiology allows the determination of the overall effect of a given carcinogen in the human population (*e.g.*, hepatitis B virus and hepatocellular carcinoma) but cannot prove causality in the individual tumor patient. Molecular pathology cannot determine the overall impact of a carcinogen in the population but can at times prove causality in the individual tumor patient [such as the detection of high-risk human papillomavirus (HPV) in a cervical carcinoma biopsy]. This is possible when molecular techniques have shown that the agent is required for transformation or malignant growth of human cells (such as antisense HPV strategies showing the requirement for the expression of HPV proteins for tumor cell growth) and when there is supportive experimental animal evidence. Ideally, epidemiology and molecular pathology information together with experimental evidence in animals should be available for the most reliable identification of human carcinogens. All sets of data are not always available, and a rapid identification of human carcinogens is in the best public health interest. Swift validation of a causal relationship when followed by a rapid deployment of preventive and therapeutic approaches should lead to a favorable public health impact (such as hepatitis B virus vaccination to prevent hepatocellular carcinoma).

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Introduction

Infectious agents, chemical substances, and physical factors have all been associated with disease and cancer causation. For acute diseases associated with microorganisms, the association and causative relationships are usually readily established. Koch's postulates (1) provided a framework for pinpointing the associated bacterium with the specific illness it caused. More recently, in situations where such clarity cannot be obtained experimentally, epidemiologists have used the Hill's criteria (2) to link various diseases with extrinsic causative factors. For chronic diseases such as cancer, where there may be a long latent period between the initiation of the disease and overt illness, these approaches have generally been unsatisfactory. Thus, there is a need to clarify and delineate the significant factors that may enable us to establish causal relationships in a more rapid fashion. The goal of this meeting was to bring together leading scientists representing various disciplines to determine whether the integration of classical criteria with recent technological advances may allow us a more rapid and accurate identification of human carcinogens. The meeting was organized and chaired by Michele Carbone (Cardinal Bernardin Cancer Center, Loyola University Chicago, IL) and by May Wong, (Cancer Etiology Branch, National Cancer Institute, Bethesda, MD), and it was attended by 40 invited participants from the United States, Canada, and Europe. To keep the discussion focused on the process of identification of human carcinogens, only those already studied and accepted by International Agency for Research on Cancer (IARC) were discussed. The meeting was a unique opportunity for leading scientists in different disciplines to meet and openly discuss and challenge strengths and weakness of various approaches to identify human carcinogens. This exchange among scientists from a variety of disciplines resulted in an exciting open forum where different perspectives and approaches were debated. A general consensus was often reached on several topics such as the need to integrate molecular pathology and epidemiology for a more accurate and rapid identification of human carcinogens. Other topics produced more contrasting views such as the need to have different criteria to identify viral and bacterial carcinogens *versus* those used to identify chemical carcinogens. Concerning the predictive value of mechanistic studies, the meeting set the initial stage to begin to identify the molecular epidemiological results and the molecular pathology evidence that can serve as a reliable indicator of a carcinogenic agent.

Opening Remarks

In introductory remarks, Michele Carbone pointed to the fact that despite great advances in our understanding of cancer at the cellular and molecular level, there has been little improvement in our ability to treat advanced solid tumors. Therefore, cancer prevention should be emphasized. In the recent past, several infectious agents have been linked to tumor development. This has led to the implementation of preventive and therapeutic measures that have had a tremendous impact in decreasing cancer incidence [*e.g.*, hepatitis B virus (HBV) vaccination and hepatocellular carcinoma (HCC)]. This fact underscores that the ideal intervention is to prevent cancer from developing

at all. For HBV and HCC, the link was established epidemiologically in the 1980s, and subsequently, in the 1990s, molecular pathology has provided a mechanistic rationale for HBV carcinogenesis. For HPV and cervical cancer, the opposite is true; molecular pathology demonstrated a causal association between virus and cancer and epidemiological evidence lagged by 5–7 years. The lack of epidemiological evidence caused confusion about human papillomavirus (HPV) causality in cervical cancer, and it may have delayed the implementation of preventive vaccine strategies. These facts indicate that depending on the circumstances, different scientific approaches can be used to establish causality and that the integration of epidemiological criteria with modern molecular pathology techniques should allow for a more rapid identification of human carcinogens. Ideally, before labeling an agent as a human carcinogen, it is important to have epidemiological, experimental animals, and mechanistic evidences (molecular pathology). Not all of the evidence is always available, and, at times, it may be prudent to identify a human carcinogen earlier rather than later.

Session I: Current Criteria to Establish Causation, Use, and Limitations

Chair, May Wong, National Cancer Institute, Bethesda, MD. Speakers, Vincent J. Cogliano, Head, IARC Monographs Progamme, Lyon, France; and Eduardo L. Franco, McGill University, Montreal, Quebec, Canada.

Summary. Carcinogen identification at IARC (3) considers epidemiological studies, studies in experimental animals, and other data relevant to carcinogenicity and its mechanisms. In epidemiological studies, the relevance is clear, but there are often limitations in the characterization of exposure, and the ability to identify and adjust for confounding exposures or genetic susceptibility. As a result, epidemiological studies find associations but bring into question whether these associations are causal. Moreover, epidemiological studies require that the effect, cancer in this case, has already occurred, when of course it would be more desirable to identify potential carcinogenic substances at an earlier stage before they have caused a large number of malignancies and thus become identifiable by epidemiological studies. Experimental animal studies present a complementary set of strengths and limitations: exposure is clearly defined but the question of relevance must be addressed. As a result, animal bioassays can demonstrate causality, but the question is whether these causal relationships are relevant to humans. IARC also considers mechanistic studies generally as an aide in interpreting the results of positive animal bioassays. It is important to develop criteria to identify potential carcinogens primarily from mechanistic information, even in the absence of epidemiological or experimental animal studies in which the tumors are observed. This implies a new paradigm for risk assessment: identifying the key precursor events and processes in human cancer, then asking whether an agent can affect these key events and processes. The issue of cofactors and direct *versus* indirect carcinogens was debated at length, and there was a general consensus that more emphasis should be put in investigating tumor promoters because initiators require cofactors to cause cancer. Several infectious agents have been conclusively red-flagged as playing a causal role in cancer, *e.g.*, HBV, hepatitis C virus, HPV, EBV, HIV-1, human T-cell lymphotrophic virus-1, *Helicobacter pylori*, *Schistosoma hematobium*, and *Opisthorchiasis viverrini*. Decisions about causality have taken into account the Hill's criteria (2) and the Evans' modified guidelines (4). A few of the Hill's criteria have not stood the test of time and cannot be considered essential: specificity, analogy, plausibility, and coherence. The long latency of $\geq 20-40$ years from time of exposure to cancer development is one of the main obstacles for a rapid assessment of carcinogenicity. When these carcinogens are identified and eliminated, such as asbestos, their carcinogenic effects continue to cause cancer in those previously exposed. The goal is to identify carcinogens earlier rather than later before they have caused a large number of cancers in the population and therefore become identifiable epidemiologically. The issue is how molecular pathology, combined with tissue culture and animal experiments, can provide reliable evidence for the identification of human carcinogens.

Session II: Molecular Mechanisms of Malignant Transformation

Chair, Michele Carbone, Loyola University, Chicago, IL. Speakers, Michele Carbone, Hans Schreiber, University of Chicago, Chicago, IL; Lisa Coussens, University of California in San Francisco, San Francisco, CA; Carol Prives, Columbia University, New York, NY; Regina Santella, Columbia University, New York, NY; and Andrew Feinberg, Johns Hopkins University, Baltimore, MD.

Summary. The speakers underscored the importance of cofactors in carcinogenesis. Because the ultimate goal of the identification of human carcinogens is cancer prevention, the important biological question of whether a given agent is an initiator or a promoter becomes less relevant from a public health and clinical perspective. As stated by Hans Schreiber, "initiated cells are innocuous until promotion occurs," thus preventive therapeutic approaches against tumor promoters can be very effective. The answer to the challenging question posed by Vincent J. Cogliano, "What is a carcinogen? An agent that causes tumors or an agent that simply plays a role in tumor development?" appears to include the latter. There was an overall consensus that in the future we should devote more resources to investigating the interactions of environmental and infectious agents, among themselves and with the genetic background, because cancer risk will vary depending on exposure and genetic predisposition. Michele Carbone and Regina Santella, underscored that analyses of single agents can at times miss their carcinogenic potential when such agents increase the risk of cancer only in subgroups of individuals because of their genetic background (5), exposure to other carcinogens or promoters, and microbial infection. Carcinogenicity is often species and cell-type specific, and it is influenced by cofactors. For example, human mesothelial cells are very susceptible to asbestos toxicity and SV40 malignant transformation, and these two agents have a synergistic carcinogenic effect in human mesothelial cells (6). Other human cell types, instead, are much less susceptible to asbestos and to SV40 carcinogenicity, thus, the mere detection of a carcinogen, such as asbestos, in a given tissue, or of a tumor virus, such as SV40, in a human biopsy, does not prove causality.

Some molecular pathways required for carcinogenesis have been identified (Fig. 1). The signal transduction field for some years has suffered from an excess of data and a deficiency of concepts. However, there was a general consensus that retinoblastoma, p53 and PP2A inactivation, nuclear factor-κB activation, immortalization, and evasion from apoptosis are central to the formation of a malignant tumor cell (7). Most often malignant tumor cells will have to induce angiogenesis to sustain tumor cell growth and become able to invade and metastasize other tissues. Can this information be used to identify human carcinogens and cocarcinogens when the epidemiological evidence is not available? It can when there is sufficient evidence of carcinogenicity in animals and when there is molecular pathology proof that in human cells the agent interferes with key molecular pathways that lead to tumor formation. Agents that interfere with any of these pathways should be suspected human carcinogens, although simple *in vitro* evidence should not be considered proof of carcinogenicity. George Klein and Michele Carbone observed that it is very likely that many new cancer genes and genetic changes required for

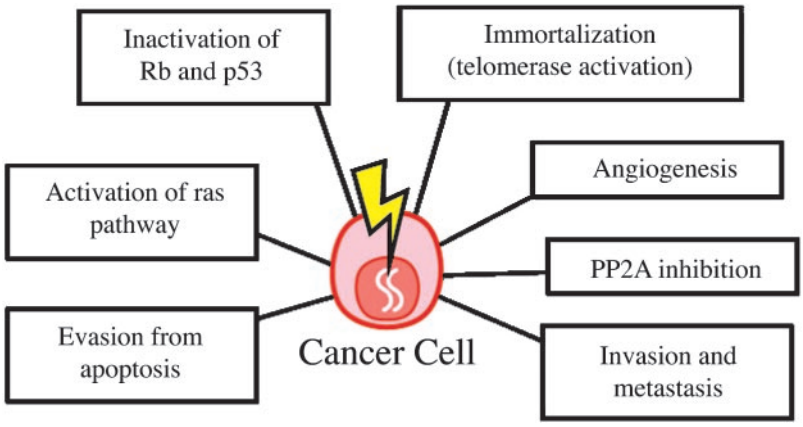

Fig. 1. Malignant growth requires the inactivation of cellular tumor suppressor genes such as Rb, p53, and possibly others, activation of growth stimulatory pathways, such as ras, phosphorylation changes of several cellular proteins such as those obtained by inactivating phosphatase 2A, evasion from apoptosis, immortalization, angiogenesis, and invasion and metastasis. Moreover, interactions among the malignant cells with the tissue stroma and the immune system will influence tumor growth.

tumor formation will be discovered in the future, apparently adding to the complexity of an already complex picture. However, it can be predicted that these new cancer genes will affect those pathways required for tumor formation shown in Fig. 1. Thus, the apparent complexity of tumor formation can be reduced to a much simpler picture. Regardless of the specific mechanism by which a given gene pathway is altered, carcinogenesis requires the activation or inactivation of the pathways shown in Fig. 1. Andrew Feinberg proposed a novel epigenetic hypothesis of cancer, suggesting that epigenetic modification of normal cells is responsible for the age-dependent increase in cancer risk and that these epigenetic alterations determine the effect of subsequent genetic insults. Even assuming that genetic alterations are necessary for cancer initiation by this model, the frequency of neoplasia depends upon the presence of a preexisting epigenetic alteration. An example may be a common epigenetic variant involving loss of imprinting of *IGF2* associated with a personal and family history of colorectal cancer (8). He also suggested that epigenetic alterations are largely responsible for tumor progression. The implications of this model are that assessment of carcinogens will require epigenetic measurement in addition to mutational effects. Hans Schreiber and Lisa Coussens underscored the role of the microenvironment in tumor growth and invasion. Acute inflammation can be beneficial in certain cases, as observed by the use of live *Bacillus Calmette-Guérin* instillation in the therapy of superficial bladder carcinoma. Chronic inflammation, however, usually promotes tumor growth, most notably, HCC, gastric and colon cancer. T-Cell-mediated immune surveillance against the development of primary cancers is restricted to certain virally associated malignancies and possibly certain UV-induced cancers (nonmelanoma), although T-cell-mediated, tumor-antigen-specific responses may be observed in patients with cancer.

Session III: Currently Accepted Microbial Associations

Chair, Bernard Roizman, University of Chicago, Chicago, IL. Speakers, Harald zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Germany; Marie-Annick Buendia, Institut Pasteur and INSERM, Paris, France; Nancy Raab-Traub, University of North Carolina at Chapel Hill, Chapel Hill, NC; Martin J. Blaser, New York University, New York, NY.

Summary. Whenever human cancers are analyzed for the presence of viruses, dependent on the method used for detection, a number of different agents may show up. Particularly the sensitivity of the PCR permits the discovery even of very small concentrations of DNA or RNA of infectious agents. Does this mean that these agents are causally involved in the development of those cancers? Clearly the answer is no: viral carcinogenicity is cell type specific, and it is influenced by cofactors. Thus, the simple detection of a virus in a given tumor biopsy in the absence of mechanistic and experimental evidence is not proof of causality. However, detection of an infectious agent in a given tumor type, especially in the presence of supportive experimental evidence of possible causality, should be carefully investigated. Harald zur Hausen noted that cervical cancer was the first case of a common human cancer where molecular techniques directly proved a necessary role of viral proteins for the maintenance of the malignant phenotype (9). Necessary does not mean sufficient, and viral oncogene expression is not sufficient for cell immortalization and transformation; additional modifications of the host cell genome are required to develop invasive growth properties. Marie-Annick Buendia observed that hepatitis B vaccination represents the first case of an efficient preventive immunological treatment for combating cancer (10). The enormous public health benefit derived from the identification of HBV as one of the causes of HCC and the subsequent implementation of preventive vaccine strategies underscore the importance of a rapid identification of human carcinogens. In this regard, infectious agents are particularly important because it is often possible to design specific treatment options to prevent or eradicate infection and thus diminish cancer incidence. HBV as a cause of HCC is the most egregious example, and it is hoped that HPV vaccination and eradication of *H. pylori* infection will have similar beneficial effects. It was noted that until the 1980s, infectious agents were not seriously considered as causes of human cancer. It appears likely that more infectious agents will be identified in the future as causative agents, cofactors, or promoters in other types of human cancer. Research in this area should be intensified because it has the potential to be very beneficial to public health. The issue of direct *versus* indirect carcinogenesis was discussed. It was noted that HBV appears to cause cancer predominantly through indirect mechanisms (and even more so HCV and *H. pylori*) by promoting a chronic inflammatory response that drives tumor growth. This indicates that the eradication of direct (such as HPV) and indirect carcinogens may be equally beneficial. Nancy Raab-Traub proposed an additional mechanism of viral carcinogenesis. She noted that in some tumors EBV drives the malignant growth, in others it modifies the tumor phenotype as shown by a series of recent articles from the laboratory of Joseph Pagano (11). Perhaps the role of the virus in such conditions may be to modify tumor cell behavior so that it becomes more aggressive and has an enhanced malignant phenotype. Martin J. Blaser discussed the importance of viral host interactions, indicating that risk varies among infected individuals. He noted that the *H. pylori* microbial strain differences and the host polymorphisms appear to

be synergistic in causing adenocarcinoma of the stomach. Therefore, individuals of particular genotypes who carry particular strains are at highest risk of gastric cancer development.

Session IV: Environmental and Chemical Carcinogenesis

Chair, James S. Felton Lawrence Livermore National Library, University of California, Livermore, CA; Speakers, Allan H. Conney, Rutgers University, Piscataway, NJ; Stephen S. Hecht, University of Minnesota, Minneapolis, MN; Gerald N. Wogan, Massachusetts Institute of Technology, Cambridge, MA; Lawrence A. Loeb, University of Washington, Seattle, WA.

Summary. Epidemiological and experimental data clearly demonstrate that lifestyle and environmental agents are major causes of human cancer. Several human carcinogens have been identified according to the criteria discussed in Refs. 2 and 3. The IARC monographs and United States Department of Health and Human Services, Reports on Carcinogens, provide an authoritative guide to cancer induction by exogenous chemicals. Nevertheless, the working group recognized that there are gaps in our ability to evaluate individual susceptibility to such carcinogens. Environmental and chemical carcinogenesis is strongly influenced by cofactors, diet, and individual susceptibility. The relevance of DNA mutations in carcinogenesis was underscored and thus of agents capable of inducing such mutations. James Felton noted that it is difficult to evaluate the impact of the different carcinogens to which we are exposed because they come from numerous sources, have different potencies, and are affected by genetics, environmental modulators of carcinogen metabolism, and lifestyle factors such as the ingestion of rare *versus* well-done meat products. Allan H. Conney observed that many chemical carcinogens are not carcinogenic *per se* but are metabolized by multiple cytochrome P450 enzymes with characteristic but often overlapping substrate specificities to chemically reactive electrophiles that react with DNA before initiating a carcinogenic response. These same cytochrome P450 enzymes, as well as Phase II enzymes (*e.g.,* glucuronyl transferase, glutathione *S*-transferase and others), also metabolize chemical carcinogens by inactivation pathways, and the relative amounts of enzymes that metabolically activate and detoxify the chemical will determine whether it is carcinogenic. Both genetic and environmental factors influence the levels of enzymes that metabolically activate and detoxify chemicals, and these factors influence carcinogenic risk. Stephen S. Hecht noted that individual susceptibility in tobacco-induced cancers must be evaluated more thoroughly. All speakers agreed that among exposed individuals cancer risk will vary and that interactions among carcinogens and among carcinogens and the genetic background will determine individual risk. Gerald N. Wogan underscored the importance of studying possible interactions among different carcinogenic substances. He noted that there was a relative risk of 3.4 for HCC cases in whom aflatoxin biomarkers, but no evidence of HBV infection, were detected. For HbsAg (the HBV surface antigen)-positive individuals without aflatoxin biomarkers, the relative risk was 7, whereas for those positive for both aflatoxin and HBV biomarkers the relative risk was 59 (10). Moreover, carcinogens and anticarcinogens can have different effects in different situations. As shown by the example of addition of β-carotene in the diet, β-carotene has chemopreventive effects in many experimental systems, yet it appears to have increased the incidence of lung cancer in heavy smokers. Animal experiments can be very useful in predicting the carcinogenicity of a given chemical. However, there are significant differences in susceptibility among species and within organs in the same species, and differences in the metabolic pathway of a given chemical among human and animals could lead to error. The marked multiplicative effect among aflatoxin and HBV in causing HCC underscores that viral infections can strongly influence chemical and environmental carcinogenesis and *vice versa*. Lawrence A. Loeb considered the hypothesis that cancer is manifested by a mutator phenotype. He argued that in normal somatic cells mutations are rare and recent evidence suggests that in stem cells mutations occur even less frequently. Thus, it seems likely that the large number of mutations in tumor cells cannot be accounted for by the low mutation rates observed in normal somatic cells. Rather, it must be a manifestation of a mutator phenotype present early during the tumorigenic process, providing a mechanism for the selection of cells with increased proliferative advantage. Evidence for large numbers of mutations in tumors include microsatellite instability, gene amplification, alterations in comparative genomic hybridization, loss of heterozygosity, and aneuploidy, each of which is characteristically elevated in tumors. Loeb estimated that each malignant cell contains tens of thousands of random mutations.

Discussion

Twelve issues were identified and discussed by the participants. The participants were divided into two groups, chaired by Brooke T. Mossman (group 1) and H. zur Hausen (group 2). Each group was further subdivided in subgroups. Expertise in different disciplines was mixed to produce an overall balance of expertise. The conclusions of the two groups (and various subgroups) were debated in an open forum.

1. With regard to current criteria to identify human carcinogens and to improve these criteria to reflect new advances in scientific knowledge and state-of-the-art techniques (transcriptional profiling, proteomics, and so on). J. Cogliano had outlined the strengths and weakness of current criteria in his presentation (see summary of Session I and Refs. 1–3), and there was no need for further discussion. There was a general consensus that the hope for the omics technology is to enhance our understanding to discriminate among the diversity of potential environmental, chemical, dietary, physical, infectious, and other biological agents that can trigger the carcinogenic process.

2. Should the criteria be the same for different agents (viruses, chemicals, physical agents, promoting agents *versus* initiating DNA-damaging agents)? There were different opinions. Group 1 debated this issue and concluded that the current listing of criteria should remain the same because we lack sufficient evidence to develop a separate classification. Group 2 strongly supported the view that it is useful to separate the biological or infectious agents from chemical and physical carcinogens due to their frequently entirely different mode of action. Some biological agents were designated as direct carcinogens, characterized by the persistence of their nucleic acid and by their modification of host cell DNA and interference with specific cellular functions. Others were viewed as indirect carcinogens. Their function does not require the persistence of their nucleic acid, they act as indirect carcinogens by inducing immunosuppression, causing chronic inflammation and the release of growth factors, causing DNA damage, amplifying genomes of other persisting small DNA viruses, or by preventing apoptosis after DNA damage exerted by other carcinogens. The group agreed on the following criteria for direct biological carcinogens: the regular presence of the genome or parts of it in every cancer cell; excision of this nucleic acid from transfected or cancer cell harboring this DNA or inhibition of the function of this nucleic acid should lead to a reversion of the immortalized or malignant phenotype of these cells; and epidemiological

studies should identify the agent as a risk factor for the respective tumor type. In addition, transfection of this nucleic acid into tissue culture cells or suitable laboratory animals should result in cell immortalization or tumor induction, respectively (e.*g.*, HPV-16, HPV-18; EBV, human T-cell lymphotrophic virus-1, and so on). Similarly, criteria were developed for indirect carcinogens: clinical observations, experimental and animal studies should point to a role of the respective agent as cocarcinogenic factor; epidemiological studies should identify these infections as risk factors for cancer development; vaccination against the agent or other successful treatment of the respective infection should provide significant protection against cancers suspected to be coinduced by these infections (*e.g.*, HBV, HCV, HIV, *H. pylori*, and so forth). Some infectious agents may cause cancer by both direct and indirect mechanisms.

3. Should the criteria be the same for the elderly, children, different sexes and genetic background? There was general consensus that criteria should be the same for the population at large. There is insufficient evidence to stratify populations based on susceptibility, although it is recognized that there are individuals with unique susceptibility (*e.g.*, those who are immunocompromised such as with AIDS or because of specific genetic polymorphisms, *e.g.*, p53 codon 72 in HPV and cervical carcinoma). Thus, not everyone is at equal risk.

4. How can we integrate our new knowledge of molecular biology/pathogenesis with previous criteria to establish cancer etiology more accurately and more promptly? It is hoped that the new genomic technology will lead to a better understanding of markers of exposure (early pathways or profiles) that may decrease the number and cost of epidemiological investigations and make them more efficient and precise.

5. Knowing the genetic/epigenetic changes that take place in cancer, can this information help us to identify the carcinogens that caused those changes? It was agreed that although such changes might help to identify the carcinogens, more information is needed. Specifically, (*a*) there is not enough information on the specific genetic alterations linked to specific exposure pathways. In addition, there is a lack of information on epigenetic alterations linked with exposure (*i.e.*, smoking and diet). The scientific community needs to understand the epigenetic state of the population/host to evaluate a putative carcinogen. (*b*) More research is needed to clarify the role of carcinogens in both genetics and epigenetics in premalignant tissues. (*c*) Surrogate/intermediate endpoints and markers of early detection are needed. (*d*) It will be difficult to perform comprehensive experiments showing that putative carcinogens definitely cause mutations in genes because there are so many unknowns pathways alternatives that could explain the observation (*i.e.*, p53 somatic mutations). (*e*) Most carcinogens are weak and nonspecific in their effect on the tumor. (*f*) The genetic tumor progression model is not proven, and more measures/ways of thinking about changes are needed. For example, consider linking epigenetics with dietary states and conducting more experiments on target tissue. (*g*) More research on expression and activation of genes is needed. Consider a panel of genetic markers for cancer as well as epigenotypes of a population. It was noted that genetic and epigenetic changes caused by chemicals generally are not specific, except for AA 249 p53 mutation (which leads to G-T substitution) caused by aflatoxin in liver cancer cells, and for thymine dimers and skin cancer after sunlight exposure. At the moment, except for these two examples, we cannot link a chemical/environmental carcinogen to a specific genetic alteration. Because two specific associations have been identified, it is likely that more will be identified in the future. It is expected that in some instances multiple agents will cause the same lesions and that single agents will cause multiple lesions.

6. What is the hierarchy of state-of-the-art approaches needed for confirmation criteria, and which bioassays are critical for decisions: epidemiology, animal testing, cell culture, genomics, and so forth? There should be no such hierarchy. Epidemiology, animal, tissue culture and molecular pathology should be seen as integrating evidences in the determination of human carcinogenicity.

7. If a given agent alters key cellular mechanisms required for carcinogenesis (including inactivation of Rb and p53, activation of ras and of telomerase, tumor invasion, angiogenesis, and metastasis), should such an agent be considered a human carcinogen? There is insufficient knowledge that effects on any gene product *in vitro* can be a major factor in designation of a carcinogen. By itself the information is insufficient. However, there are set of genes, such as p53, Rb, nuclear factor-κB, and so forth, that are altered in most human cancers. Thus, when there is evidence that a given carcinogen alters these key regulatory cellular gene pathways (Fig. 1) in the specific target human cell type, together with evidence of carcinogenicity in animals, especially in the same target organ, this should be considered evidence for carcinogenicity in humans.

8. What is the present value of using tissue culture and animal experiments to identify human carcinogens? Tissue culture and animal experiments are complementary approaches that can be very useful to identify human carcinogens. Two aspects of the role of tissue culture and animal experiments in the study of carcinogenesis to be considered are:

Screening. Consider how to determine whether a new compound is a human carcinogen. Begin screening with a tissue culture and animal experiments, then use the assays to gain insight into whether these agents might be carcinogenic in humans. There must be enough information on mechanisms to assure that the information gained from the tissue culture and animal experiments makes sense in terms of human carcinogenesis.

Causation. Epidemiological studies have identified the association between exposure and the development of cancer. Animal and tissue culture experiments can augment this information and lead to the conclusion of causation through the mechanistic realm. Does the exposure make sense in terms of its ability to cause cell transformation? Use this information to look at more specific targets in the human population to gain better insight into causation.

Recommendations for the future:

(*a*) To increase the specificity of the predictive value of tissue culture experiments, it is important that the carcinogen is tested in human cells of the specific target organ. The same concept applies to animal models. The stronger predictive value of animal experiments is when a carcinogen is given in such a way that it reached the target organ (*e.g.*, the pleura if asbestos is tested). Moreover, the tumors that develop in animals should be of the same histotype as those that have been associated in humans to that specific carcinogen.

(*b*) The stronger predictive value of transgenic models is when transgenes are constructed using the natural promoter expressing the gene in the target organ.

(*c*) Improving the current battery of tissue culture tests—stages of transformation, mutagenesis, cell proliferation, invasion, angiogenesis, and migration. Validate with known carcinogens and create a useful profile for testing new agents.

(*d*) Integrating new technologies and determining whether to use rodent or human cells. The use of small interfering RNA allows scientists to address mechanistic and human environment interaction questions.

(*e*) Additionally increasing the value of animal testing used to test target-organ, metabolism steps. The value could be increased through the expansion of available models with different target-organ specificity into other areas. Mouse and primate genetics could allow mechanistic bacteria/virology studies and gene/environment interactions.

(*f*) Determining how to study complex mixtures of agents including those that when given as single agents to animals do not appear to be carcinogenic. A significant knowledge gap exists in the area of combined exposures.

(*g*) Encouraging integration of new technologies into tissue culture and animal models. Industry, government, and academic laboratories are applying the new technologies (microarrays and proteomics), and all groups must cooperate and share their knowledge in these areas to validate the results of such studies.

9. Can we integrate genetic predisposition to cancer to identify the carcinogens that individuals with these genetic changes are most susceptible to? This is possible because there are definable genetic susceptibility groups, and there is variation. The following recommendations were made: (*a*) integrate epigenetics into studies of cells or animals that already have one known genetic hit; (*b*) investigate the role of chromatin in existing genes (*i.e.*, Rb, p53) and other proteins that interact; (*c*) study the role of epigenetic inheritance (*i.e.*, loss of imprinting) and familial clustering/vertical inheritance in families; and (*d*) generate new information using high throughput genotyping by instituting epigenetic analysis of cohorts. It was noted that this is a very promising area of current research and it should be accelerated. However, the public should be educated that from well defined clinical and epidemiological studies there is potential for great benefit by permitting testing for genetic markers that will be identified in the future to reduce disease risk and burden.

10. We are screening populations using new molecular approaches (proteomics, methylation changes, and so forth) to identify high-risk individuals. Can we use this information to identify the causes (*i.e.*, the carcinogenic substances) responsible for these molecular changes? There were different opinions. Group 1 indicated that high-risk individuals could be defined by a variety of approaches: accurate exposure measurements, genetic predisposition analysis, known previous history of tumors, early detection by biospecimen analysis or imaging with new technology, and so on. These represent good models for identifying the causes, including association, epigenetic outcomes, and association or lack of association with exposures, be it cadmium, alcohol, smoking, and so forth. Recommendations included (*a*) experiments on markers of susceptibility and exposure are needed to validate genetic, proteomic, or molecular assays; (*b*) interaction between genetic and environmental factors must be considered; (*c*) genotyping and phenotyping are important areas to pursue; and (*d*) nongenetic factors influencing risk should be examined. Group 2 did not share this optimism and concluded that presently there is nonspecificity in gene expression and proteomic changes.

11. What are the most important biases and confounders in these bioassays? What are the main problems in interpretation of data, especially if agents demonstrate threshold effects and not conventional linear-dose responses? The following problems were identified: (*a*) *in vitro*, testing the agent in the wrong cell type; (*b*) *in vivo*, testing the substance/agent in the wrong animal model or administering it in such a way that does not reach the target organ; (*c*) single species testing and not considering differences in metabolic pathways; and (*d*) single-agent testing that does not consider the promoting or antagonizing effects of cofactors.

12. Modern epidemiological studies often depend on genetic, biochemical, or viral assays that had not been developed in the 1960s when Hill's criteria to identify carcinogens were developed. How can we incorporate this information to improve the accuracy of Hill's epidemiological criteria to identify human carcinogens? The two groups addressed this question, and all participants accepted the recommendations. It was stated that modern epidemiological studies augmented by these assays will enhance Hill's criteria.

Strength. Measuring and quantitating with greater accuracy and precision, resulting in smaller studies producing larger relative risk estimates.

Consistency. Improved techniques with reduced measurement error will contribute to greater consistency among studies and potentially decreased publication bias.

Plausibility and Biological Coherence. Defining downstream pathways more accurately.

Analogy. Comparing at a broader level the events found in new technologies and, potentially, discovering new analogies never before suspected. This may lead to an easier path for use of these technologies.

Specificity. The new studies are unlikely to help in its assessment that requires the verification of external models to be useful.

Temporal Relationships. Defining relationships through these new studies, will enable a more accurate assessment of exposure onset, leading to a better definition of latency.

Dose-Response Relationships. Lowering the threshold of detection through new technologies and therefore helping to expand the range of dose-response relationships. This will lead to more accurate measurements of the exposure dose-risk relation.

Experimental Evidence. Defining pathways earlier and more quickly. They then can be expanded to prospective human epidemiological trials for more rapid validation.

Summary. To our knowledge, this is the first workshop devoted solely to a discussion of the etiology of human cancer that takes into consideration infectious microbial agents, chemical carcinogens, and other exogenous physical and environmental factors. The workshop could not encompass the whole multitude of factors involved in cancer etiology, but it is our hope that the conclusions and guidelines that we have proposed will provide a framework of acceptable criteria for establishing causation. We are rapidly gaining insight into the nature of cancer causation from the study of the genetic and endogenous cellular and tissue aspects, and hopefully this workshop will assist in the consideration of the exogenous factors.

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