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Meeting Report

National Cancer Institute Workshop on Tumor Promotion and Cofactors in Carcinogenesis[1]

Although the important role of environmental factors in the causation of human cancer is now well recognized, it is not clear whether chemically diverse environmental agents operate through a common mechanism. Since carcinogenesis appears to be a multistep process, it is probable that various environmental factors act at different steps in this process. One of the best-studied models in this regard is the so-called "2-stage carcinogenesis" system in mouse skin. In this system, 2 distinct stages in the carcinogenic process have been identified, the 1st designated "initiation" and the 2nd, "promotion." There is considerable evidence that additional stages and cofactors play a role in cancer induction in other tissues and species, and, probably, the interaction of multiple factors is required for the induction of many human cancers.

For assessment of the current status of knowledge in this area and for stimulation of speculation on possible mechanisms and future directions of research, an informal meeting titled, "Workshop on Tumor Promotion and Cofactors in Carcinogenesis," was held October 12 to 14, 1976, at The Marine Biological Laboratory, Woods Hole, Massachusetts. The meeting was sponsored by the Division of Cancer Research Resources and Centers of the National Cancer Institute and included the following participants: R. K. Boutwell, J. H. Clark, B. A. Cunningham, T. J. Domanski, P. Feigelson, E. W. Hecker, M. Lippmann, S. Mondal, C. Peraino, R. A. Pledger, J. D. Scribner, A. Sivak, T. J. Slaga, W. Troll, B. L. Van Duuren, and I. B. Weinstein.

The initial discussion, led by R. K. Boutwell, focused on the general biological properties of initiating agents and promoting agents as revealed by mouse skin carcinogenesis studies. Basic properties of initiating agents include the following. (*a*) At sufficient dosages they are usually carcinogenic by themselves, *i.e.*, they can serve as "solitary carcinogens." (*b*) At lower doses that are not carcinogenic or are only weakly carcinogenic ("subthreshold" or "threshold" amounts), they can cause a process termed "initiation," resulting in a cryptic state, manifested by the fact that subsequent exposure of the initiated tissue to promoting agents leads to the occurrence of benign and malignant skin tumors. At present no specific morphological or biochemical feature has been identified with the process of initiation. It probably involves a somatic cell mutation, although a stable epigenetic change has not been excluded. (*c*) A single exposure to the agent is sufficient for initiation. (*d*) Initiation appears to be a rapid, irreversible process, since application of the promoting agent can be delayed for many months and cancers will still be induced. (*e*) There does not appear to be a threshold dose below which the initiating effect is completely lost. (*f*) The administration of 2 initiating agents or repetitive small doses of the same agent appears to be additive. (*g*) Most initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules. Their activated forms are generally mutagenic.

The general properties of promoting agents highlighted during this discussion included the following. (*a*) When given after a low dose of an initiating agent, they increase cancer formation, increasing the number of skin tumors that appear in the treated area and shortening the lag time for tumor appearance. (*b*) They are not carcinogenic (or at most are only very weakly carcinogenic) when given without prior application of an initiating agent. (*c*) To exert their promoting effect, they must be given after, not before, the initiating agent. (*d*) Their action requires prolonged exposure, is reversible at early stages, and appears to have a threshold. (*e*) They induce proliferation, but it is not clear whether this is the mechanism by which they act. (*f*) There is no evidence that metabolic activation or covalent binding is required for their action, nor is there evidence that they are mutagenic.

In the early studies by I. Berenblum and coworkers, the promoting agent used in mouse skin experiments was the irritant croton oil from the seeds of the plant *Croton tiglium*. The following discussion was led by E. W. Hecker (Heidelberg), who reviewed evidence from his own and other laboratories that the active principle present in croton oil is represented by the complex substituted diterpene, TPA.[2] It resembles the most active skin tumor promoter known so far. Many structurally similar phorbol esters bearing various substituent groups, as well as compounds with modifications in the structure of the tetracyclic phorbol nucleus (*i.e.*, ingenol, hexadecanoate milliamine, resiniferonol esters, etc.), have now been identified in various plant species. Many of these are extremely potent promoters on mouse skin. Certain structure-activity relationships are emerging from the study of these compounds in mouse skin and in cell culture systems. Of particular interest is recent epidemiological evidence by E. W. Hecker and his group suggesting that the use of a tea made from *Croton flavens* may be associated with the high incidence of esophageal cancer on the island of Curaçao. Although the evidence on this point is not yet conclusive, this example suggests that the worldwide distribution of plants containing potent

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[2] The abbreviation used is: TPA, tetradecanoyl phorbol acetate.

promoting agents, as shown by E. W. Hecker and his group, should be considered in further human cancer epidemiological studies.

Although the precise mechanism of action of TPA and related promoting agents is not known, several interesting biochemical events associated with their action were discussed. R. K. Boutwell and T. G. O'Brien have found that application of TPA to mouse skin leads to the induction of a key enzyme involved in polyamine synthesis, ornithine decarboxylase. This is associated with an increased intracellular level of putrescine. Although a 2nd enzyme, *S*-adenosylmethionine decarboxylase, is also induced by TPA, there appears to be a better correlation of promoting activity with ornithine decarboxylase induction. TPA induction of ornithine decarboxylase in epidermal cell cultures has also been studied by S. Yuspa and associates at the National Cancer Institute. The role of proteases in promotion and hormone-induced hyperplasia and the ability of protease inhibitors to inhibit the process of skin tumor promotion were reviewed by W. Troll. An intriguing recent finding of S. Meyn, T. Rossman, and W. Troll is that proteases appear to play an important role in *Escherichia coli* during the process of UV induction of the lysogenic bacteriophage λ, the SOS error-prone DNA replication mechanism, and mutagenesis. If this finding extends to higher organisms, it suggests a role for proteases in DNA repair and replication associated with DNA damage and carcinogenesis. Other biochemical events associated with the exposure of mouse skin and/or cell cultures to TPA and related substances that were discussed include: (*a*) alterations in cell surface, including increased phospholipid synthesis and membrane phosphorylation; (*b*) induction of the enzyme ornithine decarboxylase; (*c*) enhanced RNA and protein synthesis; (*d*) an early inhibition of DNA synthesis, followed by a later stimulation of DNA synthesis; and (*e*) the recently discovered induction of the synthesis of a protease, plasminogen activator (see below).

The development of cell culture systems for the study of the action of promoting agents is a relatively new field and one that holds considerable promise in terms of elucidating their molecular mechanism(s) of action. These systems may also provide convenient bioassays for the rapid detection of environmental agents that have promoting activity. S. Mondal summarized recent studies conducted by himself and C. Heildelberger, indicating that TPA and related compounds have an enhancing effect on *in vitro* transformation of the mouse cell line C3H/10T½. The effect resembles in several respects the action of promoting agents on mouse skin. Cultures treated with TPA alone are not transformed, whereas cultures previously treated with a single dose of an initiating carcinogen, such as methylcholanthrene or UV irradiation, and then maintained for several weeks in media containing TPA show an increased number of transformed foci when compared to cultures exposed only to methylcholanthrene or UV irradiation. TPA causes an early inhibition followed by a later enhancement of [^{3}H]thymidine uptake in these cultures; other investigators have found that TPA increases the saturation density of certain, but not all, cell cultures. The significance of the latter effects in terms of the process of transformation requires further study. I.B. Weinstein reviewed the discovery made by himself and M. Wigler that extremely low concentrations of TPA (10^{-8} M) cause the induction of the protease, plasminogen activator, in avian and mammalian cell cultures. These findings lend weight to the hypothesis that protease production may be an important aspect of the carcinogenic process. Evidence was also presented that there is a good correlation between the ability of a series of phorbol-like diterpenes to induce plasminogen activator in cell culture and their known activity as promotors on mouse skin. It appears that, in addition to inducing plasminogen activator, TPA induces several changes in the phenotype of cells in culture that mimic other features of transformed cells; the major difference is that in tumor cell these phenotypic changes are stable or "locked in," whereas in normal cells they revert when TPA is removed from the medium.

Several participants made the point that, in many respects, TPA resembles a hormone in its action on cells and tissues. Therefore, further studies to identify specific TPA cellular receptors, alterations in levels of secondary messengers, or alterations in gene expression associated with TPA action may provide important insights into its mechanism of action.

A striking finding in the skin tumor promotion system is the fact that the promoting phase can be inhibited by topical application of protease inhibitors or by a variety of potent synthetic glucocorticoid hormones. Recent studies by T. Slaga, M. Wigler, and I. B. Weinstein indicate that this effect of the synthetic glucocorticoids is paralleled by the ability of these agents to inhibit plasminogen activator production in tumor cell cultures. The development of pharmacological agents that might block specific phases of the carcinogenic process is of obvious interest in cancer prevention. The effects of synthetic glucocorticoids in these model systems are, therefore, provocative and should encourage further studies in this area.

Distinct from the promoting agents, which can be given after the initiating agents, is a group of cofactors described by B. Van Duuren that, although not carcinogenic when given alone, markedly enhance the carcinogenicity of benzo(*a*)pyrene on mouse skin when given simultaneously with the initiating agent. A number of aliphatic hydrocarbons that have this property, such as undecane, decane, and tetradecane, have been identified in cigarette smoke condensates. Benzo(*e*)pyrene and pyrene have a similar cofactor effect, although compounds like benzo(*a*)pyrene are not promotors in the 2-stage mouse skin carcinogenesis system. Studies are in progress to determine whether these cofactors act by inducing aryl hydrocarbon hydroxylase systems, influencing absorption or distribution of the initiating agent, or producing alterations in cell membrane permeability, DNA repair, or other cellular functions.

A longstanding question has been to what extent the 2-stage mouse skin system applies to other tissues. Dr. C. Peraino reviewed evidence from his own laboratory that the chronic administration of phenobarbital, butylated hydroxytoluene, or dichlorodiphenyltrichloroethane (DDT) to rats previously fed a small amount of acetylaminofluorene markedly enhanced liver tumor induction. Although it is not yet clear that these agents act in the same manner as do the phorbol esters in mouse skin, this appears to be a promising system for studying the interaction of various cofactors

in carcinogenesis in a tissue that is well suited for extensive biochemical studies.

Because of the importance of hormonal agents as cofactors in the carcinogenic process as well as certain similarities between the actions of promoting agents and of hormones, M. Lippman, J. H. Clark, and P. Feigelson led discussions on current research related to cellular and molecular events associated with hormone action. The key roles of specific hormone receptors and the modulation of their synthesis were stressed.

Recent studies by J. H. Clark suggest that the treatment of newborn rats with estrogen antagonists, such as Nafoxidine and Clomid, causes marked abnormalities of the female reproductive tract and uterine carcinoma in the adult animal. These abnormalities may be caused by a form of hyperestrogenization that results from the retention of the estrogen receptor by the nucleus for long periods of time. Evidence that androgens and glucocorticoid hormones alter the level of specific mRNA's in target tissues and the use of subcellular protein-synthesizing systems to quantitate the abundance of specific mRNA's in normal and tumor tissues were reviewed by P. Feigelson. Possible mechanisms by which hormones might enhance tumor formation that were discussed included: (*a*) hormone-induced growth and hyperplasia of the target tissue; (*b*) hormone-induced alterations of drug-metabolizing enzymes or drug-excretory mechanisms, which influence the activation, inactivation, or excretion of a 2nd substance that has the potential of being converted to an ultimate carcinogen; (*c*) induction of latent viruses (glucocorticoids can induce the synthesis of mouse mammary tumor virus); (*d*) suppression of immunological surveillance or other host defense mechanisms; and (*e*) actual biotransformation of the hormone to a reactive derivative that binds covalently to cellular macromolecules. It was agreed that a number of experimental systems are now available for exploration of these and other hypothetical mechanisms related to the role of hormones in carcinogenesis.

Because of the increasing evidence that changes in the cell surface are an important aspect of the carcinogenic process, B. A. Cunningham led a discussion on current research related to cell surface structure. G. M. Edelman's concept of a cell surface-modulating assembly, in which the binding of specific agents to cell surface receptors might alter, via transmembrane proteins, the intracellular organization of microfilaments and microtubules, was reviewed. Although this scheme is speculative, it has important implications in terms of the mechanism by which signals received at the cell surface could alter cell function and proliferation. Fluorescent antibody-staining techniques have shown that, in cells transformed by oncogenic viruses, there is a disruption of the normal architecture of the actin- and myosin-containing microfilaments. These changes may relate in ways that are not yet apparent to defects in cell surface recognition and growth control.

In the final summary discussion, A. Sivak emphasized that several model systems are now available for in-depth studies of the mechanisms of tumor promotion and the role of various cofactors in carcinogenesis. The experimental systems include not only mouse skin, but also the skin of rats, hamsters, gerbils, and rabbits, in which the promotion phenomenon has been observed. The liver acetylaminofluorene plus phenobarbital system described by Peraino appears extremely promising, and there is suggestive evidence for similar 2-stage phenomena in other organs, including the lung, colon, and bladder. Hormone-induced tumor systems are available in rodents, in which the interaction between hormones, chemical carcinogens, viruses, and genetic factors can be studied. Studies on mechanism in these systems should be greatly facilitated by recent advances in our understanding of hormone action. The development of cell culture transformation systems, in which the interaction between initiating agents, promoting agents, hormones, and other factors can be studied *in vitro*, holds great promise in terms of elucidating cellular and molecular mechanisms, as well as providing rapid screening tests for potential carcinogens.

The participants agreed that, although a number of biochemical markers of the response of tissues or cells in culture to promoting agents are now available, further research is required to reveal additional markers, as well as to identify which of these biochemical effects is critical to the action of promoting agents. Studies in subcellular systems containing purified enzymes, membrane fractions, or other cellular organelles may provide a direct approach to understanding the mechanism(s) of action of promoting agents. Obviously, key questions in the field of tumor promotion are: (*a*) is there a specific cellular receptor for the phorbol-type compounds, and (*b*) how are pleiotropic responses of the cell to its interaction with these agents mediated?

The participants agreed that, to an increasing extent, human clinical and epidemiological studies must take into account the probable role of promoting agents, hormones, and other cofactors as possible rate-limiting determinants in the incidence of specific human cancers. The increasing number and diversity of both long- and short-term bioassay systems should facilitate the intelligent monitoring of the human environment for not only initiating agent carcinogens but also for promoting agents and other factors that play a role in the multistep carcinogenic process. The ability of protease inhibitors and glucocorticoid hormones to modify the responses of cells to promoting agents provides encouragement for the development of additional approaches for interrupting the carcinogenic process at an early stage. This area of research has obvious practical implications in terms of cancer prevention, particularly in those instances where individuals have already been exposed to a carcinogenic agent or where it is not feasible to remove completely a carcinogenic agent from the human environment.

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