The Multistep Nature of Cancer Development¹

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In this communication, I will discuss multistage carcinogenesis in tumor models other than skin. It is becoming increasingly apparent that cancer development in many tissues or organs is a stepwise process involving altered cell populations at many steps (31, 34, 61). Some of these new cell populations function as the site of origin for yet another “new” or altered noncancerous cell population, and with each step, the process shows an increased probability of evolving ultimately into a malignant neoplastic population. Thus, the carcinogenic process (with many types of etiological agents) appears to be one of cellular evolution (23) based upon repeated selection of rare cells (4, 31, 57), with increasing relevance to cancer as the system evolves through the noncancerous steps. It must be emphasized that the different cell populations that constitute the long precancerous period show no evidence of cancer and that the appearance of cells with any properties of cancer is very late in the process. Once a malignant neoplastic cell population does appear, its further progression is most easily understood as a manifestation of repeated new clonal selections for increasing growth rate and autonomy (4, 57). The term “neoplastic” is applied selectively to those cells or cell populations showing growth that is relatively independent of their environment, i.e., showing some degree of autonomy.

What mechanisms might be operating to encourage the progressive selection of cells for increasing relevance to cancer? If “spontaneous” growth or autonomy of growth were a common property acquired by a few target cells at initiation, then the multistep carcinogenic process could be explained by the progressive selection of new genetically altered cells for increasing ability to grow “spontaneously” (4, 57).

This simple formulation, however attractive it may be, is not consistent with the many observations on carcinogenesis in different organs including the skin. The efficient initiation of carcinogenesis with a relatively small dose of a carcinogen in a single or brief exposure need not generate altered cells that show any autonomy of growth. This is so not only in normally quiescent organs such as the liver, pancreas, urinary bladder, kidney, brain, etc., but also in some proliferating organs such as the skin and the colon. Thus, initiation can occur often without any manifestations of spontaneous or autonomous growth.

An apparent exception may appear to be the induction of cancer with a relatively large single dose of a carcinogen. However, even under these conditions, cancer development is still slow and prolonged and associated with “preneoplastic” or “precancerous” changes in cell populations. It is highly probable that the large dose of carcinogen is exerting not only its initiating effect but also a promoting one on the whole organ or tissue.

This lack of spontaneous growth or autonomy of initiated cells is reflected in the well-established need for another operation, the imposition of a promoting environment, for cancer development in most systems.

What, then, is the biological or physiological nature of the cells that are generated during initiation and that are selectively stimulated to proliferate during promotion, and how do they contribute to the ultimate development of cancer? What changes do the cells undergo, as new populations appear, that favor their subsequent role in the further progression toward cancer? Since this preneoplastic-preneoplastic phase is by far the slowest and most prolonged sequence in most carcinogen processes and thus contains the major rate-limiting step or steps in cancer development, it is the most puzzling with respect to both phenomena and mechanism.

Models for the Multistep Nature of Cancer Development

The past decade has seen the development of increasing numbers of models for the stepwise analysis of carcinogenesis (see Ref. 31 for references until 1980). Although the major emphasis in these studies has been on the agents and their immediate effects on cells and tissues, that is, on identifying and studying an increasing number of carcinogens, initiators, and promoters, increasing attention is being directed to the nature and properties of the tissue changes as they relate to particular steps. Pancreas, urinary bladder, trachea, colon, central nervous system, and liver, in addition to skin, are among the organs and tissues that have received major emphasis.

This phase in carcinogenesis has been catalyzed by two major developments: (a) the discovery of a reproducible initiation-promotion regimen (2-AAF³ plus phenobarbital or other promoters) for the liver by Peraino et al. (59) in 1971; and (b) the organ orientation in carcinogenesis that became increasingly popular at about the same time. These developments have encouraged an increasing attention to the multistep nature of carcinogenic processes in vivo and to the similarities and differences in the response to carcinogens, initiators, and promoters in different tissues and organs.

These in vivo developments have been paralleled by in vitro studies that have reinforced the multistep nature of neoplastic development (e.g., Refs. 2, 3, 21, and 54). The in vitro systems have not led so far to ready and reproducible ways to obtain distinct separable new cell populations for detailed biochemical analyses of sequences. When these become available, a new dimension in the mechanistic analysis of the individual steps in cancer development should become possible.

Another set of systems that is receiving more attention is the combined in vivo-in vitro models in which some steps are induced in a whole tissue or organ and others following in vitro manipulation of the organ or tissue. This approach has been particularly successful in the central nervous system (47), the kidney (42), and the respiratory tract (55).

Among the in vivo systems, those of the urinary bladder (8, 35, 36, 43, 86) and liver (16, 26, 45, 62, 67, 76, 83) are probably the most advanced, mainly because of the availability of an increasing number of models for study. In both organ systems, two types of models have

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received most attention: (a) the continuous or intermittent relatively long-term exposure to one or more carcinogens; and (b) initiation with a relatively brief exposure to a carcinogen and promotion with one of several types of promoters. In the bladder, initiation with N-(4-[5-nitro-2-furyl]-2-thiazolyl)-formamide, W-butyl-N-(4-hydroxybutyl-1-[(4-hydroxybutyl)-nitrosamine, and N-methyleneisourea and promotion with saccharin; cyclaminate; ascorbate; 2-(3)-tert-butyl-4-hydroxyanisole; 2,6-di-tert-butyl-4-methylphenol; or ethoxyquin are being used as models. In the liver, initiation has been carried out with many different types of carcinogens including aromatic amines, nitrosamines, and nitrosamides, and poly-cyclic aromatic hydrocarbons. Promoters used include phenobarbital, 1,1,1-trichlorodibenzo-p-dioxin, \( \alpha \)-hexachlorocyclohexane, 2,3,7,8-ter-trachlorodibenzo-p-dioxin, cyproterone acetate, polychlorinated biphenyls, 2,6-di-tert-butyl-4-methylphenol, etc., as well as feeding a choline-deficient methionine-low diet (77) or dietary orotic acid (9, 67). In addition, a third type of model, the RH model, selects initiated hepatocytes with dietary 2-AAF or other compounds plus partial hepatectomy after initiation with one of approximately 40 carcinogens (79, 80, 87).

With the RH model, it has become possible to follow day by day the origin, growth, biological behavior, and biochemistry of nodules, since they develop rapidly and synchronously. They arise from hepatocytes randomly throughout the liver and not from any special region or zone of the organ, lobule, or acinus.

For a variety of reasons, the liver systems among the in vivo models have become the most versatile and have generated many new insights into the carcinogenic process. An important advantage of the liver is the ability to turn on, at will, cell proliferation in an otherwise quiescent organ. This makes it possible to offer some control over a major variable in carcinogenesis, cell proliferation. Such a control may become readily available in the urinary bladder as well (8, 36, 66).

Steps in Cancer Development

Initiation. The gestalt of the initiation process with many chemicals and with radiation is now becoming reasonably clear in outline. The metabolism of carcinogens, the nature of their active forms, the interactions of such derivatives with DNA and other cellular constituents, the resultant alterations in structure and function of DNA, and the repair of such alterations are now being studied intensively. These studies have told us and should continue to tell us much about the physicochemical changes that could be a basis for the appropriate change in phenotype in initiated cells.

However, certain aspects appear to stand out as being in need of further scrutiny. These relate to: (a) the essential molecular basis for the induction of the rare altered initiated cells; (b) the mechanistic role of cell proliferation in initiation; and (c) the essential physiological or metabolic nature of the initiated cells that enables them to act as the original progenitors for cancer.

Although not yet conclusive, the evidence for the importance of some change in DNA being related to the initiation events is impressive. This was formulated early in terms of the induction of an altered base, a miscoding lesion, and a mutation in the classical sense. However, the developments in "gene dynamics" during the last decade or so have opened up the possibilities for alternative mechanisms for initiation. Rearrangements of segments of DNA, the activation and modulation of gene expression by neighboring base sequences, and the increasing evidence for gene duplication are but three aspects of genetic organization that could have an important role to play in some forms of initiation. This becomes particularly interesting as more carcinogens are identified with no measurable immediate interaction with DNA, even after time for metabolism is provided. Several hypolipidemic agents (mostly "peroxisome proliferators" (69), phthalates, and other types of chemicals fall into this group. Also, dietary deficiencies such as that of choline and methionine appear to initiate without the need for a "classical" carcinogen (38, 52). How do such agents initiate the carcinogenic process?

One of the most important aspects of initiation relates to cell proliferation. In the liver, there is an established requirement for a round of cell proliferation with at least three end-points: (a) cancer (12); (b) islands of hepatocytes with altered histochemical properties (16); and (c) induction of resistant hepatocytes that can be selected to generate nodules, a few of which become precursor lesions for cancer (6, 10, 87, 90). The mechanistic role of cell proliferation in initiation is not understood. It may be related to the "permanent" fixation of some chemical change in DNA by DNA replication and/or to metabolic properties, such as repair processes, associated with different phases of the cell cycle. This is one of the major challenges in the analysis of the initiation process. Although well-established in the liver, the dependence of initiation on cell proliferation appears to be common to many organs or tissues including urinary bladder (8, 36) and pancreas (14, 65, 71).

The implications of this generalization for human cancer are far reaching. With nonnecrogenic carcinogen stimuli (a majority for any specific organ or tissue), the rate-limiting step may not be exposure to the carcinogen, but rather occurrence of cell damage and resultant cell regeneration. This may be important for many quiescent organs such as the pancreas, salivary glands, liver, urinary bladder, thyroid, etc. Cancer in some of these organs has been related to chronic tissue damage. Its possible importance in initiation with chemicals is an interesting area for exploration.

The liver models have also thrown some new light upon the relative importance of "short-lived" versus "long-lived" or "persistent" carcinogen-DNA interactions (DNA adducts) in carcinogenesis. With two different models of liver carcinogenesis, it has been possible to show that the changes that are repaired or reversed within 72 to 96 hr are the most relevant to initiation of liver cancer development (6, 10, 73, 90). The possible importance of long-lived adducts is based on the previous work, begun by Goth and Rajewsky (41) in studies on adducts in the brain, using correlations between adducts and the ultimate cancer development. They did not assay for initiation independently (58). The studies in liver indicate the importance of two properties, cell proliferation and presence of repair. In the central nervous system, neither property is very active, and therefore the persistence may allow a much longer time span for cell proliferation to occur. In the liver, and probably other organs and tissues as well, repair is active, and cell proliferation must occur soon after the initial interaction of carcinogen with DNA for the interaction to be effective.

The essential nature of an initiated cell that allows it to be expanded by a promoting environment is known in only one model in the liver, the RH model. In 1973, it was suggested that the phenotype of one kind of precursors, the putative initiated hepatocyte, would have had to acquire this resistance. Fortunately, it was possible to develop an assay for resistant hepatocytes (79) and to show that approximately 40 different chemical carcinogens, with varying structures and properties, induce resistant hepatocytes during initiation (45, 84, 87).

An exciting new development is the observation that mouse skin after initiation contains cells that no longer undergo terminal differentiation to keratin on exposure to calcium (91, 92). This may well indicate that some initiated cells in the skin have acquired a resistance to an inhibitory environment. The possible importance of resistance to an appropriate environmental influence in many types of initiation is worthy of exploration (31).

Promotion. By promotion, I refer to the process whereby the initiated cells become expanded into nodules, papillomas, or polyps, one or more of which can act as a population or origin for the ultimate development of cancer. These focal proliferations are the immediate indices for promotion that could have an important role to play in some forms of initiation. Although not yet conclusive, the evidence for the importance of some "short-lived" versus "long-lived" or "persistent" carcinogen-DNA interactions (DNA adducts) in carcinogenesis is far reaching. With nonnecrogenic carcinogen stimuli (a majority for any specific organ or tissue), the rate-limiting step may not be exposure to the carcinogen, but rather occurrence of cell damage and resultant cell regeneration. This may be important for many quiescent organs such as the pancreas, salivary glands, liver, urinary bladder, thyroid, etc. Cancer in some of these organs has been related to chronic tissue damage. Its possible importance in initiation with chemicals is an interesting area for exploration.

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Promotion. By promotion, I refer to the process whereby the initiated cells become expanded into nodules, papillomas, or polyps, one or more of which can act as a population or origin for the ultimate development of cancer. These focal proliferations are the immediate indices for promotion, even though they are often only "distantly" related to the appearance of the ultimate malignant neoplasia. There appear to be certain critical issues to be clarified before a real
understanding of the process of promotion is possible.

1. The first relates to how the promoter or promoting stimulus creates an environment that selectively favors the expansive growth of the initiated cells vis-à-vis the surrounding ones. By what overall means does the expansion occur in different initiated tissues or organs? Does this occur by the creation of a mitogenic stimulus plus a selective or differential inhibition of the surrounding cells, as occurs in the RH model? The evidence available indicates that this differential inhibition is not a general phenomenon by any means. Promotion by orotic acid, a very effective promoter, does not favor nodule formation in the liver by this overall mechanism. What property or properties have initiated cells lost or gained that enable them to respond to a promoting environment differently or selectively than the surrounding cells? The scientific analysis of promotion involving nodules, papillomas, polyps, and such lesions would seem to require not only the study of possible biochemical and molecular effects of promoters on cells and tissues but also as a prerequisite, the study of how the promoter creates an environment that has a selective effect on initiated cells to favor their net gain in cell number.

2. The second relates to another important aspect, is expansion (clonal expansion?) of initiated cells to form a stable new focal proliferation sufficient to set in motion the further evolution to cancer, or does the promoter add some additional effect on the genome or elsewhere that is essential for the further progression? In the liver, the rapid expansion of initiated hepatocytes to form visible nodules (within 2 weeks) is sufficient to induce an incidence of hepatocellular carcinoma within 12 to 18 months of from 70 to 90%, depending upon the nature of the initiating carcinogen (78). If an effect of a promoting environment is necessary, what is its nature vis-à-vis gene expression?

Progression. By progression, I refer to the stepwise process whereby the occasional expanded initiated cell, the nodule, the polypl, or the papilloma, evolves into a cancer and the further steps the cancer undergoes as it becomes progressively more malignant. The nodule-to-cancer, polypl-to-cancer, or papilloma-to-cancer sequences are the least understood of any segment of the carcinogenic process.

In the skin, it has been reported periodically that a papilloma can act as a site for cancer development. In the liver, nodules have been shown to be sites of origin for hepatocellular carcinoma with five different carcinogens in different models (18, 22, 39, 63, 66, 72, 80). Thus, the relevance of nodules to cancer resides not only in their constant presence before the appearance of unequivocal malignant neoplasia but more convincingly in their demonstrated role as one precursor for hepatocellular carcinoma.

In the experimental liver models, two patterns of promotion-progression appear to exist. One pattern is seen in models in which promotion as well as progression are brought about by exposure to carcinogens. With long-term continuous or intermittent exposure to a carcinogen and most clearly, in the resistant hepatocyte model, a large number of initiated hepatocytes expand to form visible hepatocyte nodules. However, only a small number of these nodules persist and may become sites for further progression to cancer while the vast majority undergo remodeling by redifferentiation to normal-appearing liver (84). A second apparent pattern is seen in those models in which promotion and further evolution to cancer are affected by noncarcinogenic or "weakly" carcinogenic promoters such as phenobarbital, orotic acid, and probably α-hexachlorocyclohexane (75, 76). Under these conditions, many initiated cells expand to only a moderate degree to form stable foci or islands that remain microscopic in dimensions, and only a relatively few slowly expand to become visible persistent nodules. Whether the persistent nodules generated in these two different patterns are entirely equivalent remains to be established. These considerations place an important perspective on the attempts to isolate initiated hepatocytes or other initiated cells as a group. Since the expanded initiated cells, i.e., the nodules, have at least two options (redifferentiation and persistence to cancer), it is probable that the study of the initiated cells as a group may not generate information that can be related to the pathway leading to cancer.

The persistent nodules show slow progressive growth over the many weeks during which malignant neoplastic cells are evolving (17, 84). Is this new property of the nodules a reflection of: (a) some basic change in the control of the cell cycle; (b) cell death followed by compensatory regeneration of scattered hepatocytes in the nodules as a common feature of this cell population; or (c) the local production of growth factors by the nodule hepatocytes? The increasing availability of models to generate the persistent nodules makes it now possible to begin to pose such questions in a scientific manner.

Also, are there steps in the persistent nodule-to-cancer sequence where it may now be possible to grow hepatocytes in vivo and thereby to study their properties? Assuming that the properties of transforming in vitro systems, such as increasing growth rate, changes in serum requirements, loss of anchorage dependence, and tumorigenicity, also occur in the in vivo sequence, in what steps do they appear and in what order?

It has been shown recently that the persistent nodules all grow well on transplantation into the spleen, and all ultimately show the development of hepatocellular carcinoma (50). The early nodules, the vast majority of which remodel, do not show these properties. This new approach may make it possible to begin to analyze the nodule-to-cancer sequence in the absence of the many confounding variables that are virtually impossible to control when the nodules remain in situ in the liver. Another aspect of the nodule-to-cancer sequence is the basis for the appearance in nodules of rare altered hepatocytes that generate the new cell populations by forming "nodules in nodules" (27, 63). Operationally, in the liver and in other organs as well (31), no further external stimulus is necessary for the progression to cancer. How is this "self-generating" process carried out? Are we dealing with a "multi-hit" phenomenon (16, 64), in which the "hits" are generated endogenously, or a totally different mechanism, such as the activation or alteration of an oncogene?

A major limitation to the stepwise analysis of the carcinogenic process is the paucity of specific markers for new cell populations and lesions. This limitation relates to all steps including those involved in the progression of a cancer. Although many changes have been found in different cells during cancer development, few become established as reliable (53, 82). The observation of a polypeptide of about M, 21,000 in nodules generated in different models of liver carcinogenesis (20) is encouraging in the search for specific markers for selected steps. The search for such indices of discrete steps might be particularly rewarding in the nodule-to-cancer sequence.

Some Fundamental Issues in Carcinogenesis

The study of the stepwise analysis of how cancer develops in many organs or tissues is still early. Despite this, some interesting questions concerning the fundamental principles underlying the processes are beginning to emerge. Among the issues are: (a) states of differentiation, new phenotypes, and cancer; (b) physiological adaptation as an important component during cancer development; and (c) heterogeneity and diversity versus commonality in the early steps in carcinogenesis.

States of Differentiation, New Phenotypes, and Cancer. One of the most interesting aspects of the multistep process of carcinogenesis relates to the nature of the new cell populations (papillomas, polyps, nodules) that regularly appear. They are readily distinguished from both the normal tissue of origin and any ultimate cancer. In most systems, they have not been studied sufficiently to allow even a tentative suggestion or conclusion as to their fundamental nature.

In the liver, the hepatocyte nodules can be readily distinguished both from normal adult liver and from liver cell cancer. Unlike normal adult liver, the hepatocytes in nodules are arranged in plates usually two or three cells thick or as acini. Thus, the sinusoids are more widely separated than in adult liver. The blood supply is also different in that nodules show a relative decrease in portal blood supply and a normal or increased arterial blood supply (11, 32). Although the individual hepatocytes in the very early developing nodules resemble hepatocytes in regenerating adult liver, the hepatocytes in the grossly visible nodules rapidly acquire distinctive cytoplasmic and nuclear changes with proliferation of smooth endoplasmic reticulum and an apparent increase in euchromatin. In composite, the hepatocyte nodules do not resemble liver at any stage of growth.
normal development or maturation. They are not similar to embryonic, fetal, neonatal, or regenerating liver but show a unique phenotype. Thus, they are not dedifferentiated (60).

These special architectural and structural features are accompanied by a special biochemical pattern that again is not similar to any developmental phase (19, 29). The nodules show large decreases (75 to 80%) in total microsomal cytochromes P-450 and cytochrome b$_{5}$ and in several mixed-function oxygenase activities and relatively large increases (2- to 15-fold) in glutathione (reduced, oxidized, and bound) (1), cytosolic glutathione S-transferases and DT-diaphorase, microsomal epoxide hydrolase, and UDP-glucuronoyl transferase-1 and membrane $\gamma$-glutamyl transferase. In addition, the nodules show a special polypeptide of Mr 21,000 in the cytosol (20). These patterns of architecture, cytological appearance, and biochemical pattern are seen in nodules in six different models and in nodules from the resistant hepatocyte model 16 months after they have been transplanted to and growing in the spleen of normal syngeneic animals (29). Thus, the phenotype is constitutive and appears to represent a totally new "state" of liver with a mixture of features, some of adult liver, some of embryonic or fetal liver, and some special to this new state. The different models studied involved short- or long-term continuous or intermittent exposure to 2-AAF or 3'-methyl-4-dimethylamino-azobenzene, initiation with DENA, 2-AAF, or 1,2-dimethylhydrazine, or one of approximately 40 carcinogens (16, 67) and promotion with phenobarbital, choline-deficient low-methionine diet, or dietary orotic acid (29). Thus, under quite diverse conditions, a major cell population, the hepatocyte nodules, show an unusual degree of commonality in several aspects of their phenotype.

In addition, with the one model studied intensively, the resistant hepatocyte model, the nodules (95 to 98%) undergo a complex process of redifferentiation (so called "regression") back to normal-appearing liver with only a very small subset persisting (84). Thus, the majority are programmed for a complicated redifferentiation process with a change in architecture, cytological appearance, and biochemistry. This process of "regression" was seen in many studies of liver carcinogenesis since the first liver model was described in 1933 (84, 85). In the DENA-phenobarbital model, Goldsworthy et al. (40) have reported that the enzyme-altered foci of hepatocytes are stable in number and do not appear to regress. It must be emphasized that the remodeling of nodules involves a far more expanded lesion than is represented by microscopic foci. The nodules are grossly visible and may measure up to several mm in diameter. It is possible that nodules during their increase in size show a phenomenon similar to developing muscle and other cells (44). In these developing systems, new options for cells, such as differentiation to mature myocytes, appear after a predetermined number of cell divisions. The appearance of a new option, redifferentiation, when the nodules reach a certain size, remains an attractive hypothesis.

The architecture, cytological appearance, and biochemistry of the persistent nodules are so far indistinguishable from those in the majority of nodules before redifferentiation (remodeling). Since the persistent nodules are at least one precursor population for liver cancer (18, 22, 39, 63, 66, 72, 80), it is conceivable that the persistence of the "nodule phenotype" may be relevant to the subsequent development of cancer. Thus, a critical aspect of the carcinogenic process could be the inability of the small subset of persistent nodules to undergo redifferentiation, i.e., a block in redifferentiation. This is somewhat analogous to the block in normal development and maturation proposed by Potter (64).

"Regression" of papillomas is a common feature in skin carcinogenesis in mice, especially with noncarcinogenic promoters. Whether the phenomenon is similar in mechanism to the liver is unknown. Regression has also been reported in polyps in the colon in humans (13).

These findings concerning the new state of differentiation of hepatocytes throw into a different perspective an important hypothesis of carcinogenesis, that relating to "oncofetal development" and cancer. Based on carcinogenesis in the liver, it would appear that liver cancer does not arise from mature adult liver cells, and is not a "throwback" to a prenatal condition ("dedifferentiation"). Rather, it may arise from a population that represents a totally new state of differentiation ("redifferentiation") special to the reaction of the liver to certain types of xenobiotics (5). It would be appropriate to reexamine critically the old concepts that cancer cell populations somehow have a close relationship or analogy to one or more steps in the normal maturation (60, 64) or represent examples of dedifferentiation.

Parenthetically, it should be pointed out that the hepatocyte model may be a useful one to study some fundamentals of differentiation and the control of gene expression. Early nodules, remodeling nodules, and persistent nodules represent three populations of hepatocytes the origin, behavior, options, and fates of which are highly predictable and readily available. Conditions for the study of possible genetic rearrangements and/or alterations in methylation or other secondary modifications of DNA would seem to be available in this liver system.

Physiological Adaptation as a Possible Component in Cancer Development. The observations concerning the special architecture, cellular structure, and biochemical pattern of nodules and the built-in program for remodeling of the nodules through redifferentiation to normal-appearing liver indicate quite clearly that this phase of the carcinogenic process in the liver is a common response pattern to carcinogens. This suggests that this is an additional type of physiological adaptation (5, 31) to a more severe form of exposure to xenobiotics or environmental hazards.

Since one form of selection pressure (promotion) is differential resistance to the toxicity effects of 2-AAF and other carcinogens, the overall phenotypic pattern in nodules seen in the resistant hepatocyte model is not surprising. The low levels of activated enzyme components in the microsomes and the high levels of DT-diaphorase and epoxide hydrolase, glutathione, glutathione-S-transferases, and one UDP-glucuronoyl transferase are all consistent with a decreased ability of the cells to generate reactive moieties from some xenobiotics and an increased ability to inactivate whatever becomes activated. This is manifested in vivo as a more efficient excretion of one carcinogen, 2-AAF, by animals with many liver nodules (81), a resistance to the induction of cell death in vivo by CG$_{4}$ and dimethylnitrosamine (33), a resistance of isolated nodule hepatocytes to some hepatotoxins such as aflatoxin B$_{1}$ and 2-AAF (46, 48), and most importantly an ability to grow in an environment that severely inhibits cell proliferation of the surrounding liver (79, 80). Also, nodules show a resistance to some cytotoxic effects of DENA (37).

However, the finding of a common pattern in nodules in several different models of liver carcinogenesis indicates that this new population may have a more general significance in the adaptive responses of the liver. In one case, the orotic acid model, selection of nodules by differential resistance to inhibition of cell proliferation seems to have been ruled out (67). What is the physiological significance of the appearance of a new population of hepatocytes with a distinctive phenotype? A positive evolutionary role with survival value has been suggested for nodules, since in some respects the animal with nodules in its liver is better able to handle some toxic xenobiotics (28, 29, 31). Also, it is conceivable that, as in the case of another common reaction to many hazardous stimuli, the heat shock pattern (51, 74), the new pattern in the nodules could be under the control of a common gene or family of genes that somehow become triggered at or shortly following the time of initiation. Studies leading to the ultimate identification and isolation of such a hypothetical gene or gene family might be appropriate.

In this respect, it may be of interest to point out that cancers, especially carcinomas, often arise in atrophic or severely damaged organs or tissues, not in hyperplastic or hypertrophied ones. Stomach, liver, colon, urinary bladder, and pancreas are some sites in which this principle seems to apply. Under these conditions, it is not unreasonable to suggest that a few cells may acquire some type of resistance to the offending agent or environment and may initiate a new sequence of events that might relate ultimately to neoplasia.

These phenomena also raise an important perspective concerning promoters and promoting environments. At least in respect to the liver and probably also the urinary bladder (35, 86), promotion appears to be as much an effect on the whole organ or tissue as on the initiated cells. We are increasingly impressed with the need to view promoting environ-
ments as creating or exploiting differential properties between the initi-
ated cells and their surroundings as a basis for the selective growth of
the former. Such differential properties could conceivably relate to re-
sistance to stimuli relevant to the particular tissue or organ (31).

If the nodule is fundamentally a physiological response pattern to a
special kind of toxic damage, exemplified by carcinogens, and if this
response pattern is not special or unique to liver but is seen in other
organs as well, then the early steps in carcinogenesis take on a
different perspective. Clearly, ultimate cancer development appears to
be related to only a very small subset of persistent nodules. What is
special about these persistent nodules that makes them demonstrate or
acquire a new life history? The description of hepatocyte nodules gen-

erally as benign neoplasms (adenomas), "neoplastic" nodules, or even
"hyperplastic" nodules does not seem appropriate. Only the few nodules
that persist may be truly "preneoplastic" or "premalignant."

Heterogeneity versus Commonality. There is increasing realization
today that many if not all cancers are very heterogeneous and diverse
with respect to the phenotype of their constituent cells. This pertains not
only to the histological and cytological appearance of the cells but also
to their karyotype, content of hormone receptors, expression of surface
antigens, immunogenicity, responsiveness to the host, capacity for met-
astatic spread, and many biochemical properties (89). Are the divers-
ity and heterogeneity of the early phenomena of cancer inherent in the
carcinogenic process or do they appear relatively late as advanced
malignant neoplasia is approached? The probable multiplicity in chemical
interactions of activated carcinogens with DNA and the wide spectrum
of resultant phenotypic variants that might be expected might favor an
early appearance of heterogeneity or diversity. The scientific strategy
toward the cellular metabolic analysis of cancer and its multistep devel-

opment could be radically different depending upon which of these
general patterns seems to predominate.

The evidence from the liver models and the less well-documented
evidence from other organs or tissues indicate that several of the early
steps in the carcinogenic process show a commonality, not a diversity
or heterogeneity. This also pertains to different individual nodules in any
single liver. Not only are the nodules remarkably similar to each other
architecturally and morphologically, but with several biochemical prop-
erties relating to xenobiotic metabolism (glutathione, glutathione-S-trans-
ferase, epoxide hydrolyase, γ-glutamyltransferase, and DT-diaphorase)
(19, 29, 32), over 90% of all nodules and the majority if not all persistent
nodules show a common pattern of elevated concentrations or activities.

It is possible that the diversity and heterogeneity shown by advanced
malignant neoplasms may represent noise accompanying a common
theme (30). Since the diversity becomes more evident with increasing
autonomy of growth, it remains attractive to view the fundamental
alterations in growth and invasion and metastasis of cancers as having
a corresponding commonality at the biochemical level. 

Oncogenes and the Multistep Development of Cancer

Given the current interest in oncogenes, it is interesting and important
to attempt to relate their activity to the carcinogenic process. At one end
of the spectrum, it is possible that oncogenes may play key or essential
roles at each of the major steps in the process. Three to six or more
oncogenes would then have to participate in tandem, in a way that could
account for the very long time span required in most carcinogenic
systems. This possibility is also consistent with the increasing evidence
that, even in highly proliferating cell systems in vitro, two or more
oncogenes may be required for transformation (49, 56, 70).

At the other end of the spectrum, it is possible that one or more
oncogenes might play a role only in the relatively late steps in the
progression of a malignant cell population to "more malignant" properties.
Since only a maximum of 15% of human cancers have so far been
positive for the expression of an oncogene, as assayed by transfection,
and since the further selection of such cancers by cell culture facilitates
such expression, it may be that the oncogenes may play some special
role once a malignant cell population has developed.

Perhaps something in between the two extreme possibilities may
occur. Conceivably one or more oncogenes could be involved in the slow
progressive growth of the persistent nodules and in the further new
populations that arise as nodules in nodules. The special new state of
differentiation of the nodules might favor this development.

An interesting facet of the recent developments in the oncogene area
is the observation that some may be closely related to one or more
growth factors (15, 88). Since the new cell populations appearing during
the development of cancer, especially the epithelial ones, may not
manifest any growth in the absence of promotion, and since any "spontane-
ous" or autonomous growth is usually a relatively late acquisition, it
is difficult at this time to relate the growth factors to the carcinogenic
process. The more intensive study of oncogene activity in discrete
populations that are likely steps in the multistep development of cancer
would be most appropriate.

Comparisons of Different Systems

Is the emerging pattern seen in these new models of carcinogenesis
relevant to other models? Is the multistep carcinogenic process in the
liver representative of the process in other organs and tissues? With
respect to a comparison with other liver models, it must be emphasized
that the stepwise sequential analysis of any multistep process including
carcinogenesis requires a highly synchronized system in which similar
cells or cell populations undergo changes simultaneously. This is seen
to a large degree in the early steps in the resistant hepatocyte model
but not in the initiation-phenoantibolital and initiation choline-deficient
methionine-low diet models. Because of this, it is currently difficult to study
any possible sequence beginning with initiation in these models. In
the newer initiation-organic acid model (67), the tissue response appears to
be synchronous. On comparing the two synchronous models, a pattern
is emerging with both similarities and differences. In the organic acid
model, small microscopic foci of altered cells do appear synchronously
and are similar to the microscopic lesions seen with all the other models.
However, only a few foci grow into grossly visible nodules, and these
appear to be closely related to the ultimate cancer development. Thus, in
a comparison of two liver models, the patterns seen are somewhat
different, even with the same initiating carcinogen. In one, many nodules
rapidly appear, the majority undergo redifferentiation to normal-appearing
liver, and a very small subset persist to become involved in the further
evolution to cancer. In the other, the putative-initiated hepatocytes only
show minimal early proliferation, but a small subset selectively grow to
form persistent nodules which again appear to be closely related to the
subsequent cancer. This comparison suggests that the options available
to altered hepatocytes induced during initiation with carcinogens may be
determined at that time, with only a very small minority of initiated
hepatocytes being altered in a manner relevant to the ultimate cancer.
With continuous exposure to a carcinogen or carcinogens, it is likely
that the number of potentially relevant hepatocytes may increase so that
a larger number of nodules may show interruption of redifferentiation.
Under the conditions of prolonged exposure to carcinogens, increasing
target cells could be recruited for relevance to cancer by the continual
"bombardment" with activated molecules coupled with increasing
cell proliferation as a result of the tissue damage. Conceivably, the
system under these conditions may become so much more complex as
to magnify excessively the difficulties in sequential analysis.

With respect to the comparison between different organs, tissues,
and species, only the mere outlines of some of the facets can be
examined at this early time period. The similarities in principle between
the response patterns to carcinogens of liver and of other organs (often
also between species) have been indicated at several points in this
discussion. The many similarities between papillomas and hepatocyte
nodules suggest that the processes leading to epithelial neoplasms in
these tissues may well be analogous in many respects. This may also
pertain to differences in the options expressed by papillomas under
different conditions. In mouse skin, in an apparent analogy to the liver
nodules, more papillomas appear to persist when induced by treatment
with carcinogens than with an initiator-promoter regimen (68).

Another comparison which is potentially of great interest concerns the
rat liver cell cancer and human melanoma. In an unusual study of the biology of the development of melanoma in relation to naevi, Clark et al. have proposed a sequence of steps involving selection pressures.

Some General Considerations

It is evident by the developments that have occurred in studies on carcinogenesis in several sites that it is possible to identify discrete steps in the process and to begin to orient these steps to each other and to initiation and cancer. Although in vitro approaches will no doubt be essential in analyzing many of the steps, it is also evident that the experimental animal is very useful or perhaps even essential in generating the new cell populations associated with some steps.

There is compelling evidence that some of the steps are common with several different models in the same organ. This commonality relates to several levels of organization—architecture, appearance, blood supply, and several biochemical and biological properties. This realization has important implications in the analysis of the genesis of heterogeneity and diversity of advanced cancer.

The commonality also points to some of the early steps being physiological, as a form of physiological adaptation rather than as “abnormal” reactions. If further evidence supports this concept, then a part of the carcinogenic process is highly programmed with presumably one or more key controlling genes being involved. The current availability of genetic technology makes the pursuit of such controlling genes within the realm of the possible. It also offers a realistic prospect of interrupting or reversing the process of cancer development at one or more steps before the “truly” malignant cells appear. The possibility of generating practical approaches to cancer therapy through understanding the multi-step nature of carcinogenesis is interesting and intriguing.

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References

The Multistep Nature of Cancer Development

Emmanuel Farber

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