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Progression: The Terminal Stage in Carcinogenesis

Henry C. Pitot

McArdle Laboratory for Cancer Research, The Medical School, Departments of Oncology and Pathology, University of Wisconsin, Madison, Wisconsin 53706, USA

INTRODUCTION

Our knowledge of the multistage nature of neoplastic development has expanded substantially during the last two decades. The original concepts of the multistage nature of neoplastic development evolved from the experiments of Rous and Kidd,[1] Mottram,[2] and Berenblum and Shubik.[3] These three reports were based on studies of epidermal carcinogenesis in mouse skin, and the endpoint of most of the experiments was the development of benign papillomas, although in most experiments, if the animals were allowed to remain alive for a sufficiently long period, some carcinomas would develop as well. The term initiation was coined to designate the initial alteration in individual cells within the tissue, usually induced by a single administration of a sub-carcinogenic dose of a known carcinogen, whereas promotion involved a subsequent, chronic multi-dose regimen of an agent that by itself was essentially noncarcinogenic.

Within a decade following these experiments, with an entirely different system — the development of mammary adenocarcinoma in the mouse — Foulds[4] proposed that, whereas initiation was effected by a single dose of a carcinogenic agent, all subsequent processes in the development of cancer were part of a process he termed progression. In fact, his description of the process emphasized changes characteristic of malignant neoplasia and its evolution to higher degrees of autonomy and malignancy. Thus, Foulds' concept of tumor progression emphasized the later progressive development of malignant neoplasia; the earlier two-stage concept of initiation and promotion in mouse skin, largely because of the format of the experiments, had emphasized the early development of the neoplastic process prior to frank malignant changes. In both concepts, the first event was initiation.

During the last decade a number of other multistage models of carcinogenesis have been developed.[5] These studies have led to an understanding that both the original two-stage concept of initiation and promotion and the concept of progression by Foulds are part of the same process but viewed in different experimental circumstances and systems. Experiments in the two most extensively studied multistage carcinogenesis systems, mouse skin and rat liver, now allow the combining of the two-stage concept with that of Foulds' description of tumor progression to a three-stage phenomenon involving initiation, promotion, and progression.

Some of the characteristics of the three stages noted are listed in Table I. These define the first and last stages as irreversible and probably involving structural changes in the genome along with other characteristics that follow, at least for initiation, from such characteristics. In contrast, the stage of promotion, when it can be clearly identified in multistage carcinogenesis, involves reversible expansion of the initiated cell population as well as reversible changes in genetic expression. Thus far the definition of reversibility has been primarily operational, although both the contraction of expanded clones[6] and the changes in the regulation of genetic expression seen on withdrawal of the promoting agent[7] are not understood at the mechanistic level.

In the past a number of issues have clouded the extension of the multistage concept to carcinogenesis in tissues other than mouse skin and rat liver. These include the apparent "complete carcinogenicity" of promoting agents,[8] the lack of any distinct reversible stage of promotion following the application of a single large dose of a carcinogenic agent,[9] and the "non-genotoxic" chemical carcinogens that have now been identified in long-term chronic bioassay tests.[10] Now, however, through a more complete understanding of the multistage nature of neoplastic development and its relation to the whole animal, including factors within the internal environment of the organism including hormones, growth factors, and other potential endogenous promoting agents, as well as exogenous factors, initiating agents, complete carcinogens, and promoting agents, such an extension is possible. Several factors, which are usually not considered in experiments on the multistage nature of neoplastic development, can now be shown to be critical in these previously misunderstood experiments in carcinogenesis. Thus, promoting agents may mimic complete carcinogens by promoting spontaneously or fortuitously initiated cells, the latter resulting from such uncontrolled factors as background radiation, carcinogens contaminating dietary regimens, or mechanisms as yet not understood.[11] Similarly, administration of a single, even moderately low dose of a complete carcinogen can give rise to malignant neoplasms as a result of endogenous or uncontrolled exogenous promoting agents, including endog-

Table I. Biological Characteristics of the Stages of Initiation, Promotion, and Progression in Carcinogenesis[a)]

Initiation	Promotion	Progression
Irreversible, with constant stem cell potential	Reversible increase in replication of progeny of the initiated cell population	Irreversible — demonstrable alterations in cell genome
Efficacy sensitive to xenobiotic and other chemical factors	Reversible alterations in gene expression	Evolving karyotypic instability
Spontaneous (fortuitous) occurrence of initiated cells can be demonstrated	Promoted cell population existence dependent on continued administration of the promoting agent	Relatively autonomous malignant neoplasia
Requires cell division for "fixation"	Efficacy sensitive to dietary and hormonal factors	Induction by progressor agents and/or complete carcinogens
Dose response does not exhibit a readily measurable threshold	Dose response exhibits measurable threshold and maximal effects dependent on dose of initiating agent	Spontaneous (fortuitous) progression of cells in the stage of promotion
Relative effect of initiators depends on quantitation of focal lesions following defined period of promotion	Relative effectiveness of promoters depends on their ability with constant exposure to cause an expansion of the progeny of the initiated cell population	

a) Adapted from Pitot.[5)]

enous hormones[12)] and growth factors as well as exogenous, undefined dietary components.[13)] Through such an understanding of complicating factors in multistage carcinogenesis, one can more carefully delineate the characteristics of the first two stages, initiation and promotion, not only in the well-studied systems of mouse epidermal carcinogenesis and rat hepatocarcinogenesis, but also in epidermal[14)] and hepatocarcinogenesis[15)] in other species and multistage carcinogenesis in other organs.[16–18)] Critical in such an understanding, however, is the knowledge of the final stage of neoplastic development, progression — its induction, characteristics, and modification. These are the subject of this review.

PROGRESSION — A DISTINCT STAGE IN THE DEVELOPMENT OF CANCER

In later discussions of tumor progression, Foulds[19)] emphasized the "characters" of a neoplasm. These characters included growth rate, invasiveness, potential and actual metastases, hormonal responsiveness, and histologic appearance. Foulds noted that any one of these characters could undergo progression independently of the others and that each primary or secondary neoplasm within any one host might progress independently of others in that host.[19)] Such a degree of variable development within a neoplasm is characteristic of the stage of progression in multistage carcinogenesis, as seen in Table I. In fact, many of the characters described by Foulds are a direct function of, or closely associated with, demonstrable changes in the genome of the cell. Karyotypic alterations in neoplasms are directly correlated with increased growth rate,[20)] invasiveness,[21)] metastatic potential and capability,[22,23)] hormone responsiveness (cf. ref. 24), and morphologic characteristics.[25)] Therefore, the "characters" and their changes during progression as described by Foulds[19)] are a reflection of the genetic heterogeneity characteristically seen in the stage of progression[22,26)] by both karyotypic analyses and more detailed molecular studies (cf. ref. 27). Furthermore, although significant phenotypic heterogeneity has been described during the stage of promotion in hepatocarcinogenesis in the rat,[28,29)] significant biochemical homogeneity[30)] and a lack of demonstrable genetic heterogeneity and instability characterize the stages of initiation and promotion. Unlike the relatively limited phenotypic characteristics of cells in the stages of initiation and promotion, those in the stage of progression may undergo a continued evolution towards increased autonomy from host influences. This process is accompanied by, if not a reflection of, the continued evolution of karyotypic changes that accompany the evolution of the stage of progression, as has been described in a variety of systems, both experimental[31,32)] and in the human.[26)]

A number of the more critical characteristics distinguishing the stage of progression from the stages of initiation and promotion in the development of malignant neoplasia are listed in Table I. The irreversibility of this stage is emphasized by the demonstrable alterations in the cell genome that accompany this stage. Such genomic changes clearly distinguish the stage of progression from the reversible preceding stage of promotion. However, under certain circumstances, cells in the stage of progression may be induced, by treatment with specific

chemicals, to terminal differentiation, thereby removing them from continued progression to a more malignant state.[33] Although one may argue that this latter process is a manifestation of "reversibility" of progression, in fact the process is quite different from the reversible nature of the stage of promotion. In the latter instance it is the removal of the promoting agent that causes the loss and/or change of the cells in the stage of promotion, thus decreasing or eliminating lesions in that stage. The active administration of "differentiating" agents that may alter the stage of progression is more analogous to active cytotoxic treatment of malignant cells, since terminal differentiation usually results ultimately in cell death.

That readily demonstrable alterations in the genome are associated with the malignant state has been known since the early part of this century, initially from the comprehensive studies of Boveri.[34] Utilizing the modern techniques of chromosome banding and/or premature chromosome condensation,[35] karyotypic abnormalities have been shown to be almost ubiquitous in malignant neoplasms. For example, in the human acute nonlymphocytic leukemia[36] and in the animal the "minimal deviation" hepatomas[37] exhibit chromosome abnormalities in apparently diploid neoplasms when banding techniques are used.

Recently Aldaz and his associates[31] demonstrated that the karyotype of early-appearing papillomas after initiation and promotion with tetradecanoylphorbol acetate (TPA) showed normal banding during epidermal carcinogenesis in the mouse. However, with continued frequent applications of TPA, karyotypic abnormalities appeared with increasing complexity as the time of treatment with the promoting agent was extended. Earlier studies in mammary carcinogenesis[20] showed a very similar effect, and recent studies in our laboratory[38] have demonstrated that cells isolated from altered hepatic foci (AHF) in the Peraino protocol of multistage hepatocarcinogenesis[29] exhibit no significant chromosomal abnormalities. This protocol utilizes very low, nontoxic doses of an initiator, diethylnitrosamine (DEN), administered to newborn animals, followed by promotion with phenobarbital (PB) after weaning. However, when hepatocytes were isolated from AHF induced by the Solt-Farber protocol,[39] the majority of hepatocytes from AHF exhibited significant chromosomal abnormalities. This latter protocol involves the administration of a necrogenic dose of DEN followed by a "selection" procedure employing a second carcinogenic agent, usually acetylaminofluorene. These results can be correlated with the fact that the Solt-Farber protocol induces hepatocellular carcinomas relatively rapidly when animals are subjected to promotion with PB,[40] whereas promotion with PB according to the Peraino protocol requires a considerably longer period of treatment with the promoting agent.[29] Since virtually all hepatocellular carcinomas in the rat exhibit chromosomal abnormalities,[40,41] one may conclude that the development of malignancy is the result of karyotypic genetic alterations, different from those which produced initiated cells and their progeny in the stage of promotion.

A number of investigators have demonstrated that readily measurable alterations in the cellular genome are associated with genetic instability of neoplastic cells in the stage of progression (cf. refs. 22 and 42). Karyotypic instability results in a variety of consequences for the neoplastic cell in the stage of progression. These include gene amplification,[43] gene and chromosomal translocations and rearrangements (cf. ref. 44), gene deletions (cf. ref. 45), proto-oncogene activation (cf. refs. 46 and 47), and more efficient transfection of genes into neoplastic cells that are in the stage of progression.[48] Of all of the characteristics of the three stages in neoplastic development seen in Table I, karyotypic instability is unique to the stage of progression and is probably the major factor distinguishing this stage from initiation and promotion. Furthermore, it is not unreasonable to suggest that such genomic instability is the basis for the malignant transformation of cells in the stage of progression.

A neoplasm has been defined as a "heritably altered, relatively autonomous growth of tissue."[49] Relative autonomy during the stage of progression usually develops into complete autonomy of cells from environmental regulatory factors such as hormones, growth factors, and cell-cell interactions. In contrast, neoplasms in the stage of promotion exhibit complete dependence on the presence of hormones and/or other regulatory factors.[50] Thus, it is in the transition from promotion to progression that the relative autonomy of neoplastic cells from the effects of promoting agents develops. This is especially true of the responsiveness and autonomy of neoplasms to hormones during the stage of progression (cf. ref. 51). Another potential mechanism for increasing the relative autonomy of neoplastic cells in the stage of progression is alteration of the methylation of DNA, a process shown to be important in the regulation of gene expression.[52] For example, administration of the "demethylating" agent, 5-azacytidine, to neoplastic cells in culture results in an alteration of their growth rates and morphologic characteristics.[53,54]

PHENOTYPIC ALTERATIONS IN THE DEVELOPMENT OF THE STAGE OF PROGRESSION

Morphologic Changes

In the "classical" two-stage carcinogenesis format involving mouse epidermis,[1-3] the endpoint in most experiments was the development of benign papillomas. How-

ever, the implication of these studies was that continued chronic administration of the promoting agent to the skin would result in the appearance of malignant neoplasms. Later a similar rationale was applied to analogous stages in hepatocarcinogenesis in the rat, regardless of the fact that nodules of relatively benign-appearing hepatocytes were monitored as endpoints in several such models (cf. ref. 55). In a more practical sense, although regulatory agencies in the United States tend to equate benign and malignant neoplasms as endpoints in chronic animal bioassays for carcinogens, the use of intermediate lesions, such as papillomas in epidermal carcinogenesis and altered hepatic foci or nodules in hepatocarcinogenesis as endpoints in such bioassays, has not met with general acceptance. On the other hand, it is evident that such intermediate lesions do represent transient, unstable cell populations derived from initiated cells from which carcinomas may arise with a frequency greater than that in uninitiated cells. Furthermore, such transient, unstable intermediate lesions may be considered in many instances to be the cellular expression of the reversible stage of promotion.[49,56] The genesis of the major neoplasms of the human — lung, breast, prostate, and uterus — probably involves promotion, exogenous or endogenous, as the principal stage in the history of their development.[49] Many lesions have been described in the human that have many of the biological characteristics of the intermediate, transient lesions seen during multistage carcinogenesis in the rodent. Some examples of these lesions are seen in Table II. However, not all malignant neoplasms, either in the human[57,58] or in the experimental animal,[59,60] can be shown to have arisen from such intermediate lesions. Presumably, in such instances, the initial carcinogenic insult is sufficient to convert the target cell to the stage of progression from the very onset of carcinogenesis. However, morphologic evidence that a secondary change occurs in a group of cells within the intermediate lesion can frequently be demonstrated. In many instances cells showing such a secondary change assume the histologic characteristics of malignancy; e.g., carcinoma *in situ* of the uterine cervix may arise within areas of preneoplastic cervical epithelium,[61] and the adenoma-carcinoma sequence is well known in the development of intestinal neoplasia in the human.[62] In experimental systems carcinomas can be seen to arise both in areas of preneoplasia[63] and in benign neoplastic lesions of the liver and intestine.[64]

As suggested above, when cells of a different morphology or phenotype arise in areas of preneoplasia or benign neoplasia, further genetic alterations have probably taken place in one or more cells of the initial lesion, leading to the development of the secondary lesion. Model systems that mimic this phenomenon of malignancy arising in preneoplasia are those employing the "initiation-promotion-initiation" format first enunciated by Potter.[66] Scherer has developed an experimental model system in rat liver in which focal carcinomas can be induced within pre-existing altered hepatic foci and/or nodules.[63] Such lesions have been designated "foci-in-foci." In our laboratory we have utilized quantitative techniques[67] to monitor the appearance of "foci-in-foci" as phenotypically heterogeneous foci identified in livers of animals undergoing such initiation-promotion-initiation protocols.[65] By means of such quantitative studies it is now potentially possible to identify and characterize agents that act specifically to convert cells in the stage of promotion to the stage of progression. Such agents have been termed "progressor agents."[65]

Table II. Preneoplastic Lesions in the Human and Their Counterparts in Rodents[a]

Tissue	Human	Rodent
Skin	Keratoacanthoma	Papilloma
Tracheobronchial epithelium	Atypical metaplasia	Atypical metaplasia
Esophagus	Moderate to severe dysplasia	Moderate to severe dysplasia
Stomach	Intestinal metaplasia	Glandular dysplasia
Colon	Polyp	Polyp
Pancreas	Focal acinar cell dysplasia	Atypical acinar cell foci
Liver	Liver cell dysplasia	Altered hepatic foci
	Focal nodular hyperplasia	"Neoplastic" nodules
Bladder	Moderate to severe dysplasia	Papillary hyperplasia
Adrenal	Adrenocortical nodules	Adrenocortical hyperplasia
Mammary gland	Atypical lobule type A	Hyperplastic terminal end buds

a) From Pitot.[65]

Identification of "Progressor" Agents through Morphologic Endpoints

Agents that act only to induce cells to enter the stage of progression have not been definitively characterized, as have promoting and initiating agents, but because of the action of complete carcinogens, their action as "progressor" agents with this function is assumed. To date perhaps the best known example of a "progressor" agent that is not a complete carcinogen is the free radical generator, benzoyl peroxide, an agent capable of inducing the stage of progression in experimental epidermal carcinogenesis.[68] Theoretically, progressor agents should be capable of inducing the genetic changes characteristic of the stage of progression and thus should exhibit some degree of clastogenic activity. That putative progressor agents can act in this manner has now been demonstrated with an "initiation-promotion-initiation" format such as proposed earlier by Potter[66] and experimentally demonstrated in the mouse epidermis by Hennings *et al.*[69] and by Scherer in rat hepatocarcinogenesis.[63] These investigators demonstrated that, when the usual initiation-promotion format was followed by the application of a second complete carcinogen, such as an alkylating agent, a rapid and high incidence of carcinomas resulted, unlike that seen in the standard initiation-promotion format in either tissue, the latter usually resulting primarily in benign neoplasms during the time span of the experiment.

With these two models, proposals for model systems designed to identify and characterize "progressor" agents have been developed. In the skin, O'Connell *et al.*[70] have utilized a system quite similar to that originally described by Hennings *et al.*[69] in order to study the mechanisms and potency of agents in enhancing the stage of progression. Utilizing a slight modification of this system, Rotstein and Slaga[71] identified acetic acid as a potential "progressor" agent when administered in the format described by O'Connell *et al.*[70] for an extended period of time after application of the promoting agent, TPA.

By combination of the Peraino protocol of initiation of neonatal rats[72] with that of Pitot *et al.*,[73] the latter involving partial hepatectomy at the time of initiation, it has been possible to develop a model system for the identification and characterization of putative "progressor" agents in multistage hepatocarcinogenesis in the rat.[65] Furthermore, such a model format can demonstrate the occurrence of foci-in-foci that occur "spontaneously" in animals subjected only to the initiation-promotion format.[65, 73] This would be expected since, as indicated from the characteristics of the stage of progression seen in Table I, spontaneous or fortuitous progression should occur, just as does spontaneous initiation.[11] The occasional focus-in-focus occurring prior to the application of the progressor agent probably represents a spontaneous genetic change, just like altered hepatic foci arising spontaneously. In both instances the focal lesion reflects the development of a genetically new population of cells. In the case of the focus-in-focus, however, since the genetic alteration, i.e., gross chromosomal alterations, reflects more extensive changes in the genome, one might expect that the phenotype of these cells in the stage of progression would be different from those in the stage of promotion. Evidence that this is so is presented below.

Molecular Characteristics of the Stage of Progression

With the exception of the abnormal regulation of genetic expression, no ubiquitous biochemical or molecular abnormality common to all neoplasms has thus far been reported. On the other hand, with a greater understanding of a specific set of genes, known as proto-oncogenes, it has been proposed that malignant neoplasms may universally exhibit one or more abnormalities in the expression of one or more proto-oncogenes. It is clear, however, that abnormality in the expression of any single proto-oncogene is not universal in all malignant neoplasms. Therefore, just as with the induction of neoplasia by a variety of retroviruses exhibiting one or more viral oncogenes, many pathways involving the abnormal expression of one or more different proto-oncogenes may accompany chemical and radiation carcinogenesis.

The "activation" of proto-oncogenes may occur by a variety of mechanisms involving either direct mutation of the proto-oncogene or alterations in the regulation of its expression.[65] To date, most studies have demonstrated the activation, both mutational and transcriptional, of proto-oncogenes as a phenomenon characteristic of malignant neoplasms and thus of the stage of progression. Exceptions to this phenomenon include the mutational activation of the Ha-*ras* proto-oncogene in mouse papillomas of the skin[74] and adenomas of the mouse liver[75]; this suggests that such activation occurs during the stage of initiation in these two tissues. However, no such mutational activation of this or any other proto-oncogene is seen in significant numbers at the stages of initiation and/or promotion in multistage hepatocarcinogenesis in the rat. Transcriptional activation of proto-oncogenes in the mouse, rat, human, and other species during the stage of progression is quite common.[76–78]

In multistage hepatocarcinogenesis in the rat, transcriptional activation of proto-oncogenes occurring in altered hepatic foci following initiation with a necrogenic dose of DEN has been demonstrated for the Ha-*ras* proto-oncogene,[79] as well as the *myc*[80, 81] and the *src* proto-oncogenes.[82] On the other hand, when initia-

tion was carried out with a non-necrogenic dose of DEN, the resulting foci actually showed lowered levels of the protein products of a number of proto-oncogenes in the rat (M. Neveu, J. Hully and H. Pitot, unpublished observations). However, occasional AHF, especially those resulting from the initiation-promotion-initiation protocol, did exhibit an increased expression of the *fos* or *raf* proto-oncogenes in the internal or new focus. One interpretation of these results is in accord with the finding of transcriptional activation of proto-oncogenes within hepatocellular carcinomas in the rat.[83-85] Therefore, on the basis of findings both in the human and in several experimental systems, one may propose that a molecular characteristic of the stage of progression is the transcriptional activation of proto-oncogenes and that such activation may be mechanistically associated with the development of the stage of progression, as might be predicted from carcinogenesis by acutely oncogenic retroviruses. Since karyotypic changes are among the major mechanisms for the transcriptional activation of proto-oncogenes,[65] the critical nature of karyotypic instability in the genesis and maintenance of the stage of progression is further reinforced.

CONCLUSIONS

There is now substantial evidence, some of which has been presented in this discussion, to distinguish the stage of progression as a distinct and final stage in multistage carcinogenesis and the development of malignant neoplasia. Morphologic and karyotypic changes as well as alterations in genetic expression that are both absolutely and relatively unique to this stage, distinguishing it from the stages of initiation and promotion, have been described in one or more systems both in the animal and in the human. It should be emphasized again, however, that high doses of complete carcinogens, acutely transforming oncogenic viruses, as well as high doses of ionizing radiation may convert normal cells to cells already in the stage of progression without any demonstrable intervening stage of promotion. However, where the stage of promotion occurs, progression follows, either spontaneously or induced, before the appearance of malignant neoplasia.

The relationship of the three-stage concept of carcinogenesis to the requirement for two genetic events as proposed by Knudson[86] becomes obvious. Although the actions of proto-oncogenes are usually dominant, it is only with acutely transforming oncogenic viruses that such dominance is apparent *in vivo* without any known further genetic change. Even in artificial transfection experiments with activated proto-oncogenes, it appears that in most instances more than one genetic change is necessary.[87, 88] In the genetic systems originally forming the basis for Knudson's hypothesis, such as retinal blastoma, the second genetic change was presumed to be an allelic variation of the first. Subsequent recent findings have borne out this hypothesis (cf. ref. 89). Such gross chromosomal changes characteristic of the stage of progression would give an even greater chance for the second mutational change, especially if occurring in the same allele of the critical gene. It is of interest that recent investigations have demonstrated that cells of altered hepatic foci are largely diploid,[38] in contrast to normal hepatocytes, which are largely tetraploid. The two-event genetic hypothesis of Knudson could not function in a tetraploid cell; this suggests that the progenitors of carcinomas in the liver are this diploid population, a finding borne out by other investigators.[90]

An understanding of the characteristics and nature of the stage of progression is critical not only to the treatment, but also to the prevention of human neoplasia. As seen above, experiments now suggest that agents may exist which specifically act to convert cells from the stage of promotion to progression but which do not act to initiate cells. Such progressor agents could be very important in human health considerations, especially since humans exist in an environment, largely self-made, in which tumor promotion is predominant, especially in relation to the major human cancers.[5] In fact, it is likely that some known human chemical carcinogens should be classified primarily as progressor agents, such as benzene and asbestos.[5] Identification of other such progressor agents in the human environment may become very important both in our understanding of the genesis of human cancer and in its prevention.

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