Critical Aspects of Initiation, Promotion, and Progression in Multistage Epidermal Carcinogenesis (43511A)

HENRY HENNINGS,[1] ADAM B. GLICK, DAVID A. GREENHALGH, DAVID L. MORGAN, JAMES E. STRICKLAND, TAMAR TENNENBAUM, AND STUART H. YUSPA

Laboratory of Cellular Carcinogenesis and Tumor Promotion, Division of Cancer Etiology, National Cancer Institute, Bethesda, Maryland 20892

Abstract. **Carcinogenesis in mouse skin can be divided into three distinct stages: initiation, promotion, and progression (malignant conversion). Initiation, induced by a single exposure to a genotoxic carcinogen, can result from a mutation in a single critical gene (e.g., *ras*^{Ha}), apparently in only a few epidermal cells. The change is irreversible. Promotion, resulting in the development of numerous benign tumors (papillomas), is accomplished by the repeated application of a nonmutagenic tumor promoter. The effects of single applications of tumor promoters are reversible since papillomas do not develop after insufficient exposure of initiated skin to promoters or when the interval between individual promoter applications is increased sufficiently. The reversibility of promotion suggests an epigenetic mechanism. Promoter treatment provides an environment that allows the selective clonal expansion of foci of initiated cells. The conversion of squamous papillomas to carcinomas (termed progression or malignant conversion) occurs spontaneously at a low frequency. The rate of progression to malignancy can be significantly increased by treatment of papilloma-bearing mice with certain genotoxic agents. These progressor agents or converting agents are likely to act via a second genetic change in papillomas already bearing the initiating mutation. Progression in the skin is characterized by genetic changes that result in several distinct changes in the levels or activity of structural proteins, growth factors, and proteases. The mechanisms involved in progression are being studied in epidermal cell culture. In order to determine the *in vivo* phenotype of cultured cells, a grafting system was developed in which the cells were transferred from culture to a prepared skin bed in athymic mice. Introduction of an activated v-*fos* oncogene into initiated cells bearing an activated *ras*^{Ha} gene produced cells with a carcinoma phenotype, i.e., carcinomas formed when the cells were grafted as part of reconstituted skin. Grafted keratinocytes containing the *ras*^{Ha} gene alone produced papillomas; with v-*fos* alone, normal skin formed when grafted. The *ras*^{Ha}/*fos* carcinomas showed changes in differentiation markers characteristic of chemically induced carcinomas. A cell culture assay utilizing cells initiated by the introduction of an activated *ras*^{Ha} oncogene was developed to study progression. After exposure of initiated cells to progressor agents under conditions in which the proliferation of the *ras*^{Ha}-initiated cells was suppressed, proliferating foci developed, with a good correlation of activity in the assay with activity in the progression stage *in vivo*. The cell culture assay provides a quantitative model to study chemically induced neoplastic progression and may be useful to identify potential progressor agents.**

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Historical perspective. For over 50 years, the mouse skin model of experimental carcinogenesis has been important in defining the multiple stages in the neoplastic process (1). Since carcinogens were isolated from coal tar in the 1930s, repeated topical application to the skin and observation of tumor development with time was a convenient assay system (2). However, little was learned about mechanisms of action because high carcinogen doses were used. In 1941, studies of the cocarcinogenic activity of croton resin led Berenblum (3) to suggest that there were three phases in skin carcinogenesis: (i) a latent, precarcinogenic phase; (ii) a phase of papilloma development; followed by (iii) a phase of conversion to

[1] To whom requests for reprints should be addressed at National Cancer Institute, NIH, Building 37, Room 3B26, Bethesda, MD 20892.

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malignancy. Similar ideas had been expressed by Deelman (4) in the 1920s based on experiments in which mice were painted with tar, then wounded.

Protocols defining the first two discrete stages in mouse skin tumor formation, initiation and promotion, were developed in the mid-1940s (5–7). The first stage, initiation, was accomplished by a single topical application of a carcinogen at a dose which alone induced no skin tumors. Subsequent croton oil treatment of the mice initiated by the single application of carcinogen resulted in skin inflammation and hyperplasia, followed by development of many benign squamous papillomas and a few malignant squamous cell carcinomas. Promotion, the second stage in epidermal carcinogenesis, required repeated applications of croton oil for papillomas to appear, and many of the papillomas were dependent upon the continued presence of the promoter. The first promoter exposure could be delayed for several months with no diminution of tumor response, which indicates the irreversibility of initiation (8). Thus, from the earliest initiation-promotion studies, basic differences in the action of initiators and promoters were apparent (Table I).

As early as 1950, it was recognized that protocols with repeated carcinogen treatment were much more effective than initiation-promotion protocols for inducing malignant tumors (9). Thus, additional carcinogen-induced irreversible changes appeared likely to be important in the progression to malignancy. Shubik (9) recognized the irreversibility of the stage of conversion of papillomas to carcinomas, stating "the changes necessary . . . seem to be identical with the change involved in initiation." Even with this insight available in the literature for many years, a protocol demonstrating the third stage in experimental skin carcinogenesis, the malignant conversion of papillomas to carcinomas, was not developed until 1983 (10). After induction of papillomas on mouse skin by initiation and promotion, the papilloma-bearing mice were exposed to a tumor initiator, and carcinomas developed from papillomas at an increased rate and frequency. This stage has been termed malignant conversion or progression and active agents have been termed converting agents or progressor agents. These terms will be used interchangeably here. Fifteen to twenty percent of the squamous cell carcinomas progress further to metastasizing lesions (11); progressor agents vary in their ability to increase metastases (11). However, a specific treatment regimen to enhance this stage of epidermal progression has not been reported.

Initiation

It is generally accepted that initiation is accomplished in the skin by mutation in a critical gene or genes in only a few epidermal cells. A role for cell proliferation either before or soon after exposure to the initiator has been demonstrated (12) that perhaps results in fixation of the mutation. Examples of initiating carcinogens, including hydrocarbons and alkylating agents, are shown in Table II. Cell culture studies of initiated epidermal cell lines have indicated that the critical mutation alters the cells' ability to respond to signals that induce terminal differentiation (13). A single mutation may accomplish initiation, as indicated by initiation by introduction of a virus containing an activated ras^{Ha} gene (14, 15). More than 90% of the papillomas initiated by application of the hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA) or by injection of urethane contain an activated ras^{Ha} gene with a single A to T transversion at the second position of codon 61 (16–18). Other mutations must also produce the papilloma phenotype; less than 50% of the papillomas initiated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) contain an activating ras^{Ha} mutation (19). The heterogeneity of papillomas in appearance, growth rate, and persistence, as well as spontaneous conversion to malignancy, is consistent with the idea that different mutations or multiple mutations will be found in subpopulations of papillomas with different phenotypes.

Promotion

In contrast to the genetic changes necessary for initiation, promoter-induced changes are largely reversible and appear to be epigenetic. Promotion, accomplished by repeated treatments at the proper dosage and frequency (1), is characterized by selection for the growth of initiated cells resulting in their clonal expan-

Table I. Characteristics of Initiation and Promotion in Mouse Skin

Initiation
1. Rapid, single application effective
2. Irreversible
3. Additive
4. Induced by mutagens
Promotion
1. Requires weeks of exposure
2. Reversible
3. Epigenetic
4. Continued exposure required for maintenance of some papillomas
5. Accompanied by changes in gene expression
6. Selection for growth of initiated cells

Table II. Tumor Initiators for Mouse Skin

DMBA
Urethane (ethyl carbamate)
4-Nitroquinoline-*N*-oxide
MNNG
β-Propiolactone
Benzo(a)pyrene

sion to a papilloma. The most frequently studied tumor promoter (originally isolated from croton oil), the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is not directly mutagenic. Alterations in gene expression, induction of inflammation, proliferation, and terminal differentiation result from exposure to promoters, but no single critical change has been defined. A list of promoters for mouse skin is shown in Table III.

Progression

Progression of a benign tumor to a malignant tumor is characterized by an increased autonomy from both the environment and the host. This stage of conversion to malignancy, associated with an increased frequency of genetic changes (20), is irreversible. In 1980/81, Potter presented his initiator + promoter + initiator protocol for carcinogenesis in rat liver (21). His hypothesis (22) was stated as follows: "Cancer results from two or more relevant mutations: Promoters enhance proliferation of cells with one relevant mutation; this increases the probability of obtaining one cell with two relevant mutations." Potter used the term conversion to represent the second mutation caused by a second dose of initiator acting on a cell in the expanded population of initiated cells (21). Based on epidemiologic studies in man, Moolgavkar and Knudson (23) suggested that cancer could result from two mutations, that promotion is the expansion of the population of initiated cells, and that protocols to demonstrate these ideas should be developed in the mouse skin model of experimental carcinogenesis.

Independently, a protocol was developed to induce carcinomas in mouse skin by treating papilloma-bearing mice with a tumor initiator (10). Initiation by DMBA followed by promotion with TPA induces the development of multiple papillomas on mouse skin. However, few of these benign tumors progress to malignancy, although nearly all malignant tumors induced by the initiation-promotion regimen develop from benign papillomas (9). Genetic changes, seldom seen in early papillomas, are much more frequent at the time of spontaneous conversion to malignancy (20). The rate of progression to malignancy (malignant conversion) was increased by treatment of DMBA-initiated, TPA-promoted, papilloma-bearing mice with mutagenic agents. In the first experiments, when the genotoxic initiators urethane, MNNG, and 4-nitroquinoline-*N*-oxide were given once weekly for 20 to 30 weeks, carcinomas appeared earlier and in larger numbers than in mice treated with TPA or with the solvent acetone (10). This result emphasized the basic differences between the action of progressor agents (converting agents) and phorbol ester-type promoters. TPA efficiently promotes the development of papillomas, but, once papillomas have appeared, continued TPA treatment does not affect conversion to malignancy. Exposure to TPA accomplishes the expansion of clones of initiated cells to papillomas, greatly increasing the target population for the action of progressor agents. Progressor agents do not generally act to produce papillomas, but enhance the progression from papillomas to carcinomas. An exception is benzoyl peroxide, which is active both as a tumor promoter and a progressor agent (24).

In addition to the chemicals already mentioned, other agents reported to be active in progression include ionizing radiation (25), hydrogen peroxide (inactive as an initiator, weakly active as a promoter) (24), benzo(*a*)pyrene diol epoxide I, ethylnitrosourea (a weaker initiator than MNNG, but more effective than MNNG as a progressor agent) (26), and cisplatin (27) (Table IV). The induction of free radicals and the clastogenicity of both benzoyl peroxide and hydrogen peroxide may be important to their activity as progressor agents. The most likely common mechanism of action involves a further mutation in a papilloma cell; genetic changes defining malignant conversion are being sought (20). A specific genetic change is suggested by the finding that some progressor agents (MNNG, ethylnitrosourea, and benzoyl peroxide) are active when given as few as four times (26, 28), and cisplatin (27) and benzo(*a*)pyrene diol epoxide I (29) are active when given just once.

Papilloma Heterogeneity

The study of induced conversion of papillomas to carcinomas is complicated by the fact that subpopulations of papillomas vary substantially in their potential for progression. Many papillomas require continued exposure to TPA to prevent regression; some papillo-

Table III. Tumor Promoters for Mouse Skin

Croton oil
TPA
Benzoyl peroxide
Mezerein
Teleocidin
Aplysiatoxin
Okadaic acid
Chrysarobin
Wounding

Table IV. Tumor Progressor Agents (Converting Agents) for Mouse Skin

Urethane
MNNG
4-Nitroquinoline-*N*-oxide
Benzoyl peroxide
Hydrogen peroxide
Cisplatin
Ethylnitrosourea
Ionizing radiation
Benzo(*a*)pyrene diol epoxide I

mas regress even in the presence of TPA (11). Other papillomas can persist for the lifetime of the host without progressing to carcinomas. A short-term TPA promotion protocol, in which TPA treatments were ended after 5 weeks, produced only 20–40% of the papillomas found with promotion for 10 to 40 weeks. However, a similar number of carcinomas developed in mice promoted for 5, 10, 20, or 40 weeks, which indicates that the population of papillomas that appears first during promotion by a strong promoter contains all of the papillomas that will spontaneously progress to carcinomas (30). Apparently, the papillomas that developed later during TPA treatment did not possess the potential to progress to carcinomas. The "high-risk" papillomas that appear early during TPA promotion differ from the later-appearing papillomas in their persistence and their lack of dependence on TPA (Table V). These papillomas could have more than one critical mutation resulting from initiation, and may be destined for progression to malignancy from the time of initiation. High-risk papillomas, with a high spontaneous progression to malignancy, can also be promoted by mezerein (30), chrysarobin (31), or by wounding (32). The high-risk papillomas are also more responsive than later-appearing papillomas to the progressor agents urethane and 4-nitroquinoline-*N*-oxide (33).

Table V. Characteristics of High-Risk Papillomas

1. Selectively induced by limited exposure to a strong promoter
2. Selectively induced by "weak" promoters
3. Persistent
4. Arise early
5. More sensitive to mutagen-induced progression

Proteins Altered during Progression

A number of markers of epidermal growth and differentiation are altered during neoplasia in the skin (34). Some of the many changes reported in structural proteins, extracellular matrix receptors, growth factors, specific enzymes, and proteases are summarized in Table VI. In normal skin, two keratins, K5 and K14, are transcribed in basal cells, but the proteins persist in the suprabasal layers (35). The commitment of cells to differentiate is associated with the transcription of two suprabasal keratins, K1 and K10, in the spinous layer. In the granular layer, new proteins are expressed, including filaggrin, an interfilamentous matrix protein, and loricrin, a major component of the cornified envelope (36, 37).

In benign tumors induced by a DMBA initiation-TPA promotion protocol, the amount and distribution of K14 is similar to that in normal skin. Levels of K1 and K10 are reduced, although the tissue distribution remains suprabasal (35). Unlike normal epidermis, some suprabasal cells expressing K1 and K10 are capable of proceeding through S phase. Thus, the usual pattern of inhibition of proliferation-specific function while differentiation-specific genes are transcribed is altered. In carcinomas, the expression of several different keratins and other markers of epidermal differentiation is greatly altered (35). K14 protein and transcripts are diffusely expressed, whereas protein and transcripts for K1 and K10 are essentially absent from carcinomas. Levels of loricrin and filaggrin, present in both normal skin and papillomas, are greatly diminished in carcinomas. By immunofluorescence analysis, foci negative for expression of the suprabasal markers of keratinocyte differentiation can be noted prior to detection of a change in cellular phenotype by light microscopy, which suggests that these are early events that characterize malignant conversion (35). In addition, carcinomas express keratins, such as K8, that are not expressed in normal skin (38). K13, a marker of dysplastic, progressing papillomas, is abundant in well-differentiated carcinomas, but is lost from poorly differentiated carcinomas (39). As K13 disappears in poorly differentiated carcinomas, K8 is found more frequently.

Recently, we have shown alterations in transforming growth factor (TGF)-α and TGF-β during progression in mouse skin carcinogenesis (40). The level of TGF-α was increased in both papillomas and carcinomas compared with normal skin. TGF-β1 was not found in normal skin, but was abundant in the basal

Table VI. Expression of Marker Proteins in Normal Skin and Skin Tumors

Marker protein	Normal skin	Papillomas	Carcinomas
Structural proteins			
K5	++	++	+
K14	++	++	+
K1	++	+	—
K10	++	+	—
K6	++[a]	++	++
K13	—	+[b]	+[c]
K8	—	—	+
Loricrin	++	++	—
Filaggrin	++	++	—
Growth factors			
TGF-α	+	++	++
TGF-β1	—	++	—
TGF-β2	++	++	—
Proteases			
Stromelysin (transin)	—	±	++
Urokinase	—	+	++
Enzymes			
γ-Glutamyltranspeptidase	—	+	++
Integrin receptors			
$\alpha 3\beta 1$	+	+	±
$\alpha 6\beta 4$	+	++	++

[a] Hyperproliferative only.
[b] Focal in dysplastic papillomas.
[c] Well-differentiated carcinomas.

and spinous layers of low-risk papillomas. TGF-β2 was found both in normal skin and in the differentiating layers of low-risk papillomas. In contrast, 75–80% of high-risk papillomas did not express TGF-β1 or TGF-β2, a phenotype also found in most squamous cell carcinomas. Thus, the loss of TGF-β expression is an important marker for benign tumors with a high risk for malignant conversion.

Interaction of cells with the basement membrane is important in the control of epidermal proliferation and differentiation. Disruption of the basement membrane is an early event in the progression from benign to malignant tumors; malignancy is generally defined by invasion through the basement membrane. Cell-matrix interactions are altered during tumor development and progression. The cell surface integrin receptor $\alpha 6\beta 4$, present on the basal surface of basal cells in normal skin, was expressed in suprabasal as well as basal layers of dysplastic papillomas, and became the predominant receptor expressed in most cells in carcinomas (39). The integrin receptor $\alpha 3\beta 1$ was expressed in the differentiating cells of normal epidermis as well as along the basal side of cells in contact with the basement membrane. In papillomas, $\alpha 3\beta 1$ antibody staining was increased in areas of advanced differentiation, but was decreased in more dysplastic papillomas, and nearly absent in most carcinomas. The localization of K13 appears to be linked to changes in matrix receptors; K13 is found in suprabasal differentiating cells, but only in $\alpha 6\beta 4$ negative cells. The distribution of $\alpha 6\beta 4$ in the proliferating cells of dysplastic papillomas as well as in carcinomas is associated with the decreased expression of K13.

Changes in the activity of enzymes such as γ-glutamyltranspeptidase and various proteases have been associated with progression in mouse skin. γ-Glutamyltranspeptidase is present in carcinomas, but absent from most papillomas (41). Similarly, stromelysin (transin) is commonly elevated in mouse skin carcinomas, but not papillomas (42). Collagenase activity is increased in many malignancies and has been associated with invasion and metastasis (43). These secreted enzymes are components of a cascade that includes other classes of proteases. Members of this cascade are secreted as proenzymes, requiring activation by partial proteolysis. A critical activating function is attributed to plasmin, the product of the action of urokinase on the plasmin precursor, plasminogen (44). Our preliminary studies indicate that high levels of both stromelysin and urokinase transcripts are found in carcinomas but not papillomas when compared with normal skin levels (34).

Activation of *ras*^{Ha} and *fos* in Keratinocytes Induces a Carcinoma Phenotype

Initiation of skin carcinogenesis can be accomplished by infection with retroviruses carrying activated *ras*^{Ha} genes (14, 15). Treatment with some initiating chemicals, particularly DMBA (16) and urethane (17, 18), results in papillomas and carcinomas with an activated *ras*^{Ha} gene. Thus, this single mutation is sufficient to produce the papilloma phenotype. Introduction of an activated *ras*^{Ha} gene into mouse basal keratinocytes in culture increases the proliferation rate of the cells 5-fold in medium with 0.05 mM Ca^{2+} (45). The infected cells are resistant to terminal differentiation induced by 1.4 mM Ca^{2+} in the medium, a property shared by a number of initiated keratinocyte cell lines (46). Transfer of cultured *ras*^{Ha}-infected keratinocytes to a prepared graft bed on recipient athymic mice produced papillomas, consistent with the complete initiating activity of this single genetic change (15).

Studies of fibroblasts in cell culture have indicated that two different oncogenes can act together to achieve malignant transformation (47). The introduction of certain viral oncogenes into fibroblasts immortalized previously by chemical carcinogens caused malignant progression (48), which suggests that chemically induced mutations can cooperate with oncogenes to produce the malignant phenotype.

Keratinocyte cell lines have been established from initiated mouse skin or from mouse skin papillomas; these cells produce benign tumors when grafted as part of a reconstituted skin to the backs of athymic mice (49). Two cell lines, designated 308 and SP-1, have been used as recipients for transfected oncogenes to study malignant conversion in cell culture (50). Both cell lines contain an activated *ras*^{Ha} gene with an A to T transversion at codon 61, as expected from their origin in skin treated with DMBA. Plasmid DNA, encoding a specific oncogene construct, were stably transfected into each cell line. All plasmids were used in cotransfections with a neomycin phosphotransferase gene (*neo*^{R}) contained in pSV$_2$*neo* to select for transformants in the presence of G418. The oncogenes *myc* and *E1A* were chosen for study because they had been shown to cooperate with a *ras* oncogene in other cell lines. The *fos* oncogene was chosen because of its expression during embryonic skin development (51). The products of these oncogenes are localized to the nucleus and are involved in the transcriptional control of other genes.

After transfection and selection in G418 medium, the uptake and expression of exogenous DNA in recipient cells were confirmed by DNA and RNA hybridization analysis (50). The transfected cells were then grafted to nude mice and the tumors were examined histologically and characterized immunohistochemically. The v-*fos* construct caused malignant conversion in both cell lines, as demonstrated by the squamous cell carcinoma histology of tumors from grafted cells (Table VII). The benign tumor phenotype found with untreated 308 or SP-1 cells was not altered by transfection of either pSV$_2$*neo*, c-*fos*, *myc*, or *E1A*. The tumors derived by introduction of v-*fos* lacked the suprabasal

Table VII. Influence of Specific Oncogenes on Progression of Keratinocytes

Exogenous gene	Phenotype	
	In vivo skin graft	*In vitro* Ca^{2+}-induced differentiation
Transfection into SP-1 and 308 cells		
None	Papilloma	Resistant
neo^{R}	Papilloma	Resistant
myc	Papilloma	Resistant
E1A	Papilloma	Resistant
c-*fos*	Papilloma	Resistant
v-*fos*	Carcinoma	Resistant
Retroviral infection into normal keratinocytes		
None	Normal skin	Sensitive
v-*fos*	Normal skin	Sensitive
v-*ras*^{Ha}	Papilloma	Resistant
v-*fos*/v-*ras*^{Ha}	Carcinoma	Resistant

keratin markers K1 and K10 using specific antisera for indirect immunofluorescence staining. Although the grafted tumor phenotype was clearly altered by the introduction of v-*fos*, no changes in the *in vitro* phenotype could be detected. The transfected cell lines were similar to parental cells in growth rate, lack of response to Ca^{2+}-induced terminal differentiation, and anchorage dependence.

Since 308 and SP-1 cells may have acquired genetic changes other than the *ras*^{Ha} mutation, it was possible that a gene other than *ras*^{Ha} contributed to the action of the *fos* oncogene in malignant conversion. To directly test the cooperativity of *ras* and *fos* oncogenes in causing progression to malignancy, primary cultures of keratinocytes were used as targets for these genes. Replication-defective retroviruses were prepared by transfecting plasmids containing *ras*^{Ha}, v-*fos*, or the *neo*^{R} gene into Psi 2 cells, selecting transformant cells, and collecting supernatants. After exposure of keratinocytes to retroviral supernatants, the *in vivo* phenotype of the cells was determined by grafting in reconstituted skin grafts (Table VII). Either uninfected or *neo*^{R}-infected keratinocytes were sensitive to Ca^{2+}-induced differentiation in culture and produced normal skin when grafted. The v-*fos*-infected keratinocytes could not be distinguished from these control cells in culture, producing normal skin in grafts. Exposure to *ras*^{Ha} alone produced squamous papillomas as expected; the combined exposure to *ras*^{Ha} and v-*fos* resulted in squamous carcinomas in the graft test. The benign tumors resulting from infection with *ras*^{Ha} alone contained K1 and K10, but both of these suprabasal cell markers were lacking in the tumors resulting from combined infection with *ras*^{Ha} and v-*fos* (52).

The induction of the carcinoma phenotype by cooperation of two oncogenes, v-*fos* and v-*ras*^{Ha}, provides a model to analyze a possible mechanism of malignant conversion. The v-*fos* gene product appears to be the critical element in progression. The v-*fos* protein modulates transcription by forming a heterodimer complex with the transcription factor AP1. This *fos*/AP1 complex has been shown to enhance the expression of two secreted proteases that are increased in carcinomas, stromelysin, and collagenase (53). Thus, as a selective transcriptional enhancer, v-*fos* may induce malignant conversion in the epidermis by altering the expression of specific genes.

Cell Culture Assay for Malignant Conversion

The analysis of the genetic targets responsible for carcinogen-induced neoplastic progression would be facilitated by the development of cell culture models in which the process is rapid, focal, and quantitative. For this purpose, primary newborn mouse keratinocytes were initiated in culture by the introduction of the v-*ras*^{Ha} oncogene via a defective retrovirus. Recipient cells produce squamous papillomas when grafted to athymic mice and proliferate rapidly in culture medium with 0.05 mM Ca^{2+}. The v-*ras*^{Ha}-infected cells persist, but fail to grow in medium with 0.5 mM Ca^{2+}, conditions favoring the growth of malignant keratinocytes. When keratinocytes infected with v-*ras*^{Ha} were exposed to mutagenic converting agents *in vitro*, proliferating foci emerged after culture in 0.5 mM Ca^{2+} for 4 weeks (54). These foci stained intensely and were easily quantitated. Numbers of foci increased with the dose of several converting agents tested (Table VIII). Cisplatin, benzo(*a*)pyrene diol epoxide I, MNNG and 4-nitroquinoline-*N*-oxide, progressor agents in mouse skin, were all active in this assay. *N*-Acetoxyacetylaminofluorene, a strong mutagen that has not been tested as a progressor agent, was inactive, as was the promoter TPA. Benzoyl peroxide, effective as both a promoter and progressor agent in mouse skin, causes excessive cytotoxicity with chronic exposure at biologically active concentrations *in vitro*. Thus, we tested only a single exposure to 10 mM benzoyl peroxide, and no foci were induced. This test may have been inadequate; alternatively, the assay may be deficient in detecting agents that may act via generation of free radicals.

Table VIII. Comparison of Agents Tested to Induce Proliferative Foci in *ras*^{Ha}-Infected Keratinocytes

Agent	Dose range (μM)	Foci/dish
Benzo(*a*)pyrene diol epoxide I	0.1–0.3	1–2
Cisplatin	2–4	1–6
4-Nitroquinoline-*N*-oxide	3–8	1–2
MNNG	6–20	1–10
N-Acetoxyacetylamino-fluorene	1–30	None
TPA	0.16–0.80	None

Of the cell lines derived from foci, a subset produced malignant tumors when grafted to nude mice while others were not tumorigenic. Analysis of DNA from cell lines and tumors revealed that tumorigenic cells maintained the v-*ras*^{Ha} genome. However, the viral sequences were frequently lost from nontumorigenic cell lines. Cells in foci, but not the surrounding v-*ras*^{Ha} cells, expressed keratin 13, a marker strongly associated with malignant progression of skin tumors in mice. This cell culture assay provides a quantitative model to study chemically induced focal neoplastic progression at the cellular level and to identify potential progressor agents.

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