Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6389–6393, September 1988
Cell Biology

Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells

(multistep carcinogenesis/*ras* oncogene/dermal–epidermal interactions)

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Contributed by Robert A. Weinberg, May 17, 1988

ABSTRACT **The activated *ras* oncogene that is present in Harvey sarcoma virus is able to induce malignant transformation of pure cultures of mouse primary keratinocytes. Malignant transformation of these cells is demonstrated by their ability to form carcinomas when grafted back onto syngeneic animals. However, expression of the malignant phenotype by the *ras*-transformed keratinocytes is drastically inhibited by the presence of normal dermal fibroblasts. This inhibitory effect depends on the ratio of fibroblasts to keratinocytes. It can be observed with mitomycin C-treated growth-arrested dermal fibroblasts and not with other cells, such as normal keratinocytes or established fibroblasts. Thus, a cellular environment approximating normal tissue can suppress tumor formation triggered by a single oncogene.**

Recently attempts have been made to associate the genetic lesions occurring during specific steps of carcinogenesis with the molecular events leading to activation of identified oncogenes (1–6). In particular, in the mouse skin model, good evidence has been produced that points to the specific role of *ras* activation in the initiation of carcinogenesis (4, 5, 7, 8). In this system, additional genetic changes, acting in concert with the *ras* oncogene, appear to be required for carcinoma formation (7). Contrasting with these studies is older work showing that oncogenic retroviruses such as Harvey sarcoma virus (HaSV) are able on their own to rapidly induce full-blown tumors (9). Because a virus such as HaSV carries only a *ras* oncogene, this would suggest that a single oncogene is indeed able to trigger full tumor formation on its own.

In an attempt to reconcile these observations, we have examined the response of mouse skin keratinocytes to an introduced *ras* oncogene in reconstituted tissue, thinking that the behavior of these cells depends critically on the environment around them. Using this system, we have come across evidence that behavior of the oncogene-transformed keratinocytes depends both on their complement of active oncogenes and on the cellular context in which these cells find themselves.

MATERIALS AND METHODS

Cells. Primary keratinocytes from newborn BALB/c mice were grown in medium at low calcium concentration as previously described (10, 11). Infection with Moloney murine leukemia virus (MuLV) or HaSV plus helper was carried out 2–3 days after plating: 2 ml of virus (1×10^6 transforming units/ml) per 10-cm dish, in Polybrene (Aldrich) at 8 μg/ml, for 2 hr. Cultivation was continued for 7 more days.

Primary dermal fibroblasts were prepared from newborn BALB/c mice at the same time as keratinocytes, by treatment of dermis with collagenase (0.4 mg/ml, Boehringer Mannheim) for 30 min at 37°C. Cells were plated (2×10^6 cells per 10-cm dish) in Dulbecco's modified Eagle's medium/10% fetal bovine serum (HyClone, Logan, UT). One or 2 days later they were split 1:4, infected with MuLV (in Polybrene at 8 μg/ml for 2 hr), and grown again to confluence. Cells were split once more 1:4 and again grown to confluence and kept until used for grafting. Successful MuLV infection of fibroblasts, keratinocytes, and BALB/c 3T3 cells was confirmed by XC assays (12).

Grafting. Grafting was performed as described in refs. 13 and 14. The surgical procedure involved two steps. In step one, a glass disk was implanted subcutaneously. Step two was carried out 2–4 weeks later by replacement of the glass disk with a dome-shaped, open-bottom silicone chamber (Renner, Heidelberg). Cells were brought into suspension in low-calcium (0.05 mM) minimal essential medium (MEM), mixed in various combinations, and immediately injected into the silicone chambers. Rapid execution ensured minimal nonspecific killing of cells during experimental manipulations prior to grafting. In all experiments positive controls were included, with HaSV-infected keratinocytes grafted alone always after grafting of the same cells admixed with fibroblasts. Viability of cells was also controlled by replating some of them at the end of each experiment on collagen-coated dishes in MEM. HaSV production from the replated keratinocytes (normal or mitomycin C-treated) was controlled by collecting medium 3 days after replating and using it, after appropriate dilution, for focus assays with Fisher rat 3T3 fibroblasts. Similar HaSV titers were produced by normal and mitomycin C-treated keratinocytes. Mice were sacrificed 10–14 days after engraftment and tissue was fixed in Formalin for histologic analysis.

Immunoblotting. Tumors were isolated from the grafts and separated from contaminating surrounding skin. Specimens were minced while still frozen and extracted in 9.5 M urea/10 mM Tris·HCl pH 7.5/1% 2-mercaptoethanol. Similar amounts of proteins—as determined by colorimetric reactions (Bio-Rad)—were analyzed by $NaDodSO_4$/10% acrylamide gel electrophoresis. Immunoblotting with a mixture of AE1 and AE3 anti-keratin monoclonal antibodies (Hybritech, La Jolla, CA) and peroxidase staining was as described by Woodcock-Mitchell *et al.* (15).

***In Situ* Hybridization.** *In situ* hybridization studies were performed as described in refs. 16–18. Tissue sections were hybridized to ^{35}S-labeled DNA probes, prepared by labeling gel-purified specific fragments with deoxycytidine 5′-[α-[^{35}S]thio]triphosphate (>1000 Ci/mmol, Amersham; 1 Ci =

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Abbreviations: HaSV, Harvey sarcoma virus; MuLV, murine leukemia virus.

37 GBq) by use of random oligonucleotide primers (P-L Biochemicals). Slides were washed under high-stringency conditions and exposed for autoradiography in Kodak NTB-2 emulsion.

RESULTS AND DISCUSSION

Carcinoma Formation by *ras*-Transformed Primary Keratinocytes

To study skin carcinogenesis, we have chosen an *in vitro–in vivo* protocol that depends on grafting cultured keratinocytes onto the back of a mouse. This results in the reconstitution of well-stratified epidermis (13, 14). The grafting technique that we have adopted, developed by N. Fusenig and coworkers (13, 14), involves the use of dome-shaped silicone transplantation chambers that are placed onto the back of the mouse in a way that prevents invasion of the graft bed by the surrounding host skin.

In a set of control experiments, we observed that normal primary keratinocytes formed a thin layer of skin as early as 10–14 days after grafting (Fig. 1*A*). Consistent with results of others (14), concomitant addition of dermal fibroblasts to keratinocytes at the time of grafting resulted in better take of grafts, with more layers of cells in the newly formed epidermis (Fig. 1*B*).

In experiments aimed at exploring the role of the *ras* oncogene in keratinocyte transformation, HaSV was used to convey the activated oncogene into cells. Primary keratinocytes were infected with HaSV 2–3 days after plating at a time when cells were 30–40% confluent. The presence of helper MuLV ensured spread of infection to all cells in the culture (19) and resulted in the production of virus-encoded *ras* p21 protein in amounts comparable to those detected in HaSV-infected NIH 3T3 fibroblasts (data not shown).

Grafting of HaSV-infected keratinocytes onto syngeneic BALB/c mice was performed 7 days after exposure to the virus and experiments were terminated 14 days thereafter. At that time, macroscopic examination was already sufficient to detect differences between grafts of HaSV-infected keratinocytes and those of control cells infected with helper virus alone. The helper virus does not transduce an oncogene and has no apparent effect on the phenotype of the keratinocytes; in MuLV-infected controls, the graft bed was covered by a thin film, often difficult to visualize. In contrast, large, erosive, and often (>70% of cases) hemorrhagic tumors were evident after grafting of HaSV-infected cells.

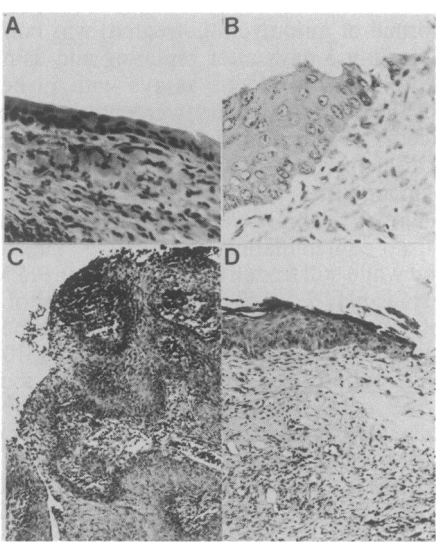

FIG. 1. Grafting assay: Histologic analysis of grafts, 14 days after grafting. (*A* and *B*) MuLV-infected keratinocytes grafted alone (*A*) or in association with dermal fibroblasts (1:4 ratio) (*B*). (×220.) (*C* and *D*) HaSV-infected keratinocytes grafted alone (*C*) or in association with dermal fibroblasts (1:4 ratio) (*D*). (×55.)

Histological examination revealed heterogeneous tumors that had infiltrated surrounding tissues (Fig. 1*C*, Fig. 2, Table 1). Epithelial characteristics were evident in many cases, including areas of well-conserved differentiation (grade I and grade II carcinomas, as defined in Table 1) (Fig. 2 *A* and *B*). Next to these areas there were large areas of marked anaplasia, for which a diagnosis of origin on purely histological criteria was not possible (grade III carcinomas) (Fig. 2 *C* and *D*). In particular, we note that the morphology observed in these cases is equally suggestive of spindle cell carcinomas, commonly seen in mouse skin tumors (20), and fibrosarcomas.

The heterogeneous histological picture of these tumors could be explained by infection by replication-competent HaSV of several keratinocyte cell types (stem cells, partially committed cells, etc.) present originally in the primary cultures. Alternatively, the anaplastic parts of the tumors could result from horizontal spread of transforming virus from infected keratinocytes to surrounding cells of other kinds, either contaminating the engrafted cultures or present as host tissue at the site of grafting.

Positive identification of the origins of these anaplastic regions was provided by immunohistochemical techniques (Table 2). Use of immunohistochemical stains for vimentin, factor VIII, and *Ulex* lectin ligand, which are specific markers for cells of mesenchymal and vascular origin, yielded completely negative results in all cases (Table 2, Fig. 2). Conversely, extensive reaction was detected upon testing with a mixture of anti-keratin AE1 and AE3 monoclonal

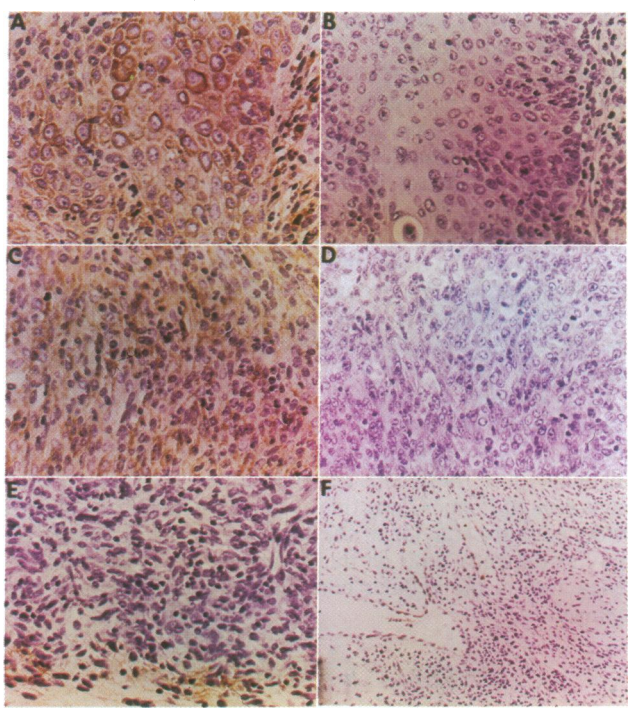

FIG. 2. Keratin production in tumors as detected by immunoperoxidase staining. (*A* and *B*) Grade II carcinoma, plus a small area of grade III, stained with keratin-specific monoclonal antibodies (AE1/AE3) (*A*) or with vimentin-specific monoclonal antibodies (*B*). (×280.) (*C* and *D*) Grade III carcinoma stained with keratin-specific monoclonal antibodies (AE1/AE3) (*C*) or with vimentin-specific monoclonal antibodies (*D*). (×280.) (*E*) Periphery of a tumor (grade III carcinoma), with anti-vimentin antibodies staining surrounding fibroblasts. (×280.) (*F*) Grade III carcinoma, with anti-factor VIII antibodies staining vascular cells. (×140.) The antibodies used in these studies are as described in the legend of Table 2.

Table 1. Malignant conversion of primary keratinocytes as detected by grafting

Treatment and additions to HaSV-keratinocytes	Macroscopic tumors/mice	Histologic diagnosis of carcinoma
None	30/30 (11)	Grades I–III
Mit. C, 0.4 mg/ml	0/7 (2)	No Car.
Mit. C, 1.2 mg/ml	0/4 (1)	No Car.
D. Fs./MuLV (1:4)	3/29 (10)	No or little Car.
D. Fs./MuLV (1:0.4)	3/3 (2)	Grades I–III
D. Fs./MuLV (1:0.1)	2/2 (1)	Grades I–III
D. Fs./MuLV/Mit. C (1:4)	0/11 (4)	No or little Car.
D. Fs./Mit. C (1:4)	0/16 (5)	No Car.
D. Fs. (freeze-thawed)	2/2 (1)	Grades I–III
Krtcs./MuLV (1:4)	3/3 (1)	Grades I–III
3T3 Fs./MuLV (1:4)	6/6 (2)	Grades I–III

All mice received 6×10^5 HaSV-infected keratinocytes and the indicated additions. Mit. C, mitomycin C (0.4 mg/ml where no concentration is indicated). Ratio of HaSV-infected keratinocytes to dermal fibroblasts (D. Fs.), normal keratinocytes (Krtcs.), and 3T3 fibroblasts (3T3 Fs.) is indicated in parentheses. The number in parentheses in the second column is the number of independent experiments performed for each combination of cells. Mice were sacrificed 10–14 days after grafting and tissues were fixed in Formalin for histologic analysis. Tumors were classified as follows: grade I, well-conserved epithelial morphology and organization, lower mitotic index, and various degrees of squamous differentiation; grade II, large cells with less evident epithelial organization, with big and anomalous nuclei; grade III, markedly anaplastic areas characterized by high cellularity, fusiform shapes, or both. Histologic examination was supplemented by immunohistochemical analysis (see Table 2 and Fig. 2).

antibodies (15). Eighty to 100% of tumor cells showed good cytoplasmic staining even in anaplastic areas containing spindle-shaped cells (Fig. 2 *A–D*).

The specificity of antibody reactivity and keratin production in various tumors was confirmed by gel electrophoresis and immunoblotting of tumor-derived extracts with AE1/AE3 anti-keratin monoclonal antibodies (15). As shown in Fig. 3 *Upper*, Coomassie blue staining of the polyacrylamide gel revealed that urea extracts of tumors (lanes 1–5), skin (lane 6), and cultured keratinocytes (lane 7) contained several specific bands that were absent from similar extracts from cultured dermal fibroblasts (lanes 8 and 10) or subcutaneous tissue (lanes 9 and 11). One or two of these bands, with an approximate molecular mass of 50 kDa, were specifically detected after immunoblotting of the same gel with AE1/AE3 anti-keratin monoclonal antibodies (Fig. 3 *Lower*, lanes 1–7). In the case of one tumor (lane 2), in spite of large keratin production (*Upper*), only weak reactivity could be detected in the corresponding immunoblot (*Lower*; a weak band, hard to detect after photographic reproduction, was clearly visible in the original blot).

Table 2. Immunohistology of tumors formed by HaSV-transformed keratinocytes

Antigenic marker	No. of positive tumors	% positive cells
Keratins	20/20	80–100
Vimentin	0/11	—
Factor VIII-related antigen	0/9	—
Ulex lectin ligand	0/11	—

Immunohistochemistry in all cases used the biotin-avidin method (21). Appropriate negative controls with nonimmune sera or ascitic fluid (for comparison with monoclonal antibodies) were always included. Sources of antibodies against the corresponding antigens were as follows: anti-keratin monoclonal antibodies AE1/AE3, Hybritech; anti-vimentin, Biogenics; anti-factor-VIII, Dako (Santa Barbara, CA); and anti-*Ulex europaeus* lectin I, and the lectin itself, Vector Laboratories (Burlingame, CA).

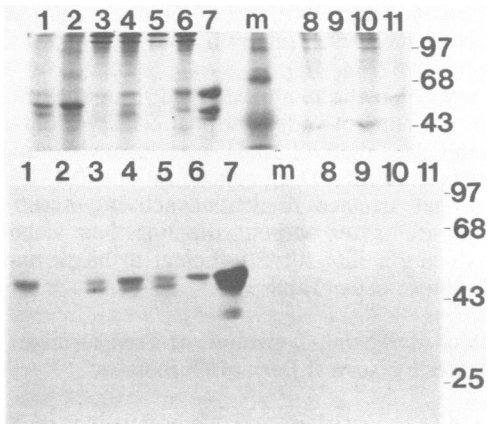

FIG. 3. Keratin production in tumors as detected by immunoblotting. (*Upper*) Coomassie blue staining of gel. (*Lower*) Immunoblot of the same gel tested with AE1/AE3 anti-keratin monoclonal antibodies (15). Extracts were from tumors in lanes 1–5, normal skin in lane 6, cultured keratinocytes in lane 7, cultured dermal fibroblasts in lanes 8 and, in double amounts, 10, and subcutaneous tissue in lanes 9 and, in double amounts, 11. Lane m contains size markers, whose molecular masses are indicated on the right in kilodaltons.

The epithelial origin of tumors, as judged by keratin production, was independently confirmed by *in situ* hybridization with keratin-specific cDNA probes (22, 23). Strong and extensive positivity was detected even in the anaplastic areas of tumors, with no reactivity in surrounding tissues (Fig. 4 *A–C*). Viral Ha-*ras* RNA expression was also easily detectable in the same tumors by use of a specific v-Ha-*ras* DNA probe (24) (Fig. 4*D*). Again, no reactivity could be detected in the surrounding tissues (Fig. 4*E*). An unrelated

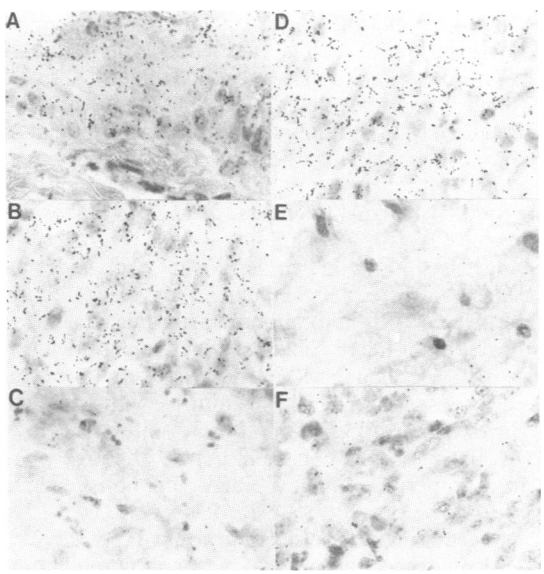

FIG. 4. *In situ* hybridization of tumors with keratin- or *ras*-specific probes. (×670.) (*A–C*) Normal skin (*A*), grade III carcinoma (*B*), and underlying normal tissue (*C*) hybridized with a keratin-specific cDNA probe [corresponding to human epidermal 50-kDa type I keratin; KB-2 clone (22); similar positivity was observed with another cDNA clone, KA-1 (23), corresponding to human epidermal 56-kDa type II keratin (data not shown)]. (*D* and *E*) Grade III carcinoma (*D*) and underlying normal tissue (*E*) hybridized with a v-Ha-*ras*-specific probe (24); similar positivity for v-Ha-*ras* RNA expression was detectable in the limited neoplastic areas formed in some of the grafts of HaSV-transformed keratinocytes together with dermal fibroblasts (data not shown). (*F*) Grade III carcinoma (same tumor as *B* and *D*), hybridized with a cDNA probe specific for rubella virus, kindly provided by T. K. Frey (Georgia State University, Atlanta).

but similarly prepared DNA probe (complementary to rubella virus) yielded negative results in both neoplastic and surrounding tissues (Fig. 4*F*).

Final proof that the tumors derived from engrafted keratinocytes and not from adjacent host cells was obtained by mitomycin C treatment of HaSV-transformed keratinocytes 24 hr prior to grafting. Drug concentrations (0.4–1.2 mg/ml for 2 hr) that arrested replicative activity of the HaSV-infected keratinocytes without affecting their viability and virus-producing ability were sufficient to block tumor formation by these cells (Table 1).

Inhibition of Malignant Growth of *ras*-Transformed Keratinocytes by Normal Dermal Fibroblasts

Conversion of primary keratinocyte populations into aggressive carcinomas occurred with great rapidity. These cells were infected and expanded *in vitro* for only 10 days. After grafting, they yielded clearly observable tumors within 10–14 days in all 30 hosts. This rapid development of tumorigenic cell populations makes it unlikely that genetic alterations beyond acquisition of the *ras* oncogene were required for tumor formation. Such a conclusion conflicts with the model that tumorigenesis requires the involvement of multiple distinct oncogenes (1–3). Moreover, the data contrast with results of a very similar set of experiments using skin engraftment described recently by others (8).

In these other experiments, keratinocytes were infected *in vitro* by HaSV that lacked helper virus and were grafted in the presence of an excess of normal dermal fibroblasts (8). This experimental design was likely to result in the engrafting of a relatively small number of initially infected keratinocytes together with large excess of uninfected keratinocytes and fibroblasts. The absence of helper virus prevented recruitment of transformants by spread of HaSV.

In our own experiments, in contrast, use of helper virus allowed infection of a high percentage of keratinocytes, which were then grafted in the absence of dermal fibroblasts. Accordingly, we speculated that the composition of the engrafted cell population is a strong determinant of the subsequent behavior of these cells and could result in formation of either benign papillomas, as seen by Roop *et al.* (8), or frankly malignant cancers, as seen in our own experiments.

To address this model, experiments were undertaken in which HaSV-infected keratinocytes were engrafted alone or after admixture with normal dermal fibroblasts. Inadvertent transformation of the fibroblasts, which might occur through release of infectious HaSV from the keratinocytes, was blocked by infecting fibroblast cultures with MuLV helper virus prior to grafting. This MuLV infection, in which virtually 100% of cells can be infected, effectively protects cells from superinfection by HaSV (unpublished observations).

We undertook a new series of grafting experiments, using HaSV-infected keratinocytes alone or in combination with the protected dermal fibroblasts. As mentioned before, in the absence of admixed fibroblasts, keratinocytes proceeded to yield highly invasive tumors (30 out of 30) (Table 1). However, in 26 of 29 mice engrafted with an identical number of these *ras*-transformed keratinocytes together with a 4-fold excess of dermal fibroblasts, malignant growth of the keratinocytes was drastically inhibited (Table 1). In these 26 cases, implanted cells yielded no macroscopically visible tumors or only very thin and diffuse areas of enduration, which differed at least 10- to 20-fold in weight from the tumors induced in the absence of fibroblasts. In the three cases in which tumor formation could be detected in spite of admixed dermal fibroblasts, tumors consisted of small single knobs at the periphery of the graft that might have been due to uneven mixture of cells at the time of grafting. A decrease in fibroblast number to 1/10th abolished the inhibitory effects of these cells on *ras*-transformed keratinocytes (Table 1).

Inhibition of malignant growth by dermal fibroblasts, initially observed macroscopically, was confirmed histologically. In many cases (>50%), only reactive tissue was present at the site of grafting. In other cases, however, nests or small areas of hyperplastic cells were detected, but proliferation was clearly contained and poorly invasive (Fig. 1*D*).

In no cases could we observe papilloma formation as described by Roop *et al.* (8). These authors performed their grafting experiments in *nude* mice, while we used syngeneic immunocompetent animals. Thus, it is possible that the different immune status of the host has an important role in determining whether fibroblast inhibition of *ras*-transformed keratinocytes results in virtual suppression of growth or in its diversion to a controlled benign pattern.

Effect of Fibroblast Growth on Tumor Inhibition

As mentioned before, mitomycin C treatment of cells causes growth arrest while leaving metabolic activity intact. By inhibiting growth of dermal fibroblasts with mitomycin, we could block their ability to compete for limiting space and nutrients. Moreover, these mitomycin-treated cells did not require the protection conferred by MuLV infection, since they had lost the proliferative capacity that is necessary for HaSV-induced transformation. Accordingly, they could be used in an uninfected state.

Dermal fibroblasts, either infected with MuLV as described before or uninfected, were treated with mitomycin C (0.4 mg/ml for 2 hr) 24 hr prior to grafting. These cells were then grafted after mixing with HaSV-transformed keratinocytes. In parallel, experiments were conducted with keratinocytes grafted alone or in association with MuLV-infected mitomycin-untreated fibroblasts. As shown in Table 1, mitomycin C treatment did not affect the ability of dermal fibroblasts to block carcinoma formation by HaSV-transformed keratinocytes. Similar effects were observed with both MuLV-infected and uninfected fibroblasts. Thus, the inhibitory effect of dermal fibroblasts on *ras*-transformed keratinocytes is not dependent on either their replicative activity or their production of MuLV.

Effects of Other Cells on the Growth of *ras*-Transformed Keratinocytes

Given the important role of dermal–epidermal interactions in the physiology and pathology of skin (14, 25, 26), it was of interest to ascertain whether the inhibitory effects observed with dermal fibroblasts are specific to these cells or can be observed with other types of cells as well.

Accordingly, HaSV-infected keratinocytes were grafted alone or in 1:4 admixture either with normal untransformed keratinocytes or with cells of an established fibroblast line (BALB/c 3T3). As in the case of dermal fibroblasts, prior infection of admixed cells with MuLV was used to prevent their infection with HaSV released by the transformed keratinocytes.

Carcinoma formation by *ras*-transformed keratinocytes was unaffected by addition of untransformed keratinocytes or 3T3 fibroblasts (Table 1). Histological and immunohistochemical analysis confirmed carcinoma formation in all cases (data not shown). We conclude that only specific cell types, such as dermal fibroblasts and possibly primary fibroblasts of other origin, are able to exert an inhibitory effect on *ras*-bearing keratinocytes.

CONCLUSIONS

The data presented here show that activation of a single *ras* oncogene is sufficient, under special conditions, to cause malignant conversion of normal primary keratinocytes. Our findings contrast with the benign tumor formation reported by two other groups (7, 8) after exposure to the same transforming agent (HaSV) that was used in these studies. The remarkable difference in results is consistent with our demonstration that the cellular environment of a keratinocyte plays a crucial role in its responsiveness to transformation by an oncogene. The results that were obtained by grafting have been recently confirmed by use of an alternative assay, that of subcutaneous injection into *nude* mice (G.P.D., unpublished results).

The inhibitory effects of normal cells on the growth of transformed neighbors have been well documented in a number of *in vitro* monolayer models (24, 27–29). In addition, embryonal tissues seem able to suppress tumorigenicity of teratocarcinoma (30) or melanoma (31) cells. The present work establishes a strong parallel with the previous studies and provides direct proof of the inhibitory effects of normal cells on tumor formation *in vivo* in an environment in which the influence of tissue organization and heterotypic cell interactions may be felt.

Prompted by such findings, we now attempt to reconcile the apparent discordance between the proposed essential role of multiple oncogenes in tumorigenesis (1–3) and the observed ability of single oncogenes to induce full malignant conversion of cells (9). We suggest that multiple oncogenes are indeed required for malignant tumor formation when this process is initiated by genetic damage occurring to a single cell located in the context of a normally structured tissue. However, this requirement for multiple oncogenes can be abrogated when normal intercellular interactions and tissue architecture are destroyed.

The latter situation was approximated here by engraftment of pure populations of transformants. Similarly, *in vitro* transformation of primary cultures with single oncogenes can be achieved either by mass infection with oncogene-bearing retroviruses or by introduction into these cells of an oncogene together with a selectable marker, with subsequent elimination of untransformed populations (27, 32).

The nature of the inhibitory influences of normal cells operating *in vivo* and *in vitro* are unclear. In the present system, transformed keratinocytes are inhibited by cells of heterotypic origin (dermal fibroblasts) and not by cells of homotypic origin (normal keratinocytes) or established cells (BALB/c 3T3). In addition, similar inhibition was observed with normal and growth-arrested dermal fibroblasts. Because of these facts, we consider it unlikely that simple nonspecific competition between cells for physical space or nutrients underlies the inhibition. More likely, instead, are mechanisms that depend upon intercellular exchange of specific actively inhibitory substances. We suggest that these substances and the inhibitory influences that they provoke represent, as do oncogenes, centrally important elements in the process of multistep carcinogenesis.

We gratefully acknowledge N. E. Fusenig and P. Boukamp for instructions in their grafting technique. We thank C. Conti, J. O'Connell, J. Rosai, K. Stenn, and G. Stanta for helpful discussions and D. Stern for critical reading of the manuscript. This work was supported by funds of the Yale Pathology Department and, in its initial part, by National Institutes of Health Grant CA39826 and a grant of the ABC Research Foundation to R.A.W. In addition, G.P.D. is a recipient of a Hull Cancer Research Award. R.A.W. is a Research Professor of the American Cancer Society.

1. Rassoulzadegan, M., Naghashfar, Z., Cowie, A,. Carr, A., Grisoni, M., Kamen, R. & Cuzin, F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4354–4358.
2. Land, H., Parada, L. F. & Weinberg, R. A. (1983) *Nature (London)* **304**, 596–601.
3. Ruley, H. E. (1983) *Nature (London)* **304**, 602–606.
4. Balmain, A. & Pragnell, I. B. (1983) *Nature (London)* **303**, 72–74.
5. Balmain, A., Ramsden, M., Bowden, G. T. & Smith, J. (1984) *Nature (London)* **307**, 658–660.
6. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D. & Barbacid, M. (1985) *Nature (London)* **315**, 382–385.
7. Brown, K., Quantanilla, M., Ramsden, M., Kerr, I. B., Young, S. & Balmain, A. (1986) *Cell* **46**, 447–456.
8. Roop, D. R., Lowy, D. R., Tambourin, P. E., Strickland, J., Harper, J. R., Balaschak, M., Spangler, E. F. & Yuspa, S. H. (1986) *Nature (London)* **323**, 822–824.
9. Bishop, J. M. & Varmus, H. E. (1982) *RNA Tumor Viruses*, Molecular Biology of Tumor Viruses, eds. Weiss, R., Teich, N., Varmus, H. & Coffin, J. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Part 3, pp. 999–1108.
10. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. & Yuspa, S. H. (1980) *Cell* **19**, 245–254.
11. Dotto, G. P., Gilman, M. Z., Maruyama, M. & Weinberg, R. A. (1986) *EMBO J.* **5**, 2853–2857.
12. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42**, 1136–1139.
13. Worst, P. K. M., MacKenzie, I. C. & Fusenig, N. E. (1982) *Cell Tissue Res.* **225**, 65–77.
14. MacKenzie, I. C. & Fusenig, N. E. (1983) *J. Invest. Dermatol.* **81**, 189s–194s.
15. Woodcock-Mitchell, J., Eichner, R., Nelson, W. G. & Sun, T.-T. (1982) *J. Cell Biol.* **95**, 580–588.
16. Angerer, L. M. & Angerer, R. C. (1981) *Nucleic Acids Res.* **9**, 2819–2840.
17. Hayashi, S., Gillam, I. C., Delaney, A. D. & Tener, G. M. (1978) *J. Histochem. Cytochem.* **26**, 677–679.
18. Sarkar, S., Kacinski, B. M., Kohorn, E. I., Merino, M. J., Carter, D. & Blakemore, K. J. (1986) *Am. J. Obstet. Gynecol.* **154**, 390–393.
19. Yuspa, S. H., Vass, W. & Scolnick, E. (1983) *Cancer Res.* **43**, 6021–6030.
20. Turusov, V. S., ed. (1979) *Tumours of the Mouse*, Pathology of Tumors in Laboratory Animals (Int. Agency Res. Cancer, Lyon, France), Vol. 2.
21. Taylor, C. R. (1986) *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist* (Saunders, Philadelphia).
22. Hanukoglu, I. & Fuchs, E. (1982) *Cell* **31**, 243–252.
23. Hanukoglu, I. & Fuchs, E. (1983) *Cell* **33**, 915–924.
24. Dotto, G. P., Parada, L. F. & Weinberg, R. A. (1985) *Nature (London)* **318**, 472–475.
25. Sengel, P. (1976) *Morphogenesis of Skin* (Cambridge Univ. Press., Cambridge, U.K.).
26. Saiag, P., Coulomb, B., Lebreton, C., Bell, E. & Dubertret, L. (1985) *Science* **230**, 669–672.
27. Land, H., Chen, A. C., Morgenstern, J. P., Parada, L. F. & Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 1917–1925.
28. Herschman, H. R. & Brankow, D. W. (1986) *Science* **234**, 1385–1388.
29. Mehta, P. P., Bertram, J. S. & Loewenstein, W. R. (1986) *Cell* **44**, 187-196.
30. Pierce, G. B., Lewis, S. H., Miller, G., Moritiz, E. & Miller, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6649–6651.
31. Gerschenson, M., Graves, K., Carson, S. D., Wells, R. S. & Pierce, G. B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7307–7310.
32. Spandidos, D. A. & Wilkie, N. M. (1984) *Nature (London)* **310**, 469–475.