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Restoration of growth potential in paraclones of human keratinocytes by a viral oncogene

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ABSTRACT **Human diploid keratinocytes may be divided into three clonal types with differing capacities for proliferation. The paraclone, which has the shortest life span, is limited to 15 divisions, after which all the cells undergo programmed terminal differentiation. By means of a retroviral vector, paraclones which have not completed their life span and which consist of not more than a few hundred cells can be transduced at a high frequency with DNA complementary to the 12S transcript of the adenovirus early region 1A gene. Transformation can be detected within a single cultivation by the formation of progressively growing colonies. The transformants appear to have an unlimited growth potential, and they form a disorganized epidermis when they are grafted as an epithelium onto athymic mice. These experiments clearly show that, in order to be transformed by a viral oncogene, the target cell need not be a stem cell.**

Under suitable conditions, human diploid keratinocytes can be serially cultivated (1, 2). Three clonal types with differing capacities for proliferation have been described (3). The holoclone has the most growth potential and is likely to be an epidermal stem cell. The paraclone has a short replicative life span, not exceeding 15 cell generations, after which all the cells stop growing and terminally differentiate. The third type, the meroclone, has intermediate growth potential and gives rise to paraclones. The progressive conversion of holoclone to meroclone to paraclone is normally a unidirectional process. It occurs during the serial cultivation of keratinocytes and probably accounts for the finite life span of this cell type. It also occurs in the epidermis during the course of aging (3).

Fibroblasts obtained from rodents convert to established lines spontaneously (4). The expression of viral genes such as the adenovirus early region 1A (E1A) or polyomavirus large tumor (T) antigen can facilitate this process (5–10). Serially cultivated human cells are more stable than rodent cells and very rarely convert spontaneously into established lines (11, 12); however, they may be induced to do so by the influence of viral genes (13). Human keratinocytes have been transformed with simian virus 40 (SV40) (14), the transforming region of that virus (15), an adenovirus type 12–SV40 hybrid (16), and human papillomavirus type 16 DNA (17). They also have been transduced with a complementary DNA for the 12S transcript of the adenovirus type 5 E1A region by means of an amphotropic defective recombinant retrovirus; the transduced keratinocytes acquire some of the properties expected of a cell line (J.R.M. and R.C.M., unpublished experiments).

It is difficult to assay transformation of mass cultures of diploid human fibroblasts or keratinocytes. These cells already possess so much growth potential that numerous subcultivations are required to determine whether the growth potential of the cells has been increased by a transforming agent. To overcome this difficulty we have used keratinocyte paraclones as the target cells. Two weeks after their infection by a recombinant retrovirus encoding adenovirus E1A, paraclones form colonies of transformants that can be easily and quantitatively scored. The transformants are released from the program of obligatory terminal differentiation inherent in the paraclone.

MATERIALS AND METHODS

Cell Culture. Human keratinocytes were grown on a lethally irradiated feeder layer of mouse 3T3-J2 cells (1) in a 3:1 mixture of the Dulbecco–Vogt modification of Eagle's medium and Ham's F12 medium. Supplements were as described (18, 19). Human recombinant epidermal growth factor (Chiron) was added at 10 ng/ml beginning at the first refeeding.

Keratinocyte strain GMA was derived from a biopsy of skin from a 78-year-old woman undergoing reconstructive surgery. The clonal composition of this strain was previously evaluated (3) and was found to consist of a high proportion of paraclones.

Adenovirus-transformed human embryonic kidney 293 cells (13), Swiss mouse 3T3 cells (J2), and ΨAM cells (NIH 3T3 origin) were grown in Dulbecco–Vogt modification of Eagle's medium supplemented with 10% calf serum or fetal calf serum.

Single Cell Isolation. Cells obtained by trypsinization of 7-day secondary keratinocyte cultures were centrifuged and 10^3 cells were suspended in 10 ml of medium in a 100-mm Petri dish. A single cell was isolated by aspiration into an elongated Pasteur pipette under an inverted microscope, using a ×10 objective (20), and carefully deposited at the center of a 35-mm Petri dish already containing lethally irradiated 3T3 cells. One hundred single cells were isolated and cultivated per experiment. Their colony-forming efficiency was about 50%.

Recombinant Retrovirus. The details of the 12S E1A and ZipneoSV(X) retroviruses (Fig. 3*A*) have been previously described (21, 22).

Infection. Human keratinocytes were infected with the recombinant retroviruses by cocultivation with lethally irradiated NIH 3T3 ΨAM producer cells (2.5×10^4 cells per cm^2) as described by Morgan *et al.* (23). By 3 or 4 days of cultivation most of the ΨAM cells had detached, so freshly irradiated uninfected Swiss 3T3-J2 cells were added to provide the keratinocytes with optimal growth conditions. All resulting keratinocyte lines were tested for the production of infectious recombinant retroviruses and were found to be negative.

Southern and Northern Hybridizations. For Southern blots, high molecular weight DNA was prepared by using the SDS/proteinase K method (24), digested with the appropriate restriction enzyme, electrophoresed through a Tris acetate/

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Abbreviations: E1A, early region 1A; SV40, simian virus 40.

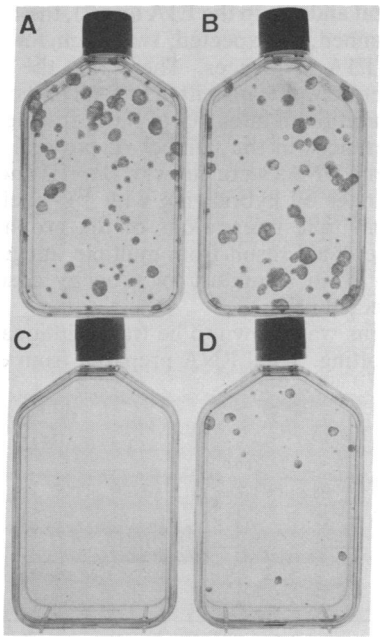

FIG. 1. Detection of transformants in a single cultivation. A single keratinocyte was isolated from a secondary culture of the strain GMA and subcultivated. When a clone formed and contained 300–600 cells it was trypsinized and the cells were divided equally between four flasks, two containing an irradiated feeder layer of 3T3-J2 cells (control) and the other two containing ΨAM cells producing the recombinant retrovirus. After 14 days of cultivation, one culture of each type was fixed and stained with rhodamine B. (*A*) Uninfected meroclone 55 formed a mixture of growing and aborted colonies. (*B*) Infected meroclone 55 formed a similar mixture. (*C*) Uninfected paraclone 85 formed uniformly aborted colonies containing terminally differentiated cells (average colony size 0.53 mm^2). (*D*) Infected paraclone 85 formed numerous progressively growing colonies (average size 34 mm^2) as well as aborted colonies.

1% agarose gel (pH 7.8), and transferred to a Zetabind membrane (CUNO). Filters were hybridized according to the procedure supplied by the manufacturer, using a ^{32}P-probe labeled by the random primer method (25).

For Northern blots, total RNA was isolated by the guanidinium thiocyanate procedure (26), fractionated by electrophoresis in a 1% agarose/formaldehyde gel, and transferred to nitrocellulose. Filters were hybridized with ^{32}P-labeled probes. The *neo* gene probe was a *Bam*HI/*Bgl* II fragment from pSV2neo (27) and the E1A probe was a *Bam*HI fragment from a rescued provirus encoding the 13S E1A gene (21).

Immunoblot Analysis. Protein extracts were prepared according to Cone *et al.* (28), electrophoresed onto an SDS/10% polyacrylamide gel, transferred to nitrocellulose membrane, and stained according to standard procedures (29, 30).

Injections and Grafting. Female athymic mice (19–20 g, strain NIH Swiss *nu/nu*) were obtained from Taconic Farms. Keratinocytes were resuspended at 2×10^6 in 0.3 ml of medium and injected subcutaneously on the back. Usually two or three sites were injected per mouse and two or three mice were injected per cell line. The animals were observed for 6 months.

For grafting, a confluent culture of human keratinocytes was detached as a sheet from the vessel surface with Dispase (Boehringer Mannheim) (31), and the epithelium was grafted according to Barrandon *et al.* (32). Briefly, a skin flap was incised on the back of the mouse and the cultured epithelium was grafted onto the subepidermal connective tissue deep to the panniculus carnosus. The flap was then replaced in its original position and sutured. The grafts were harvested 10 days later, fixed in Formalin, embedded in paraffin, and processed for histological examination.

RESULTS

Transformation of a Paraclone Results in Growing Colonies

Single keratinocytes were isolated with a Pasteur pipette from a secondary culture of strain GMA (20) and each was inoculated into a 35-mm culture dish containing irradiated 3T3-J2 cells. If, by 7 days, a colony formed and contained 300–600 cells, it was trypsinized and the cells were centrifuged and resuspended in medium. Aliquots of the cell suspension were inoculated into two flasks containing either 3T3-J2 cells (control) or ΨAM 3T3 cells producing retrovirus able to express the 12S E1A gene. After cocultivation for 14 days, the cultures were fixed and stained with rhodamine B. The clonal type to which the target cells belonged was determined by scoring the number of aborted, terminally differentiated colonies in the control flask (3). A clone was scored as a paraclone if 100% of the colonies aborted, as a meroclone if more than 5% but less than 100% aborted, and as a holoclone if 5% or less aborted.

As expected from the age of the donor of the keratinocytes (78 years), no holoclones were formed in these experiments; all clones scored were meroclones or paraclones. Whether infected or not, meroclones gave rise to a mixture of progressively growing and aborted colonies (Fig. 1 *A* and *B*). If transformation occurred in the infected cultures, it was impossible to score at this stage.

In contrast, whereas a paraclone, when uninfected, gave rise only to aborted colonies (Fig. 1*C*), its infection with the recombinant retrovirus led to the formation of a number of progressively growing colonies (Fig. 1*D*), macroscopically visible after 9–10 days of cultivation. When the cells from such colonies were trypsinized and reinoculated onto a freshly irradiated feeder layer of 3T3-J2 cells they were able to form new growing colonies (Fig. 2*B*). When uninfected cells of the indicator flask were subcultured, they did not form colonies (Fig. 2*A*). In control experiments, paraclones infected with the ZipneoSV(X) recombinant retrovirus lacking the E1A gene did not form progressively growing colonies. It can be concluded that the infection of a paraclone with a recombinant retrovirus containing the E1A gene results in the formation of progressively growing colonies. The transformants can be detected in a single cultivation because the uninfected cells are programmed to terminally differentiate after a few divisions and yield only aborted colonies.

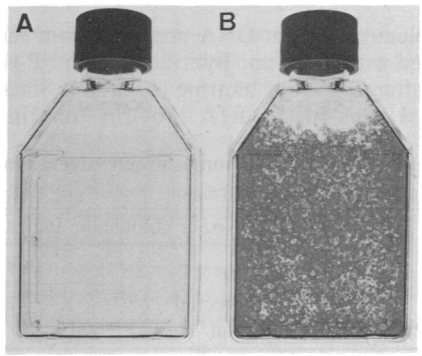

FIG. 2. Subcultivation of the keratinocytes of progressively growing colonies formed by infected paraclones. Sister cultures of the uninfected paraclone shown in Fig. 1*C* and the infected paraclone shown in Fig. 1*D* were trypsinized and transferred, each to a single flask containing an irradiated feeder layer of 3T3-J2 cells. The cultures were fixed and stained after 12 days. (*A*) The uninfected cells failed to form colonies. (*B*) The infected cells gave rise to numerous progressively growing colonies.

Table 1. Formation of progressively growing colonies by paraclones infected with recombinant retrovirus bearing the E1A gene

Exp.	No. of paraclones infected	No. of infected paraclones yielding progressively growing colonies
1	4	4
2	6	4
3	5	1
4	6	2
5	7	2
Total	28	13

Mass cultures of normal human keratinocytes can be grown through over 140 cell doublings (12), an average cultivation time of at least 4 months; scoring of immortalization through transformation of mass cultures is therefore difficult and time-consuming. By using a paraclone as target cells, the scoring time is sharply reduced, as a progressively growing colony can be readily scored after 10–12 days. Furthermore, as each colony results from a single cell, the frequency of transformation can be precisely evaluated.

Frequency of Transformation

A total of 28 paraclones in five experiments were isolated and infected with the retrovirus bearing the E1A gene. Thirteen gave rise to progressively growing colonies (Table 1). The absence of transformants in about half the experiments may be due to poor survival of the irradiated ΨAM cells and limitation of the period of infection to the first few days of the cocultivation. In the case of successfully infected paraclones, the fraction of colonies growing progressively varied between 0.06 and 1.0 (Table 2), whereas in the absence of infection, all colonies aborted. These experiments demonstrate that human keratinocytes transduced with the recombinant retrovirus were transformed so efficiently that the frequency could be scored even though the target for infection was fewer than 100 colony-forming cells.

Integration and Expression of the Recombinant Retrovirus

Five cell lines were derived from five paraclones infected with the recombinant retrovirus. Every line consisted of the progeny of several progressively growing colonies, each presumably resulting from a separate infection and transformation.

High molecular weight DNA prepared from each cell line was digested with *Xba* I and hybridized with ^{32}P-labeled E1A sequence after Southern blotting (Fig. 3*B*). Since there are three *Xba* I sites in the E1A provirus (one in each long terminal repeat and one in the E1A insert), the DNAs of each cell line contained, as expected, two fragments that hybridized to the E1A sequences. Therefore, the correct E1A proviral structure was transmitted to each of the keratinocyte lines. The variation in intensity of these fragments probably reflects differences in the number of proviral integrations. When the same DNAs were cut with *Bam*HI and analyzed in a similar manner by hybridizing with ^{32}P-labeled *neo* gene sequences, multiple integrations of the provirus were detected. These could result from multiple integrations in the same cell or from a mixture of progeny descended from different infected cells.

The provirus was shown to be transcriptionally active by Northern blotting. Total RNA prepared from each cell line

Table 2. Frequency of formation of progressively growing colonies by individual paraclones

Exp.	Paraclone	No. of colonies in indicator flask: Uninfected total*	Infected: Total	Infected: Progressively growing
1	2	63	64	34 (0.53)
	8	17	26	21 (0.81)
	10	83	77	41 (0.53)
	11	4	5	5 (1.0)
5	85	76	57	12 (0.21)
	115	42	47	3 (0.06)

Numbers in parentheses are the fraction of total colonies.
*All aborted.

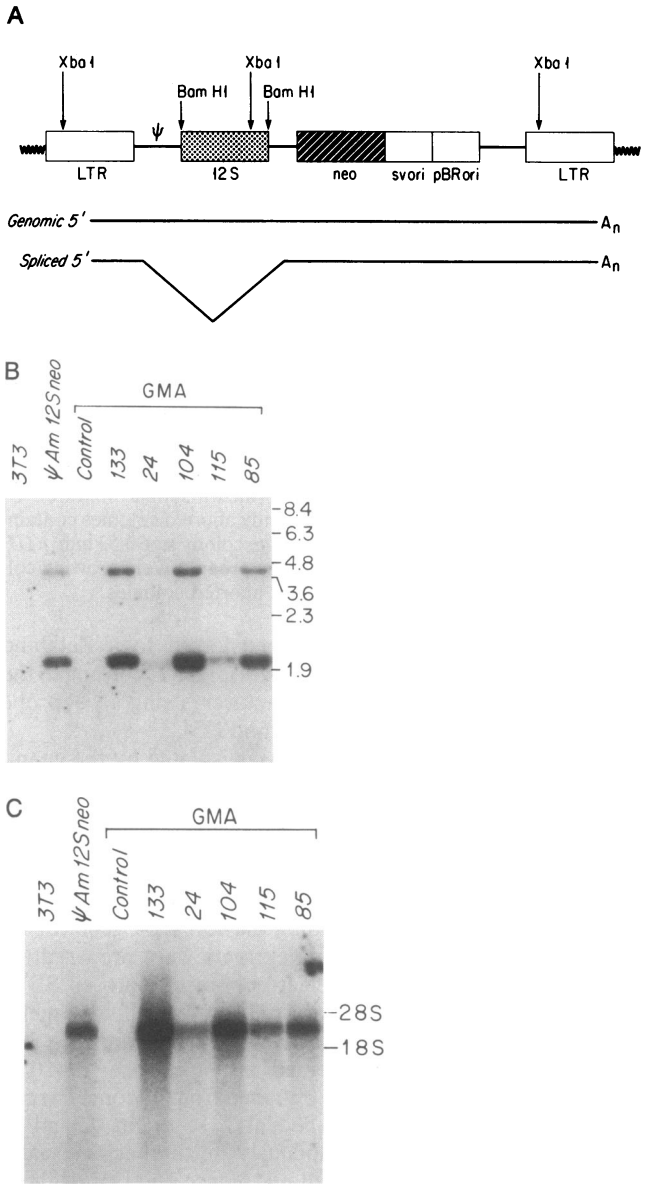

FIG. 3. Integration and expression of the E1A 12S recombinant retrovirus. (*A*) Diagram of the 12S E1A provirus, showing the *Xba* I and *Bam*HI sites and the expected RNA transcripts. LTR, long terminal repeat. (*B*) Southern blot of cellular DNA (10 μg) digested with *Xba* I and probed with ^{32}P-labeled E1A gene sequences labeled to high specific activity with deoxycytidine 5'-[α-^{32}P]triphosphate by the random primer method. Each lane is labeled to indicate the cells from which the DNA was isolated. Numbers on right are size in kilobases. (*C*) Northern blot of total RNA (5 μg) probed with ^{32}P-labeled E1A gene sequences. Lanes are labeled to indicate the cells from which the RNA was isolated.

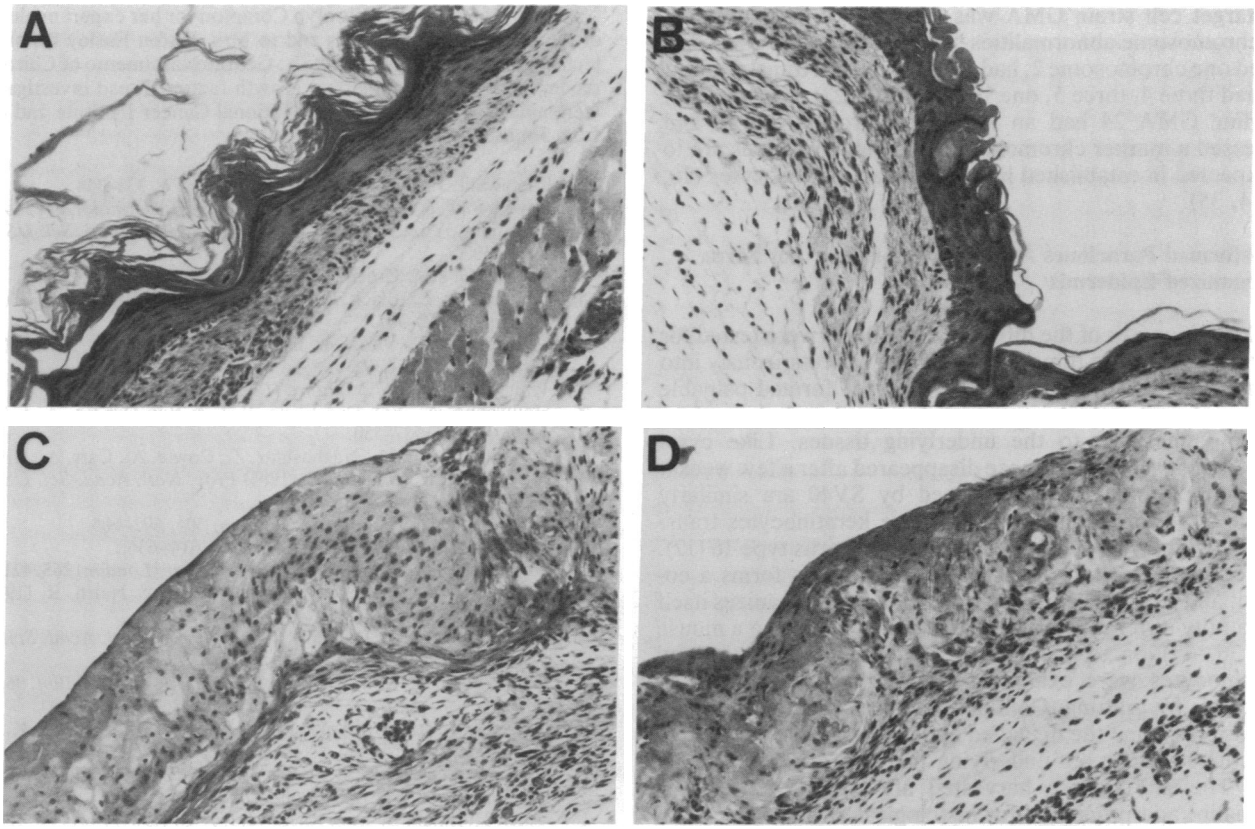

FIG. 4. The transformed keratinocytes are unable to form a normal epidermis. Confluent cultures of normal keratinocytes or E1A-transformed keratinocytes were grafted onto athymic mice. The grafts were harvested after 10 days and sections were prepared and stained with hematoxylin/eosin. (*A*) Epidermis generated by keratinocytes of strain GMA. Note the regular architecture with well-defined stratum granulosum and the mostly orthokeratotic stratum corneum. (*B*, *C*, and *D*) Epithelia generated from transformed lines GMA 85, GMA 24, and GMA 104, respectively. Note thicker but architecturally disorganized living layers containing dyskeratotic cells and keratin pearls. The stratum granulosum and the stratum corneum are generally absent or patchy.

was fractionated by formaldehyde/agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled E1A sequence (Fig. 3*C*). RNA from each cell line contained the full-length mRNA of the provirus. The amount of this mRNA correlated with the number of proviral copies seen on the Southern blot (Fig. 3*B*). When the same RNAs were hybridized with ^{32}P-labeled *neo* sequence, both the full-length mRNA and the spliced subgenomic mRNAs were detected (data not shown).

Three GMA lines, 133, 24, and 85, were examined for the presence of the E1A 243 protein by Western blotting, using a rabbit polyclonal antiserum to this E1A protein kindly provided by J. Lillie and M. Green (Harvard University). In all transformants, small amounts of the protein were detected; 293 cells were more strongly positive, and the parent strain GMA was negative.

Properties of the Transformants

Growing keratinocyte colonies resulting from the infection of five different paraclones were serially cultivated on an irradiated layer of 3T3-J2 cells. The cells of each transformant were cultured through at least 80 generations before they were frozen. Two clones, GMA 133 and GMA 24, were serially cultivated for more than 150 generations with no evidence of deterioration. Therefore, all transformants have acquired a greatly extended capacity for growth and are likely to be established lines.

After the lines GMA 133 and 24 had undergone at least 110 and 128 doublings, respectively, single cells were isolated and individually cultivated on a feeder layer of lethally irradiated 3T3-J2 cells for 7 days. Cells from both lines formed clones with high frequency (70–90%). Sixteen clones from each line were chosen at random and trypsinized, and the cells were transferred to indicator dishes. Cultures were fixed after 16 days and the colonies were classified as progressively growing or aborted. Of 1220 colonies from the line GMA 24 and 207 colonies from the line GMA 133, 98% were progressively growing. Although the rate of growth varied from clone to clone and colonies were of various sizes, no typical paraclone was observed. Transformants therefore have no ability to give rise to paraclones. Similar experiments performed on keratinocyte lines derived from squamous cell carcinomas (unpublished experiments) showed that they too failed to contain typical paraclones.

When plated at clonal density, most transformants were dependent upon supporting 3T3 cells for maximal growth. In the case of one transformant, GMA 24, 2% of the cells gave rise to progressively growing colonies in the absence of 3T3 cells. These cells could thereafter be subcultivated without 3T3 support. Such independence has been reported for SV40-transformed keratinocytes (14) and cell lines derived from squamous cell carcinoma (33) as well as for the very rare cases of spontaneously transformed keratinocytes (34, 35).

All transformants produced by E1A, like cell lines derived from authentic keratinocyte tumors (33), grew more slowly than normal keratinocytes. During exponential growth, cells of strain GMA had a doubling time of about 17 hr, whereas the doubling time of transformants was 25–35 hr. Transformant colonies differed from normal colonies in their variably reduced tendency to stratify, line GMA 24 showing the least stratification.

The karyotypes of the different lines were kindly examined by S. Latt and D. Shook of the Children's Hospital (Boston).

The target cell strain GMA was diploid. All transformants had chromosome abnormalities. For instance, line GMA 133 lacked one chromosome 2, had 3p involved in a translocation, and had three 4, three 5, one 16, and a t(21,22) chromosome. The line GMA 24 had an abnormal chromosome 10 and possessed a marker chromosome. Such abnormalities are to be expected in established lines of human keratinocytes (16, 17, 34, 35).

Transformed Paraclones Are Not Tumorigenic but Form Disorganized Epidermis

The keratinocytes of the different GMA lines were tested for their ability to form tumors by injection subcutaneously into athymic mice. After a few days, the lines formed palpable epidermal cysts which were well localized and showed no sign of adherence to the underlying tissues. Like cysts formed by normal cells, these disappeared after a few weeks. Human keratinocytes transformed by SV40 are similarly unable to form neoplasms (15), as are keratinocytes transformed with DNA of the human papillomavirus type 16 (17).

A confluent culture of normal keratinocytes forms a coherent and stratified epithelium which readily organizes itself into a fully differentiated epidermis when grafted to a mouse (32, 36, 37) or to a human (38). Thus the transformed keratinocytes were examined for their ability to form an epidermis by grafting. Confluent cultures were treated with Dispase (31), and the detached epithelia were then grafted to the connective tissue underlying the skin of athymic mice (32). The grafts were harvested after 10 days, fixed in Formalin, and processed for histological examination.

The uninfected keratinocytes of strain GMA formed a normal epidermis whose layers were correctly organized. There was a stratum granulosum and an orthokeratotic stratum corneum (Fig. 4*A*). In contrast, the transformed keratinocyte lines formed a highly abnormal epidermis, always smaller in area than the control, and whose degree of disorganization varied with the line (Fig. 4 *B–D*). There was dyskeratosis and other evidence of dysplasia, and occasional keratin pearls, but no obvious invasiveness.

It could be concluded from these experiments that, while the transformed keratinocytes were not tumorigenic, their program of differentiation was sufficiently altered to prevent them from forming a normal epidermis. This is also true of keratinocytes derived from authentic tumors (ref. 39; K. Lindberg and J. G. Rheinwald, personal communication), although these usually show more evidence of invasiveness.

Concluding Remarks

It has been suggested that malignant tumors arise from tissue stem cells (40). Although the paraclone is a multiplying cell, it is not a stem cell, for it is programmed to terminally differentiate after a maximum of 15 divisions (3). This program can be erased by introduction of the E1A gene; as a result the cells become indefinitely cultivable, but they acquire a disorder in terminal differentiation most clearly shown by the disorganized epithelium they generate when grafted to athymic mice. As a paraclone is not a stem cell and yet can be immortalized by a viral oncogene, we suggest that neoplastic transformation might similarly not be restricted to stem cells. Although the paraclone consists of only a few cells, the high efficiency of gene transfer by the recombinant retroviruses makes it an ideal target for assaying transforming genes effective on human cells.

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