Immortalized keratinocyte lines derived from human embryonic stem cells

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Cells of the human embryonic stem (hES) cell line H9, when cultured in the form of embryoid bodies, give rise to cells with markers of the keratinocyte of stratified squamous epithelia. Keratinocytes also form in nodules produced in scid mice by injected H9 cells; the hES-derived keratinocytes could be recovered in culture, where their colonies underwent a peculiar form of fragmentation. Whether formed from embryoid bodies or in nodules, hES-derived keratinocytes differed from postnatal keratinocytes in their much lower proliferative potential in culture; isolated single keratinocytes could not be expanded into mass cultures. Although their growth was not improved by transduction with the hTERT gene, these keratinocytes were immortalized by transduction with the E6E7 genes of HPV16. Clonally derived lines isolated from E6E7-transduced keratinocytes continued to express markers of the keratinocyte lineage, but the frequency with which they terminally differentiated was reduced compared with keratinocytes cultured from postnatal human epidermis. If other hES-derived somatic cell types also prove to be restricted in growth potential, not identical to the corresponding postnatal cell types, and to require immortalization for clonal isolation and expansion, these properties will have to be considered in planning their therapeutic use.

Immortalization

Since the derivation of the first human embryonic stem (hES) cell line (1), it has been evident that, like murine ES cells (2, 3), hES cells are able to differentiate into a great variety of somatic cell types, and many of these have been proposed as candidates for human cell therapy. The somatic cell type of interest, derived from hES cells, will be used to replace cells lost or damaged in disease. Before such a procedure can be carried out, two problems must be dealt with. The first is that the hES cells that give rise to somatic cell types can also give rise to teratomas (1). Inhomogeneity of ES-derived somatic cell types has been recognized in several recent reviews (4–7). The last of these contains the statement, “No approach to the differentiation of (human) ES cells has yet yielded a 100% pure population of mature progeny.” The only way to purify with complete confidence is to clonally isolate the desired cell type and grow it in culture to usable quantities. The second problem is the assumption that the hES-derived somatic cell type will be identical to the corresponding cell type that forms during normal development; the possibility that this assumption may be incorrect does not seem to have been considered in the literature.

We have studied both of these problems using keratinocytes derived from H9 cells. We have found that these keratinocytes do not proliferate nearly as well as do postnatal keratinocytes. To increase their proliferative potential, we immortalized them by transduction with the E6E7 genes of HPV16.

Results

Initial Stages of the Cultivation. It was shown earlier that an embryoid body (EB) prepared from H9 cells and attached to a vessel surface gave rise to migrating cells in which markers of the keratinocyte appeared in the order: p63, K14 and basonuclin1, and involucrin, the latter a marker of keratinocyte terminal differentiation (8). These keratinocytes could not be serially subcultured.

In cultures derived from H9-induced nodules, keratinocyte proliferation seemed to be inhibited by the presence of contaminating H9 cells and their non-keratinocyte derivatives. This was particularly evident in cultures inoculated at high cell density. To avoid the problem, we performed the initial cultivation of disaggregated nodule cells in a low Ca²⁺ medium optimized for the growth of postnatal keratinocytes (EpiLife medium, Cascade Biologics, Portland, OR). This eliminated most, although not all, non-keratinocytes, but the H9-derived keratinocytes proved not to be serially subcultivable in this medium. We then combined this medium with 3T3 feeder cell support. Irradiated 3T3 cells were inoculated at a density of 5 × 10⁵ per 60-mm plate in DME medium containing 10% calf serum. On the following day, the medium was changed to EpiLife and the ES-derived keratinocytes were inoculated. Presumably because of the low Ca²⁺ concentration and absence of serum in EpiLife medium, the irradiated 3T3 cells began to detach, but at a slow rate, so that their supporting function continued for a period of 7–10 days. Under such conditions, colony-forming keratinocytes were recovered from the nodules with a frequency of 0.5 × 10⁻³ of cells plated, 5-fold higher than that reported earlier (8). One such colony is shown in Fig. 1.

Subsequent Cultivation. Although the initial passage in EpiLife medium with supporting 3T3 cells removed most non-keratinocyte cell types and improved the proliferation of the keratinocytes, these conditions still did not permit their serial propagation. We then transferred the cells to culture conditions that best support the proliferation of postnatal keratinocytes (co-cultivation with 3T3 feeder cells in FAD medium (9, 10)). Under these conditions, keratinocytes derived from postnatal epidermis formed colonies of tightly packed, cohesive cells (Fig. 2). Colonies formed by keratinocytes derived from hES nodules behaved quite differently. They grew more slowly and tended to fragment into two or more parts that formed subcolonies (Fig. 3), suggesting a failure to form desmosomal junctions or a greater cell motility.

The fragmentation was even more clearly evident in cultures fixed and immunostained with an antibody specific for the transcription factor p63 (Fig. 4). Postnatal keratinocytes produced typical compact colonies, whose intensity of p63 staining was greatest at the colony periphery. By day 8, such colonies contained 1,000–2,000 cells. The colonies of hES-derived keratinocytes shown in Fig. 4 were considerably older

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Abbreviations: EB, embryoid body; hES, human embryonic stem.

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but contained far fewer cells. Their marked tendency to fragment made it difficult to relate colony number to number of keratinocytes inoculated or to estimate the rate of proliferation from the increase in number of cells in a colony over time.

When such cultures were serially transferred, the proportion of colonies containing multiplying cells quickly declined and the cultures became completely senescent within six passages. The feeder cells remained as abundant as the keratinocytes, so it was difficult to calculate the total population doublings undergone by the keratinocytes. The total proliferation potential was certainly <30 cell generations and <15 population doublings.

Failure of hTERT to Extend Replicative Lifespan. Seeking to extend the replicative lifespan of the hES-derived keratinocytes, we transduced a fourth passage culture derived from nodule-derived keratinocytes (Nod3) with a retroviral vector containing the gene for hTERT, the catalytic subunit of telomerase. This was done in two separate experiments with different retroviral vectors, as described in Materials and Methods. In the first experiment, no chemical selection for the hTERT gene was applied. In the second experiment, we used blasticidin to select for BABE-Bsd-hTERT transductants. In neither experiment did the hTERT confer a greater replicative lifespan, more rapid growth rate, or higher colony-forming efficiency than untransduced, control cells; the cells failed to increase in number above the plating density and formed only small, senescing colonies. This was so despite feeder-layer support. Failure of hTERT to increase the replicative potential of the hES-derived keratinocytes may be the result of the p16INK4A/p14ARF-dependent senescence mechanism and immortalization barrier that becomes activated in postnatal keratinocytes during growth in culture, independent of their telomere status (11–14).

Transduction with E6E7 of HPV16. Expression of the E6E7 genes of HPV16 has been shown to disable p53 and Rb-mediated checkpoints (15–19) and to induce expression of the endogenous TERT gene in keratinocytes (20), thereby providing an efficient method for producing immortalized human keratin-
In contrast to the untransduced controls, the basis for coherence of adjacent keratinocytes in a colony is the formation of desmosomal junctions with their attached cytoplasmic filaments. In small growing colonies of Nod3/E6E7-c2 keratinocytes, the basis for coherence was the presence of keratinocyte markers (8). Before the transduction, such keratinocytes had practically no ability to multiply on subculture. Within days of the transduction, many growing colonies appeared, and about one-half had keratinocyte-like morphology.

**Clonal Isolation.** In contrast to the untransduced controls, the E6E7-transduced cells of both origins were clonable with high efficiency. In the case of nod3-derived keratinocytes, eight single cells were isolated from the trypsinized cells of the mass culture (Fig. 5). A colony in which cleavage furrows have developed, anticipating fragmentation. There are numerous small clusters of keratinocytes separated from the rest of the colony. All keratinocytes stain strongly for p63.

**Formation of Coherent Epithelium.** As noted above, the basis for coherence of adjacent keratinocytes in a colony is the formation of desmosomal junctions with their attached cytoplasmic filaments. Small growing colonies of Nod3/E6E7-c2 keratinocytes have a relatively smooth perimeter, where the staining intensity is greatest.

**Fig. 4.** Two keratinocyte colony types, stained for p63 (×4 objective). (A) Postnatal (strain YF29), day 7. Colonies are compact and have predominantly well delimited perimeters, where the staining intensity is greatest. (B) Nod3, day 12. Shown is a colony in which cleavage furrows have developed, anticipating fragmentation. There are numerous small clusters of keratinocytes separated from the rest of the colony. All keratinocytes stain strongly for p63.

**Fig. 5.** Colonies of postnatal and of hES-derived, E6E7 transduced keratinocytes. Eight-day colonies are shown for each cell type (×10 phase objective). The colony of YF29 is composed of tightly packed cells, with a smooth perimeter at the site of excavation of the 3T3 supporting cells. Colonies of the Nod3/E6E7 clone and the EB/E6E7 clone are of comparable size but contain fewer cells, owing to their tendency to spread. Their colony perimeters are irregular.
tinocytes, the cells do not pack tightly (Fig. 5) and would probably not form extensive intercellular junctions. But when a culture was maintained in the confluent state for 12 days, all cells were seen to be linked to their neighbors by “intercellular bridges” (Fig. 8A), the old term used to describe what is seen by light microscopy to result from the presence of desmosomes.

A coherent epithelium of postnatal keratinocytes grown to confluence in culture can be detached as an intact sheet through the action of the proteolytic enzyme dispase (23). Such sheets are graftable to mice (24) and to humans, where they are able to generate a permanent epidermis on areas of full thickness burns (25, 26). Nod3/E6E7-c2 keratinocytes possessed this property of coherence. When the line was inoculated at high density into a tissue culture dish with 3T3 feeder cells and treated 8 days later with dispase, the confluent culture detached as a single sheet (Fig. 8B), in the same manner as postnatal keratinocytes. Even the degree of shrinkage after detachment was similar.

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Fig. 6. Keratinocyte markers in EB/E6E7-c2A2. Cultures were grown for 8 days in FAD medium in the presence of supporting 3T3 cells. Each frame shows a region at the expanding perimeter of a growing colony. As can be seen from the identical pattern of the DAPI-stained nuclei, p63 and K14 stained nearly all of the same cells. The p63 is nuclear, and the K14 is cytoplasmic. Different regions of the expanding perimeter were stained for K5 and basonuclin 1. Like K14, K5 is cytoplasmic. As in the epidermis (43), basonuclin 1 is usually nuclear, but sometimes cytoplasmic. (Scale bars: 100 μm.)

Fig. 7. Terminal differentiation in colonies of YF29 and Nod3/E6E7-c2. Seven-day colonies were fixed and stained for K14, K10, TG1, and involucrin. All colonies contained K14. In contrast to YF29, very few Nod3/E6E7-c2 cells contained K10 or TG1, and none contained involucrin. At 18 days, involucrin became evident in suprabasal squames (red), whereas p63 persisted in basal cells (lower right). (Scale bars: 50 μm.)

Fig. 8. Cohesion of Nod3/E6E7-c2 cells. (A) Formation of “intercellular bridges.” Shown is a 12-day confluent culture (×40 objective). (B) Detachment of the cell layer as an integral sheet by dispase. A culture dish of diameter 3.5 cm was inoculated with 3.5 × 10^5 cells of Nod3/E6E7-c2 on supporting fibroblasts. Eight days later, the culture was washed and treated with dispase. One hour later, all of the keratinocytes detached as a single sheet that shrank to 1.3 cm in diameter.

Rb in the hES Cell-Derived Keratinocytes and Their E6E7-Immortalized Progeny. A well characterized activity of HPV16 E7 is the sequestration and destruction of the cell cycle regulator Rb
Discussion

The discovery of the culturability of postnatal human keratinocytes in the presence of 3T3 feeder support (32) had its origin in the earlier discovery that feeder support was necessary to support proliferation of keratinocytes originating from the stem cells of a transplantable mouse teratoma (33). The experiments described here show that, just as murine teratomal keratinocytes differed from postnatal murine keratinocytes in their reduced cohesiveness and ability to form a superficial layer of differentiated cells, hES-derived keratinocytes differed from human postnatal keratinocytes. The most surprising difference was their restricted proliferative capacity, which did not permit their multiplication from isolated single cells to mass cultures, a simple matter for postnatal keratinocytes that, depending on the type of stratified epithelium and the age of the donor, typically have lifespans of 60–150 cell generations and 30–100 population doublings (12, 34, 35).

This problem was dealt with by transducing the cells with the E6 and E7 genes of HPV16. The resulting immortalization of both EB- and node-derived keratinocytes permitted the isolation of numerous clones. These varied in proliferation rate and colony morphology, indicating that they were the products of independent transduction events and emphasizing the importance of clonal isolation for obtaining homogeneous material for either investigation or practical use.

It had been shown previously that E6E7 transduction provides an efficient method for producing immortalized human keratinocyte lines (refs. 21, 22, 36, and 37; also see refs. 11 and 38). Such lines retain the terminal differentiation characteristics of the primary keratinocyte type of origin (39). The E6E7-immortalized, node-derived keratinocytes were capable of terminal differentiation but not with the same frequency as postnatal keratinocytes.

In the absence of transduction by E6E7, we did not obtain any serially cultivable epithelial cell types from hES-derived nodules or embryoid bodies. We did observe and preserve some slowly growing cells of fibroblastoid morphology, presumably mesenchymal cell types, but did not determine their replicative lifespans.

To date, no clonally isolated somatic cell types derived from hES cells have been reported. Attention should be directed to the question of whether other somatic cell types derived from hES cells, like keratinocytes, lack sufficient replication potential for investigative or therapeutic use and will have to be immortalized. E6E7 transduction is a powerful method of immortalization and has made it possible to obtain cloned hES-derived keratinocytes that cannot be obtained by ordinary cultivation. But for prospective therapeutic purposes, it will be preferable to use a set of immortalizing genes that are less likely to perturb keratinocyte differentiated function, such as a combination of the nonviral genes p16-resistant mutant cdk4, dominant-negative mutant p53, and TERT (14).

Materials and Methods

Retroviral Transduction. hTERT. PA317 amphotropic packaging cells were transduced with pTERT-Bsd-WZL (40) (provided by John Sedivy, Brown University Medical School, Providence, RI). Overnight supernatants were incubated with cultures of node-derived keratinocytes plated 1 day previously in supplemented Gibco ker-sfm medium (Invitrogen) in the presence of 2 μg/ml polybrene for 6 h, as described for transduction of postnatal human keratinocytes (14). The cells were subcultured the next day to dishes with feeder cells and FAD medium, without drug selection.

By using a different vector and transduction method, supernatants were prepared from PA317 cells producing BABE-Bsd-hTERT vector (provided by Woodruff Wright, University of Texas Southwestern Medical Center, Dallas). The supernatants were incubated with 1-day-old cultures of node-derived keratinocytes in EpiliLife medium in the presence of 8 μg/ml polybrene for 2 h and then overnight in the presence of 2 μg/ml polybrene. The cells were then transferred to dishes with blastocidin-resistant feeders, cultivated for 2 days in FAD medium without antibiotic, and selected for 5 days in the presence of blastocidin at 20 μg/ml.

E6E7. PA317 cells producing the L(HPV16E6E7)SN vector (41, 42) were provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle). Cultures of nodule- or EB-derived keratinocytes plated 1 day previously in Gibco ker-sfm medium were incubated with retroviral supernatants (prepared in the same medium) for 6 h in the presence of 2 μg/ml polybrene and...
subcultured the next day to feeders and FAD medium without drug selection.

**Clonal Isolation.** Each cell to be cloned was isolated under direct vision, aspirated into a pipette, and transferred to a tissue culture dish containing supporting feeder cells.

**Western Blotting of Rb.** Cells were trypsinized, centrifuged, rinsed in PBS, resuspended in 80 mM Tris-HCl buffer (pH 7.5) plus 0.1% Triton X-100 plus a protease inhibitor mixture (Complete, Roche Diagnostics), and sonicated. Proteins were resolved on a 7.5% acrylamide gel by SDS/PAGE and transferred to a nitrocellulose membrane (Bio-Rad), which was incubated for 1 h in 1% Western blocking reagent (Roche Diagnostics) and then overnight at 4°C with monoclonal antibody to Rb (BD Pharmingen) diluted 1:600 in 0.5% Western blocking reagent. The membrane was further incubated for 1 h at room temperature with goat anti-mouse IgG conjugated to Alexa Fluor 680 (Molecular Probes) diluted 1:10,000 in 0.5% Western blocking reagent and then washed in Tris-buffered saline plus 0.05% Tween 20. In parallel, Western blotting for K14, using mouse monoclonal antibody to K14 (CBL 197; Chemicon) diluted 1:500, was carried out to normalize total protein in different cell extracts.

Additional methods for cell cultivation and immunocytology can be found in the supporting information, which is published on the PNAS web site.