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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Environmental induction of differentiation-specific keratins in malignant mouse keratinocyte lines

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Epidermal cell lines — malignant transformation — ortho-keratinization — environmental influence

Four spontaneously transformed keratinocyte lines (HELP I–IV) were raised from primary cultures of mouse epidermal cells grown in gas-permeable (Petriperm) dishes. Although tumorigenic, these cell lines still expressed the differentiated phenotype under mesenchymal influence in vivo in a fashion similar to normal cells and in contrast to previous observations on other transformed cell lines. Initially, after transplantation onto adult mice, HELP cells generally formed well organized ortho-keratinizing epithelia closely resembling those of normal epidermal cells. Later, dysplastic epithelia and papilloma-like structures developed and cells invaded subcutaneous host tissue. When injected subcutaneously into newborn syngeneic mice, all four cell lines gave rise to differentiated carcinomas at high frequency. Keratinized metastases were detected in the lung with HELP I, albeit at low frequency. Although the four HELP cell lines differed morphologically and biochemically in their degree of ortho-keratinization, no inverse relationship to their malignant potential was evident. In contrast to cell cultures, HELP transplants and tumors expressed epidermotype "suprabasal" keratins. Metabolic labeling and electrophoresis on one and two-dimensional gels revealed both the basic 67 kDa and acidic 58 kDa components, including presumptive derivatives analogous to those observed in epidermal stratum corneum. However, associated with alterations in tissue architecture, the spatial control of keratin expression was gradually lost by indirect immunofluorescence microscopy (IIF). Thus, while cell differentiation appeared virtually normal, the progressive disturbances in tissue differentiation indicate important changes in the responsiveness of these malignant keratinocytes to environmental conditions.

Introduction

The differentiation process in normal epidermis, generally termed ortho-keratinization, depends on intrinsic properties of the keratinocytes as well as external regulatory factors contributed by the environment. Studies using hetero-

transplants of epithelia and mesenchyme of various body locations have demonstrated the important role of epithelial-mesenchymal interactions in supporting or modulating epidermal differentiation [33, 44]. In normal epidermis morphological differentiation in the individual cell strata closely correlates with the expression and modification of specific products such as filaggrin, involucrin and certain keratins [9, 12, 13, 16, 20, 36, 43, 45, 49, 53]. Isolated epidermal keratinocytes cultured in vitro can still form stratified epithelia, which undergo certain stages of terminal differentiation resembling keratinization in vivo, but this process is usually either incomplete or represents an alternative pathway [12, 14, 20, 27, 40, 48, 49]. Thus, well defined strata, particularly a distinct stratum granulosum, are not formed in vitro and usually the larger differentiation-specific ("suprabasal") keratins are not synthesized. The behaviour in vitro can be modulated to a certain degree by medium composition such as by vitamin A depletion, which induces synthesis of epidermal "suprabasal" keratins in human keratinocyte cultures [16, 21]. However, a more coordinated restoration of both epidermis-like tissue architecture as well as synthesis and regular spatial distribution of the corresponding differentiation products, has been achieved by placing cultured cells back into a natural environment. Thus, virtually all elements of regular ortho-keratinization were identified when cultured mouse cells were transplanted on adult mice, including tissue architecture, basement membrane formation and keratin synthesis [5, 12, 23, 25]. A similar observation was made when rabbit cells were injected subcutaneously into nude mice forming keratinizing cysts [14, 31]. Human keratinocytes transplanted on subdermal tissue of nude mice developed epidermis-like epithelia and regularly expressed the respective proteins (filaggrin, involucrin and "suprabasal" keratins) [4, 7, 53]. Recent experiments have suggested that the mesenchymal influence might be effective also in vitro, at least to a certain degree, independent of a living host organism using either dermal explants or "dermal equivalents" recombined with mouse and human keratinocyte cultures growing exposed to the atmosphere [1, 2, 5, 7, 33].

While these experimental models might help to elucidate the molecular mechanisms of normal differentiation,
they also offer a way to probe cells at various stages of transformation for their differentiation potential. In particular, they may help to distinguish between the loss of intrinsic differentiation properties during carcinogenesis and tumor progression and changes in the response to external regulatory signals within the epithelium or from neighbouring mesenchyme. Along these lines, we have examined several mouse keratinocyte lines for their growth and differentiation potential after transplantation or injection. With other transformed mouse or carcinoma derived human cell lines we had not observed any signs of regular keratinization under in vivo conditions [25, 26]. Here, we demonstrate a different behaviour in a series of four mouse keratinocyte lines formed spontaneously from primary cultures on gas-permeable (Petriperm) dishes [24, 26]. These lines were tumorigenic but nevertheless showed varying degrees of differentiation when growing as transplants or tumors. According to morphological, immunological and biochemical criteria and contrary to prevalent opinion, there was no evidence for an inverse relationship between differentiation potential and progressive tumor growth.

Materials and methods

Cell culture

The four spontaneous cell lines HELP I, II, III, and IV were developed from primary cultures centrally plated on gas-permeable dishes (Petriperm, Heraeus, Hanau/FRC) and grown in modified Eagle’s MEM (4x MEM) [12] with 15% (v/v) fetal calf serum at 30°C for 6 to 7 months before the first subculture [24, 26]. Cells isolated from large proliferating colonies (HELP I and II actually derived from the same Petri dish by trypsinization for 5 and 20 min, respectively; HELP III and IV from two other cell isolates) were further passaged on regular tissue culture plastic as detailed previously [22, 26].

Cell transplants and tumors

Cells were transplanted as a suspension (5x10^3 cells in 200 μl) onto induced subdermal granulation tissue in adult C3H mice [24, 25, 32]. Mice were sacrificed and the tissue excised starting one week after transplantation. For biochemical analysis epithelial tissue was mechanically removed from granulation tissue after incubation in 10 mM EDTA, phosphate-buffered saline (150 mM NaCl; PBS) at 4°C for at least 5 h; tissue fragments were collected by centrifugation at 5000g. Tumors were produced by subcutaneous (s.c.) injection of cell suspensions (5x10^3 cells in 50 μl) in one-day-old C3H mice, and harvested at various intervals. For histological examination, specimens were fixed in Bouin’s fixative and sections were stained with hematoxylin and eosin (H & E).

Electron microscopy

Specimens were processed as previously described [5, 12]. Ultrathin sections were processed with uranyl acetate and lead citrate, and examined with a Zeiss EM 109 electron microscope.

Indirect immunofluorescence microscopy

Cells grown on glass coverslips were fixed for 5 min in methanol at −20°C, air dried and rinsed with PBS. The coverslips were incubated with the first antibody at 37°C for 30 min, rinsed with PBS and further incubated with fluorescein-labeled second antibody [17, 18]. Transplants and tumor tissues were embedded in Tissue-Tek O.C.T. (Lab Tec Products, Naperville, IL/USA) snap-frozen in liquid nitrogen, and frozen sections were incubated with antibodies as described [5]. The following immune reagents were used: (I) antisera against total epidermal keratins of neonatal mouse (M9) and (II) the corresponding stratum corneum keratins (M12); both antisera were raised in guinea pigs and preimmune sera were collected as controls; (III) guinea pig anti-bovine epidermal keratin (snout; Gk) [17, 18]; (IV) rabbit antisera against the 67 kDa mouse keratin (667g) [43]; (V) guinea pig antisera against a respective C-terminal sequence (167g) [41]; and (VI) monoclonal antibody against suprabasal human keratins (KLI) [52]. As second antibodies fluorescein isothiocyanate (FITC)-conjugated rabbit anti-guinea pig IgG and rabbit anti-mouse IgG (both Miles Scientific, Naperville, IL/USA) were used. On sections of skin or transplants of normal keratinocytes M9 and Gk antisera stained all epidermal layers, while generally M12, R67, S67 and KLI showed quite a uniform staining of the suprabasal layers. The R67 antisera (against the intact C-terminus), however, appeared to be more selective on HELP transplants staining fewer cells than the other “suprabasal” antisera. The antisera (III–VI) were kindly provided by W.W. Franke, D. Roop, J. Schweizer and J. Thivolet’s laboratory, respectively (KLI also from Immunotech, Marseille/France).

Protein preparation

Total proteins. Tissue or cells were washed in PBS and extracted directly in 1 or 3% (w/v) SDS-sample buffer (25 mM dithioerythritol, 0.5 mM MgCl2, 20 mM Tris-HCl, pH 7.4). The samples were first homogenized on ice (glass-glass homogenizer, Potter type), heated for 20 min at 90°C, homogenized at 22°C and extracted after 2 h cleared by centrifugation (40,000g for 30 min). Cytoskeletal proteins. Cytoskeletons were prepared by sequential extraction using low and high salt buffers containing nonionic detergent (Triton X-100) at 4°C [12]. Samples were homogenized at least twice in high salt buffer, cytoskeletons washed in low salt and extracted in 1% SDS-sample buffer.

Metabolic labeling of proteins

Tissue from transplants or tumors was dissected under aseptic conditions, placed in 35 mm dishes and rinsed with PBS. Transplants were radially incised and tumor slices cut in strips of about 2 mm diameter, specimens were incubated in 2 ml RPMI medium (without cold methionine; with 1% glucose added freshly) containing 40 μCi/ml L-[35]S methionine (Amersham, Braunschweig/FRC) for 4 to 8 h as indicated (Legend, Figs. 4, 5). After incubation, samples were washed with ice-cold PBS and processed as above.

Electrophoretic procedures

One-dimensional gel electrophoresis. Samples adjusted to 3% SDS, 15% glycerol, 0.01% bromphenol blue (final concentrations), were heated at 60°C for 5 min and electrophoresed on SDS-polyacrylamide slab gels (10% or linear 7.5-17.5% acrylamide gradient) using a discontinuous Tris/glycine buffer system [9, 12]. Gels of labeled proteins were stained, further processed for fluorography using EN'hnence (NEN Chemicals, Dreieich/FRC) and exposed on Kodak XAR-5 film at −70°C. Two-dimensional gel electrophoresis. Briefly, samples dissolved in 1% SDS-sample buffer were adjusted to 9.5 M urea and diluted with two volumes sample dilution buffer containing 9.5 M urea, 4% NP40, 5% mercaptoethanol and 3% ampholines (pH 5–8; LKB, Bromma/Sweden). Separation in the first dimension was achieved by non-equilibrium pH gradient electrophoresis (NEPHG) in tube gels (ampholine range pH 2–11) and in the second dimension by SDS-PAGE [9, 37].

Results

Cell culture results

When mouse local cell cultures were established and serially subcultured [26], Subculturing led to Petriperm-on-Petriperm cloning. Similarly, positive [24], cornified cells expressing HELP I (Fig. 1b) were nearly absent.

Cell transplant experiments

Cells of all transplantation sources induced granulation tissue.
Results

Cell culture morphology

When mouse epidermal keratinocytes were plated at high local cell density on gas-permeable dishes (Petriperm, no. 112); both adhered and proliferating and stratifying cultures could be maintained and serially passaged for 6 to 7 months after plating [25, 26]. Subcultured cells grew equally well on both substrata, Petriperm or plastic, and were further propagated on plastic dishes. Gross morphology was typical for “differentiated” epidermal cell lines [10, 22] and remained virtually unchanged from early to higher passages (Figs. 1a, c) [24, 30]. Generally, cells reacted strongly with keratin antibodies (Fig. 1b) only a few (< 1%) being in addition vimentin positive [24]. Stratification and formation of superficial cornified cell squames were noted more pronouncedly in HELP I (Fig. 1d) and IV and less in HELP III and were clearly absent in HELP II cultures.

Cell transplants

Cells of all four lines grew readily when transplanted on adult mice irrespective of the

Tab. 1. Growth and differentiation of HELP transplants on adult C3H mice.*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage no.</th>
<th>Observation period</th>
<th>Transplants taken (positive/total no.)</th>
<th>Morphology</th>
<th>Keratin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HELP I</td>
<td>16</td>
<td>14–26</td>
<td>10/10</td>
<td>++ +</td>
<td>nd</td>
</tr>
<tr>
<td>31</td>
<td>12–34</td>
<td>10/10</td>
<td>++ +</td>
<td>++ + +</td>
<td>+</td>
</tr>
<tr>
<td>HELP II</td>
<td>9</td>
<td>15–28</td>
<td>9/10</td>
<td>++ + +</td>
<td>–/–</td>
</tr>
<tr>
<td>20</td>
<td>7–28</td>
<td>9/9</td>
<td>++ +</td>
<td>–/–</td>
<td></td>
</tr>
<tr>
<td>HELP III</td>
<td>12</td>
<td>12–29</td>
<td>10/10</td>
<td>++ + +</td>
<td>nd</td>
</tr>
<tr>
<td>18</td>
<td>7–28</td>
<td>10/10</td>
<td>++ +</td>
<td>++ + +</td>
<td>+ +</td>
</tr>
<tr>
<td>HELP IV</td>
<td>9</td>
<td>7–28</td>
<td>9/10</td>
<td>++ +</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>14–27</td>
<td>14/14</td>
<td>++ +</td>
<td>++ + +</td>
<td></td>
</tr>
</tbody>
</table>

* Two × 10⁶ cells were transplanted. — * Days after transplantation when animals were sacrificed. — * Range of keratinization, arbitrary rating according to gross morphology and light microscopy of frozen and paraffin-embedded sections. Stratified epithelium: (++) virtually not keratinized, (+++) with per and ortho-keratinized areas, (++) predominantly ortho-keratinized, with distinct stratum granulosum and large horny layer. Transplants of all lines except HELP II developed large papilloma-like structures at later stages. — * Presence of “superbasal” epidermal keratins (1D and 2D gels: acidic 67 kDa component, basic 67 kDa component and 69 to 64 kDa derivatives); (−) not detectable; (+) to (+++) weak to strong expression (close to the level of normal keratinocyte transplants).

Fig. 1. Morphology of HELP cultures at early and high passage levels. Confluent culture of HELP II at the 2nd passage (a) and reaction of corresponding cells with anti-keratin (bovine snout) antibodies (b). Similar appearance of HELP III at the 34th passage (c) and formation of superficial cell squamae in stratifying culture of HELP I, 38th passage (d). In the latter case the culture was preextracted with Tritton X-100 and high salt buffer for better visibility of cell squamae (some of their contours are marked by arrows). — a, c, d. Phase-contrast. — b. Indirect immunofluorescence microscopy. — Bars 40 μm.
weeks after inoculation, squamous epithelial sections of the implantation site had normal-looking cells (Fig. 6b, c). The numbers of these cells increased, as seen in Figs. 9b, c. The tumors were also classified into four grades: Grade I, II, III, and IV (Fig. 10). Tumors in Grade I are characterized by highly differentiated squamous cells and normal-looking tissue architecture. Tumors in Grade II are characterized by moderately differentiated squamous cells and more disorganized tissue architecture. Tumors in Grade III are characterized by poorly differentiated squamous cells and more disorganized tissue architecture. Tumors in Grade IV are characterized by undifferentiated squamous cells and highly disorganized tissue architecture.

Tumorigenicity

Tumorigenicity of the 10 cell lines in the TA2 line was assessed by converting them into tumor xenografts in nude mice. In most tumors, the 10 cell lines exhibited high degrees of differentiation, with the exception of one cell line, which had a highly undifferentiated phenotype. The tumors were classified into four grades: Grade I, II, III, and IV. Tumors in Grade I are characterized by highly differentiated squamous cells and normal-looking tissue architecture. Tumors in Grade II are characterized by moderately differentiated squamous cells and more disorganized tissue architecture. Tumors in Grade III are characterized by poorly differentiated squamous cells and more disorganized tissue architecture. Tumors in Grade IV are characterized by undifferentiated squamous cells and highly disorganized tissue architecture.

Electron microscopy

Electron microscopy was performed on tissue samples from the TA2 line and the 10 cell lines. The results showed that the TA2 line and the 10 cell lines exhibited similar ultrastructural characteristics, including well-defined cell membranes, mitochondria, and rough endoplasmic reticulum. However, the 10 cell lines exhibited more pronounced nuclear abnormalities, including nuclear enlargement, nucleoli, and coiled bodies, compared to the TA2 line.

weeks after transplantation. At these early stages histological sections revealed stratified epithelia resembling hyperplastic mouse epidermis (Fig. 2a). In this regard, HELP cells behaved like normal cells [12, 23, 33] (compare also Figs. 6b, d), while most other transformed keratinocyte lines did not form organized epithelial structures nor did they exhibit signs of ortho-keratinization in transplants [25]. Distinct layers, including stratum granulosum and corneum, were most noticeable in transplants of HELP I and IV (Figs. 2a, b); they were somewhat less pronounced in HELP III transplants (compare Fig. 6d), and rather poorly developed in transplants of HELP II cells (Tab. I). At later stages, these epithelia, except those of HELP II, increased in thickness and were often covered by large plaques of loosely adherent cornified cells. HELP II cells developed only a few cell layers with partially parakeratinized areas. Thick transplants revealed a papilloma-like morphology with papillary growth cones or large cell masses invading the host connective tissue (Fig. 2c) and with further progression, large invasive tumors with nests of cornified cells. The growth and differentiation behaviour of the individual lines was quite consistent through serial passages (Tab. I).

Tumorigenicity test

Tumorigenicity was assayed by s.c. injection of cell suspensions in newborn C3H mice. All four cell lines were tumorigenic at the lowest passage levels tested (between 6 and 16) with high efficiency as summarized in Table II. In most tumors, except those of HELP II, areas with a high degree of cytodifferentiation, including continuous stretches of stratum granulosum-like cells (Fig. 2d), were abundant (Tab. II). In the case of HELP I tumors, at later stages metastases were detected in the lung by macroscopic examination at autopsy. Both primary tumors (Figs. 2e, f) and metastatic lesions (Fig. 2g) had the appearance of highly differentiated carcinomas.

 Electron microscopy of cell transplants

Electron microscopy of fully developed transplants further underlined the large spectrum of disturbances in overall tissue architecture and morphological differentiation. Cells were not regularly aligned, showed a wide variety in shape and, most striking, a mosaic of lighter and darker cells (Fig. 3a). The latter presumably represent so-called "dark cells", rare in normal epidermis but increased in papillomas and carcinomas [30, 38]. In cells with cornified envelopes (Fig. 3b) tonofilaments were either loosely arranged in bundles or individually dispersed, in contrast to the dense packing ("keratin pattern") observed in stratum corneum of epidermis and transplants of normal cells. Further, HELP cells produced basement membrane components and formed, in vivo, a visible basal lamina (Hornung et al., in preparation). The apparent discontinuities in the basal lamina (Fig. 3c) might indicate another defect in epithelial differentiation or be caused, at least partially, by the irregular contours of the epithelial-mesenchymal junction.

**Keratin analysis**

When keratins (cytoskeletal preparations) of HELP I-IV cultures were compared by electrophoresis on SDS-polyacrylamide gels (SPAGE) the patterns were virtually identical, as shown in Figure 4c (lane 1), and resembled closely those of primary keratinocyte cultures (Fig. 4a, lane 1). Two-dimensional gels (NPHG/SPAGE) also revealed the corresponding isoelectric variants of the basic 60 kDa and 59 kDa as well as of the acidic 53 kDa keratins, analogous to normal cells (Figs. 5a, b). Only the 49 kDa keratin was significantly reduced in HELP cultures, while one acidic 51 kDa keratin (minor in primaries) was enhanced and additionally accompanied by a slightly more acidic spot of similar size (Fig. 5b).

Transplants of normal keratinocytes forming ortho-keratinizing (hyperplastic) epithelia reexpressed the larger epidermal keratins (mouse: basic 67 kDa and acidic 58 kDa) together with the respective modified products (62-64 kDa range) seen in newborn mouse, all usually absent in culture (compare Fig. 4a, lanes 1-3 and Figs. 5a, c, d). Since these keratins are almost exclusively expressed in the suprabasal, differentiating epidermal layers (stratum spinosum and corneum), being also precursors of the modified stratum corneum keratins, they are herein called differentiation-specific or "suprabasal" keratins [9, 12]. Generally, their expression in the epidermis (also during ontogenesis) correlates closely to the development of stratum granulo-

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**Tab. II.** Properties of tumors grown in C3H mice after s.c. injection of HELP cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage no.</th>
<th>Tumor rate (positive/no. injected)</th>
<th>Latency period *</th>
<th>Degree of differentiation</th>
<th>Keratin *</th>
</tr>
</thead>
<tbody>
<tr>
<td>HELP I</td>
<td>16</td>
<td>5/5</td>
<td>12-28</td>
<td>+/+/ +</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>7/7</td>
<td>22-40</td>
<td>+/ +</td>
<td>++</td>
</tr>
<tr>
<td>HELP II</td>
<td>9</td>
<td>3/5</td>
<td>17-51</td>
<td>+/+ +</td>
<td>+/++</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4/8</td>
<td>56-65</td>
<td>+/+ +</td>
<td>+/++</td>
</tr>
<tr>
<td>HELP III</td>
<td>9</td>
<td>7/7</td>
<td>6-50</td>
<td>+/+ +/+</td>
<td>++</td>
</tr>
<tr>
<td>HELP IV</td>
<td>6</td>
<td>6/6</td>
<td>20-57</td>
<td>+/++/+</td>
<td>++/++++</td>
</tr>
</tbody>
</table>

* Days after injection, when growing nodules became palpable. — * Keratinization according to histology and electron microscopy: (+ +) small to larger horn pearls within tumor tissue; (+ + +) partially polarized epithelium around large keratinized areas, abundant stratum granulosum-like cells. — * Presence of "suprabasal" epidermal keratins, arbitrary rating as in Table I. — * Lung metastases, visible macroscopically and diagnosed on histological sections.
sum, making them suitable markers for ortho-keratinization [49]. However, in transplants and tumors of cell lines previously isolated from mouse skin carcinomas (e.g. HD II [22]) or transformed spontaneously in vitro (HEL I-30 [18, 22]), the culture-type keratin pattern remained virtually unchanged, although these cells formed (morphologically) keratinizing surface epithelia and squamous cell carcinomas, respectively (Fig. 4b). In contrast, and comparable to normal cells, most transplants of HELP cells showed on 1D-gels significant amounts of 67 kDa keratin and varying 62 to 66 kDa components, in addition to the respective culture pattern. The patterns (Fig. 4c, lane 2 and Fig. 4d, lanes 1, 2) correlated to the morphological degree of keratinization, which was least pronounced in HELP II transplants.

In addition, 2D-gels revealed the corresponding “suprabasal” 58 kDa keratin (Figs. 5e, g, h). Evidence for the identity of the respective keratins described above was supported by co-electrophoresis with epidermal keratins (Figs. 5i, k) and comparable reactions on Western blots (preliminary data not shown here). The level of 62 to 66 kDa components usually increased with age of HELP transplants (similar to those of normal cells, Fig. 5c), suggesting that they were modified, proteolytic derivatives of the 67 kDa keratin as seen in epidermal stratum corneum. This was further supported by radioactive labeling, which always gave rise to stronger bands in the 67 kDa and only traces at most in the 62 to 66 kDa region (Fig. 4d, lanes 1, 2; Figs. 5g, h). On the other hand, the comparison to total protein content during differentiation due to this result.

When the cell lines were generally also cultured on different 18, 22, specimens ranged from 3 days in size. Whole section examination of excised specimens, the control, revealed, a high amount of labeling.

Accurate, excluding “unusual” keratins.

Indirect immunofluorescence

The indirect immunofluorescence (with some cells per section) is shown in epithelial cells of the epidermis, using antikeratin antibodies, which also recognize epidermal keratins.

Fig. 3. Electron microscopy of vertical sections of HELP I transplant. — a. Survey micrograph showing transition from preferentially cuboidal cells in the lower layers to elongated cells in upper layers. Cells exhibit a striking heterogeneity regarding the electron density of their cytoplasm (abundance of “dark cells”). Some adjacent cells show extensive interdigitations heavily decorated with desmosomes (arrows), reminiscent of thick stratum spinosum in bovine snout or human plantar epidermis. — b. Cells from upper layers at higher magnification with cornified envelopes, abundant tonofilaments (TF) and desmosomes (arrows). — c. Junction zone between epithelial cell and host mesenchyme with distinct but discontinuous lamina densa (LD) and hemidesmosomes (arrowheads). — Bars 10 μm (a), 1 μm (b), 0.5 μm (c).
protein extracts (not shown) demonstrated that degradation during the cytoskeletal preparation was negligible in this respect.

When tumors were examined, the larger keratins were generally abundant and in this case HELP II tumors were also clearly positive (Fig. 4c, lanes 3–6; Fig. 5f). On the average, the degree of keratinization (at the protein level) ranged in the same order as in transplants: HELP I = IV > III = II (Tab. II). However, the heterogeneity in tumor specimens of the same cell line was much higher than in transplants, and seemed not to depend on tumor age or size. While some samples were almost negative, differentiation-specific keratins were also found in tumor nodules excised from lung metastases (not shown). Also in tumors, the conversion of 67 kDa keratin to smaller derivatives was high as seen in Figure 4e (lanes 3–6), Figure 5f and, after labeling of excised tissue in vitro, in Figure 4d (lanes 3, 3’).

According to the 2D-patterns the ratio of the 67 kDa (including presumptive derivatives) to the 58 kDa “suprabasal” keratin seemed to be roughly unity in most samples.

Indirect immunofluorescence microscopy

The incubation with antisera against epidermal keratins (with broad specificity) showed strong staining in frozen sections of HELP cell transplants including invading epithelial cell masses (Fig. 6a). In order to evaluate the degree of regular tissue organization transplants were examined using antisera against differentiation-specific keratins which reacted almost exclusively with suprabasal cells in epidermis and in transplants of normal keratinocytes (Fig. 6b). Consistent with the protein analysis, transplants of the previously tested malignant cell lines were either negative or contained only a few scattered positive cells, as exemplified by a well differentiated transplant of HEL I-30 cells (Fig. 6c). In HELP transplants, however, the regular compartmentalization of “suprabasal” keratins was often maintained initially, concomitant with a rather normal (epidermis-like) tissue organization (Fig. 6d). Both features were gradually lost with further growth. In parallel with increased folding of the dermal-epithelial junction zone or invasive growth, respectively, primarily the unstained compartment of basally located cells increased and later on positive single cells or small cell nests became interspersed within negative or weakly stained epithelium (Figs. 6e–g). The staining patterns correlated well with the histological appearance, labeling also keratinized areas within invasive cell masses (Fig. 6h). Comparable results were obtained with various “suprabasal” antisera, except with R67-antibodies staining somewhat more selectively (see Materials and Methods and Discussion). All tumors examined reacted with these “suprabasal” antisera in highly keratinized areas (seen by histology, H & E, compare Fig. 2d) exhibiting foci and single strongly reacting cells (not shown).

In conclusion, the immunofluorescence microscopy shows (i) strong expression of “suprabasal” keratins corresponding to the biochemical analysis in both transplants and tumors, but also (ii) severe disturbances in the spatial distribution of these differentiation markers, thus further substantiating the histological and ultrastructural observations.

Fig. 4. One-dimensional electrophoresis of keratins on 10% (a) and 7.5 to 17% acrylamide gradient gels (b, c, d). — a. Induction of epidermal “suprabasal” keratins in transplants of normal keratinocytes: lane 1, normal keratinocytes in culture and, lane 2, three weeks after transplantation; lane 3, backskin epidermis of neonatal mouse. — b. Absence of differentiation-specific (“suprabasal”) keratins in mouse cell lines: lane 1, culture of HD II cell line, lane 2, HD II transplant, lane 3, lung metastasis of corresponding injection tumor (keratin pattern virtually identical to primary tumor, also on two-dimensional gels; not shown); lane 4, injection tumor of HEL I-30 cells in nude mouse. — c. Reactivation of “suprabasal” keratins in transplants and tumors of HELP cells: lane 1, HELP III culture, lane 2, HELP III transplant, lanes 3 to 6, tumors of HELP I, II, III, and IV, respectively. — d. Metabolic labeling of “suprabasal” keratins with [35S]metionine: Coomassie stain (lanes 1–3), corresponding fluorographs (lanes 1’–3’). — Lanes I, 1’, HELP I transplant and lanes 2, 2’, HELP IV transplant (48 and 18 h label, respectively), lanes 3, 3’, HELP I tumor (10 h label). Numbers at left (a) apparent mol. wt. in kDa, marked by dots in (b–d). — A (dashes) Actin.

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Discussion

Generally, epithelial skin malignancies are considered to be linked to defects in keratinocyte differentiation. Numerous attempts to transform animal or human keratinocytes in vitro have also suggested that loss of differentiation functions accompanies or precedes the manifestation of the malignant phenotype [22, 26, 28, 47, 57]. This relates to a more general concept developed in other systems (for review see also [46]), and to earlier observations in clinical pathology, postulating an inverse correlation between differentiation and malignancy (for discussion see [8]). Here we present examples of mouse keratinocyte lines, which are malignant, but still are able to differentiate in response to environmental stimuli. Comparable to normal keratinocytes [1, 2, 3, 12, 14, 23, 31] these cells can be induced to express most, if not all, epidermal differentiation markers.

![Diagram of keratinocyte differentiation](image)
Characteristics of spontaneous cell lines

With the goal to develop spontaneously transformed cell lines as one way to study carcinogenesis, different strategies for long-term growth and immortalization of mouse keratinocytes have been followed [22, 25, 26]. One successful way was to use gas-permeable “Petriperm” dishes for initial growth which gave rise to the four cell lines HELP I, II, III, and IV. In culture, HELP cells exhibited properties comparable to other terminally “differentiating” epidermal cell lines [10, 25]. They reacted strongly with “general” keratin antibodies [24], expressed keratin patterns resembling those of primary cultures, and gave rise to (parakeratotic) cell squames during stratification. Interestingly, the two variants most remote phenotypically, HELP I and II, were derived from the same primary culture and, as we assume, also in regard to earlier studies [24, 25], that variants diverge at a rather early phase (see also [42]). This may relate to an originally unstable karyotype, which usually stabilizes during further passaging [15, 26].

Tissue organization under environmental influence

When transplanted, three of the four cell lines (HELP I, III, IV) initially developed stratified, ortho-keratinizing epithelia, however, with time transplants showed increasing alterations in tissue architecture, assumed a papilloma-like appearance and grew invasively. Finally, large tumor-like cell masses developed in the deep connective tissue, frequently with no connections to the original transplant, thus already revealing malignant properties. Apparently, the balance between differentiation and proliferation was irreversibly lost and growth continued “autonomously” in a non-physiological environment (deep connective tissue).

After s.c. injection all four cell lines (including HELP II) formed moderately to highly differentiated squamous cell carcinomas (SCCs), again indicating a different environmental response.

Induction of epidermis-type “suprabasal” keratins

Generally, keratin expression is closely correlated with morphological differentiation. This has been demonstrated experimentally by transplantation of tissue recombiantants [44], by transplantation or injection of normal cells [3, 12, 14, 23, 31] or in true in vitro-systems [1, 2]. For clarity we like to refer in this context to the catalog of human keratins [35]. The basic murine keratins 67 kDa, 60 kDa and 59 kDa are analogous to human 68 kDa (no. 1), 60.5 kDa (no. 5), 58 kDa (no. 6) and the acidic murine keratins 58 kDa, 53 kDa and 49 kDa to human 57 kDa (no. 10/11), 51 kDa (no. 14), and 49 kDa (no. 16), respectively (apparent mol. wt. according to our gel system [9, 12]). In good correlation to morphological keratinization all four HELP cell lines are able to express (at least after s.c. injection) both differentiation-specific keratins (identified by coelectrophoresis on 2D-gels), namely the acidic 58 kDa (type I) and the basic 67 kDa (type II). In most specimens the 67 kDa keratin was modified to a large extent (increasing with age of transplant), being analogous to proteolytic processing in upper epidermal layers [9, 12]. Labeling experiments comparing newly synthesized with total keratin were consistent with a product-precursor relationship between the 67 kDa keratin (strongly labeled) and the smaller more acidic components (mostly unlabeled) in the size range of 62 kDa to 64 kDa and 66 kDa (Figs. 4c, d and Figs. 5e-h). The comparably larger acidic charge shift (also seen in older transplants of normal cells, Fig. 5c) presumably reflects a higher proteolytic susceptibility due to the loose filament association in cornified cells (in contrast to the dense packing in epidermal stratum corneum). Also, as in transplants of normal keratinocytes, none of the major keratins present in culture is completely turned off even in highly differentiated tissues (transplants or tumors). Thus, according to Sun et al. [49], the subsets indicative for “stratified epithelia” (53/60 kDa), “keratinization” (58/67 kDa) as well as a “hyperproliferative” state (49/59 kDa), are all present. The persistence of the “hyperproliferative” type might correlate to the hyperplastic morphology and/or an alternative differentiation pathway. This is also observed in vivo under physiological (e.g. human palmor and plantar epidermis) as well as pathological conditions (34, 36, 49, 54) and our unpublished data.

Other mouse cell lines with some residual differentiation functions have been described previously [39, 40] and similarly, as shown here, the induction of “suprabasal” keratins by environmental factors (vitamin A depletion in vitro; nude mouse tumors) has been observed in human cell lines from SCCs [29, 56].

Relationship between keratin expression and histological appearance

Using various antisera against “suprabasal” keratins [41, 43, 52] histologically “normal” transplants revealed also a
regular or even quite highly regular. The very weak response of the KL1 antibody (Fig. 6) suggests that this cell surface component is expressed in very low amounts on the cell membranes of the infiltrating cells. However, the KL1 antibody also stains a network of filaments in the extracellular matrix, indicating the presence of KL1-like molecules in the extracellular environment. This suggests that KL1 is not exclusively restricted to the cell membranes of the infiltrating cells, but is also present in the extracellular matrix. Overall, these findings provide evidence for the role of KL1 in the regulation of cell migration and plasticity in the developing nervous system.

**Conclusions**

The findings presented in this study suggest a novel role for KL1 in the regulation of cell migration and plasticity in the developing nervous system. The observation that KL1 is expressed in very low amounts on the cell membranes of the infiltrating cells, as well as in the extracellular matrix, indicates that KL1 may play a dual role in the development of the nervous system. On the one hand, KL1 may act as a cell surface component, regulating cell migration and adhesion. On the other hand, KL1 may act as an extracellular matrix component, influencing the organization and function of the extracellular environment. These findings provide new insights into the complex role of KL1 in the development of the nervous system, and suggest potential avenues for the study of KL1 function in disease states.

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**Fig. 6.** Antibodies raised against KL1 (KL1-antibodies raised against KL1), stained for HELP II antibodies (HEL-antibodies raised against HELP II), stained for HELP III antibodies (HEL-antibodies raised against HELP III), and stained for HELP IV antibodies (HEL-antibodies raised against HELP IV).
regular compartmentalization of keratins. Although this is not definitive proof, it strongly suggests an initially well orchestrated onset of differentiation by an apparently quite homogeneous cell population. With increase in cell mass and morphological disturbances, however, the continuity of suprabasal staining (IIF) became gradually interrupted and further on single positive cells ("dyskeratoses") or small foci were dispersed within virtually negative epithelium as seen likewise in the subcutaneous tumors. This apparently reflects a gradual escape of individual cells from environmental (mesenchymal and/or intraepithelial) control of growth and differentiation.

Compared to other "suprabasal" antibodies (such as KLI the M12 antiserum was slightly less specific, showing very weak staining in usually negative areas. On the other hand, R67 antibodies raised against the C-terminal sequence of 67 kDa, were more selective. Because, during proteolytic processing terminal sequences are lost, these antibodies react neither with respective derivatives of 67 kDa on protein-bLOTS nor with epidermal stratum corneum by immunofluorescence [41]. Thus, the differential staining with the various antiseras in HELP tissues is consistent with the high level of 67 kDa turnover products. It also underlines the tissue heterogeneity of HELP transplants, some cells being far more differentiated than their neighbours.

Concluding remarks and implications

The failure of transplanted HELP cells to reach a steady-state (homeostasis) and to maintain tissue polarity might reflect (i) reduction in self-regulatory constraints (feedback and positional control), (ii) defects in the response to mesenchymal signals, or (iii) an alteration in mutual mesenchymal-epithelial interactions. All three mechanisms might be involved, possibly in a certain sequence or cycle as proposed recently [19]. A strong indication for a mutual tissue-interaction is the increased vascularization of the graft bed compared to normal transplants, which was most apparent by staining (IIF) of ingrowing capillaries with anti-type IV collagen (Hornung et al., in preparation).

In conclusion, our data show, in agreement with earlier observations in vivo [32, 36] and in vitro [7, 8, 29, 39, 56], that maintenance of differentiation functions and malignancy are not mutually exclusive and further, that differentiation is not necessarily inversely proportional to the malignant potential. Thus, the well differentiating HELP I cells gave rise not only to tumors but also metastases in contrast to the poorly differentiating HELP II cells. On the other hand, certain defects in the regulatory mechanism of differentiation seem to be mandatory for malignancy (as discussed [57]), since the well differentiating lines also showed discrete alterations in spatial expression. These still might represent exceptional cases, so that the more common situation in malignant tumors is characterized by rather severe disturbances in regard also to keratin expression [6, 11, 35, 36, 50, 51, 55]. Nevertheless, these HELP cell lines represent suitable models for regulation of epidermal differentiation and its relevance for the malignant phenotype. The present study demonstrates that despite normal differentiation at the cellular level, differentiation at the tissue level might be severely disturbed. Although complex molecular mechanisms are involved in regular tissue architecture, subtle alterations at the molecular level, such as cell surface changes [53], might have profound consequences on tissue organization. Therefore, all of the established criteria of differentiation should be considered in the overall context of normal differentiation since reliance on one or a few criteria may lead to erroneous conclusions regarding normal versus aberrant differentiation.

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