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Stem Cell Patterning and Fate in Human Epidermis

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Summary

Within human epidermis there are two types of proliferating keratinocyte: stem cells, which have high proliferative potential, and transit-amplifying cells, which are destined to undergo terminal differentiation after a few rounds of division. We show that, in vivo, stem cells express higher levels of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins than transit-amplifying cells and that this can be used both to determine the location of stem cells within the epidermis and to isolate them directly from the tissue. The distribution of stem cells and transit-amplifying cells is not random: patches of integrin-bright and integrin-dull cells have a specific location with respect to the epidermal-dermal junction that varies between body sites and that correlates with the distribution of S phase cells. Stem cell patterning can be recreated in culture, in the absence of dermis, and appears to be subject to autoregulation.

Introduction

Within the epidermis, proliferation takes place in the basal layer of keratinocytes that are attached to the underlying basement membrane, and cells undergo terminal differentiation as they migrate through the suprabasal layers, finally being shed from the tissue surface as dead, cornified squames (reviewed by Watt, 1989). Three subpopulations of basal keratinocytes have been defined by cell kinetic analysis (reviewed by Potten and Morris, 1988): stem cells, transit-amplifying cells, and committed cells. Stem cells retain a high capacity for self-renewal throughout adult life and are ultimately responsible for epidermal maintenance and repair. The daughters of stem cells can either be stem cells themselves or cells known as transit-amplifying cells. Transit-amplifying cells divide a small number of times, but have a high probability of producing daughters that withdraw irreversibly from the cell cycle and are committed to differentiate terminally. Committed cells detach from the basement membrane and move upward from the basal layer via a mechanism that involves inactivation of β_1 integrin extracellular matrix receptors on the cell surface (Hotchin et al., 1993).

Although evidence for the existence of epidermal stem cells and transit-amplifying cells dates back more than 20 years (reviewed by Potten, 1974), studies of their properties have been hampered by the lack of any molecular markers that could be used to isolate viable subpopulations directly from skin. We reported previously that in cultures of human epidermal keratinocytes, cells with characteristics of stem and transit-amplifying cells can be identified and isolated on the basis of their adhesive properties (Jones and Watt, 1993). Stem cells express high levels of three of the β_1 family of integrins ($\alpha_2\beta_1$, receptor for collagen and laminin; $\alpha_3\beta_1$, receptor for laminin and epiligrin/kalinin; and $\alpha_5\beta_1$, fibronectin receptor) and adhere rapidly to type IV collagen, fibronectin, and the extracellular matrix deposited by cultured keratinocytes. Basal cells that express lower amounts of β_1 integrins on the cell surface adhere more slowly to the extracellular matrix and undergo terminal differentiation after one to five rounds of division, thus displaying characteristics of transit-amplifying cells.

Our experiments with cultured keratinocytes raised the question of whether or not differences in integrin levels could be used to determine the location of stem cells and transit-amplifying cells in vivo and to isolate each subpopulation directly from human epidermis. We now show that this is indeed the case. Stem cells and transit-amplifying cells, distinguished on the basis of β_1 integrin expression, have a nonrandom distribution within the basal layer of the epidermis from different body sites. The cells can be isolated directly from the epidermis and their fate can be followed in culture. Patches of integrin-bright and integrin-dull cells similar in size to those observed in the epidermis can be recreated in culture in the absence of dermis, suggesting that stem cell patterning may be an intrinsic property of keratinocytes.

Results

Variation of Integrin Levels within the Basal Layer of the Epidermis

The differences in integrin levels that reflect differences between stem cells and other basal cells in culture are 2- to 3-fold (Jones and Watt, 1993). Conventional immunofluorescence techniques, involving a primary antibody and a secondary fluorochrome-conjugated antibody, are designed to maximize the signal from the primary antibody and might therefore mask small, but potentially relevant, differences in integrin levels in tissue sections. To overcome this problem, we labeled frozen sections of skin with anti-integrin antibodies that had been directly conjugated to fluorescein isothiocyanate (FITC), thereby obviating the amplification provided by a secondary antibody. We also viewed the sections with a confocal microscope that allowed a uniform optical thickness of material (1 μm) to be examined, thus controlling for any variation in the total thickness of each tissue section and also allowing quantitation of fluorescence levels. Sections in which the plane of sectioning was not vertical through the epidermis were excluded from analysis.

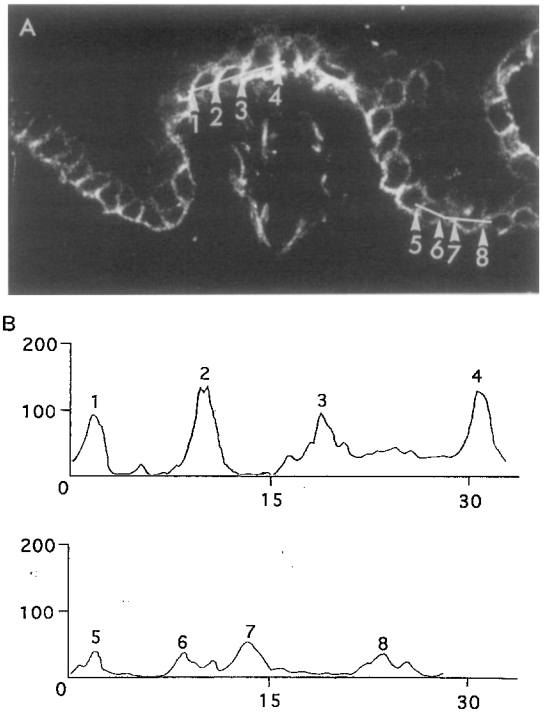

Figure 1. Quantitation of Integrin Levels by Confocal Microscopy

(A) Section of human scalp stained with FITC-conjugated antibody to α_3 integrin subunit. (B) Fluorescence (y axis shows pixel intensity, arbitrary units, linear scale) was measured along lines drawn through individual cells, each numbered peak corresponding to one numbered cell–cell border. The x axis shows distance along the basal layer in micrometers. Peaks 1–4, brightly stained cells at tip of dermal papilla. Peaks 5–8, weakly stained cells at tip of rete ridge. Mean fluorescence of cell–cell borders is 105 U (peaks 1–4) and 42 U (peaks 5–8).

Figure 1 shows how fluorescence levels were measured. Frozen sections of skin were labeled with FITC-conjugated antibodies to the α_2, α_3, α_6, and β_1 integrin subunits. α_5 was not examined because $\alpha_5\beta_1$ levels are very low in mature, undamaged epidermis (Hertle et al., 1991). The α_6 subunit, which forms a heterodimer with β_4 and is a component of hemidesmosomes (Carter et al., 1990), was included in the study because α_6 levels are not correlated with proliferative potential in vitro (Jones and Watt, 1993). For the subunits of β_1 integrins, fluorescence was measured along a line drawn through the lateral borders of cells within the basal layer. Each cell–cell border appears as a peak of pixel intensity in arbitrary units on a linear scale (Figure 1). For the α_6 subunit, fluorescence was concentrated on the basal rather than the lateral or apical cell surfaces (Figure 2D), and fluorescence was therefore measured across the basal surface of individual basal cells.

We examined skin from three body sites: neonatal foreskin, because this was the source of keratinocytes for our earlier in vitro experiments on stem cells (Jones and Watt, 1993); adult palm, because firm predictions about the location of epidermal stem cells in palm have been made from cell kinetic analysis (Lavker and Sun, 1983); and adult scalp, since the location of stem cells within hair follicles has been determined with some precision (Yang et al., 1993; Rochat et al., 1994). In epidermis from each site, there was variation in the levels of the α_2, α_3, and β_1 integrin subunits (Figures 1 and 2; Table 1) but not in the level of the α_6 subunit (Figure 2D; data not shown). Although the specimens were studied using a confocal microscope, the variation in fluorescence levels could also be seen by using a conventional fluorescence microscope (for example, Figures 2E and 2F). In each site stained with antibodies to the α_2, α_3, or β_1 subunits, patches of brightly fluorescent basal cells were interspersed with stretches of basal cells of lower fluorescence.

The junction between the epidermis and underlying dermis is not flat, but undulates; sites where the dermis comes closest to the skin surface are known as dermal papillae, and sites where the epidermis projects furthest into the skin are known as rete ridges (see Figure 2A). In foreskin and scalp, rete ridges are relatively uniform in size, but in palm they have been classified as deep or shallow (Lavker and Sun, 1983). In foreskin (Figure 2B) and interfollicular epidermis of scalp (Figure 1; Figures 2C and 2E), the integrin-bright cells were found adjacent to the tips of the dermal papillae, whereas in palm (Figure 2A) they were found at the tips of the deep rete ridges. In scalp, a "cuff" of bright cells was also seen where hair follicles opened out onto the surface of the skin (Figure 2F).

Table 1 presents quantitation of the data for sections stained for the α_2 or α_3 integrin subunits. The proportion of brightly fluorescent cells in the basal layer was higher in foreskin and interfollicular scalp epidermis than in palm. The mean patch size for anti-α_2-stained foreskin was 14 cells, compared with nine cells in scalp and palm (the difference was statistically significant; $P < 0.001$, using the Mann–Whitney U test); for anti-α_3-stained sections, the mean patch size was 13 cells in foreskin and palm and 11 cells in scalp (this difference was not statistically significant). In foreskin the difference in the size of bright patches labeled with anti-α_2 or anti-α_3 antibodies was not significant; in palm and scalp, the α_3-bright patches were significantly larger than the α_2-bright patches ($P = 0.030$ and 0.0015, respectively, using the Mann–Whitney U test). Staining of serial sections with anti-α_2 and anti-α_3 antibodies showed that the α_2- and α_3-bright patches colocalized. In all sites and with both antibodies, the variation in fluorescence intensity between bright and dull cells was approximately 2-fold.

One region of the epidermis for which there is strong evidence for the location of stem cells is the hair follicle. In rodents, the stem cells lie in a well-defined bulge in the outer root sheath (Cotsarelis et al., 1990). In humans, the upper limit of the bulge is defined by the point of insertion of the arrector pili muscle, below which the outer root sheath broadens, but its lower limit is less clearly defined and stem cells are reported to be either close to the muscle (Yang et al., 1993) or slightly lower down (Rochat et al., 1994). Both regions of the human outer root sheath that are reported to contain stem cells can be labeled with an antibody to keratin 19 (Lane et al., 1991). We therefore double labeled sections of scalp with antibodies to keratin

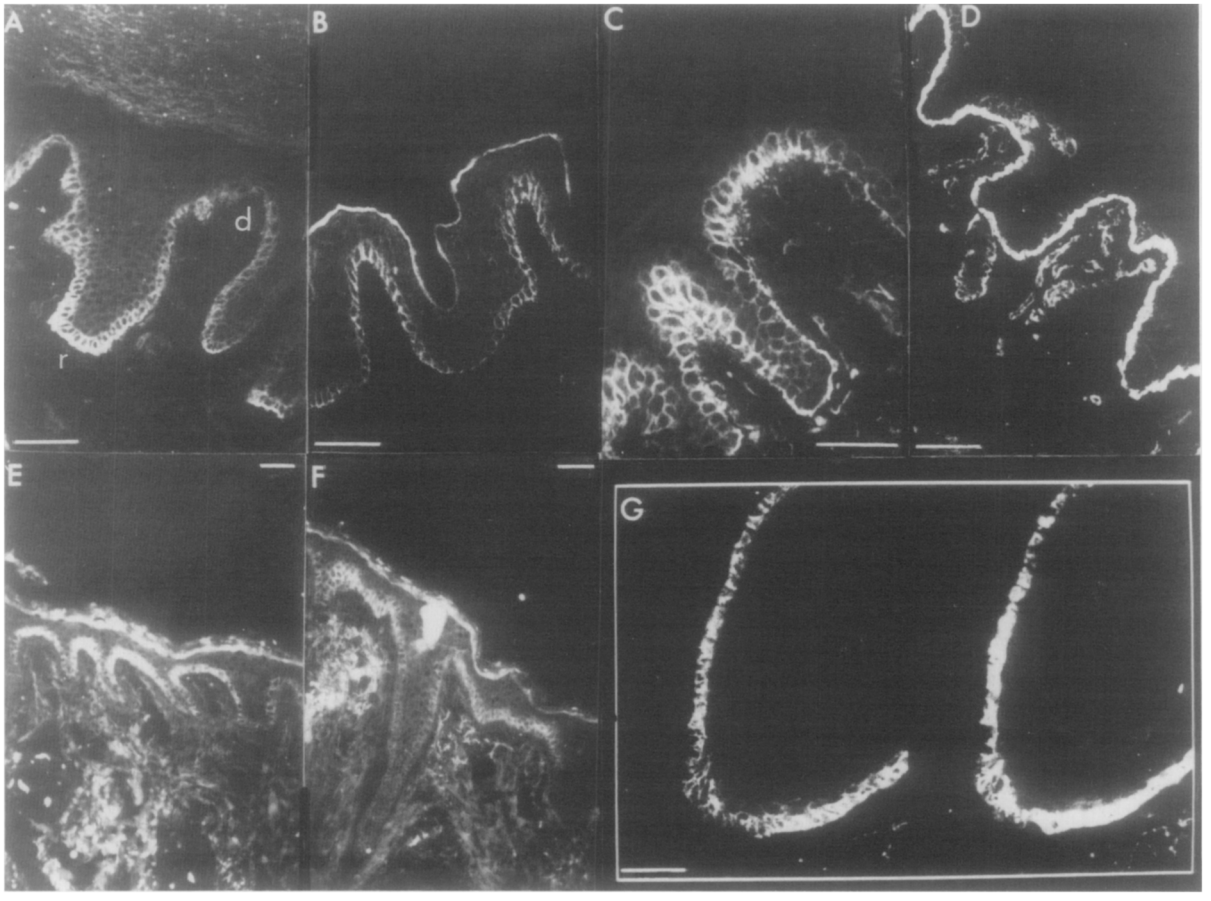

Figure 2. Variation in Integrin Levels in Human Skin from Different Body Sites

Frozen sections stained with FITC-conjugated antibodies to the integrin subunits indicated were viewed by confocal (A–D and G) or conventional (E and F) microscopy.

(A) Palm, α_3 subunit. Abbreviations: r, (deep) rete ridge; d, tip of dermal papilla.

(B) Foreskin, α_3 subunit.

(C) Interfollicular scalp, α_3 subunit.

(D) Palm, α_6 subunit.

(E) Interfollicular scalp, α_2 subunit.

(F) Scalp, including hair follicle, α_2 subunit.

(G) Hair follicle outer root sheath double labeled with an FITC-conjugated antibody to the α_3 integrin subunit (shown on left) and an antibody to keratin 19 visualized with a rhodamine-conjugated second antibody (shown on right).

Scale bars, 50 μm.

19 and the α_2 or α_3 integrin subunits (Figure 2G). Keratin 19–positive outer root sheath cells had 1.4-fold (± 0.1 SEM) greater mean α_2 integrin subunit fluorescence and 1.6-fold (± 0.1 SEM) greater mean α_3 integrin subunit fluorescence than keratin 19–negative outer root sheath cells (both $P < 0.001$, Mann–Whitney U test). There was no significant difference in α_6 integrin subunit fluorescence between keratin 19–positive and keratin 19–negative cells.

Isolation and Cultivation of Integrin-Bright and Integrin-Dull Cells

In cultured keratinocytes our operational definition of a

Table 1. Integrin Expression in Basal Cells in Different Body Sites

	α_2 Subunit			α_3 Subunit		
Measurement	Foreskin	Scalp	Palm	Foreskin	Scalp	Palm
Percentage of bright cells	43.0 ± 1.7	39.0 ± 1.4	25.0 ± 2.2	44.0 ± 1.4	48 ± 2.5	25.0 ± 1.2
Patch size	14.0 ± 1.4	8.7 ± 0.5	8.7 ± 0.7	13.0 ± 1.5	11 ± 0.7	13.0 ± 0.8
Intensity ratio	2.1 ± 0.1	2.2 ± 0.3	1.8 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	1.9 ± 0.1

Values are means ± SEM. Percentage of bright cells, percentage of all basal cells with high integrin fluorescence. Patch size, number of cells in each integrin-bright patch. Intensity ratio, ratio of average fluorescence of cell–cell borders in bright patches to average fluorescence of cell–cell borders in dull patches.

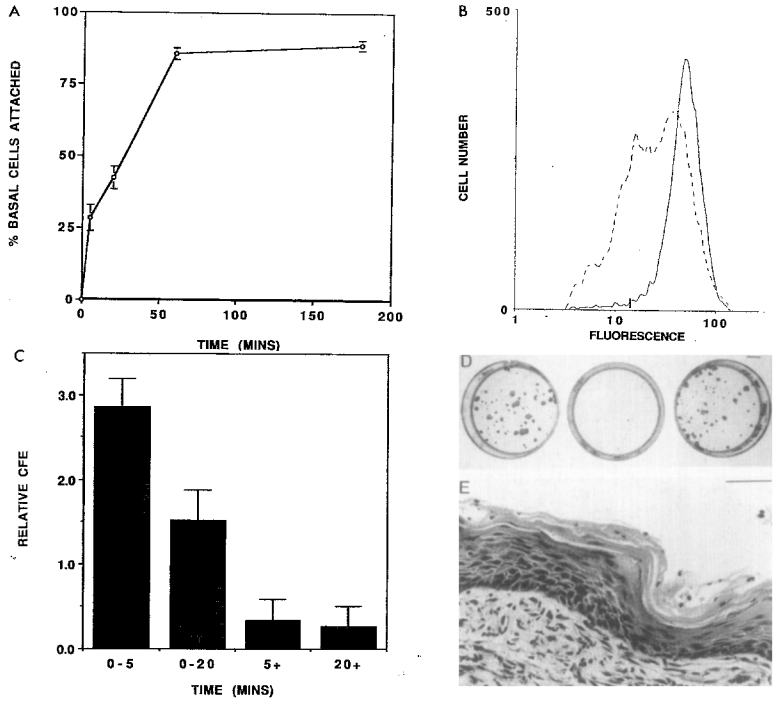

Figure 3. Relationship between Integrin Expression and CFE in Keratinocytes Isolated Directly from Neonatal Foreskins

(A) Percent basal cells that attached to 100 μg/ml type IV collagen with time after plating.

(B) Keratinocytes were labeled with an FITC-conjugated antibody to the α_2 integrin subunit and analyzed by flow cytometry. Fluorescence (in arbitrary units on a log scale) is shown on the x axis and cell number on the y axis. Broken line, total keratinocyte population; solid line, cells that adhered to 100 μg/ml type IV collagen within 5 min. The marker on the x axis shows the upper limit of fluorescence of a control antibody (CD8–FITC). The double peak in the total population comprises suprabasal, integrin-negative cells (left-hand peak) and basal, integrin-positive cells (right-hand peak).

(C) Keratinocytes were plated onto 100 μg/ml type IV collagen for 5 min (0–5) or 20 min (0–20), and the nonadherent cells were then transferred to fresh collagen-coated dishes (marked as 5+ and 20+, respectively) and allowed to attach overnight. After 14 days in culture, all colonies larger than 32 cells in size were counted, and the CFE of each population was calculated and expressed relative to that of unfractionated basal cells (which equals 1.0). Results shown are the means of three experiments; error bars show the SEM. Cells that adhered within 5 min had a significantly higher CFE than those that adhered more slowly ($P = 0.007$, Student's t test).

(D) Colonies from one experiment performed as described in (C), visualized by staining for involucrin and keratins. Left-hand dish, colonies formed by cells that adhered to type IV collagen in 5 min; center dish, cells adhering after 5 min; right-hand dish, unfractionated cells. Scale bar, 1 cm.

(E) Hematoxylin- and eosin-stained section of epidermis formed by grafting nude mouse with cell sheet generated by keratinocytes selected from foreskin by adhesion to 100 μg/ml type IV collagen for 5 min. Basal, granular, spinous, and cornified layers are present. Scale bar, 50 μm.

stem cell is its ability to form a clone that is large enough to be scored using a dissecting microscope 14 days after plating; many of the clones contain more than 10^4 cells, but the minimum size that can be detected in this way is about 32 cells (Jones and Watt, 1993). We used the same criterion to determine whether or not the integrin-bright population of cells isolated directly from the epidermis had high proliferative potential and might therefore be stem cells. Keratinocytes were isolated from foreskin epidermis and plated onto 100 μg/ml type IV collagen for different lengths of time. Maximal attachment of freshly isolated basal keratinocytes occurred within 1 hr (Figure 3A), compared with 3–4 hr for keratinocytes maintained in culture through several passages (Adams and Watt, 1991). Adhesion of freshly isolated cells was mediated by the $\alpha_2\beta_1$ integrin, since it could be inhibited with antibodies to the α_2 and β_1 subunits (data not shown; see Adams and Watt, 1991). Keratinocytes were distinguished from other epidermal cells, such as melanocytes, by staining with an anti-keratin antibody, and basal keratinocytes were distinguished from suprabasal, differentiating cells by staining with an antibody to the β_1 integrin subunit (see Adams and Watt, 1991; Hertle et al., 1991).

The keratinocytes that adhered to 100 μg/ml type IV collagen within 5 min were 28% (mean; range 13%–43%) of basal cells and had a higher modal $\alpha_2\beta_1$ fluorescence than the total (unselected) basal population (Figure 3B). We could therefore use rapid adhesion to collagen to isolate integrin-bright cells directly from the epidermis and to examine the relationship between integrin levels and proliferative ability.

As shown in Figures 3C and 3D, most of the cells that founded actively growing colonies adhered to type IV collagen within 5 min; the cells that took longer to adhere formed few colonies. The maximum colony forming efficiency (CFE) we obtained from cells that attached to 100 μg/ml type IV collagen within 5 min was 33% (mean 26% ± 4.7% SEM; four experiments). By reducing the collagen concentration to 10 μg/ml, we observed CFEs of up to 63% (mean 57% ± 3% SEM; three experiments). However, whereas 77% (± 10% SEM) of all colony-forming cells attached to 100 μg/ml type IV collagen within 5 min, only 40% (± 8% SEM) attached to 10 μg/ml, the remainder being found in the more slowly adherent fractions. Therefore, 100 μg/ml type IV collagen was chosen for further experiments because it gave greater separation of colony-forming cells from other basal cells than the lower collagen concentration. These experiments show that the relationships among elevated β_1 integrin expression, enhanced adhesiveness, and high proliferative potential that exist in cultured human keratinocytes (Jones and Watt, 1993) also hold for keratinocytes isolated directly from the skin.

Keratinocytes selected by rapid adhesion to type IV collagen not only had high proliferative potential in vitro but were able to form a fully differentiated epidermis when

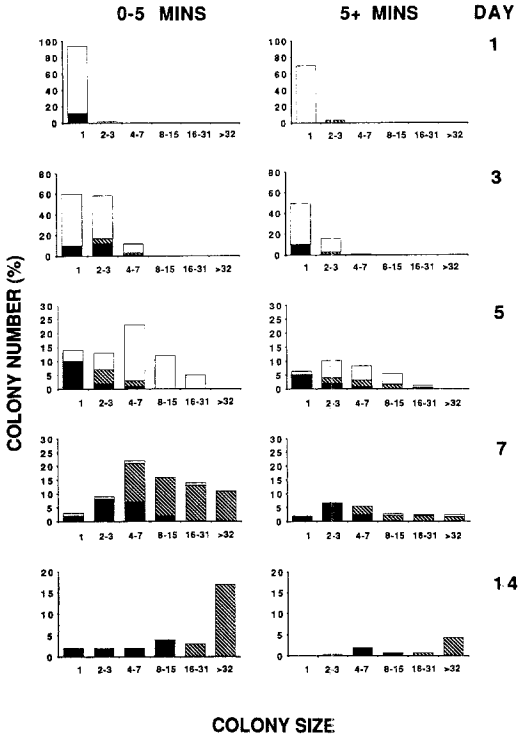

Figure 4. Fate of Individual Cells Isolated from Foreskin Epidermis

Keratinocytes that adhered to 100 μg/ml type IV collagen within 5 min (0–5 MINS) were compared with the remaining basal keratinocytes (5+ MINS). The graphs show the number of cells per colony, 1–14 days after plating. Open bars, all cells per colony were involucrin negative; closed bars, all cells were involucrin positive; hatched bars, mixture of involucrin-positive and involucrin-negative cells. The y axis shows colony number expressed as a percentage of basal cells seeded per dish.

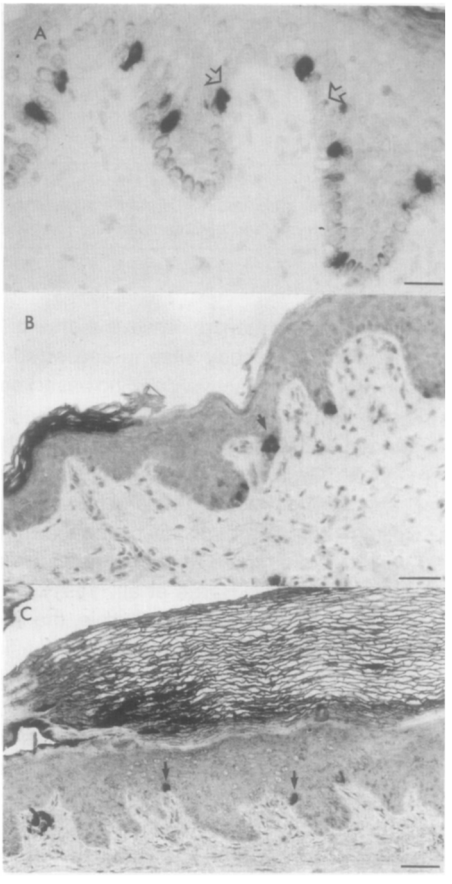

Figure 5. Distribution of S Phase Cells within the Epidermis

In situ hybridization using probes against mRNAs for histones $H2^b$, H3, and H4 on paraffin sections of epidermis.

(A) Neonatal foreskin. Open arrows define group of cells at the tip of a dermal papilla, corresponding to the size and location of an integrin-bright patch (see Table 1).

(B) Interfollicular scalp. Arrow indicates group of S phase cells.

(C) Palm. Arrows indicate individual S phase cells.

Scale bars, 25 μm in (A) and 50 μm in (B) and (C).

grafted onto nude mice (Figure 3E). Fourteen confluent cultures generated by cells selected directly from three foreskins were grafted onto mice and examined 1 week later. In 11 grafts, an epithelium containing all four cell layers characteristic of epidermis (basal, spinous, granular, and cornified) was formed. In the remaining three cases, no epithelium was recovered, and a dense inflammatory infiltrate surrounding amorphous eosinophilic material was seen.

The fate of individual keratinocytes isolated from foreskin epidermis was also examined, and the results of one experiment are shown in Figure 4. We have previously defined committed cells as cells that undergo terminal differentiation without dividing and transit-amplifying cells as those that form small (<32 cell) colonies of differentiating cells by 14 days in culture (Jones and Watt, 1993). Dishes of cells that had adhered to type IV collagen within 5 min or that took longer than 5 min to adhere were fixed at intervals between 3 hr and 14 days. All adherent keratinocytes were visualized by staining with a broad spectrum anti-keratin antibody, and cells that were undergoing terminal differentiation were detected with an anti-involucrin antibody. Almost all the single cells that originally attached had either divided or differentiated by day 5, and many of the differentiated cells had detached from the dishes. At day 5, the number of 8- to 31-cell colonies that were completely involucrin negative provided an accurate prediction of the number of large (≥32 cell) colonies at 14 days: the final CFEs were 16.5% for the rapidly adhering cells and 4.4% for the remaining basal cells. By day 14, the majority of colonies with fewer than 16 cells were entirely involucrin positive in each population. The data show that both rapidly and slowly adhering populations contained transit-amplifying and committed cells, but that the proportion was higher in the slowly adherent population.

Cell Cycle Kinetic Analysis

Although epidermal stem cells can proliferate extensively in culture or after an appropriate stimulus in vivo (Barrandon and Green, 1987; Potten and Morris, 1988; Yang et al., 1993; Rochat et al., 1994), there is evidence that under steady-state conditions stem cells divide infrequently in vivo (Potten and Morris, 1988; Cotsarelis et al., 1990).

Table 2. Distribution of S Phase Cells in the Basal Epidermal Layer, as Determined by In Situ Hybridization with Histone Probes

Tissue	Integrin Bright[a]	Integrin Dull[a]	Combined Ratio[b]	95% Confidence Interval	P[b]
Foreskin (n = 4)	7.1	10.9	1.27	1.10–1.48	0.001
Scalp (n = 3)	1.9	5.4	2.97	1.43–6.15	0.003
Palm (n = 4)	0.78	2.2	4.00	2.00–7.69	<0.0001

[a] Percentage of basal cells in S phase (typical specimen from each site).
[b] Ratio of S phase cells in integrin-dull:integrin-bright patches (combined values from all specimens). Combined ratios, confidence intervals, and P values calculated using method of Greenland and Robins (1985).

We therefore examined the proliferative status of basal keratinocytes in different body sites to see whether, as predicted, the proportion of actively cycling basal keratinocytes in integrin-bright regions was lower than the proportion in integrin-dull regions. Cells in S phase of the cell cycle were located by in situ hybridization with a cocktail of deoxyoligonucleotide probes to histone H2[b], H3, and H4 mRNAs. Cellular concentrations of histone mRNAs increase 20- to 100-fold during S phase and rapidly decline in G2 (Zhong et al., 1983; Harris et al., 1991). In palm, foreskin, and interfollicular scalp epidermis, the integrin-dull regions of the basal layer contained a significantly higher proportion of S phase cells than the integrin-bright regions. Thus, in palm, more S phase cells were found at the tips of the dermal papillae and sides of rete ridges, whereas in scalp and foreskin there were more S phase cells at the tips of the rete ridges (Figure 5; Table 2).

Reconstitution of Stem Cell Patterning In Vitro

The nonrandom distribution of stem cells within the basal layer of the epidermis could reflect a response of keratinocytes to differences in the local microenvironment present at rete ridges and dermal papillae or could be maintained in a keratinocyte-autonomous fashion. To distinguish between these possibilities, we investigated whether or not patches of integrin-bright and integrin-dull cells formed in confluent sheets of keratinocytes cultured on plastic in the absence of dermis. Cells isolated from foreskin were passaged once prior to plating, and stem cells were therefore selected by adhesion to 100 μg/ml type IV collagen for 20 min, as described previously (Jones and Watt, 1993); these rapidly adhering cells were compared with unselected cells. Intact sheets were detached from the plastic using dispase and either sectioned prior to staining with FITC-conjugated anti-integrin antibodies (Figure 6A) or stained as whole mounts (Figure 6B). Labeling with antibodies to the α_2 or α_3 integrin subunits revealed patches of integrin-bright cells whether the sheets were generated by an unselected cell population or by rapidly adherent cells (Figure 6; Table 3). The variation in fluorescence intensity was similar to that observed in vivo (see Table 1): the ratios of mean fluorescence intensity of cells in the bright patches to cells in the dull patches were 1.7 (± 0.04 SEM) and 1.9 (± 0.09 SEM) for the α_2 and α_3 integrin subunits, respectively. As shown in Table 3, the size of the integrin-bright patches was the same whether unselected or rapidly adhering cells were plated and was not affected by a 10-fold difference in plating density.

We investigated the relationship between patch size and plating density in more detail using rapidly adherent cells. Keratinocytes were seeded onto 100 μg/ml type IV collagen at densities ranging from 200 to 10^5 per 8 cm^2 dish, and nonadherent cells were removed after 20 min. The density of attached cells ranged from 6×10^3 to 3.2×10^3 per square centimeter. As shown in Table 4, there was no significant difference in patch size or percentage of bright cells at any density, and there was no significant difference in the size of α_2- and α_3-bright patches.

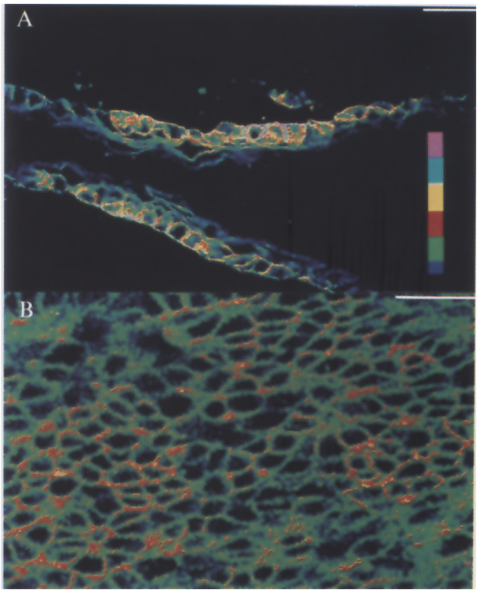

Figure 6. Integrin Levels in Cultured Epidermal Sheets

Keratinocytes grown on tissue culture plastic to form a confluent stratified sheet were removed from the culture dish by incubation with dispase and were either sectioned prior to staining (A) or stained as a whole mount (B).

(A) Two vertical sections stained for α_3 integrin subunit (sheet folded so that basal layer is uppermost in top section). Sheet was generated by cultured keratinocytes that adhered to 100 μg/ml type IV collagen within 20 min (2×10^4 cells plated per 35 mm dish).

(B) Stained for α_3 integrin subunit. We plated 10^5 cells onto 100 μg/ml type IV collagen and allowed the cells to attach for 24 hr. Specimens were examined with a confocal microscope; color indicates fluorescence intensity, increasing from dark blue to pink, as shown.

Scale bars, 50 μm (A) and 87.5 μm (B).

Discussion

We have shown that in vivo, as previously observed in vitro (Jones and Watt, 1993), high levels of β_1 integrins

Table 3. Integrin-Bright Patches Formed in Sheets of Cultured Keratinocytes: Comparison of Unselected and Rapidly Adhering Cells

Cells	Plating Density (per 8 cm² Dish)	Days to Confluence[b]	Patch Size[c,d]	Percentage of Bright Cells[c,d]
Unselected	2×10^5	4	11.0 ± 1.1	44.6 ± 1.3
Rapidly adhering[a]	2×10^5	4	10.6 ± 1.4	46.9 ± 1.5
Unselected	2×10^4	8	11.9 ± 1.2	46.5 ± 1.3
Rapidly adhering	2×10^4	8	11.4 ± 3.1	48.1 ± 1.3

[a] Selected by attachment to 100 μg/ml type IV collagen for 20 min (see Jones and Watt, 1993).
[b] Cultures were harvested 3 days postconfluence.
[c] Vertical sections through the cutures were labeled with antibodies to the α_2 integrin subunit.
[d] Values are mean ± SEM.

on the surface of human epidermal keratinocytes correlate with high proliferative potential. When a single cell suspension of keratinocytes was prepared directly from the skin, it was possible to isolate the cells that were capable of founding actively growing colonies by selecting for high surface expression of the $\alpha_2\beta_1$ integrin and rapid adhesion to type IV collagen. The cells with the highest levels of $\alpha_2\beta_1$ also expressed the highest levels of $\alpha_3\beta_1$, but expression of $\alpha_6\beta_4$ did not correlate with proliferative potential; this is also observed in cultured keratinocytes (Jones and Watt, 1993). Those keratinocytes isolated directly from the epidermis that had lower levels of $\alpha_2\beta_1$ and adhered more slowly to type IV collagen had characteristics of transit-amplifying cells and committed cells, undergoing terminal differentiation within a few rounds of division. The differences in integrin levels that were significant in vivo were the same as in vitro (2- to 3-fold). The maximum enrichment for colony-forming cells within the integrin-bright population was 63% (range 52%–63%), which is comparable with the CFE of keratinocytes dissected from a region of the hair follicle enriched for stem cells (18%–45%; Rochat et al., 1994). The integrin-bright population not only had high colony-forming ability but was also able to reconstitute an epidermis when grafted onto nude mice. We were not able to obtain a pure population of transit-amplifying cells free from contaminating colony-forming cells and were thus unable to test their behavior as nude mouse grafts.

About 40% of cells in the basal layer of the epidermis were integrin bright, yet kinetic analysis would predict that the percentage of stem cells is closer to 10% (Potten and Morris, 1988); the CFE of unselected basal cells in our experiments was 9.5% (± 2.3% SEM). Although the integrin-bright population of basal cells contained almost all the stem cells, it also contained some transit-amplifying cells (see Figure 4). It is hardly surprising that quantitative differences in the expression of a single family of cell surface markers are not sufficient to define the epidermal stem cell compartment uniquely; hemopoietic stem cells are isolated to progressively higher purity by combinations of monoclonal antibodies to a variety of surface antigens (see, for example, Uchida et al., 1993). Nevertheless, integrins do provide a surface marker of epidermal stem cells, and confocal microscopy of epidermal sections with fluorochrome-conjugated antibodies to other antigens will provide a rapid and sensitive screen for additional markers.

Integrin-bright cells within the epidermis were arranged in groups, 9–14 cells in diameter, indicating a clustering of stem cells within the basal epidermal layer. In mouse dorsal skin, there are morphological and kinetic data to support the epidermal proliferative unit model of Potten (1974) in which a single stem cell lies at the base of a column of suprabasal cells and is surrounded by transit-amplifying and committed cells. In the epidermal proliferative unit model, suprabasal terminally differentiating keratinocytes are the progeny of the stem cells directly beneath them. In contrast, the clustering of stem cells we have observed suggests that in human epidermis there is some degree of lateral cell movement, either in the basal or suprabasal epidermal layers (see also Lavker and Sun, 1983). Lineage analysis, in which the fate of individual stem cell progeny can be followed, will be required to resolve the spatial relationships between proliferating keratinocytes and differentiating cells in the layers above.

The distribution of integrin-bright and integrin-dull cells in sections of epidermis from different body sites was not random. Integrin-bright cells lay above the dermal papillae

Table 4. Integrin-Bright Patches Formed in Sheets of Cultured Keratinocytes: Comparison of Rapidly Adhering Cells Plated at Different Densities

Plating Density[a] (per 8 cm² Dish)	Days to Confluence[b]	α_2 Patch Size[c]	α_2 Percentage of Bright Cells[c]	α_3 Patch Size[c]	α_3 Percentage of Bright Cells[c]
10^5	7	10.1 ± 2.4	45 ± 1.4	11.5 ± 1.8	53 ± 1.5
10^3	16	10.8 ± 1.3	52 ± 1.4	9.6 ± 1.6	49 ± 1.6
200	19	9.6 ± 1.5	51 ± 1.3	12.0 ± 2.0	49 ± 1.1

[a] After attachment to 100 μg/ml type IV collagen for 20 min, nonadherent cells were removed (see Jones and Watt, 1993).
[b] Cultures were harvested 2–3 days postconfluence.
[c] Values shown are mean ± SEM. There was no statistically significant difference between patch size and percentage of bright cells at any plating density and for either integrin subunit (Mann–Whitney U test).

in foreskin and interfollicular scalp (and in breast and abdominal skin; data not shown) but were found at the tips of the deep rete ridges in palm. Cell kinetic data suggest that although epidermal stem cells have high proliferative potential, they may divide only rarely under normal steady-state conditions (Potten and Morris, 1988; Cotsarelis et al., 1990), and in the three body sites we examined, there were fewer S phase cells in the regions of the basal layer where integrin-bright cells were found. Indeed, the location of stem cells in the deep rete ridges of the palm was previously suggested by the fact that few of these cells incorporate [^{3}H]thymidine in pulse-labeling experiments (Lavker and Sun, 1983). It is interesting that in palm the morphology of basal keratinocytes differs between deep and shallow rete ridges (Lavker and Sun, 1983), as does the subcellular distribution of $\alpha_3\beta_1$, which is relatively less abundant on the apical surface of cells in the deep rete ridges and on the basal surface of cells in the shallow ridges (Symington et al., 1993).

In human hair follicles, keratinocytes with high proliferative potential are reported to lie in the outer root sheath, either at the point of insertion of the arrector pili muscle (Yang et al., 1993) or lower down (Rochat et al., 1994). Both regions can be labeled with an antibody to keratin 19 (Lane et al., 1991). Keratin 19–positive outer root sheath cells expressed higher levels of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ than keratin 19–negative cells. The fact that the difference in fluorescence (1.4- to 1.6-fold) was lower than observed in interfollicular epidermis (approximately 2-fold) would be consistent with only a subset of keratin 19–positive cells being stem cells; staining longitudual sections of hair follicles with anti-integrin antibodies may therefore help to determine the location of the stem cells more precisely.

How and when are the patches of integrin-bright and integrin-dull cells established? Integrin expression in developing human epidermis has so far only been examined by conventional double-labeled immunofluorescence microscopy; nevertheless, a patchy distribution of $\alpha_2\beta_1$ is observed prior to the onset of stratification (that is, prior to 9.5 weeks estimated gestational age) (Hertle et al., 1991). Epidermal–dermal interactions could be important in establishing the position of integrin-bright and integrin-dull patches, since these interactions are crucial for establishing the spatial distribution of cutaneous appendages (Sengel, 1990). Nevertheless, the $\alpha_2\beta_1$-bright patches appear well before hair follicles and sweat glands develop, and the epidermis shows no evidence of rete ridge formation until the third trimester of gestation (Holbrook, 1991).

The mechanism by which the distribution of integrin-bright and integrin-dull patches is maintained in adult epidermis is also of considerable interest. Positional information in embryos can either be imparted by graded external signals or a population of cells can have autonomous pattern-forming ability (for recent discussion see O'Farrell, 1994). Research on stem cells in adult mammalian tissues has tended to emphasize the importance of the cellular microenvironment or "niche": the idea that specific combinations of growth factors, extracellular matrix molecules, and neighboring cell types provide the conditions necessary for maintenance of the stem cell phenotype and that the progeny of stem cells will differentiate unless there is also a suitable niche to accommodate them (Hall and Watt, 1989). The niche hypothesis is analogous to the graded signals model of positional information and is attractive because of the evidence that proliferation and terminal differentiation of keratinocytes can be affected by external stimuli (see, for example, Barrandon and Green, 1987; Watt et al., 1993). Nevertheless, the reconstitution of integrin-bright and integrin-dull patches that we have observed in vitro in the absence of any obvious environmental cues would argue that stem cell patterning in the epidermis may be, at least to some extent, an intrinsic property of keratinocytes.

The size of the integrin-bright patches formed in post-confluent sheets of cultured keratinocytes was independent of the number of cells plated and did not depend on whether the starting population consisted of stem cells or consisted of a mixture of stem cells and other keratinocytes. At the lowest density analyzed, only six stem cells attached per square centimeter and so the progeny of a single stem cell must give rise to multiple bright and dull patches. Our data suggest that patch size and distribution are subject to some form of autoregulation that may be analogous to the phenomenon of embryonic lateral inhibition (see, for example, Campuzano and Modolell, 1992).

A final consideration is whether integrins are simply markers of stem cells or whether they play a direct role in determining the location and proliferative status of keratinocytes. It seems reasonable that the cells destined to remain in the basal epidermal layer throughout adult life should be the most adhesive to the underlying basement membrane. Integrins are capable of transducing signals between the cell surface and the nucleus (Hynes, 1992), and the proportion of β_1 integrins occupied by ligand does regulate the onset of terminal differentiation (Watt et al., 1993); whether increased integrin levels affect keratinocyte proliferation directly is, however, unknown. There is a growing awareness of the potential importance of cell adhesion molecules in embryonic pattern formation: one of the gene products that controls epidermal patterning in Drosophila is armadillo, the homolog of β-catenin in vertebrates, which regulates cell–cell adhesion through binding to the cytoplasmic domains of receptors of the cadherin family (Kemler, 1993). In view of the finding that cadherins can regulate integrin expression in keratinocytes (Hodivala and Watt, 1994), it is tempting to speculate that adhesion molecules may indeed play a role in establishing pattern and fate within adult human epidermis.

Experimental Procedures

Source of Tissue and Isolation and Culture of Keratinocytes

Human palm and scalp were obtained from adult cadavers, usually within 24 hr postmortem, and neonatal foreskins were obtained from routine circumcisions. To isolate keratinocytes, pieces of skin (approximately 4–9 mm^2) were incubated with thermolysin (Sigma; 0.5 mg/ml in 10 mM HEPES with 142 mM sodium chloride, 6.7 mM potassium chloride, 0.43 mM sodium hydroxide, 1.0 mM calcium chloride [pH 7.4]) overnight at 4°C, after which the epidermis was peeled away from the dermis using sterile forceps (Germain et al., 1993). The epider-

mis was then disaggregated into a single cell suspension by incubation with 0.05% trypsin and 0.016% EDTA for 20–30 min at 37°C in a Wheaton CelStir (Jencons).

Keratinocytes were cultured using the method of Rheinwald (1989) on a feeder layer of 3T3 mouse embryo cells, as described previously (Jones and Watt, 1993).

Antibodies

The following monoclonal antibodies were described previously (Jones and Watt, 1993). The anti-integrin antibodies were HAS6 (to the α_2 subunit), VM-2 (to α_3), GoH3 (to α_6), and anti-CD29 (to β_1). We also used LP34 (which recognizes keratins of basal and differentiating keratinocytes) and SY3 and SY5 (antibodies to involucrin).

Antibodies not described previously were LP2K (Lane et al., 1991; gift of I. M. Leigh) (to keratin 19); HAS3 (Tenchini et al., 1993) and 5E8 (Chen et al., 1991; gift of R. Bankert) (to the α_2 integrin subunit); and DH12 (de Strooper et al., 1988; gift of J.-J. Cassiman) and mAb13 (Akiyama et al., 1989; gift of K. Yamada) (to the β_1 integrin subunit).

Direct FITC conjugates of HAS6, HAS3, VM-2, and GoH3 were prepared using established procedures (Harlow and Lane, 1988). Direct FITC conjugates of anti-CD29 and anti-CD8 (negative control) were purchased from Janssen Biochimica and Sigma, respectively. Sheep anti-mouse IgG–FITC (Sigma), goat anti-mouse horseradish peroxidase (Sigma), and rabbit anti-mouse IgG–rhodamine (DAKO) were also used.

Immunofluorescent Staining of Sections, Whole Mounts, and Isolated Cells

Tissue was embedded in OCT compound (BDH Diagnostics) and frozen in an isopentane bath in liquid nitrogen; 6 μm thick frozen sections were prepared. Prior to staining, sections of palm, scalp, and neonatal foreskin were incubated with 10% fetal calf serum (FCS) in phosphate-buffered saline (PBS) containing 1 mM calcium chloride and 1 mM magnesium chloride (PBSABC) for 30 min. Sections were then incubated for 30 min with FITC-conjugated anti-integrin antibodies diluted in 10% FCS in PBSABC, washed extensively in PBSABC, fixed in 1% paraformaldehyde, washed again in PBSABC, and mounted in Citifluor (Amersham International; prepared according to the directions of the manufacturer). For double labeling of keratin 19 and integrins in hair follicles, sections were incubated first with LP2K, washed, incubated with anti-mouse IgG–rhodamine, washed, and then incubated with an FITC-conjugated anti-integrin antibody. Tissue sections were examined using a Bio-Rad MRC 600 confocal microscope or a Zeiss Axiophot microscope.

Confluent sheets of cultured keratinocytes were detached from culture dishes using dispase (Boehringer Mannheim), as described previously (Watt, 1984), and either sectioned and stained as described for tissue or stained as an intact sheet in the following way. Sheets were incubated with PBSABC containing 10% FCS for 30 min and then with HAS6–FITC or VM-2–FITC diluted in PBSABC containing 10% FCS for 1 hr at room temperature, washed extensively in PBSABC, fixed for 30 min in 1% paraformaldehyde in PBSABC, and mounted in Gelvatol (Monsanto Company).

LP34 staining was used to determine the proportion of cells isolated directly from epidermis that were keratinocytes. Cell suspensions were air dried onto coverslips, fixed with 3.7% formaldehyde, permeabilized with methanol, and stained with LP34 and anti-mouse IgG–FITC. Of cells isolated from the epidermis, 87% (± 1% SEM) were keratinocytes as determined by LP34 staining.

To determine the proportion of keratinocytes that originated from the basal layer of the epidermis, cell suspensions were fixed with 3.7% formaldehyde and incubated with anti-CD29, followed by anti-mouse IgG–FITC. Of keratinocytes, 29% ± 3% (mean ± SEM) were basal cells.

Cells were prepared and analyzed for flow cytometry as described previously (Jones and Watt, 1993). When not analyzed immediately, cells were fixed in 1% paraformaldehyde in PBS.

Quantitation of Fluorescence on Sections and Whole Mounts

Tissue sections were examined using the 25× objective of the confocal microscope with an optical section thickness of 1 μm. To ensure against artifacts due to the plane of sectioning, only rete ridges and dermal papillae in which all the cell borders of all basal cells could be seen were included. The size of integrin-bright and integrin-dull patches was calculated from at least 30 rete ridges (deep rete ridges in palm) and 30 dermal papillae for each body site and for each subunit. Fluorescence was quantitated from the confocal image by measuring pixel intensity along a line drawn through the lateral cell–cell borders (α_2 and α_3 integrin subunits) or through the basal surface of basal cells (α_6 integrin subunit). The fluorescence of at least 100 cells in dull and bright patches was determined.

The fluorescence of cultured keratinocyte sheets labeled with antibodies to the α_2 or α_3 integrin subunits was also determined. In vertical sections, the ratio of fluorescence between integrin-bright and integrin-dull patches was calculated as described above for tissue sections; the fluorescence of over 50 cells in dull and bright patches was determined. To determine patch size, we viewed sections on the confocal microscope. The histogram function was used to color bright and dull cells according to intensity: the range of 0–254 arbritary fluorescence units was divided into six bands (of increasing intensity from 1–6) of equal size apart from the lowest one, which was set just to color the dullest cell membranes. Cells that contained regions with bands 3/4 for α_2 (the brightest staining seen) or bands 5/6 for α_3 were scored as bright. Dull cells were in bands 1/2 for α_2 and bands 1–3 for α_3 and contained no areas of more intense staining. More than 50 patches in each specimen were analyzed. For whole mounts, a series of 1 μm optical sections through the entire thickness of the sheet was obtained using the 25× objective of the confocal microscope, and a composite image (Z series) was constructed from these sections.

Adhesion of Keratinocytes to Type IV Collagen

For experiments in which CFE was determined, tissue culture plastic dishes (Falcon, Becton Dickinson) were coated with 10 or 100 μg/ml human placental type IV collagen (Sigma) (Jones and Watt, 1993).

Adhesion-blocking experiments were carried out essentially as described by Adams and Watt (1991). We coated 96-well bacteriological plastic plates (Flow Laboratories) with 100 μg/ml type IV collagen. Then, 10^4 cells freshly isolated from neonatal foreskin epidermis were plated onto control and collagen-coated wells, either alone or with one of the following antibodies: mAb13 (200 μg/ml), DH12 (200 μg/ml), 5E8 (1:100 dilution of ascites). After 1 hr at 37°C, adherent cells were visualized with methylene blue. The adhesion-blocking antibodies mAb13 and 5E8 completely inhibited adhesion, whereas the non-blocking antibody DH12 did not (see Adams and Watt, 1991).

Determination of CFE

Cells isolated from epidermis were recovered from trypsin–EDTA by centrifugation in the presence of soybean trypsin inhibitor (1 mg/ml) (Sigma), washed in serum-free medium, counted in a hemocytometer, and plated onto dishes coated with type IV collagen. At intervals, dishes were washed with serum-free medium, and 3T3 feeder cells were added. In some experiments, nonadherent keratinocytes recovered during washing were replated onto fresh collagen-coated dishes and feeders were added 3 hr later. To determine the number of cells that attached, we prepared duplicate dishes and counted adherent cells after staining with the anti-keratin antibody LP34 visualized with DAB, as described above.

Dishes were fixed and stained for involucrin and keratins at 3 hr and at 1, 3, 5, 7, and 14 days after plating (Jones and Watt, 1993). The size and number of colonies were counted using a Leitz inverted microscope and scored as to whether the cells in each colony were involucrin negative or involucrin positive. CFEs were expressed as the percentage of basal cells plated; the proportion of basal cells in the population was determined as the proportion of keratin-positive, β_1 integrin–positive cells in the population.

Grafting onto Nude Mice

Keratinocytes (2×10^4 to 2×10^5) isolated directly from neonatal foreskin were plated onto 35 mm dishes that had been coated with 100 μg/ml type IV collagen. Nonadherent cells were removed after 5 min. Feeders were added to the attached cells, and the cultures were grown until they were 3 days postconfluent. Keratinocytes were removed from the dishes as intact sheets by incubation with 2.5 mg/ml dispase (Boehringer Mannheim) for 45 min (Watt, 1984) and then

placed on Silastic sheets and implanted subcutaneously in nude mice (strain Nu/Nu, female), as described by Barrandon et al. (1988) (technique 1). The mice were sacrificed 7–8 days after grafting; the grafts were removed, fixed in formol–saline overnight, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin and examined and photographed using a Zeiss Axiophot microscope.

Measurement of the Percentage of S Phase Cells in Tissue Sections

In situ hybridization for mRNA of histones $H2^b$, H3, and H4 was performed using a cocktail of nine 30 base deoxyoligonucleotides with three oligonucleotides complementary to each histone mRNA, using a modified version of the method developed by Pringle et al. (1990). Deoxyoligonucleotide design was based on previously published sequence data (Zhong et al., 1983). Specimens of skin were fixed in neutral pH 3.7% formol–saline for 48 hr and embedded in paraffin wax. Probe cocktails (obtained from Pathway Services Limited) were 3′ end labeled with digoxigenin-11-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Negative controls were these: no probe addition, RNase A1 (Sigma) pretreatment, no anti-digoxigenin antibody, substitution of histone probes with a digoxigenin-labeled random 30 base oligonucleotide cocktail (unlabeled oligonucleotides were a gift from Pathway Services) or with a digoxigenin-labeled nonhomologous probe cocktail to immunoglobulin κ light chain mRNA (Pringle et al., 1990).

Sections were photographed, and the total number of basal cells and the number of positively stained basal cells were counted in the areas corresponding to the α_2-bright integrin-staining patches in each site. Those patches were the most superficial 14 cells in foreskin or the most superficial nine cells in scalp at the tip of each dermal papilla and the nine deepest cells at the base of each rete ridge in palm.

The number of positive cells in the intervening regions corresponding to areas with dull integrin staining was also counted. All vertically sectioned areas were counted.

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