Polarized Expression of Integrin Receptors (α6β4, α2β1, α3β1, and α4β5) and their Relationship with the Cytoskeleton and Basement Membrane Matrix in Cultured Human Keratinocytes

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Abstract. In human keratinocytes cultured in conditions which allow differentiation and stratification and are suitable to reconstitute a fully functional epidermis, α6β4 and two members of the β1 integrin family (α2β1 and α3β1) were respectively polarized to the basal and lateral domains of the plasmamembrane both in growing colonies and in the reconstituted epidermis. Conversely, the αv integrin subunit, presumably in association with β5, was expressed at the basal surface in growing and migrating but not in stationary keratinocytes. The integrin αvβ4: (a) was organized in typical patches which often showed a “leopard skin” pattern where spots corresponded to microfilament-free areas; (b) was not associated with focal contacts containing vinculin and talin but rather corresponded to relatively removed contact areas of the basal membrane as shown by interference reflection microscopy; and (c) was coherent to patches of laminin secreted and deposited underneath the ventral membrane of individual cells. The two β1 integrins (α2β1 and α3β1), both endowed with laminin receptor properties, were not associated with focal adhesions under experimental conditions allowing full epidermal maturation but matched the lateral position of vinculin (but not talin), cingulin, and desmoplakin, all makers of intercellular junctions. Often thin strips of laminin were observed in between the lateral aspects of individual basal keratinocytes. The integrin complex αvβ5 had a topography similar to that of talin- and vinculin-containing focal adhesions mostly in the peripheral cells of expanding keratinocyte colonies and in coincidence with fibronectin strands. The discrete topography of β1 and β4 integrins has a functional role in the maintenance of the state of aggregation of cultured keratinocytes since lateral aggregation was impaired by antibodies to β1, whereas antibodies to β4 prevented cell–matrix adhesion (De Luca, M., R. N. Tamura, S. Kajiji, S. Bondanza, P. Rossino, R. Cancedda, P. C. Marchisio, and V. Quaranta. Proc. Natl. Acad. Sci. USA. 87:6888–6892). Moreover, the surface polarization of integrins followed attachment and depended both on the presence of Ca2+ in the medium and on the integrity of the cytoskeleton. We conclude that our in vitro functional tests and structural data suggest a correlation between the pattern of integrin expression on defined plasmamembrane domains and the mechanism of epidermal assembly.

Cell adhesion is a fundamental process in the organization of multicellular organisms (reviewed by Ekblom et al., 1986; Fleming and Johnson, 1988). The regulation of cell adhesive properties is a complex process that plays a major role in morphogenetic events and in the maintenance of tissue integrity (Edelman, 1986). Recently, major advances in our understanding of the molecular mechanisms of cell adhesion have occurred, with the identification of several intercellular and cell–substratum membrane adhesion molecules (reviewed by Albelda and Buck, 1990; Buck and Horwitz, 1987; Edelman, 1986; Ekblom et al., 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987) and extracellular matrix molecules which may assemble in the basement membrane (Martin and Timpl, 1987; Yurchenko and Schittny, 1990).

The integrins are an important class of transmembrane surface receptors involved in cell–matrix and cell–cell adhesion (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). They are heterodimers composed of noncovalently associated α and β subunits. So far, β subunits and at least 11 α subunits have been recognized (Ruoslahti and Giancotti, 1989), primarily on the basis of amino acid sequence homologies (Sheppard et al., 1990; Ramaswamy and Hemler, 1990). The α subunits tend to associate exclu-
visively with one of the $\beta$ chains, although exceptions to this rule exist (Cheresh et al., 1989; Holzmann and Weissman, 1989; Dedhar and Gray, 1990). Integrins are then divided into subfamilies, according to their $\beta$ chains (Hyne, 1987). The various integrin heterodimers share many features. For instance, all $\alpha$ chains are homologous to each other, and so are the $\beta$ chains. Many integrins bind to a common ligand motif, centered around the tripeptide Arg-Gly-Asp (Ruoslahti and Pierschbacher, 1986, 1987). Distinguishing features are the pattern of tissue-specific expression and the spectrum of ligand specificities.

One of the most recently discovered integrins, the complex $\alpha_6\beta_4$ (Sonnenberg et al., 1988; Kajiji et al., 1989; Kennel et al., 1988), has been sequenced (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990) and found to be highly expressed by epithelial cells. Moreover, two members of the $\beta$ integrin family (i.e., $\alpha_6\beta_1$ and $\alpha_6\beta_4$), have been located to cell–cell contact domains in epidermal cells (Kanter et al., 1989; Carter et al., 1990; De Luca et al., 1990b; Larjava et al., 1990; Staquet et al., 1990) while $\alpha_6\beta_4$ has been located to their basal domain in a typical pattern which suggested a polarity-dependent organization of integrins and a functional role of $\alpha_6\beta_4$ in the recognition of and in the attachment to the basement membrane both in the native and in vitro–reconstituted epidermis (De Luca et al., 1990b). A further epithelial integrin heterodimer, $\alpha_6\beta_4$, has been identified (Cheresh et al., 1989), and the $\beta_4$ chain sequenced (Ramaswamy and Hemler, 1990).

In this paper we studied the distribution and, when appropriate reagents were available, the functional role of integrins as a function of the cytoskeleton organization of epithelial cells. To study the role of integrins in the organization of epithelia, we used, as a model system, normal human epidermal keratinocytes cultured in vitro in conditions that allowed full epidermal differentiation (Rheinwald and Green, 1975). This system has been extensively characterized (Green, 1980; Barrandon and Green, 1987a,b; De Luca et al., 1988) and has many advantageous features for investigating tissue organizing determinants, such as: (a) the cultured keratinocyte is a normal cell, i.e., nontransformed; (b) it forms epithelial colonies and sheets closely resembling normal human epidermis (Green et al., 1979) and maintains virtually the same differentiation features and gene expression patterns of its in vivo counterpart (Green, 1980) such as to be used as routine grafting for large skin and mucosal defects (Gallico et al., 1984; De Luca et al., 1989, 1990a; Romagnoli et al., 1990).

In this paper we report that $\alpha_6\beta_4$: (a) has a novel type of organization in the basal plasmamembrane domain of epithelial cells; (b) a relationship with the cytoskeleton different from that displayed by any other integrins; and (c) a correspondence with organized laminn patches. The organization of $\alpha_6\beta_4$ is different from that shown by $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins and suggests that it represents the prototype of a new integrin family provided with a peculiar relationship both with the extracellular matrix and the cytoskeleton. We also propose that three different laminin receptors located to discrete surface domains may interact with their physiological ligand to support both adhesion to the basement membrane and collaborate with other molecules in the maintenance of intercellular bonds.

**Materials and Methods**

**Cell Culture**

Human epidermal keratinocytes were cultured according to the methods described by Rheinwald and Green (1975). Briefly, 2 cm² skin biopsies from healthy volunteers were minced and trypsinized (0.05% / 0.01% EDTA) by gently stirring at 37°C for 3 h. A single cell suspension was collected every 30 min. Cells were then plated (2 x 10⁶/75 cm² flask) on feeder layers of lethally irradiated 3T3-J2 mouse fibroblasts (a gift from H. Green, Harvard Medical School, Boston, MA) and cultured in keratinocyte growth medium (KGM) at 37°C in a water-saturated atmosphere of 5% CO₂. KGM composition was: Dulbecco-Vogel Eagle's and Ham's F12 media (3:1 mixture) containing 10% fetal calf serum, glutamine (4 mM), insulin (5 μg/ml), transferrin (5 μg/ml), adenosine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (20 pM), epidermal growth factor (10 ng/ml); a gift from C. Nascimento, Chiron Corp., Emeryville, CA; penicillin-streptomycin (50 IU/ml). Confluent primary cultures were trypsinized and passaged at a density of 4 x 10⁶ to 1.3 x 10⁶ cells/cm². Under these culture conditions, keratinocytes can be serially propagated in vitro for several passages (Green et al., 1979).

In some experiments, adherent keratinocyte colonies were squirted with several jets of buffer from the narrowed tip of a Pasteur pipette before fixation. The aim was to detach some of the cells, expose the underlying matrix, and loosen the bonds among cells.

For control purposes, a line of human keratinocytes was obtained from Clonetics Co., San Diego, CA and cultured according to manufacturer's instructions in growth medium with low Ca²⁺.

**Antibodies**

The murine monoclonal antibodies (mAb) S3-41 and AA3 and the polyclonal antiserum 5710 to $\alpha_6\beta_4$ have been described (Kajiji et al., 1989) and generously provided by V. Quaranta, Research Institute of Scripps Clinic, La Jolla, CA. Other murine mAb, with the investigators that kindly provided them, are as follows: TS2/7, to $\alpha_4$ (Hemler et al., 1983); B-SG10, to $\alpha_4$ (Hemler et al., 1987) from M. Hemler, Dana Farber Cancer Institute, Boston, MA; PID6 and PIFS, to $\alpha_5$ (Wayner et al., 1988) from W. Carter, Hutchinson Cancer Research Center, Seattle, WA; LM142, to $\alpha_6$, from D. Cheresh, Research Institute of Scripps Clinic; A-1AS, to $\beta_1$ (Hemler et al., 1983, 1987a), from M. Hemler; 12Fl, to $\alpha_5$ (Pischel et al., 1987) from V. Woods, University of California, San Diego, CA; IJ43, to $\alpha_3$ (Pradet et al., 1984), from L. Old, Sloan-Kettering Institute, New York; VIPI-2, to $\beta_3$, from W. Knapp, University of Vienna, Austria; CLB-54, to $\beta_3$, from R. van Liej, Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands. The rat mAb GOH3 to $\alpha_6$ (Sonnenberg et al., 1987) was a gift from A. Sonnenberg, Central Laboratory of the Netherlands Red Cross. Of two rabbit antisera to $\beta_1$, one was donated by R. Pytel, Department of Medicine, University of California, San Francisco, CA (Pytel et al., 1985), the other was raised in P. C. Marchisio’s laboratory (Dejana et al., 1986). Goat antiserum to $\beta_1$ has been described (Conforti et al., 1989). An mAb to vinculin was purchased from Bio Makers, Rehovot, Israel (VIN 11-5, cat. No. 6501) and an mAb cross-reacting with human talin (clone 8D4) was obtained from K. Burridge, University of North Carolina at Chapel Hill, Chapel Hill, NC. Rabbit antisera to laminin and to collagen type IV were respectively from Gibco Laboratories, Grand Island, NY (cat. No. 680-3019) and from Heyl GmbH, Berlin, Federal Republic of Germany. In some experiments the laminin antibodies were preabsorbed with an excess of laminin purified from EHS mouse tumor (a kind gift of G. Tarabotti, Istituto Mario Negri Bergamo, Bergamo, Italy). A rabbit antisera to cingulin (Citi et al., 1988, 1989) was obtained from S. Citi, Columbia University, New York, and an mAb to desmin (clone 1 and 2 (clone DP 2.15, code 695421) was purchased from ICN Immunobiochemicals, Lisle, IL. Finally, an mAb to cellular fibronectin (IST 9) was kindly provided by L. Zardi, IST, Genova, Italy (Borsi et al., 1987; Carmelis et al., 1987) and affinity-purified rabbit IgGs to human vitronectin were given by K. Preissner, Max Planck Institute for Thrombosis Research, Giessen, FRG (Preissner et al., 1985).

**Immunostaining**

Keratinocytes from confluent primary cultures (1.3 x 10⁶ cells/cm²) were plated onto 24-well Costar plates containing 1.1-cm² round glass coverslips.
Adhesion and Cohesion Assays

Cells were permeabilized by soaking coverslips for 3-5 min at 0°C in Hepes-Triton-X-100 buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100). This procedure of fixation and permeabilization permits immunostaining of cytoskeletal and adhesion components (for example, see Marchisio et al., 1984; Dejana et al., 1988). Indirect single-label immunofluorescence experiments were performed as reported (Marchisio et al., 1984). Briefly, the primary antibody (usually at an Ig concentration of 10-30 μg/ml) was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS-0.2% BSA, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (DAKOPATTS, Copenhagen, Denmark) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (F-PHD; Sigma Chemical Co., St. Louis, MO). Indirect double-label immunofluorescence experiments were performed essentially as reported (Dejana et al., 1988). Coverslips were mounted either in Mowiol 4-88 (Hoechst AG, Frankfurt/Main, FRG) or in 50% glycerol-PBS. Routine observations were carried out in a Zeiss Axiophot photomicroscope equipped for epifluorescence, plan-apochromatic lenses or Antiflex 63× lens for interference reflection microscopy (IRM). Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in T-Max Developer for 10 min at 20°C. In some experiments a laser confocal fluorescence imaging system (Lasersharp MRC-500) was used to evaluate the position of integrins in the thickness of keratinocyte colonies.

Cell Radiolabeling, Lysis, and Immunoprecipitation

These experiments were carried out as described (Kajiji et al., 1989). Briefly, cells were metabolically radiolabeled and detergent extracts immunoprecipitated with LM42 mAb to α, or with a rabbit polyclonal anti-vitronectin receptor antibody (both antibodies were a gift of D. Cheresh). Immunoprecipitates were washed and then eluted in sample buffer (Laemmli, 1970) at 100°C with or without reduction/alkylation by 10 mM dithiothreitol and 50 mM iodoacetamide and electrophoresed on SDS-PAGE (Laemmli, 1970), followed by fluorography (Laskey and Mills, 1975) or autoradiography. No specific bands are detectable in the anti-fl3 lanes. In the anti-txv lanes, the open arrowheads point at bands consistent with the mobility of a f15 subunit, while the closed arrowheads indicate the positions of txv bands. The higher txv band in the reducing lane is often observed and likely represents a higher molecular mass precursor of the mature αtxv chain.

Adhesion and Cohesion Assays

Costar plates (96 wells) were coated for 1 hr at 37°C with laminin (10 μg/ml in PBS) or Matrigel (either 2 μg/ml in DMEM, a gift of A. Albini, IST, Genova, Italy). Keratinocytes obtained from confluent cultures (30,000 cells/well) were plated in KGM without serum and EGF in the presence of indicated antisera or control sera from corresponding species (1:100 dilutions) and then incubated for 12 hr at 37°C. Controls of cell viability during cohesion assays were routinely carried out. Cohesion assays were repeated a minimum of six times with consistent results. The cells which had adhered after exposure to the β antigen (almost no cells adhered after treatment with the β antigen; De Luca et al., 1990b) were fixed and stained with R-PHD as described above to evaluate their morphology.

Results

Integrin Topography in Cultured Human Keratinocytes

We previously showed both by serological and biochemical means that normal human keratinocytes express the integrins α3β1, α5β1, and αvβ3 (De Luca et al., 1990b). Here we show that normal human keratinocytes express α, in association with β on a β-like molecule by SDS-PAGE mobility as demonstrated by immunoprecipitation with anti-α antibodies (Fig. 1). That the β chain associated with α, is not β, is indicated by the lack of reactivity of normal human keratinocytes with anti-β antibodies (Fig. 1) and on the basis of mobility properties, the β chain does not correspond to any of the alternative β chains reported to associate with α (Holzmann and Weissman, 1989; Dedhar and Gray, 1990). Moreover, no α chain with the mobility properties of α could be immunoprecipitated using β antibodies thus excluding the occurrence of the newly reported complex αβ, (Bodary and McLean, 1990; Vogel et al., 1990).

Here the fine topography of integrins was investigated in its relationship with cytoskeletal components in the same cells by using indirect immunofluorescence. First of all, no above control immunofluorescence signal was detected with 1. Abbreviations used in this paper: CD, cytochalasin D; F-PHD, fluorescein-labeled phalloidin; IRM, interference reflection microscopy; R-PHD, rhodamine-labeled phalloidin.
Figure 2. Distribution of $\beta_4$ (b), $\alpha_6$ (d), $\beta_1$ (f), and $\alpha_3$ (h) integrins in human keratinocyte colonies grown for 3 d on glass coverslips in complete medium without feeder layer. The corresponding staining for F-actin (A) is shown in a, c, e, and g. The cells were immunofluorescently stained with mouse mAbs and costained with fluorescein-labeled phalloidin (F-PHD). The patterns of $\beta_1$ and $\alpha_6$ staining of the ventral membrane (b and d) are very similar insofar the complex is enriched in F-actin microfilament-poor areas (see also Fig. 3). Staining is also present in footprints left by cells which had been mechanically detached during the staining procedures (a and b, see arrowhead; see also Fig. 8). This suggests that the $\alpha_6\beta_4$ complex is exposed in the ventral membrane in contact with the substrate. Staining for $\beta_3$ (f) and $\alpha_3$ (h, $\alpha_3$ is identical to $\alpha_5$) indicates that there is just a hazy staining for the $\beta_3$ complex in the ventral membrane (f, see asterisk) while fluorescence is enriched at intercellular contact rims. No F-actin staining was apparent in substrate-attached material. Bar, 5 mm.

$\alpha_1$, $\alpha_6$, or $\beta_1$ antibodies (not shown). Distinct localization patterns were observed with anti-$\beta_4$ compared to anti-$\beta_1$ antibodies. Both mAb S3-41 or AA3 and the rabbit serum 5710 to $\alpha_6\beta_4$ (Kajiji et al., 1989) stained keratinocytes on the basal surface with a pattern of polymorphic patches, somewhat granular in appearance (Fig. 2b) and often suggesting a leopard skin pattern (Figs. 3 and 4). Cell footprints, remaining on the substratum from cells detached during fixation (Fig. 2b, arrowhead; see also Fig. 8), similarly displayed granular fluorescence with $\beta_4$ antibodies. An identical pattern of reactivity (Fig. 2d) was found with an mAb reactive with $\alpha_6$; codistribution of $\alpha_6$ and $\beta_4$ in basal patches was confirmed also in double-label immunofluorescence (Fig. 3, a and b). Almost no $\alpha_6\beta_4$ immunoreactivity was detected in the contour of the cells, in the apical zones, or in association with talin at focal adhesions (Fig. 3, c and d). Such pattern of integrin distribution was virtually identical in confluent keratinocyte colonies.

A peculiar feature of $\alpha_6\beta_4$ distribution was that it was virtually absent from areas containing submembranous bundles of F-actin microfilaments suggesting a complementary distribution of the integrin complex and the submembran-
Figure 3. Double-label indirect immunofluorescence staining of human keratinocyte colonies grown for 3 d on glass coverslips in complete medium without feeder layer. Virtual codistribution of $\alpha_6$ (a) and $\beta_4$ (b) indicates that immunostaining with the corresponding antibodies (rabbit IgG to the cytoplasmic domain of $\alpha_6$ and mAb S3-41 to $\beta_4$) shows the basal location of the $\alpha_6\beta_4$ heterodimer in rather typical patches. No obviously similar pattern was observed by coimmunostaining for talin (c, rabbit antiserum to human platelet talin; T) and mAb S3-41 to $\beta_4$ (d); arrows indicate the position of some peripheral talin-positive focal adhesions (c) located in $\beta_4$-free areas (d) of the ventral attachment area. The exclusion of the $\alpha_6\beta_4$ complex from areas showing bundles of microfilaments (staining with F-PHD for F-actin; A is shown in e and f; almost negative imaging appears from the comparison of the two frames (e.g., at arrowheads). Bar, 5 $\mu$m.

The anti-$\beta_1$, as well as the anti-$\alpha_2$ and anti-$\alpha_3$ mAbs predominantly stained those areas of plasmamembrane involved in cell-to-cell contact in growing colonies (Fig. 2, e–h) as well as in the reconstituted epidermis. Little $\alpha_2\beta_1$ staining was seen in the basal surface areas found reactive with $\beta_4$ antibodies. Similarly, cell footprints that contained $\alpha_2\beta_4$ did not contain $\beta_1$ (not shown). In our hands, no specific staining was observed with $\alpha_1$ mAbs in keratinocytes but, in the same cultures, occasional human fibroblast-like cells displayed elongated streaks along stress fibers, indicating that the antibody could indeed recognize $\alpha_6\beta_4$ at focal contacts (not shown).

Further evidence for the segregation of $\beta_1$ and $\beta_4$ inte-
Figure 4. High power picture of the fluorescence staining for F-actin (a), α6β4 (b), and the corresponding IRM pattern (c) of a large keratinocyte located at the periphery of a growing colony. Irregular patches of α6β4 show a preferential location in F-actin poor areas and, conversely, α6β4 is excluded from areas where microfilaments are abundant (e.g., in the areas indicated by the asterisks and by the arrowhead). The F-actin-rich and α6β4-free areas of the ventral membrane are darker in IRM indicating a closer proximity with the substrate (see arrowhead) than those corresponding to α6β4 patches. IRM black focal contact streaks are found only at the edges of the cells and never correspond to α6β4 patches (see also Fig. 3). In general, the IRM pattern of cultured keratinocytes is completely different from that of a fibroblast-like cell (for review see Burridge et al., 1988). Bar, 5 μm.

Figure 5. Confocal sectioning images obtained with a Lasersharp MRC-500 fluorescence imaging system of human keratinocyte colonies grown for 6 d on feeder layer and stained for β1 (a and b) and β4 (c). Optical sectioning was adjusted 1.5 μm (b and c) and 3 μm (a) above the plane of adhesion. The upper two pictures, obtained from the same colony, show two digitally reconstructed optical sections showing that β1 integrins are exposed on the lateral surface at two levels 1.5 μm apart while β4 is virtually absent 1.5 μm above the adhesion plane (c). The arrow in c indicates a cell at the periphery of the colony that shows positivity to α6β4 with a slab being somewhat raised from the substrate. Bar, 10 μm.

Anti-β1 immunoreactivity could be followed a few micrometers upward from the plane of attachment within colonies, indicating that β1 integrins are enriched on lateral surfaces (Fig. 5, a and b). Anti-β4 reactivity, instead, was absent in equivalent planes of focus removed from the attachment surface, i.e., starting from 1.5 μm above the substratum (Fig. 5 c).

The different topography of β1 and β4 integrins was consistently observed in growing and stationary keratinocytes.
whether they were initially seeded on fibroblast feeder layers
or on artificial substrates like laminin, fibronectin, or vitronectin, suggesting that the sorting of β1 and β5 integrins to
discrete domains of epidermal cells was an intrinsic property
of adherent keratinocytes (see also below).

The localization of the integrin chain αv was then studied
using the mAb LM142. We assumed that the localization of
αv corresponded to that of the heterodimer αvβ3 in view of
the reported absence of β1 or other β chains from epithelial
cells (Cheresh et al., 1989; De Luca et al., 1990b). It was
found that αv was located to tiny IRM dark streaks mostly
at the periphery of the basal aspect of expanding keratinocytes
and in association with the endings of short microfila-
ment bundles (Fig. 6, a and b). A similar location was shown
by the cytoskeletal proteins vinculin (Fig. 6, c and d) and ta-
lin (see Fig. 3 c). On the basis of the similar location of αv,
vinculin and talin as the endings of microfilament bundles,
we suggest that αv, most probably in association with β3, is
the epithelial integrin chain that interacts with both vinculin
and talin (and, hence, with the microfilamentous cytoskele-
on) and forms minute focal contacts in basal keratinocytes.
Moreover, the discrete localization of vinculin and talin to
the peripheral focal contacts, that are also αv positive, high-
lights the absence of such cytoskeletal molecules from areas
containing αvβ3 (see Fig. 3, c and d). Data awaits to be
confirmed by the colocalization of αv and β3 when appro-
priate immune reagents for β3 are available.

We then looked at the cells that had migrated outward and
formed the upper layers of the colonies. We found that no
integrins were associated with obvious cellular structures
(e.g., β1, Fig. 6 e) but gained evidence that junctions of
different types had formed and that proteins like vinculin (a
marker of adhesion junctions, Geiger et al., 1985), cingulin
(a marker of tight junctions, Citi et al., 1988, 1989), and
desmoplakins 1 and 2 (markers for desmosomes, Müller and
Franke, 1982) lined the boundaries among the tile-shaped
cells of upper layers (Fig. 5, e-h). No talin immunoreactivity
was observed in keratinocytes belonging to the upper epider-
mal layers (not shown) in line with the reported absence of
talin from adhesion junctions of epithelial cells (reviewed by
Burridge et al., 1986, 1988).

In summary, these data show that αvβ3, and αvβ5 integrins
are located to different adhesion structures of the basal mem-
brane and two β3 integrins are enriched in the lateral mem-
brane of keratinocytes, in a rather mutually exclusive distri-
bution and that the interaction of these different integrins
with the keratinocyte cytoskeleton is different.

Distribution of Matrix Proteins
in Cultured Keratinocytes
Deposition and organization of fibronectin, collagen type IV,
vitronectin, and laminin were studied with specific antibod-
ies in human keratinocytes cultured with or without feeder
layer of 3T3-J2 cells. It was found that fibronectin was orga-
nized around the peripheral keratinocytes of exponentially
growing colonies and by feeder cells (Fig. 7) and formed a
circumferential meshwork around each colony. Fibronectin
was missing from underneath both growing and confluent
keratinocytes. Double-label immunofluorescence
staining was performed on cells that had been lightly
squirited by a stream of buffer before fixation to optimize
antibody access. Under these conditions αvβ4 and laminin ap-

Figure 6. Distribution of αv (b) and vinculin (d) in human keratinocyte colonies grown for 3 d on glass coverslips in complete medium
without feeder layer. The corresponding staining for F-actin is shown in a and c (A). The localization of talin is not shown and is identical
to that of vinculin (see also Fig. 3 c). The keratinocytes that had migrated outward (e-h) are devoid of integrins (e.g., β1) associated to
recognizable cellular structures (e) and show vinculin (f), cingulin (g), and desmoplakins in tiny dots (h) at cell-to-cell boundaries. No
talin was found in cells of the upper layers. Bars, 5 μm.
Effects of Substrates, \(Ca^{2+}\) Deprivation and of Cytoskeleton-disrupting Drugs on Integrin Distribution in Cultured Keratinocytes

We seeded keratinocytes on substrates composed of purified matrix proteins like laminin, fibronectin, or vitronectin (components either of the basement membrane or of the provisional matrix of regenerating epidermis) or on Matrigel which represents an artificial basement membrane and is composed of laminin, collagen type IV, nidogen, and heparan sulfate proteoglycan (Kleinman et al., 1983). The aim was to detect possible fine differences in integrin distribution as a function of different substrates of attachment. No obvious difference was shown in \(\beta_{1}, \beta_{4}\), and \(\alpha_{2}\beta_{1}\) patterns at times ranging from 12 to 72 h, indicating that the typical distribution of these integrins did not depend on the initial recognition of a well-defined substrate but rather on the interaction with the matrix that keratinocytes themselves autonomously produced and organized.

Keratinocyte stratification into epidermis is known to depend on the presence of \(Ca^{2+}\) in the culture medium (Watt and Green, 1982; Watt, 1984; Magee et al., 1987). In the absence of added \(Ca^{2+}\) or with low \(Ca^{2+}\), keratinocytes failed to form colonies and to differentiate in cells that soon moved to outward layers. We found that the sorting out of \(\beta_{1}\) and \(\alpha_{2}\) integrins to lateral and basal domains, respectively, was impaired by \(Ca^{2+}\) deficiency but rather integrins remained diffusely distributed on the cell surface (Fig. 9, b and d). A very similar pattern was shown by a secondary line of keratinocytes (Clonetics Co., San Diego, CA) that grow with low \(Ca^{2+}\) and are often used as an in vitro model for keratinocyte differentiation (Fig. 9, e-h). It must be noted that, without \(Ca^{2+}\), a fair amount of \(\beta_{4}\) but not \(\beta_{1}\), cross-reactive material is shed and found attached to the substratum (e.g., Fig. 9, b and f).

We then tested the effects of the microfilament-disrupting drug cytochalasin D (CD; Carter, 1967) and of colcemid, a microtubule-depolymerizing drug, on the integrin pattern of keratinocytes both during the spreading process and on established colonies. Attachment was fully inhibited by colcemid (1 \(\mu g/ml\)), supporting the concept that functional microtubules are required for cell adhesion (Osborn and Weber, 1976) and epithelial polarization (Eilers et al., 1989).

In contrast, CD treatment (2 \(\mu g/ml\)) allowed attachment and spreading of keratinocytes but prevented colony formation in a way similar to that produced by exposure to \(\beta_{1}\) antibodies (see below). The pattern of \(\beta_{4}\) distribution at the basal aspect of cells was not coarsely altered (Fig. 9 j) but rather followed the typical rearrangement of microfilament organization induced by CD treatment (Weber et al., 1976). Instead, CD prevented the sorting of \(\beta_{4}\) integrins to the lateral membrane. The \(\beta_{4}\) integrins were in part diffuse on the whole membrane without being enriched at lateral aspects or retained within cells (Fig. 9 I). No major changes in \(\beta_{1}\) and \(\beta_{4}\) distribution were produced by either drugs in established keratinocyte cultures.

The above experiments suggest that the sorting of integrins to appropriate membrane domains require the presence of \(Ca^{2+}\) and a functional cytoskeleton, conditions that are both prerequisites for epidermal maturation.

Effects of Antibodies to \(\beta_{1}\) on Cohesion of Keratinocytes In Vitro

The experiments were performed with human keratinocytes...
isolated and resuspended from cultures that had reached confluency for at least 24 h. We reported that the anti-β₁ serum had negligible effect on adhesion of cells while the anti-β₄ serum inhibited adhesion by >90% (De Luca et al., 1990). By observing the morphology of keratinocytes seeded in the presence of anti-β₁ serum, we found that colony organization was severely impaired and individual cells were less spread and almost devoid of cell-to-cell contacts (Fig. 10 a) while they formed regular colonies with goat preimmune serum (Fig. 10 b). This experiment suggests that β₁ integrins are required for keratinocyte aggregation, a condition required for in vitro epidermal maturation.

Discussion

The maturation of epidermis is a complex process which has been reproduced in vitro to obtain epidermal sheets that are used for repair of skin defects including severe burns (Green et al., 1979; Gallico et al., 1984; De Luca et al., 1989). Normal epidermal differentiation and the healing of skin wounds require cell proliferation and lateral migration (Barrandon and Green, 1987b) followed by outward positioning of differentiated keratinocytes that end up with the formation of a multilayered squamous epithelium. In this paper we present evidence that this complex phenomenon requires the polarized distribution of at least two integrin subfamilies that are respectively involved in the formation of bonds between cells of the basal layer with the basement membrane and in stabilizing cell-to-cell lateral recognition. To our knowledge this represents evidence for a topographically defined positioning of integrin adhesion receptors in a single cell type.

The newly described integrin α₄β₁ plays a leading role in this process because (a) specific antibodies prevent the adhesion of keratinocytes (De Luca et al., 1990b) and (b) because α₄β₁ is specifically restricted to the basal domain of keratinocytes both in vivo and in vitro and thus comes in tight contact with the basement membrane. The integrin α₄β₁ is an integrin molecule that is amazingly abundant in the basal domain of most epithelial cells such as it may be considered as the natural candidate receptor for the epithelial basement membrane. Although the actual ligand of the extracellular domain of α₄β₁ has not yet been biochemically identified (Sonnenberg et al., 1990), it is likely that it may be laminin itself or an association of laminin with another matrix component and, even if coherent distribution may not be considered as direct evidence, our data support this possibility. However, there is no codistribution of α₄β₁ with focal contacts. Focal contacts, indeed, are hardly noted in confluent keratinocytes and mostly restricted to the peripheral cells of growing colonies. By using the IRM technique we found that the basal membrane of keratinocytes adhered via scattered spots of “close contacts” (Izzard and Lochner, 1976) alternating with spots slightly more removed from the adhesion substrate on the basis of their lighter IRM signal. The latter

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**Figure 8.** Double-label immunofluorescence costaining of laminin (a and c) and β₄ (b and d) in keratinocyte colonies that had been lightly squirted by a jet of buffer from a Pasteur pipette before fixation. Laminin (LM, detected by rabbit IgG to Englebreth-Holm-Swarm mouse tumor laminin) and β₄ (detected by mAb S3-41) are codistributed underneath keratinocyte colonies (a and b). Codistributed patches of β₄ and laminin are also seen where cells have been detached and have left their footprints (c and d). In a few residual cells that had been squirted, intercellular boundaries are intensely positive for laminin (e, arrowheads). The inset (f) shows a detail of the appearance of laminin at intercellular rims. Bars, 5 μm.
correspond to areas where α4β4 and laminin are facing each other, while closer spots correspond to α4β4-free and F-actin-rich areas. A very similar pattern of adhesion where laminin is not coherent with focal adhesions has been described in transforming growth factor-β-treated thyroid cells in vitro (Garbi et al., 1990). Moreover, α5β1, a basal laminin receptor of endothelial cells (Languino et al., 1989) has never been found in association with focal contacts (Lampugnani et al., 1990). Therefore, adhesion to laminin does not occur via supramolecular structures of the focal adhesion type but rather via integrin receptors that entertain a looser relationship with the substrate and never correspond directly to the microfilamentous meshwork of the cell.

Even more elusive is the potential cytoplasmic ligand of the β4 chain which, unlike other integrin β chains, has a very large cytoplasmic domain ~1,000 amino acid residues long (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). For the reasons detailed above we feel confident in suggesting that the ligand of such long polypeptide strand exposed to the cytoplasmic environment is not any microfilament-associated molecule since β4 is almost excluded from F-actin-rich domains of the ventral membrane. The identification of the cytoplasmic interaction of β4 is a very challenging task.

Interesting and new is also the assigned role of β1 integrins in the mechanics of epidermal assembly that has been previously reported by ours and other research groups (Carter et al., 1990; De Luca et al., 1990b; Larjava et al., 1990). These integrins are members of the largest subfamily and wide is the spectrum of ligand specificities displayed by β1 integrins. In human keratinocytes we do not have any evidence that β1 integrins, including the major fibronectin receptor α5β1, are involved in basal matrix recognition but rather they are involved in cell-to-cell recognition. This goes along with recent findings that (a) α5β1 is involved in intercellular recognition (Takada et al., 1989; Campanero et al., 1990).
keratinocytes obtained from subconfluent primary cultures were passaged in secondary cultures and grown as described in Materials and Methods. They were trypsinized and immediately plated on 24-well plates in the presence of β1 antibodies. The cohesion assay was carried for 12 h. The cells that have attached were passaged in secondary cultures and grown as described in Materials and Methods. They were trypsinized and immediately plated on 24-well plates in the presence of β1 antibodies. The cohesion assay was carried for 12 h. The cells that have attached to preimmune serum. Cells were fixed, permeabilized, and stained with R-PHD for F-actin. Bar, 15 μm.

Figure 10. Inhibition of keratinocyte cohesion by goat antibodies to β1 keratinocytes obtained from subconfluent primary cultures were passaged in secondary cultures and grown as described in Materials and Methods. They were trypsinized and immediately plated on 24-well plates in the presence of β1 antibodies. The cohesion assay was carried for 12 h. The cells that have attached to preimmune serum. Cells were fixed, permeabilized, and stained with R-PHD for F-actin. Bar, 15 μm.

1990), (b) αβ1 and αβ1 are located to intracellular spaces in different cell types (Kaufmann et al., 1989; Zutter, M. M., and S. A. Santoro, 1989; J. Cell Biol. 109:106a [Abstr.]), and that (c) αβ1 and αβ1 are located to cell-cell contacts in human endothelial cells (Lampugnani et al., 1990). The integrins αβ1 and αβ1 may play roles in keeping cells of the basal layers together. Both are laminin receptors, although they bind laminin with different affinities (Geihl et al., 1988; Languino et al., 1989; Wayner et al., 1989; Kirchofer et al., 1990), and some laminin is indeed found in between the lateral domains of cultured keratinocytes such as we propose that an interaction with laminin may also be involved in αβ1 and αβ1 integrin-mediated lateral adhesion. However, we cannot rule out the possibility that they may interact with each other by hitherto undescribed homophilic bonds or with other cell adhesion molecules such as A-CAM (Geiger et al., 1985) and uvomorulin (Ekblom et al., 1986) or, finally, recognize unknown ligands.

A major point is whether integrins correspond to specific intercellular junctions in cultured keratinocytes. At the level of resolution of light microscope immunocytochemistry we cannot obtain any more detailed information but, in view of the punctate versus continuous pattern of desmoplakins and β1 integrins we can rule out a discrete integrin location to intercellular desmosomes. Even more difficult is to assign αβ1 to a specific junction. The only junctions that are found between the basement membrane and the ventral plasmamembrane of basal epidermal cells are hemidesmosomes (Farquhar and Palade, 1963). Indeed, at hemidesmosomes, the plasma membrane is somewhat removed from the attachment surface, a feature shared with αβ1 spots. It is then tantalizing to speculate that αβ1 is involved in hemidesmosome formation also in view of the fact that keratin filaments and not microfilaments converge on hemidesmosomes.

These and previous data also suggest that, while β1 integrins do not have any detectable role in cell-to-substratum adhesion in this particular cell system, αβ1 plays a major role in supporting the adhesion of keratinocytes firmly attached to their basement membrane and part of a highly differentiated cultured epidermis (De Luca et al., 1990b). As suggested by their peculiar location, β1 integrins may play an important role during histogenesis by allowing lateral recognition of keratinocytes and in building up epidermal sheets.

A further epithelial-specific integrin, αβ1, has been recently described in human carcinoma cells (Cheresh et al., 1989). We have indirectly located αβ1 using an mAb to α, since no immune reagent was available to β1 and β1 is absent in normal human keratinocytes. The location of αβ1 is restricted to peripheral cells in exponentially growing colonies in coincidence with a fibrillar network of fibronectin. Since no vitronectin is apparently produced by keratinocytes, it is likely that αβ1 binds to fibronectin at small adhesion plaques that are located at the endings of short stress fibers and contain vinculin and talin. The role of αβ1 would then be related to colony expansion and keratinocyte locomotion that have previously been ascribed to fibronectin recognition and/or deposition during epidermal growth and regeneration processes (Fakatshima and Grinnell, 1985; Adams and Watt, 1989). Antibodies inhibiting αβ1 function, when available, will clarify this point.

The keratinocyte integrin phenotype coincides with that of some malignantly transformed epithelial cell lines (Kajiji et al., 1989; Cheresh et al., 1989), suggesting that keratinocytes may also be representative of cells from other epithelial sources, e.g., from nonstratified or secretory epithelia. A distinguishing feature of this phenotype (De Luca et al., 1990) is the apparent absence from recognizable cell structures of the αβ1 fibronectin receptor and the αβ1 vitronectin receptor well characterized also for its multiple binding properties in many other adherent cell types such as fibroblasts, endothelial cells, osteoclasts, and tumor cells (Cheresh, 1987; Cheresh and Spiro, 1987; Dejana et al., 1988b, 1989, 1990; Fathi et al., 1989; Pytela et al., 1985; Singer et al., 1988; Zambonin-Zallone et al., 1989). It will be interesting to see how widespread among epithelial cells the keratinocyte phenotype is.

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