Expression of $\beta_1$, $\beta_3$, $\beta_4$, and $\beta_5$ Integrins by Human Epidermal Keratinocytes and Non-Differentiating Keratinocytes

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Abstract. We have compared the adhesive properties and integrin expression profiles of cultured human epidermal keratinocytes and a strain of nondifferentiating keratinocytes (ndk). Both cell types adhered to fibronectin, laminin, and collagen types I and IV, but ndk adhered more rapidly and at lower coating concentrations of the proteins. Antibody blocking experiments showed that adhesion of both cell types to fibronectin was mediated by the $\alpha_5\beta_1$ integrin and to laminin by $\alpha_3\beta_1$, in synergy with $\alpha_2\beta_1$. Keratinocytes adhered to collagen with $\alpha_2\beta_1$, but an antibody to $\alpha_2$ did not inhibit adhesion of ndk to collagen. Both cell types adhered to vitronectin by $\alpha_6$-containing integrins. Immunoprecipitation of surface-iodinated and metabolically labeled cells showed that in addition to $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$, both keratinocytes and ndk expressed $\alpha_6\beta_4$ and $\alpha_4\beta_5$. ndk expressed all these integrins at higher levels than normal keratinocytes. ndk, but not normal keratinocytes, expressed $\alpha_6\beta_1$ and $\alpha_6\beta_3$; they also expressed $\alpha_6\beta_4$, an integrin that was not consistently detected on normal keratinocytes. Immunofluorescence experiments showed that in stratified cultures of normal keratinocytes integrin expression was confined to cells in the basal layer; terminally differentiating cells were unstained. In contrast, all cells in the ndk population were integrin positive. Our observations showed that the adhesive properties of ndk differ from normal keratinocytes and reflect differences in the type of integrins expressed, the level of expression and the distribution of integrins on the cell surface. ndk thus have a number of characteristics that distinguish them from normal basal keratinocytes.

The integrins constitute a large family of cell surface molecules involved in cell–cell and cell–matrix interactions. Integrins are heterodimers, consisting of non-covalently associated $\alpha$ and $\beta$ subunits, both of which are transmembrane glycoproteins (Hynes, 1987). The integrins are presently classified on the basis that one $\beta$ subunit can associate with several different $\alpha$ subunits: the $\beta_1$ and $\beta_3$, subgroups are widely expressed and are principally involved in cell–matrix interactions, whereas $\beta_2$ integrins are expressed on leukocytes and are involved in cell–cell interactions (reviewed by Hemler, 1990). However, it is now apparent that one $\alpha$ subunit can associate with different $\beta$ subunits, examples being the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrins (Sonnenberg et al., 1988; Kajiji et al., 1989) and the $\alpha_6\beta_1$, $\alpha_6\beta_3$, or $\alpha_6\beta_4$ integrins (Bodary and McLean, 1990; Vogel et al., 1990; Cheresh et al., 1989; Ramaswamy et al., 1990). Heterodimer composition is important in determining ligand specificity (Cheresh et al., 1989; Bodary and McLean, 1990; Sonnenberg et al., 1990; Vogel et al., 1990), but there is also evidence that the same integrin can have different functions when expressed on different cell types (Elices and Hemler, 1989; Languino et al., 1989).

One tissue in which integrins are believed to play an important role in determining the spatial organization of the cells is the epidermis. Immunofluorescence studies have shown that integrins are expressed by the basal layer of proliferating keratinocytes attached to the basement membrane, but are largely absent from the suprabasal layers of terminally differentiating cells (Frade et al., 1984; Sonnenberg et al., 1986; Wayner et al., 1988; De Strooper et al., 1989; Peltonen et al., 1989). Keratinocytes can be grown in culture as stratified sheets that have the same basic organization as the epidermis (Rheinwald and Green, 1975; Watt, 1988). We have shown that when cells are induced to undergo terminal differentiation in culture they lose adhesiveness for extracellular matrix proteins and no longer express $\beta$ integrins. However, the reduction in adhesiveness precedes loss of integrins by several hours. In the case of $\alpha_2\beta_1$, there is direct evidence for a reduction of fibronectin-binding ability prior to loss from the cell surface. These changes may help to ensure that cells which are committed to terminal differentiation are selectively expelled from the basal layer (Adams and Watt, 1990).

We recently isolated a strain of nondifferentiating keratinocytes (ndk) (Adams and Watt, 1988). These cells

1. Abbreviations used in this paper: ECM, extracellular matrix; ndk, nondifferentiating keratinocytes.

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were identified as keratinocytes on the basis that they express the keratin profile characteristic of normal keratinocytes in culture. ndk are not immortalized or tumorigenic and yet lack the capacity for terminal differentiation. The cells do not stratify and do not require feeder cell support. Before confluence they move as individual cells and have prominent ruffled membranes; their motile phenotype appears to be a result, at least in part, of autocrine production of scatter factor (Adams et al., 1991). Normal keratinocytes are induced to undergo terminal differentiation when placed in suspension (Green, 1977), a process that can be inhibited by fibronectin or antibodies to \( \beta_1 \) integrins (Adams and Watt, 1989). When ndk are placed in suspension they undergo growth arrest but do not terminally differentiate (Adams and Watt, 1988).

The properties of ndk suggest that they have altered cell-cell and cell-matrix adhesive properties. The aim of the present report was to compare the adhesive properties and profile of integrin expression of ndk with those of normal keratinocytes to obtain further insights into the ndk phenotype.

### Materials and Methods

#### Materials

Human plasma fibronectin was obtained from Calbiochem (Cambridge, UK) or Blood Products (Elstree, Herts, UK). Human placental type IV collagen and mouse EHS laminin were obtained from Sigma Chemical Co. (Poole, UK). Bovine type I collagen (Vitrogen 100) was obtained from Collaborative Research Inc. (Lexington, MA). Human vitronectin was purchased from Telios Pharmaceuticals Inc. (San Diego, CA). GRGDS and GRGESP peptides were obtained from Novabiochem (Nottingham, UK).

#### Indirect Immunofluorescent Staining

Indirect immunofluorescent staining was used to compare the adhesive properties and profile of integrin expression of ndk with those of normal keratinocytes. The cells were grown on plastic in DMEM containing 10% FCS and were fixed in 3.7% formaldehyde for 10 min at room temperature. For involucrin staining, fixed cells were permeabilized in methanol. 6-μm sections were cut at right angles to the surface of the filter paper.

### Table I. Antibodies to Integrins Used in This Study

| Specificity | Name     | Species | Reference |
|-------------|----------|---------|-----------|
| \( \alpha_1 \) | TS2/7    | mouse   | Hemler et al., 1984 |
| \( \alpha_2 \) | J2F1     | mouse   | Pischel et al., 1987 |
| \( \alpha_3 \) | J143     | mouse   | Carter et al., 1990 |
| \( \alpha_4 \) | B-SG10*  | mouse   | Hemler et al., 1987 |
| \( \alpha_5 \) | BIIG2*   | rat     | Hall et al., 1990 |
| \( \alpha_6 \) | GoH3*    | rat     | Akiyama et al., 1989 |
| \( \alpha_7 \) | 13C2*    | mouse   | Horton et al., 1985 |
| \( \alpha_9 \) | 23C6*    | mouse   | Davies et al., 1989 |
| \( \beta_1 \) | 13*      | rat     | Akiyama et al., 1989 |
| \( \beta_3 \) | DH12     | mouse   | De Strooper et al., 1988 |
| \( \beta_4 \) | 363*     | rabbit  | Marcattonio & Hynes, 1988 |
| \( \beta_5 \) | Y2/51    | mouse   | von dem Borne et al., 1989 |
| placental vitronectin receptor | \( \alpha VNR* \) | rabbit | Suzuki et al., 1986 |
| \( \beta_4 \) | 439-9B   | rat     | Kennel et al., 1989 |

* Antibodies known to perturb cell adhesion. † Polyclonal antibodies; 363 and Rx were raised against a 39-mer peptide corresponding to the human \( \beta_1 \) subunit carboxy-terminus. See text for anti-\( \beta_3 \) antisera and anti-fibronectin receptor antisera.

### Preparation of Sections through Keratinocyte Cultures

Preparation of stratified cultures of keratinocytes was achieved by detachment of the culture dish as an intact sheet by incubation with 2.5 mg/ml dispase (Grade II; Boehringer Mannheim GmbH, Mannheim, Germany) for 30–60 min in serum-free DMEM at 37°C (Green et al., 1979). The sheet was rinsed in PBS, draped over Whatman No. 1 filter paper and frozen in isopentane in a bath of dry ice and methanol. 6-μm sections were cut at right angles to the surface of the filter paper.

### Indirect Immunofluorescent Staining

Keratinocytes and J2 feeder cells, or ndk, were plated onto glass coverslips and grown for 2–3 d. The cells were rinsed in PBS and fixed in 3.7% formaldehyde for 10 min at room temperature. For involucrin staining, fixed cells were permeabilized in methanol for 5 min on ice. The cells were incubated with primary antibody for 45 min, washed extensively in PBS, incubated with appropriate FITC-conjugated second antibody (ICN Biomedicals, High Wycombe, Bucks, UK) for 45 min, washed again in PBS, mounted in Gelvatol (Monsanto Co., St. Louis, MO), and examined under epifluorescence using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).
Cell Adhesion Assays

ECM glycoproteins, diluted in PBS, were left to adsorb to bacteriological plastic microtitre wells (Flow 76-208-05) overnight at 4°C. The plates were rinsed three times in PBS and uncoated plastic blocked by incubation for 1 h with PBS containing 0.5 mg/ml heat denaturated BSA. The plates were rinsed again, and 10^6 keratinocytes or ndk cells, prepared by trypsin/EDTA treatment, were added to each well in 100 μl of serum-free FAD medium. Cells were allowed to adhere at 37°C for varying periods of time and then the nonadherent cells were removed by gently washing the plates in PBS containing 1 mM CaCl_2 and 1 mM MgCl_2. Adherent cells were fixed, stained with methylene blue, and photographed. Adhesion was quantified by counting cells from the photographs. Adhesion-blocking antibodies, when used, were added to the wells before adding the cells.

Radioactive Labeling of Integrins

Cell surface iodination was carried out on trypsinized keratinocytes or ndk, using the lactoperoxidase–glucose oxidase method (Hynes, 1973). Typically, 1 mCi of 125I-Iodoine was used to label 10^7 cells. After labeling, the cells were washed three times in PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 and lysed for 15 min on ice in 1% NP-40 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl_2, 2 mM PMSF, and 0.01% leupeptin. For metabolic labeling of integrins, keratinocytes or ndk were incubated overnight (18–20 h) in FAD + HICE containing 1% NP-40 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl_2, 2 mM PMSF, and 0.01% leupeptin. For metabolic labeling of integrins, keratinocytes or ndk were incubated overnight (18–20 h) in FAD + HICE containing 10% dialyzed FCS, and 50 μCi/ml each of 35S-methionine and 35S-cysteine. The cells were then rinsed in PBS containing 1 mM CaCl_2 and 1 mM MgCl_2, scraped from the dish, and lysed as indicated above.

Immunoprecipitation and Gel Electrophoresis

The lysates prepared from keratinocytes and ndk were clarified by centrifugation at 14,000 rpm for 5 min. Aliquots were equalized on the basis of TCA-precipitable radioactivity; typically 2 × 10^6 to 4 × 10^6 cpm of iodinated material or 1 × 10^5 to 2 × 10^5 cpm of 35S-labeled material were used per precipitation. In the metabolic labeling experiments, this corresponded to the lysates from ~5 × 10^5 ndk or 9 × 10^5 keratinocytes per immunoprecipitation. Lysates were incubated with primary antibodies, under conditions of antibody excess, for 2 1/2 h on ice. If required, rabbit anti-rat IgG serum or rabbit anti-mouse IgG serum (ICN Biomedicals) was added for the last hour. 50 μl of a 1:1 (vol/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was then added and the mixture tumbled for 45 min at 4°C. Beads and immune complexes were washed as described (Adams and Watt, 1988), resuspended in SDS-PAGE sample buffer, boiled for 5 min, and resolved on 7.5% polyacrylamide gels according to the method of Laemmli (Laemmli, 1970). Generally, no reducing agent was added, but in some experiments 5% (vol/vol) of 2-mercaptoethanol was added to the sample buffer.

Gels were stained with Coomassie blue and destained; those containing iodinated samples were dried immediately and exposed to Kodak XAR5 x-ray film at room temperature, using an intensifying screen. Those containing metabolically labeled samples were treated with 'Amplify' (Amersham, UK) before drying and then exposed to XAR5 x-ray film at −70°C.

Preparation of mRNA and Northern Analysis

Poly A+ RNA was prepared from cultured keratinocytes, using the "Quick-prep" mRNA Purification Kit (Pharmacia Fine Chemicals), according to the manufacturer's instructions. Dami cell mRNA was a gift from Dr. Sheryl Greenberg (Department of Haematology, Brigham and Women's Hospital) (Greenberg et al., 1981). 1-μg samples were electrophoresed on a 1% agarose gel containing formaldehyde, using standard procedures (Sambrook et al., 1989). After electrophoresis, the gel was incubated in 0.05 M NaOH, 0.15 M NaCl for 30 min, and then in 0.1 M Tris-HCl, pH 7.5 for a further 30 min.

RNA was transferred from the gel to a nylon membrane (Zetaprobe, Bio-Rad Laboratories) over 1 h, using a Posiblot vacuum transfer apparatus (Stratagene) and then UV cross-linked to the membrane. 25 ng of β2 cDNA probe was radiolabeled by random priming, using a DNA multiprime labelling kit (Amersham International). Blots were prehybridized for 4 h at 42°C in 6 × SSC, 5 × Denhardt's solution, 50% formamide, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA (Sigma Chemical Co.) and hybridized overnight at 42°C in the same buffer containing 2 × 10^6 cpm/ml of β2 probe. Blots were washed three times for 15 min each at room temperature in 2 × SSC, 0.1% SDS, and then washed twice in the same solution at 60°C, for 20 min each wash. Blots were autoradiographed at −70°C using an intensifying screen and Kodak XAR5 x-ray film.

Results

Adhesive Properties of Keratinocytes and ndk

The adhesion of ndk and cultured epidermal keratinocytes to ECM glycoproteins was compared in short term, serum-free cell adhesion assays. The proteins chosen were those to which keratinocytes are exposed in vivo (Martin et al., 1988).
or in vitro when cultured on 3T3 feeder cells (Alitalo et al., 1982).

In time course experiments, irrespective of the substratum used, maximal adhesion of ndk cells was observed after 1 h of incubation, whereas maximal adhesion of keratinocytes occurred after three to four hours (Fig. 1, A–D; Adams and Watt, 1990). Under our assay conditions keratinocytes that have initiated terminal differentiation (usually 20–30% of the population) do not adhere (Adams and Watt, 1990). Between 75 and 90% of ndk adhered to each matrix glycoprotein, whereas the percentage of adherent basal keratinocytes varied depending on the matrix used: the highest numbers of keratinocytes adhered to fibronectin (60 to 65%); intermediate numbers to collagen type I and IV (40 to 45%) and the lowest numbers to laminin (33%), as reported previously (Clarke et al., 1985; Adams and Watt, 1990).

Both cell types adhered at 37°C but not at 4°C, and adhesion was not prevented by pretreatment of cells with cycloheximide. These results indicate that adhesion required metabolic energy or membrane fluidity and did not require new protein synthesis (data not shown).

In experiments in which cells were allowed to attach for 3 h to surfaces coated with increasing concentrations of each matrix glycoprotein, adhesion of both keratinocytes and ndk was found to be saturable. To achieve maximum adhesion of ndk to laminin, collagen type I or type IV, coating concentrations of about 50 μg/ml were required, whereas keratinocytes required coating concentrations of about 100 μg/ml. However, a fibronectin coating concentration of 100 μg/ml was needed to cause maximum adhesion of both cell types (Fig. 2, A–D; Adams and Watt, 1990).

Effects of Anti-integrin Antibodies on the Adhesion of Keratinocytes and ndk

To test for the involvement of integrins in cell adhesion to ECM glycoproteins, cell adhesion assays were carried out in the presence of function-blocking mAbs directed against integrin subunits (Table I). Adhesion of both cell types to fibronectin, collagen type IV and laminin was completely inhibited by an anti-β3 mAb. An anti-α3 mAb inhibited adhesion of both cell types to fibronectin but not to collagen type IV or laminin; inclusion of an anti-α5 antibody caused no further inhibition. An anti-α2 mAb inhibited keratinocyte adhesion to collagen type IV, and did not affect adhesion to fibronectin or laminin. The anti-α1 mAb used decreased keratinocyte and ndk adhesion to laminin by ~30% and did not affect cell adhesion to fibronectin or collagen type IV. Adhesion of both cell types to laminin was further inhibited when antibodies to both α2 and α1 were present. The anti-α5 antibody did not affect the adhesion of either cell type to fibronectin, laminin, or collagen (Fig. 3, A and B).

We found that both cell types could adhere to vitronectin, although the percentage of adherent ndk was greater (80% compared to 38%). The anti-α2 mAb inhibited adhesion of both cell types to vitronectin but not to fibronectin. An anti-α6β1 monoclonal, used at the same dilution, had little effect on ndk or keratinocyte adhesion. The anti-β1 mAb did not affect keratinocyte adhesion to vitronectin but did partially inhibit ndk adhesion. Thus, both cell types used α5-containing integrin(s) in adhesion to vitronectin but not to fibronectin; in ndk β1-containing integrin(s) also appeared to be involved (Fig. 3 C).

Identification of Integrins Expressed on the Surface of Keratinocytes and ndk

The adhesion assay results demonstrated that both keratinocytes and ndk used β1 integrins to adhere to fibronectin, laminin and collagen types I and IV, and α5-containing integrin(s) to adhere to vitronectin. ndk adhered and spread more quickly on all matrix glycoproteins than normal keratinocytes; keratinocytes differed in adhesiveness to the different glycoproteins whereas ndk did not. To examine the
Figure 3. Inhibition of cell adhesion with integrin subunit-specific monoclonal antibodies. (A) Keratinocyte adhesion to fibronectin (○), laminin (□) or collagen type IV (△), all used at coating concentrations of 20 µg/ml. The antibody concentrations chosen were those that were maximally effective. β1, 100 µg/ml Mab13 IgG; α2, 1:100 dilution of 5E8 ascites; α3, 1:2 dilution of PlB5 conditioned medium; α2 + α3, 5E8 ascites used at 1:100 dilution plus a 1:2 dilution of PlB5 conditioned medium (final concentrations); α3, 1:4 dilution of BIIG2 conditioned medium; α3 + α5, 1:4 dilutions of PlB5 and BIIG2 conditioned media (final concentrations); α5, 1:2 dilution of GoH3 conditioned medium. (B) ndk adhesion; conditions the same as in A, except that coating concentrations of 15 µg/ml were used. (C) Keratinocyte (○, □) or ndk (△, ◇) adhesion to fibronectin (○, □ coating concentration 20 µg/ml) or vitronectin (△, ◇ coating concentration 15 µg/ml). β1, 100 µg/ml Mab 13 IgG; α5, 1:4 dilution of BIIG2 conditioned medium; α2, 13C2; α3β1, 23C6 (both used at 1:50 dilution of ascites). Values are means from duplicate experiments, bars indicate SEM.

biochemical basis for these differences in adhesive properties, keratinocytes and ndk were surface iodinated, lysed, and aliquots equalized on the basis of TCA-precipitable radioactivity were immunoprecipitated using a panel of monoclonal and polyclonal antibodies to integrin subunits (see Table I).

An antiserum raised against a peptide corresponding to the carboxy-terminus of the β1 chain, which co-precipitates all α subunits associated with the β1 subunit, immunoprecipitated from both cell types cell surface proteins which migrated under nonreducing conditions with apparent molecular weights of 120 and 160 kD. An additional protein of 200 kD was precipitated from ndk cells (Fig. 4 A, lanes 1 and 2). The 120-kD band corresponds to the β1 subunit and the higher molecular weight bands to α subunits. The intensity of all three bands was greater in ndk, suggesting either that these integrins were more readily iodinated on ndk, or that ndk possessed higher levels of β1 integrins on the cell surface. The results also suggested that ndk cells contained a different complement of α/β1 heterodimers; therefore, we carried out immunoprecipitations using monoclonal antibodies specific for particular α subunits which are known to associate with the β1 subunit (Hynes, 1987; Hemler, 1990).

We found that both keratinocytes and ndk cells expressed α2β1, α3β1, and α5β1, and that all of these integrins were present at higher levels on ndk (Fig. 4 A, lanes 5–8, 11, and 12). Neither cell type expressed α6β1 (Fig. 4 A, lanes 9 and 10). ndk expressed α5β1, α6 corresponding to the 200-kD band seen in the ndk anti-β1 immunoprecipitation (Fig. 4 A, lane 2). In some experiments, very low levels of α5β1 could be detected in keratinocytes but this result was not consistently reproducible (Fig. 4 A, lanes 3 and 4).

The apparent molecular weights of the α1, α2, α3, and α5 subunits were in the range expected from published studies on other cell types; mean values from four separate experiments are: α1, 200 kD; α2, 160 kD; α3, 160 kD; α5, 130 kD to 160 kD (c.f., Hemler, 1990). As reported for other epithelial cells (Werb et al., 1989), the α5 band was very broad and iodinated poorly in comparison with the other α subunits. Interestingly, the β1 subunit in the ndk integrin complexes consistently migrated slightly faster than the β1 subunit in the keratinocyte integrin complexes, giving it an apparent molecular weight of 116 kD compared to 120 kD (compare Fig. 4 A, lanes 2, 4, 6, 8, and 12 with lanes 1, 5, 7, and 11). This difference was also observed when the immunoprecipitates were resolved under reducing conditions (not shown).

We used several antibodies directed against the α1, or β1 subunits to investigate the expression of β1 integrins on keratinocytes and ndk. A monoclonal to the α1 subunit precipitated proteins of apparent molecular weight 140 and 100 kD from keratinocytes; from ndk it precipitated bands of 140, 116, 90 kD and a doublet of 100 and 97 kD (Fig. 4 B, lanes 1 and 2). Monoclonals directed against either an α5β1 complex-dependent epitope or the β1 subunit did not precipitate any proteins from keratinocytes but did precipitate the 140-kD protein and a single band at 97 kD from ndk (Fig. 4 B, lanes 3–6). Affinity purified polyclonal IgG, raised against purified placental vitronectin receptor (Suzuki et al., 1986), very weakly precipitated the 140- and 100 kD proteins from keratinocytes and precipitated the same five proteins from ndk as the anti-α5 monoclonal, plus an addi-
tional protein of apparent molecular weight 130 kD (Fig. 4 B, lanes 7 and 8). The 116-kD band in ndk extracts corresponded in size to the β5 subunit and could be precipitated by the β5 peptide antiserum from dissociated integrin complexes (data not shown). The 100-kD band immunoprecipitated by anti α5 in keratinocytes and ndk had the same apparent molecular weight as the β5 subunit (Cheresh et al., 1989; Ramaswamy and Hemler, 1990). When precipitations were carried out using an antiserum raised against a synthetic peptide corresponding to the carboxy-terminus of the human β5 subunit, an α5β5 complex was clearly precipitated from ndk cells (Fig. 4 B, lane 10). In these precipitates, it was noticeable that the β5 subunit was precipitated as a doublet of rather diffuse bands. The same proteins were also precipitated from keratinocyte extracts (Fig. 4 B, lane 9). Since the latter complex was only just detectable, we confirmed the presence of β5 in keratinocytes by probing keratinocyte mRNA on a Northern blot with a human β5 cDNA. A single 3.5-kb message was detected, as observed in Dami, a human megakaryocyte line (Fig. 4 D; Ramaswamy and Hemler, 1990).

In conclusion, keratinocytes express α5β5 whereas ndk express α6β1, α5β1, and α5β5. The additional bands seen in the ndk precipitations (Fig. 4 B) may represent associated cell surface proteins, other integrin subunits, or proteolytic degradation products.

Monoclonals to the α6 or β1 subunits precipitated proteins of apparent molecular weight 200 and 180 kD, corresponding to the β1 subunit (Kajiji et al., 1989; Hemler et al., 1989; Sonnenberg et al., 1990), and 120 kD, corresponding to the α6 subunit (Sonnenberg et al., 1987, 1988a), from both keratinocytes and ndk. All three proteins appeared more abundant in ndk; there was no difference in the mo-

Figure 4. Immunoprecipitation of surface-iodinated integrins and Northern blot of the β5 subunit. (A) β1 integrins. Aliquots of lysates prepared from surface-iodinated keratinocytes (lanes 1, 3, 5, 7, 9, and 11) or ndk (lanes 2, 4, 6, 8, 10, and 12), were equalized on the basis of TCA-precipitable radioactivity and immunoprecipitated with antibodies to the β1 subunit (363 polyclonal, lanes 1 and 2) and mAbs to the α1 (TS2/7, lanes 3 and 4), α2 (12F1, lanes 5 and 6), α3 (J143, lanes 7 and 8), α5 (B-5G10, lanes 9 and 10), or α1 (BlE5, lanes 11 and 12) subunits. All lanes are from the same gel. Lanes 11 and 12 are a longer exposure than lanes 1-10. (B) α5 integrins. Lysates from keratinocytes (lanes 1, 3, 5, 7, and 9) or ndk (lanes 2, 4, 6, 8, and 10) were immunoprecipitated with mAbs to α5 (13C2, lanes 1 and 2), an α5β1 complex-dependent epitope (23C6, lanes 3 and 4), β1 (Y2/51, lanes 5 and 6), an affinity purified polyclonal IgG raised against placental vitronectin receptor (aVNR, lanes 7 and 8) or an antiserum to a peptide corresponding to amino acids 757-776 of the human β5 subunit (lanes 9 and 10). (C) β4 integrins. Lysates of keratinocytes (lanes 1 and 3) or ndk (lanes 2 and 4) were immunoprecipitated with antibodies to α6 (GoH3, lanes 1 and 2) or β4 (439-9B, lanes 3 and 4) subunits. Both pairs of lanes are from the same gel. All samples were resolved on 7.5% polyacrylamide gels under nonreducing conditions. Molecular weight markers are as indicated in kD. Markers for B, lanes 9 and 10 are the same as for A. (D) Northern blot probed with a cDNA probe for β5. 1 µg mRNA was loaded per track. Dami, a megakaryocyte cell line, served as a positive control (lane 1); normal keratinocytes (lanes 2 and 3). The blot was also probed for β actin to confirm that equal amounts of mRNA were loaded (not shown).
Figure 5. Immunoprecipitation of integrins labeled with \(^{35}\)S-methionine and \(^{35}\)S-cysteine. (A) Aliquots of cell lysates of keratinocytes (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or ndk (lanes 2, 4, 6, 8, 10, 12, 14, and 16), equalized on the basis of TCA-precipitable counts, were immunoprecipitated with nonimmune mouse IgG (lanes 1 and 2), antiserum Rx to the \(\beta_1\) subunit (lanes 3 and 4) and mAbs to \(\alpha_5\) (TS2/7, lanes 5 and 6), \(\alpha_3\) (J143, lanes 7 and 8), \(\alpha_6\) (GoH3, lanes 11 and 12), \(\alpha_6\) (13C2, lanes 13 and 14), and \(\beta_3\) (Y2/51, lanes 15 and 16). All lanes are from the same exposure of the same gel. (B) Aliquots of cell lysates of keratinocytes (lanes 1 and 3) or ndk (lanes 2 and 4) immunoprecipitated with anti-\(\alpha_2\) (12F1; lanes 1 and 2) or anti-\(\alpha_3\) (J143; lanes 3 and 4). Nonreduced samples were resolved on a 7.5% polyacrylamide gel.

Biosynthesis of Integrins by Keratinocytes and ndk

The use of surface-iodinated cells does not permit quantitative comparisons between integrins, since different heterodimers may not be equally accessible to the iodinating agents. We therefore used metabolic labeling with \(^{35}\)S-methionine and \(^{35}\)S-cysteine to obtain more information on the relative levels of integrins synthesized by each cell type and to investigate further the observation that ndk apparently expressed higher levels of integrins than keratinocytes (Fig. 5).

In metabolic labeling experiments, additional proteins were present in the immunoprecipitations, some of which correspond to \(\alpha\) or \(\beta\) subunit precursors. For example, precipitation with the anti-\(\beta_1\) serum precipitated the \(\beta_1\) precursor as well as the mature \(\alpha\) and \(\beta\) subunits (Fig. 5 A, lanes 3 and 4) and anti-\(\alpha_6\) precipitated an additional band of 140 kDa (Fig. 5 A, lanes 11 and 12). The experiments indicated that \(\alpha_6\) was synthesized by ndk but was not detectable in keratinocytes (Fig. 5 A, lanes 5 and 6) and confirmed that neither cell type made \(\alpha_6\beta_1\) (not shown). In keratinocytes, the \(\beta_1\) and \(\beta_3\) integrins were more abundant than the \(\alpha\)-containing integrin. Of the keratinocyte \(\beta_1\) integrins, \(\alpha_5\beta_1\) and \(\alpha_6\beta_1\) were labeled to approximately equal intensity and \(\alpha_5\beta_1\) was labeled less intensely, suggesting that it was indeed less abundant than the other \(\beta_1\) integrins. All integrins were present in greater amounts in ndk lysates than in keratinocyte lysates, but the relative abundance of the \(\beta_1\) integrins was the same.

Localization of Integrins on Cultured Keratinocytes

Integrin distribution was examined in small stratified colonies of human epidermal keratinocytes which had been grown on J2 feeder cells and then fixed in formaldehyde, but not permeabilized. In the center of these colonies, the basal layer of keratinocytes is covered by layers of terminally differentiating involucrin-positive cells (Fig. 6 a; Watt, 1988). Thus in nonpermeabilized preparations, staining of the basal cells can only be observed at the margins of the colony, which consist of a single cell layer.

The anti-\(\beta_1\) mAbs MabL3 and DH12 both gave intense staining of the cell–cell contact zones of the basal keratinocytes and did not stain the suprabasal cells (Fig. 6 b; Nicholson and Watt, 1991). Different mAbs to both \(\alpha_2\) (12F1, 5E8, and P1E6) and \(\alpha_6\) (J143 and P1B5) produced similar, basal cell-specific staining patterns (Fig. 6, c and d and results not shown). mAbs to \(\alpha_5\) (BIIG2 and Mab6) produced a different and somewhat varied staining pattern: a few basal cells were uniformly and diffusely stained, but in most colonies...
Figure 6. Localization of integrins in keratinocyte colonies by indirect immunofluorescence. Keratinocyte colonies were fixed in formaldehyde (b–i) or fixed and permeabilized in methanol (a). Cells were stained with a mAb to involucrin, to visualize terminally differentiating cells in the upper layers of the colonies (a), or mAbs to integrin subunits. b, Mab13, β1; c, 5E8, α5; d, P1B5, α6; e, B1G2, α5; f, 13C2, α5; g and h, 439-9B, β5; i, GoH3, α6. Arrows in a indicate the margins of the colony. Arrow in e indicates intense staining in area of cell–cell contact. Arrows in f indicate areas of intense staining at the free margins of the cells. This colony consists entirely of basal cells. Bars: (a, b, f, g–i) 50 µm; (c, d) 73 µm; (e) 36 µm.
spotty staining of varying intensity which delineated the cell-cell contact areas of basal cells was obtained (Fig. 6 e; and Nicholson and Watt, 1991). Intense, localized staining at the free edges of some cells was also observed (data not shown).

As expected from the immunoprecipitation results, no staining of keratinocyte colonies was obtained using the anti-α5 mAb. The anti-α1 monoclonal produced faint spotty staining of cell-cell contact areas between basal cells and, in some cells, intense staining of areas at the free margins of the cells. The feeder cells were also strongly stained by this antibody (Fig. 6 f), but suprabasal cells were not stained.

Antibodies directed against α6 or β1 produced basal cell-specific staining patterns in which the majority of staining was localized to cell-cell contact areas. Additionally, staining was observed at the free edges of basal cells; this was somewhat heterogeneous and appeared to be associated with cell-surface microvilli and filopodia in contact with the substratum (Fig. 6, g-i). Filopodial staining was very marked in small groups of cells (Fig. 6 h).

The observation that staining for integrins was reduced or absent on keratinocytes undergoing terminal differentiation was confirmed by staining transverse sections of confluent keratinocyte cultures with monoclonal antibodies to (a) β1 (DH12) or (b) α6 (GoH3) integrin subunits. Bar, 50 μm.

Expression of Integrins on ndk

ndk were grown on glass coverslips and stained with anti-integrin mAbs after fixation in formaldehyde. All cells were stained by these antibodies; this is consistent with the fact that the cell population does not contain any terminally differentiated cells (Adams and Watt, 1988). However, the staining patterns obtained with antibodies to different integrin subunits were markedly different. Staining with anti-β1 or anti-α1 mAbs was localized to cell surface microvilli and to areas of membrane ruffling (Fig. 8 a and c; Nicholson and Watt, 1991). In contrast, the anti-α6 and anti-α5 monoclonals stained tiny spots within areas of membrane ruffling (Fig. 8 b and data not shown). The anti-α6 mAbs gave intense spotty staining in localized regions of ruffled membrane and in areas at the trailing edge of the cells. Regions of apposed cell membranes also stained (Fig. 8 d; Nicholson and Watt, 1991). The staining obtained with anti-α5 or anti-α6 monoclonals was more heterogeneous than that observed with the anti-β1 or anti-α5 antibodies; thus, for example, not all areas of ruffled membrane were stained.

The anti-α1 mAb gave intense staining of patches of ruffled membrane. More spotty staining could be distinguished in some cells at the cell margin and underlying the spread portion of the cytoplasm (Fig. 8 g). In contrast, the anti-β1 mAb only stained membrane ruffles (Fig. 8 h). This suggests that α1β1 has a different subcellular distribution to α5β1 or α6β1; thus the different α1-containing integrins may have separate functions.

The anti-α6 and β1 mAbs produced intense staining of cell surface microvilli and filopodia in contact with the substratum (Fig. 8, e and f). These staining patterns were similar to those obtained with the anti-β1 and anti-α5 antibodies.

Discussion

In this paper we have investigated the adhesive properties and integrin expression profiles of cultured human epidermal keratinocytes and a non-differentiating strain of keratinocytes, termed ndk. The results of the adhesion-blocking experiments indicate that both cell types use α5β1 to adhere to fibronectin and α6β1 to adhere to laminin. In keratinocytes, α6β1 was involved in adhesion to collagen type IV and also functioned synergistically with α1β1 in adhesion to laminin; in ndk α6β1 appears to be only involved in adhesion to laminin. ndk also express α1β1, which has been identified as a collagen or laminin receptor in various cell types (Hemler, 1990; Hall et al., 1990); this receptor may thus be the major ndk collagen receptor. Other investigators have also reported a role for α1β1 in keratinocyte adhesion to fibronectin (Carter et al., 1990a) and for α5β1 in adhesion to collagen types I and IV (Staquet et al., 1990) or collagen type I and laminin (Carter et al., 1990a,b). Evidence regarding the role of α6β1 is conflicting: adhesion blocking experiments have indicated that it is involved in keratinocyte adhesion to fibronectin and collagen type IV (Staquet et al., 1990), or to fibronectin and laminin (Carter et al., 1990a,b). However, α6β1 is the major keratinocyte integrin which binds to fibronectin–Sepharose (Adams and Watt, 1990). α6β1 and α5β1 may also function in cell–cell adhesion (Kaufmann et al., 1989; Larjava et al., 1990; Carter et al., 1990a). It seems possible that β1 integrins in keratinocytes function in initial adhesive contacts to extracellular matrix glycoproteins and then relocate to cell–cell contact areas (Carter et al., 1990a).
Adhesion to vitronectin was mediated by $\alpha_v$-containing integrins. Consistent with a previous report, $\beta_1$-containing integrins were not involved in keratinocyte adhesion to vitronectin (Larjava et al., 1990). In keratinocytes, $\alpha_v$ was associated with the $\beta_3$ subunit; this is in agreement with the finding that these cells do not express $\beta_3$ integrins (Figs. 4 and 5; Staquet et al., 1990; Marchisio et al., 1991) and the detection of $\beta_3$ in other epithelial cells (Cheresh et al., 1989; Ramaswamy and Hemler, 1990). $\alpha_v\beta_3$ has been reported to bind fibronectin as well as vitronectin (Cheresh et al., 1989). However, the keratinocyte $\alpha_v$ integrin only mediated adhesion to vitronectin (see Fig. 3) and did not bind to fibronectin-Sepharose (Adams and Watt, 1990).

In ndk, $\alpha_v$- and $\beta_1$-containing integrins appeared to be involved in adhesion to vitronectin, and indeed ndk were found to express $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. Both $\alpha_v\beta_1$ and $\alpha_v\beta_3$ function...
as fibronectin receptors in certain cell types (Vogel et al., 1990; Cheresh et al., 1989), however, in ndk, α containing integrins only appeared to mediate adhesion to vitronectin.

The αβ4 integrin did not appear to be involved in adhesion of either cell type to fibronectin, laminin, or collagen, since an α antibody, which inhibits the adhesion of αβ4-positive cells to laminin (Sonnenberg et al., 1988b, 1990a), did not prevent adhesion to any of these matrix glycoproteins. However, in assays in which keratinocytes are allowed to adhere for 12 h, αβ4 does appear to play some role in cell–matrix adhesion (De Luca et al., 1990; Carter et al., 1990b). By this time, proteins secreted by the keratinocytes themselves form part of the extracellular matrix (Carter et al., 1990b). Recently, αβ4 has been localized to hemidesmosomes in vivo (Stepp et al., 1990), suggesting that its ligand may be a component of anchoring filaments or an as yet uncharacterized basement membrane component. It has also been proposed that this integrin may be involved in the regulation of cell proliferation (Sacchi et al., 1989).

Immunoprecipitation experiments revealed both quantitative and qualitative differences between the integrins expressed by ndk and keratinocytes and provide possible explanations for the greater adhesiveness of ndk to extracellular matrix proteins. The levels of all integrins were greater in ndk than keratinocytes, whether assessed by surface iodination or metabolic labeling, and this might account for the increased adhesiveness. However, levels of β3 integrins on the cell surface do not always correlate with the ligand binding activity, as in the case of αβ3 in keratinocytes (Adams and Watt, 1990), of αβ3, αβ3, and αβ3 in lymphocytes (Shimizu et al., 1990) and of αβ3 in neurons (Neugebauer and Reichardt, 1991). Posttranslational modifications of integrin subunits, by phosphorylation or glycosylation, have been correlated with alterations in cell adhesiveness (see Adams and Watt, 1990 for discussion). The immunoprecipitations indicate that the β3 subunit may undergo different posttranslational modifications in ndk and keratinocytes, since the β3 precursors comigrate (see Fig. 5, lanes 3 and 4), yet the mature β3 in keratinocytes has a higher apparent molecular weight than that of ndk. The keratinocyte β3 is also larger than the β3 subunit of dermal fibroblasts (Larjava et al., 1989) or M6G63 osteosarcoma cells (Adams, unpublished observations). Furthermore, in both cell types, the breadth of the β3 subunit band in the iodinated immunoprecipitations (Fig. 4) varied depending on which α subunit was being precipitated. This may indicate that the β3 subunit undergoes different posttranslational modifications depending on the α subunit with which it is associated. Finally, ndk express integrins which normal keratinocytes do not: αβ1, which binds vitronectin and, in some cases, fibronectin (Bodary et al., 1990; Vogel et al., 1990), and αβ1, which can bind vitronectin, fibrinogen, von Willebrand factor, osteopontin, and thrombospondin (Hemler, 1990).

Indirect immunofluorescent staining of the keratinocyte colonies indicated that the localization of all integrins was restricted to basal cells. This is in agreement with the distribution patterns of integrins in the intact epidermis (see references cited in Introduction), with immunoprecipitations from surface-iodinated keratinocytes (Adams and Watt, 1990) and with mRNA levels for the α3 and β3 integrin subunits (Nicholson and Watt, 1991). As previously reported, antibodies against the β1, α3, and α subunits stained cell–cell contact areas (Larjava et al., 1989; Carter et al., 1990a). Thus, suprabasal, terminally differentiating cells must use other mechanisms to adhere to each other (Watt, 1984).

α and α3 had a more patchy and variable distribution on keratinocytes than α and α1, and were sometimes located at the free margins of the cells. Antibodies to α and β also stained free cell margins and filopodia, in addition to cell–cell contact areas. The basal monolayer of keratinocytes at the periphery of a stratified colony migrates outwards as the colony enlarges (Barrandon and Green, 1987); thus the distribution of these integrins may reflect a role in cell migration (Brecher, 1989). The staining patterns we observed were of unpermeabilized cells; β1 integrins have been found in the focal contacts of permeabilized keratinocytes in short-term cultures (Carter et al., 1990a; Guo et al., 1990) and αβ3 has been localized to hemidesmosome-like structures called stable anchoring contacts (Carter et al., 1990b).

In ndk cultures, no terminally differentiating cells are present and all cells stained with every anti-integrin antibody. The distribution of the integrins did not relate in a simple way to the distribution of the extracellular matrix proteins identified as their ligands in adhesion assays: fibronectin and collagen type IV are found in a network over and around the cells, whereas laminin is localized in small patches on ruffled membranes (Adams, Nicholson, and Watt, unpublished observations). We have recently reported that when cell–cell adhesion of ndk is induced by the polyanionic detergent, suramin, the β integrins redistribute to regions of cell–cell contact (Adams et al., 1991).

Finally, our experiments have yielded more information about the phenotype of ndk cells. The presence of integrins on all ndk is consistent with a basal keratinocyte phenotype (and see Adams and Watt, 1988). However, other characteristics, such as synthesis of the αβ3 and αβ1 integrins, are not shared with normal basal keratinocytes. ndk expressed αβ1, an integrin that is expressed at moderately high levels until 15 wk of gestation in human fetal epidermis (Hertle et al., 1991) and this would suggest that ndk resemble fetal keratinocytes more closely than adult keratinocytes. Nevertheless, β1 is never detected in developing epidermis (Hertle et al., 1991), raising two other possibilities: that ndk are a minor epidermal population, perhaps found in adnexal structures, or that for some reason β1 expression has been induced in culture. Our experiments do show that the adhesive properties of ndk differ from normal basal keratinocytes and may be attributed to differences in the type of integrins expressed, the level of expression and the distribution of the receptors on the cell surface.

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