Journal of Cell Science 106, 175-182 (1993)
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Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions

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SUMMARY

Suspension-induced terminal differentiation of human epidermal keratinocytes can be inhibited by fibronectin through binding to the $\alpha_5\beta_1$ integrin. We have investigated the effect of fibronectin on expression of integrins and proteins of the actin cytoskeleton and have explored the nature of the differentiation stimulus by testing different combinations of anti-integrin monoclonal antibodies or extracellular matrix proteins in the suspension assay. Fibronectin prolonged cell surface expression of β_1 integrins but did not overcome the inhibition of intracellular transport of integrins that occurs when keratinocytes are placed in suspension. Fibronectin did not prevent the suspension-induced decline in the level of mRNAs encoding the β_1 integrin subunit, actin, filamin and α-actinin; furthermore, the inhibition of terminal differentiation did not depend on the state of assembly of microfilaments or microtubules. Terminal differentiation could be partially inhibited by an adhesion-blocking monoclonal antibody to the β_1 integrin subunit or by a combination of adhesion blocking antibodies recognising the α subunits that associate with β_1 (α_2, α_3 and α_5). Although laminin and type IV collagen do not inhibit terminal differentiation individually, they were inhibitory when added to cells in combination with a low concentration of fibronectin. We conclude that the proportion of keratinocyte β_1 integrins occupied by ligand can regulate the initiation of terminal differentiation independently of the state of assembly of the actin cytoskeleton.

Key words: adhesion, epidermis, fibronectin

INTRODUCTION

In the epidermis, proliferation is largely confined to the basal layer of cells attached to the basement membrane, and keratinocytes undergo terminal differentiation as they move through the suprabasal layers. Basal keratinocytes express a range of extracellular matrix receptors of the integrin family, including $\alpha_5\beta_1$, the fibronectin receptor (Adams and Watt, 1990, 1991; Carter et al., 1990a), $\alpha_2\beta_1$, a receptor for collagen and laminin (Adams and Watt, 1991; Staquet et al., 1990; Carter et al., 1990a,b), $\alpha_3\beta_1$, a receptor for laminin and epiligrin (Adams and Watt, 1991; Carter et al., 1990a,b, 1991), $\alpha_v\beta_5$, the vitronectin receptor (Adams and Watt, 1991; Marchisio et al., 1991) and $\alpha_6\beta_4$, a component of hemidesmosomes (Stepp et al., 1990; Kurpakus et al., 1991; Sonnenberg et al., 1991). Keratinocyte integrins not only mediate adhesion to the basement membrane, but are also believed to play a role in cell-cell adhesion (Carter et al., 1990a; Larjava et al., 1990; Symington et al., 1993; but see also Tenchini et al., 1993), lateral migration (Kim et al., 1992a,b; Tenchini et al., 1993) and stratification (Adams and Watt, 1990; Hotchin and Watt, 1992); in addition they regulate the onset of terminal differentiation (Adams and Watt, 1989) (reviewed by Watt and Hertle, 1993).

One experimental model that we have used to study the regulation of keratinocyte terminal differentiation is suspension culture (Green, 1977; Watt et al., 1988). When a single cell suspension of keratinocytes is placed in medium made viscous by the addition of methyl cellulose, the cells become committed to terminal differentiation by 5 hours (Adams and Watt, 1989) and by 24 hours the majority of cells express involucrin, a precursor of the cornified envelope that is a useful marker of terminal differentiation (Watt et al., 1988; Adams and Watt, 1989). Terminal differentiation can be inhibited in a proportion of the population by including fibronectin in the methyl cellulose at the time of resuspending the cells (Adams and Watt, 1989); however, fibronectin added to cells that have been in suspension for 5 hours has no effect, because on commitment to terminal differentiation the ability of the $\alpha_5\beta_1$ integrin to bind fibronectin is decreased (Adams and Watt, 1990;

Hotchin and Watt, 1992). Proteolytic fragments of fibronectin that include the RGD sequence that binds to $\alpha_5\beta_1$ are capable of inhibiting differentiation whereas other regions of the molecule are not (Adams and Watt, 1989, 1990). A rabbit antiserum recognising all β_1 integrins, including $\alpha_5\beta_1$, inhibits terminal differentiation, and Fab fragments are as effective as whole IgG, indicating that integrin clustering is not required for the inhibition of differentiation (Adams and Watt, 1989). In contrast to fibronectin, laminin and type IV collagen when added individually to the methyl cellulose have no effect on terminal differentiation (Adams and Watt, 1989).

The purpose of the experiments described in this report was to investigate the effect of fibronectin-mediated inhibition of differentiation on expression of integrins and proteins of the actin cytoskeleton, and to explore the nature of the terminal differentiation stimulus by testing different combinations of anti-integrin monoclonal antibodies or extracellular matrix proteins in the suspension assay.

MATERIALS AND METHODS

Cell culture

Keratinocytes were isolated from neonatal foreskin and grown in the presence of a mitomycin C-treated 3T3 feeder layer in 1 part Ham's F12 medium and 3 parts Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, 1.8×10^{-4} M adenine, 5 μg/ml insulin, 10^{-10} M cholera toxin, 0.5 μg/ml hydrocortisone and 10 ng/ml epidermal growth factor (FAD + FCS + HICE) (Rheinwald and Green, 1975; Watt, 1984; Rheinwald, 1989). Cells were used for experiments from the third passage onwards.

To induce terminal differentiation, keratinocytes were suspended in FAD + FCS + HICE supplemented with 1.3-1.8% methyl cellulose at a density of 10^5 or 2×10^5 cells per ml (Green, 1977; Watt et al., 1988; Adams and Watt, 1989). Culture dishes used for suspension culture were coated with 0.4% polyHEMA to prevent cell attachment (Folkman and Moscona, 1978; Watt et al., 1988).

Antibodies and extracellular matrix proteins

Human plasma fibronectin was purchased from Blood Products Laboratory. Mouse EHS laminin and human placental type IV collagen were purchased from Sigma.

The following anti-integrin monoclonal antibodies that lack keratinocyte adhesion blocking activity were used: DH12 which recognises the β_1 subunit (De Strooper et al., 1988); B515 which immunoprecipitates $\alpha_2\beta_1$, and E1/56 which immunoprecipitates $\alpha_3\beta_1$ (kindly provided by C. Isacke, Department of Biology, Imperial College, London); mAb16 which immunoprecipitates $\alpha_5\beta_1$ (Akiyama et al., 1989a) and GoH3 which recognises the α_6 integrin subunit (Sonnenberg et al., 1986). Monoclonal antibodies that inhibit keratinocyte adhesion to extracellular matrix proteins were: β_1: mAb13 (Akiyama et al., 1989a); α_2: 5E8 (Chen et al., 1991); α_3: P1B5 (Wayner and Carter, 1987); α_5: BIIG2 (Werb et al., 1989) or P1D6 (Wayner et al., 1988); α_v: 13C2 (Davies et al., 1989). The effects of the antibodies on keratinocyte adhesion have been described previously (Adams and Watt, 1991), with the exception of B515 and E1/56. Nonimmune control mouse IgG was purchased from Sigma.

Antibodies were used in the form of purified IgG or ascites. The concentrations used in suspension experiments were 2- to 10-fold greater than the concentrations that inhibit cell-extracellular matrix adhesion, or, for non-adhesion perturbing antibodies, equivalent concentrations (see Adams and Watt, 1991). The antibodies were not toxic as judged by keratinocyte permeability to fluorescein-conjugated second antibodies.

Measurement of the proportion of involucrin-positive keratinocytes

Single cell suspensions of keratinocytes were air-dried onto coverslips, fixed in 3.7% formaldehyde in PBS, permeabilised in methanol, and stained with a rabbit antiserum to involucrin (Dover and Watt, 1987) and a fluorescein-conjugated anti rabbit second antibody, as described previously (Read and Watt, 1988). The percentage of involucrin-positive cells was determined in the starting population and after 24 hours suspension in methyl cellulose. The increase in the percentage of involucrin-positive cells in the absence of anti-integrin antibodies or extracellular matrix proteins was taken as 100% terminal differentiation, and the percent increase in treated populations was expressed relative to the untreated cells. The absolute percentage of involucrin-positive cells was 10-30% in the starting population and 50-80% after suspension in methyl cellulose alone. The means of replicate experiments ± s.e.m. are shown.

In some experiments keratinocytes were incubated with Texas Red-conjugated phalloidin (Molecular Probes Inc.), prior to staining with a monoclonal antibody to involucrin (Hudson et al., 1992).

Surface labelling and immunoprecipitation

Keratinocyte surface proteins were labelled with biotin, using a modification of the method of Isberg and Leong (1990) as described by Hotchin and Watt (1992). Adherent cells, with or without pretreatment with 1-deoxymannojirimycin (MNJ; Boehringer), were harvested with trypsin/EDTA and recovered in the presence of 0.5 mg/ml trypsin inhibitor (Sigma). Suspended cells were recovered by centrifugation after diluting the methyl cellulose in PBS. Cells were washed twice with cell wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), resuspended at a concentration of 5×10^6 cells/ml in 100 μg/ml biotin (NHS-LC-Biotin, Pierce) in cell wash buffer and incubated for 60 minutes at room temperature with constant agitation. After three washes in cell wash buffer pellets containing 5×10^6 cells were stored at −70°C.

Lysis and immunoprecipitation were performed as follows. Cell pellets were thawed on ice and lysed 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 15 minutes on ice. Lysates were centrifuged at 16,000 ***g*** for 5 minutes and the pellets discarded. Samples equalised on the basis of equal protein content were incubated with anti-CD29 (a mouse anti β_1 monoclonal antibody purchased from Janssen Biochimica) for 90 minutes on ice. 50 μl of a 1:1 (vol/vol) suspension of Protein A-Sepharose (Pharmacia Fine Chemicals) was added and the samples mixed end-over-end for 1 hour at 4°C. The Sepharose beads with bound antibody and integrins were washed as described by Adams and Watt (1988), resuspended in SDS-PAGE sample buffer and resolved on 7.5% polyacrylamide gels (Laemmli, 1970).

Samples were transferred to Immobilon-P membrane (Millipore Corp.) at 4°C in 10 mM CAPS (Sigma; pH 11.0) for 20 hours at 0.3 A. The membrane was then incubated for 60 minutes in PBS, 0.05% Tween-20, 5% skimmed milk powder, then in horseradish peroxidase-conjugated streptavidin (Amersham International) in PBS, 0.05% Tween-20, 3% skimmed milk powder for 60 minutes and finally washed for 60 minutes in several changes of PBS, 0.05% Tween-20. Biotinylated proteins were visualised by chemiluminescence (ECL, Amersham, UK).

RNA preparation and northern blotting

RNA extraction and northern analysis were carried out as described by Nicholson and Watt (1991). Keratinocytes were washed three times with PBS and lysed in 4 M guanidine thiocyanate (Fluka Chemicals Ltd.), 0.5% sodium *N*-lauroylsarcosine, 25 mM sodium citrate, pH 7.0, 0.1% antifoam A emulsion (Sigma) and 0.1 M β-mercaptoethanol. The lysate was layered on top of a cushion of 5.7 M caesium chloride containing 25 mM sodium acetate, pH 6.0, and spun at 130,000 ***g*** for 21 hours at 20°C. The pellet of RNA was rinsed with 70% ethanol and dissolved in sterile distilled water containing 200 μg/ml heparin (Sigma).

Samples of total RNA were mixed with loading buffer and electrophoresed on 1% agarose gels containing formaldehyde. The RNA was transferred to Hybond-N (Amersham International) overnight in 20× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), rinsed in 6× SSC, baked at 80°C for 1 hour and irradiated with ultraviolet light for 4 minutes. Blots were prehybridised at 45°C for approximately 4 hours in 5× SSC (SSC is 0.18 M NaCl, 0.01 M NaH_2PO_4, 0.001 M Na_2EDTA, pH 7.7), 50% deionised formamide, 5× Denhardt's solution, 0.5% SDS and 20 μg/ml denatured salmon sperm DNA (Sigma), and hybridised overnight at 42°C in the same buffer containing 2× 10^6 cpm/ml probe, prepared using Klenow fragment and random primers. Blots were washed twice in 2× SSC, 0.1% SDS at room temperature for 30 minutes each; once in 1× SSC, 0.1% SDS at room temperature for 30 minutes; and twice in 0.1× SSC, 0.1% SDS at 65°C for 30 minutes. Blots were exposed to Kodak XAR5 film in the presence of intensifying screens. Blots were reprobed after washing at 65°C for 2 hours in 0.005 M Tris-HCl, pH 8.0, 0.002 M NaEDTA, 0.1× Denhardt's solution.

The following cDNA probes were used: human involucrin pI-2 (Eckert and Green, 1986; a generous gift from H. Green), mouse 18 S rRNA 100D9 (Edwards et al., 1987; a kind gift from D. Edwards), mouse integrin β_1 subunit pMINTβ (D. De Simone, R. Patel-King and R. Hynes, unpublished; kindly provided by R. Hynes), human filamin (1.4 kb *Eco*RI fragment; Gorlin et al., 1990; kindly provided by J. B. Gorlin), chick α-actinin C18 (Baron et al., 1987, generous gift of D. Critchley), human β actin (700 bp *Pst*I-*Ava* II insert; Gunning et al., 1983), mouse talin MT 25G and MT 26C (Rees et al., 1990, a generous gift from D. J. G. Rees), human gelsolin S4-6 (Way et al., 1989).

RESULTS

Fibronectin prolongs the half life of β_1 integrins on the cell surface

When keratinocytes are placed in suspension to induce terminal differentiation, the ligand binding ability of the $\alpha_5\beta_1$ integrin is decreased by 5 hours, when the cells become committed to terminal differentiation; by 24 hours, when the majority of cells are involucrin-positive, the receptor has largely disappeared from the cell surface (Adams and Watt, 1990). The decrease in ligand binding ability involves modulation of pre-existing receptor on the cell surface, and the subsequent loss of receptor from the surface reflects both an inhibition of transcription of the subunit genes and an inhibition of *N*-linked glycosylation and intracellular transport of newly synthesised receptor subunits (Hotchin and Watt, 1992). Addition of fibronectin does not overcome the block in *N*-linked glycosylation of β_1 integrins (Hotchin and Watt, 1992).

Since fibronectin does not overcome the block in glycosylation, it could either act by prolonging the half life of β_1 integrins on the cell surface or by allowing transport of under-glycosylated receptor to the cell surface, as occurs in fibroblasts and keratinocytes treated with the Golgi mannosidase inhibitor deoxymannojirimycin (MNJ; Akiyama et al., 1989b; Hotchin and Watt, 1992). To distinguish between these possibilities keratinocytes were surface-labelled with biotin and immunoprecipitated with an anti-β_1 antibody. As shown previously (Hotchin and Watt, 1992), normal adherent keratinocytes and cells suspended in methyl cellulose for 24 hours did not have β_1 precursor on the cell surface, whereas MNJ-treated adherent keratinocytes did (Fig. 1). When keratinocytes were placed in suspension for 24 hours in the presence of fibronectin, the level of β_1 integrins on the cell surface was higher than in the absence of fibronectin, but there was still no detectable β_1 precursor on the surface (Fig. 1). Thus fibronectin did not relieve the inhibition of intracellular transport caused by placing cells in suspension.

When keratinocytes are placed in suspension for 24 hours without added fibronectin, the level of involucrin mRNA increases and α_5 and β_1 mRNA levels decline (Nicholson and Watt, 1991); the decrease in integrin mRNAs reflects inhibition of transcription of the subunit genes (Hotchin and Watt, 1992). Fig. 2 shows that when keratinocytes were suspended for 24 hours in the presence of fibronectin, the level of involucrin mRNA was lower than in cells sus-

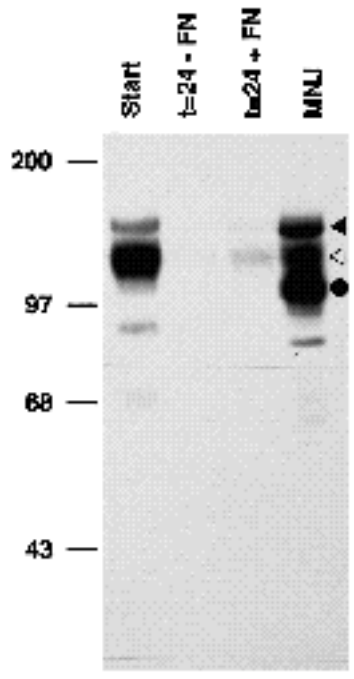

Fig. 1. Immunoprecipitation of cell surface β_1 integrins. Adherent keratinocytes were harvested with trypsin/EDTA and biotinylated immediately (start) or after 24 hours in suspension in the absence (t = 24−FN) or presence (t = 24+FN) of 100 μg/ml fibronectin; alternatively, adherent keratinocytes were treated with 50 μg/ml MNJ for 24 hours before harvesting and biotinylation. Biotinylated cells were extracted, immunoprecipitated with an anti-β_1 monoclonal antibody and resolved on a polyacrylamide gel under non-reducing conditions. Positions of molecular mass standards (kDa) are indicated. Filled arrowhead, α subunits; open arrowhead, mature β_1 subunit; dot, immature β_1 subunit.

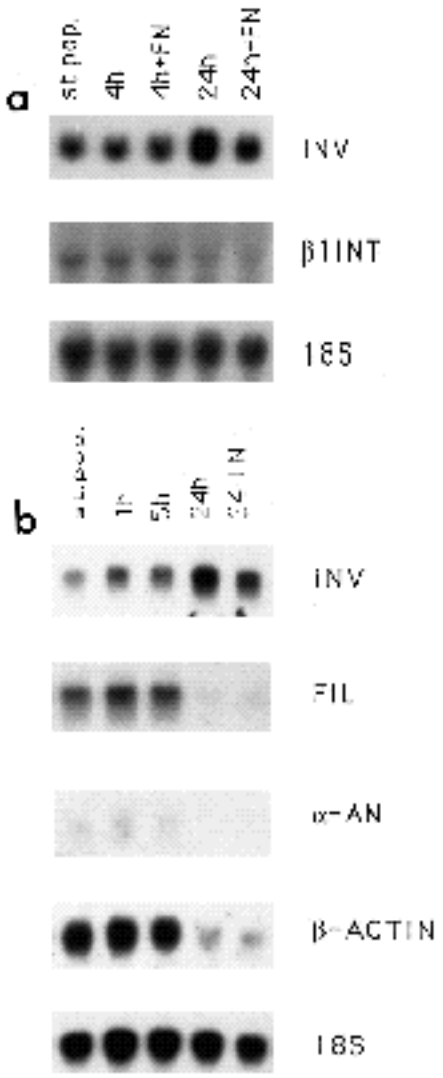

Fig. 2. Northern analysis of total RNA extracted from keratinocytes immediately after harvesting with trypsin/EDTA (st. pop) or after suspension in methyl cellulose in the presence or absence of 100 μg/ml fibronectin (FN) for the times indicated. The cDNA probes used were: involucrin (INV), β_1 integrin subunit (β_1 INT), 18 S rRNA (18S), filamin (FIL), α-actinin (α-AN) and β-actin. (a) and (b) are two separate blots.

pended for 24 hours without added fibronectin, consistent with the partial inhibition of terminal differentiation reported previously (Adams and Watt, 1989). However, β_1 mRNA levels declined to the same extent whether cells were suspended in the presence or absence of fibronectin (Fig. 2a).

Effect of fibronectin on the actin cytoskeleton

When keratinocytes adhere and spread on extracellular matrix proteins β_1 integrins localise to focal contacts in association with the ends of actin filaments (reviewed by Watt and Hertle, 1993). When keratinocytes are placed in suspension for 24 hours the cellular content of actin protein remains constant, but the levels of several proteins that are associated with focal contacts or regulate actin filament assembly decrease (Kubler et al., 1991). We therefore investigated whether the effects of fibronectin on cell surface levels of β_1 integrins were correlated with any effects on the actin cytoskeleton. We found that the steady state mRNA levels of β actin, filamin and α-actinin (and gelsolin and talin, data not shown) all decreased markedly after 24 hours in suspension (Fig. 2b) and inclusion of fibronectin in the methyl cellulose did not prevent the decline.

To test whether fibronectin influenced actin filament assembly, single cell suspensions of keratinocytes were double-labelled for filamentous actin and involucrin (Fig. 3). In freshly harvested populations (time 0) over 90% of the cells stained positive with phalloidin (Fig. 3a), whether or not they were involucrin-positive (Fig. 3b). After 24 hours in suspension, the proportion of phalloidin-positive cells decreased to approximately 60%, but again there was no correlation with involucrin expression, the percentage of involucrin-positive cells increasing from 20% to 71% (mean values) in the experiments shown in Fig. 3c. Addition of fibronectin caused some inhibition of terminal differentiation, but had no effect on the proportion of cells containing filamentous actin (Fig. 3c). Furthermore, inhibition of actin polymerisation by treatment with cytochalasin D, or of tubulin assembly by treatment with colcemid, did not overcome the inhibition of suspension-induced terminal differentiation caused by rabbit antisera to β_1 integrins (Fig. 3d and results not shown). Taken together these results demonstrate that the inhibition of suspension-induced terminal differentiation did not involve or require cytoskeletal assembly.

$\alpha_2\beta_1$ and $\alpha_3\beta_1$ can participate in the regulation of keratinocyte terminal differentiation

In order to find out more about the role of integrins in regulating terminal differentiation, we tested the effect of a range of anti-integrin monoclonal antibodies on suspension-induced terminal differentiation (Fig. 4). Since the rabbit antisera that inhibit terminal differentiation bind to both α and β_1 subunits (Adams and Watt, 1989 and unpublished observations), we began by investigating whether a monoclonal antibody that is specific for the β_1 subunit had any effect. mAb 13, an antibody to the β_1 subunit that inhibits keratinocyte adhesion to fibronectin, laminin and type IV collagen (Larjava et al., 1990; Adams and Watt, 1991), caused an inhibition of terminal differentiation comparable to that seen previously with fibronectin or the rabbit antiserum (Fig. 4a, compare with Fig. 3d). The effect was dose-dependent, with maximal inhibition at IgG concentrations of 250 μg/ml or greater. In contrast, DH12, an antibody to the β_1 subunit that does not inhibit adhesion (Adams and Watt, 1991), did not affect terminal differentiation, even at a concentration of 500 μg/ml (Fig. 4b).

We next tested monoclonals that specifically immunoprecipitate individual $\alpha\beta_1$ heterodimers, as well as α_v and α_6 subunit-specific monoclonals. None of the antibodies that lacked adhesion-blocking activity (Adams and Watt, 1991 and results not shown) had any effect on terminal differentiation (Fig. 4b). All of the α subunit-specific adhesion-blockers, including two antibodies to the α_5 subunit, also failed to inhibit terminal differentiation. However, antibodies that recognise $\alpha_3\beta_1$ and $\alpha_5\beta_1$ did cause a significant inhibition of terminal differentiation when added in com-

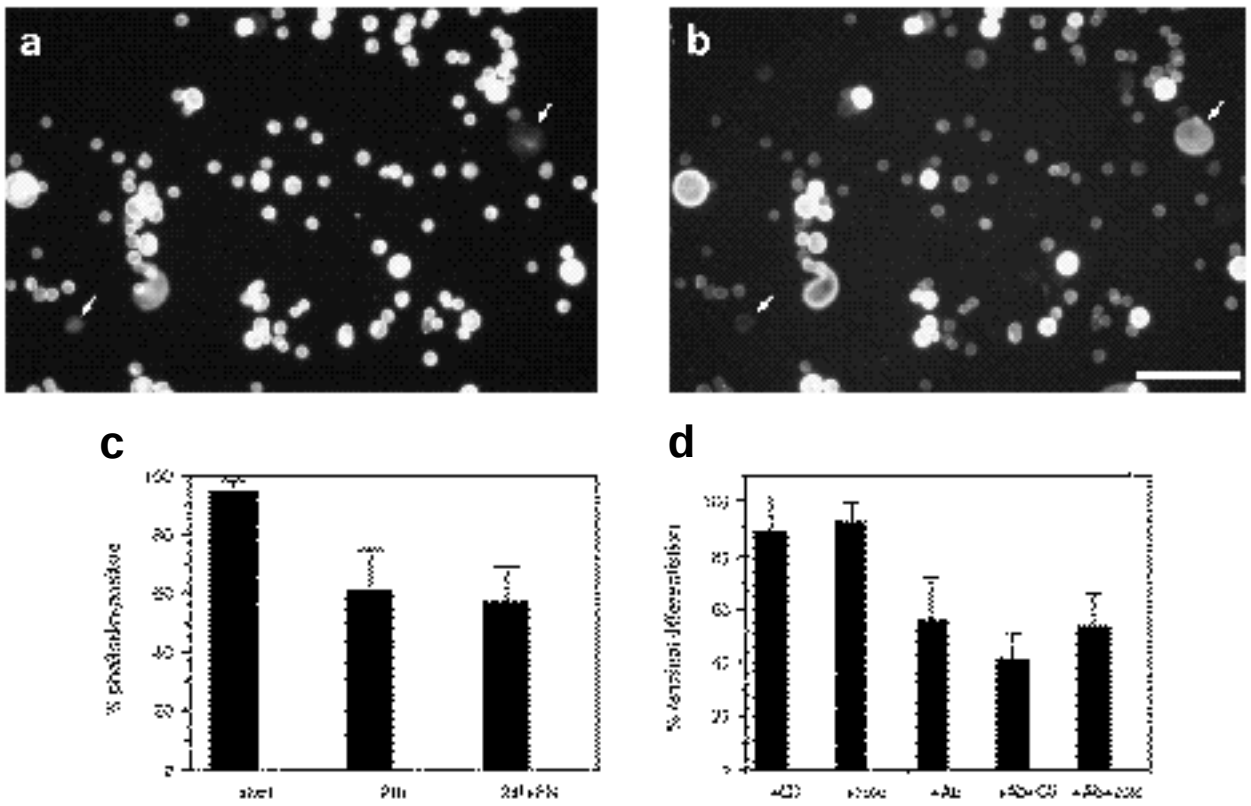

Fig. 3. The relationship between terminal differentiation and cytoskeletal assembly. (a,b) Adherent keratinocytes were harvested with trypsin/EDTA and double-labelled for phalloidin (a) and involucrin (b). Two phalloidin-negative cells are indicated by arrows; one is involucrin-positive, the other is involucrin-negative. Bar, 100 μm. (c,d) Keratinocytes were harvested with trypsin/EDTA and examined immediately (start) or after 24 hours in suspension in the presence of 100 μg/ml fibronectin (FN), 0.02 μg/ml colcemid (colc), 0.4 mg/ml rabbit antiserum raised against the human $\alpha_5\beta_1$ integrin (Ab) or 0.25 μg/ml cytochalasin D (CD). The cells were stained with Texas Red-phalloidin (c) or a monoclonal antibody to involucrin (d). Results shown are means ± s.e.m. of duplicate experiments. There is no significant difference between the percentage of phalloidin-positive cells in the presence or absence of fibronectin after 24 hours in suspension (c) nor in the % terminal differentiation in the presence or absence of CD and colc.

bination, as did a combination of antibodies to the α_2, α_3 and α_5 subunits ($P<0.05$) (Fig. 4c). In contrast, terminal differentiation in the presence of a combination of non-adhesion blocking antibodies to the α_2, α_3 and α_5 subunits was not significantly different from terminal differentiation in the presence of nonimmune IgG (Fig. 4b).

These results raised the possibility that although collagen and laminin (ligands for $\alpha_2\beta_1$ and $\alpha_3\beta_1$) do not inhibit terminal differentiation when added separately to methyl cellulose (Adams and Watt, 1989) they might have an effect when added in combination. As shown in Fig. 4d, laminin and type IV collagen in combination were not inhibitory, but when added in the presence of 25 μg/ml fibronectin (a concentration that is insufficient for full inhibition of terminal differentiation) the inhibition of terminal differentiation was comparable to that obtained with 100 μg/ml fibronectin.

DISCUSSION

The experiments we have described provide some insight into the mechanism by which integrin-mediated interactions with the extracellular matrix regulate keratinocyte terminal differentiation.

Addition of fibronectin to cells in suspension led to retention of mature β_1 integrins on the cell surface: the level of surface β_1 integrins was lower than in the starting population but higher than in cells suspended for 24 hours without fibronectin. However, fibronectin does not overcome the inhibition of *N*-linked glycosylation of newly synthesised β_1 integrin subunits (Hotchin and Watt, 1992), nor intracellular transport of under-glycosylated receptor to the cell surface. Furthermore, fibronectin did not prevent the decline in the steady state level of β_1 mRNA that occurs in suspension, which strongly implies that it does not prevent the inhibition of transcription of integrin subunit genes, since the half-life of β_1 mRNA is the same in adherent and suspended cells (Hotchin and Watt, 1992). The most likely interpretation is that fibronectin prolongs the half life of mature β_1 integrins on the cell surface and that this somehow delays the onset of terminal differentiation.

Integrin-mediated adhesion to the extracellular matrix involves clustering of integrins in focal contacts and polymerisation of the actin cytoskeleton (reviewed by Hynes, 1992). In contrast, the inhibition of suspension-induced terminal differentiation does not require receptor clustering (Adams and Watt, 1989) and the present data show that it is also independent of the state of assembly of the actin

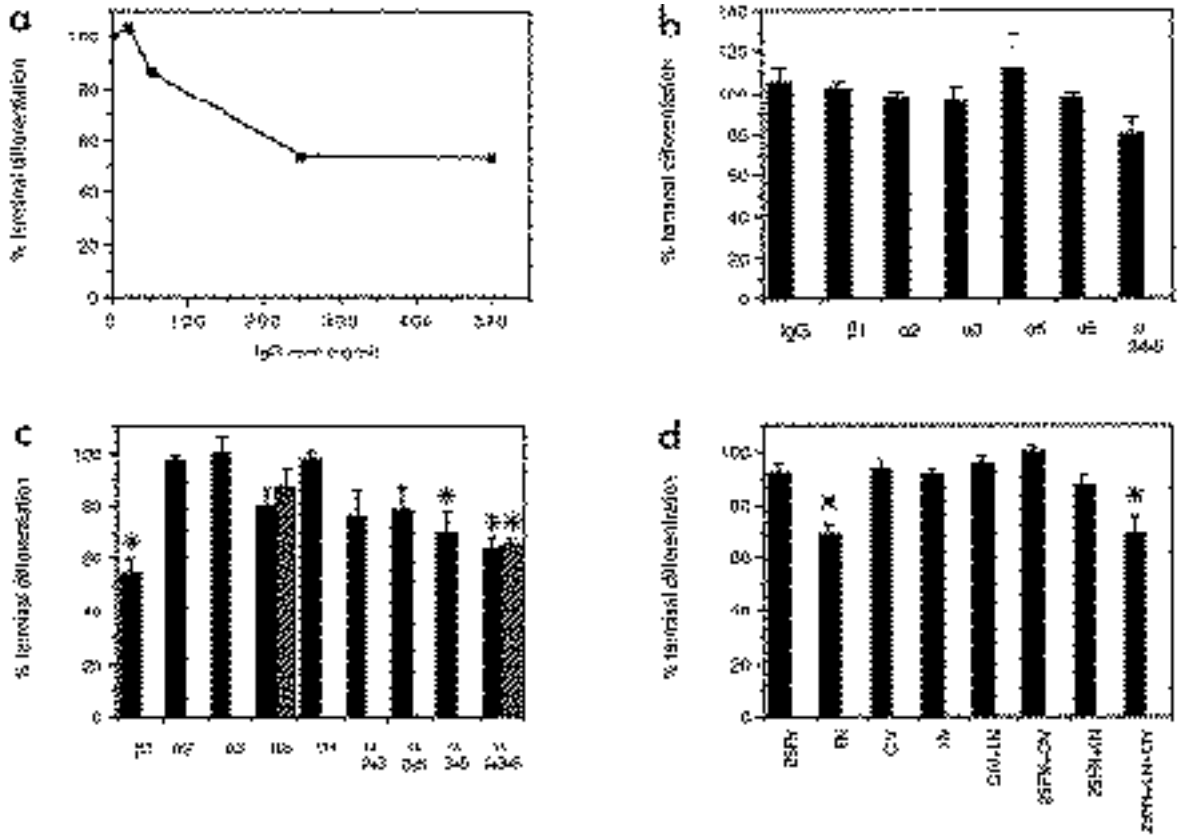

Fig. 4. The effect of anti-integrin monoclonal antibodies or extracellular matrix proteins on suspension-induced terminal differentiation. (a) Dose-response curve for mAb 13, an adhesion-blocking antibody to the β_1 subunit. (b) Antibodies that lack adhesion blocking activity. IgG, control mouse IgG (500 μg/ml); β_1, DH12 (500 μg/ml); α_2, B515 (600 μg/ml); α_3, E1/56 (500 μg/ml); α_5, mAb 16 (500 μg/ml); α_6, GoH3 (20 or 190 μg/ml). (c) Antibodies that inhibit keratinocyte adhesion to extracellular matrix proteins: β_1, mAb 13 (175 or 250 μg/ml); α_2, 5E8 (500 μg/ml); α_3, P1B5 (10 μg/ml); α_5, BIIG2 (20 μg/ml); P1D6 (hatched bars) (20 μg/ml); α_v, 13C2 (500 μg/ml). (d) Extracellular matrix proteins, laminin (LN) and type IV collagen (CIV) were added to the methyl cellulose at a final concentration of 100 μg/ml; fibronectin was added at 25 μg/ml (25 FN) or 100 μg/ml (FN). Results are means ± s.e.m. of replicate experiments. * indicates significant inhibition of terminal differentiation compared to nonimmune IgG (b,c) or 25 μg/ml fibronectin (d) (P<0.05).

cytoskeleton.In vivo, polymerised actin is detected by phalloidin staining in all layers of the epidermis (Manbridge and Knapp, 1987; Kubler and Watt, 1993), even though integrin expression is largely confined to the basal layer (Hertle et al., 1992 and references cited therein) and filamentous actin is also found in all layers of stratified keratinocyte cultures (Kubler et al., 1991). We found that the majority of cultured keratinocytes contained filamentous actin, whether or not they were involucrin-positive. The number of cells with filamentous actin declined after 24 hours in suspension whether or not fibronectin was included in the methyl cellulose, and fibronectin did not prevent the fall in steady state levels of actin, α-actinin and filamin mRNAs. Inclusion of cytochalasin D or colcemid in suspension neither inhibited terminal differentiation nor overcame the integrin-mediated inhibition of terminal differentiation.

Our data would argue that ligand binding to the β_1 integrins generates two distinct intracellular signals. One signal results in the clustering of receptors into focal contacts and polymerisation of actin filaments, providing a positive stimulus for cell adhesion and spreading. The other signal is independent of receptor clustering and cytoskeletal assembly and is a negative stimulus for differentiation. There is strong evidence that the cytoplasmic domains of both α and β integrin subunits can regulate function (reviewed by Adams and Watt, 1993) and it is therefore possible that distinct regions of the cytoplasmic domains are involved in regulating adhesion and differentiation in keratinocytes. Stable ligand-integrin binding can occur in the absence of actin polymerisation (Orlando and Cheresh, 1991). Clustering of β_1 integrins on the cell surface is associated with changes in intracellular pH (Schwartz et al., 1991) and increased tyrosine phosphorylation of a range of proteins (Kornberg et al., 1991); at present the nature of the second messenger pathways that are activated by ligand binding in the absence of clustering are unknown (Adams and Watt, 1993).

Finally, our experiments provide further insight into the nature of the terminal differentiation stimulus. The fact that only a monoclonal antibody to the β_1 subunit is sufficient, on its own, to block differentiation argues for the central importance of this subunit in regulating terminal differentiation. The inhibitory effect can be mediated by one specific $\alpha\beta_1$ heterodimer, as in the case of fibronectin binding to $\alpha_5\beta_1$ (Adams and Watt, 1989), or by all three β_1

integrins, as when laminin and type IV collagen are mixed with a low concentration of fibronectin or when α-specific monoclonals (α_3 + α_5 or α_2 + α_3 + α_5) are added to the methyl cellulose in combination. Fibronectin is more effective than laminin or collagen in supporting adhesion of cultured keratinocytes (Adams and Watt, 1990) and this may explain why individually fibronectin, but not laminin or collagen, can inhibit differentiation; it will be interesting to test the ability of epiligrin, a major component of the keratinocyte extracellular matrix and ligand for $\alpha_3\beta_1$ (Carter et al., 1991) for differentiation inhibitory activity. Taken together, our observations would suggest that terminal differentiation can be regulated by the total proportion of β_1 heterodimers occupied by ligand. This, in turn, would lend weight to the argument that the stimulus for terminal differentiation we have defined in our in vitro model is indeed physiologically relevant to intact epidermis where laminin and type IV collagen are major components of the basement membrane.

We are grateful to everyone who provided us with antibodies and cDNA probes and to Wendy Senior for excellent secretarial help.

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(Received 28 April 1993 - Accepted 3 June 1993)