

## Altered glycosylation and cell surface expression of $\beta_1$ integrin receptors during keratinocyte activation

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### Summary

We studied the mechanism by which cell adhesiveness becomes activated when keratinocytes are removed from skin and placed into cell culture. Our results suggest that activation involves altered  $\beta_1$  integrin subunit glycosylation accompanied by an increase in cell surface  $\beta_1$  integrin receptors. Activated keratinocytes contained two forms of the  $\beta_1$  integrin subunit, ~93 kDa and ~113 kDa. As shown by pulse-chase experiments, the smaller represented the cytoplasmic precursor of the larger, and only the 113 kDa mature form was detected in integrin receptors expressed at the cell surface. Pre-activated keratinocytes contained  $\beta_1$  integrin subunits ranging from ~97 to 110 kDa. These  $\beta_1$  subunits had been processed through the Golgi, based on resistance to endoglycosidase-H treatment, and were not converted to 113 kDa subunits during subsequent cell culture. Experiments with endoglycosidase-F showed that differences in the apparent sizes of  $\beta_1$  integrin subunits observed in pre-activated and activated keratinocytes could be attributed to differences in subunit glycosyla-

tion. Smaller  $\beta_1$  subunits found in pre-activated keratinocytes, like the precursor  $\beta_1$  subunits of activated cells, appeared to be less efficient in reaching the cell surface. Overall, a ~10-fold increase in the level of cell surface integrin receptors occurred concomitant with the increased proportion of 113 kDa  $\beta_1$  subunits found in activated cells. Endoglycosidase-F experiments also indicated that there were changes in keratinocyte  $\alpha$  subunits associated with  $\beta_1$ . In related experiments, keratinocytes cultured in low  $\text{Ca}^{2+}$ , serum-free MCDB medium for 4 days proliferated but their adhesiveness did not become activated. Therefore, keratinocyte proliferation and activation of adhesion are regulated separately. Finally, substantial activation of keratinocytes was observed when serum was added to cells cultured in MCDB with serum, indicating a role for serum factors in the activation process.

Key words: keratinocytes, integrins, wound repair.

### Introduction

Adhesion between cells and their extracellular matrices is important in the maintenance and regulation of cell shape, migration, growth and differentiation (Grinnell, 1978; Folkman and Moscona, 1978; Bissell et al., 1982). Studies on the biochemical mechanisms underlying cell adhesion have resulted in the identification of the integrin, selectin, cadherin and immunoglobulin families of adhesion molecules. Receptors of the integrin family mediate cell binding to matrix components such as fibronectin, vitronectin, collagen and laminin (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990; Akiyama et al., 1990; Hemler, 1990; Humphries, 1990; Springer, 1990; McDonald and Meecham, 1991). In the five years since recognition of the integrins, more than 20 distinct integrin receptors have been identified, most of which interact with more than one adhesion ligand. At least in part, the adhe-

sive behavior of cells probably depends on the timing and location of expression of these receptors.

Changes in expression of integrin receptors are likely to be of particular importance during wound repair, since normal wound healing requires that cells interact with several different extracellular matrix components (Grinnell, 1992). For instance, in normal skin, keratinocytes in the epidermal layer attach to the basement membrane by hemidesmosomes, which provide mechanical stability. Recent findings suggest that hemidesmosomes contain at least two adhesion systems: the bullous pemphigoid antigens (bp180 and bp230) (Stanley et al., 1981; Mutasim et al., 1985) and integrin  $\alpha_6\beta_4$  receptors (Kajiji et al., 1989; Carter et al., 1990; Sonnenberg et al., 1991; Jones et al., 1991). After wounding, keratinocytes switch to a migratory phenotype. There is disruption of the basement membrane, and a fibronectin-rich wound interface (Grinnell et al., 1981) provides the provisional matrix for subsequent cell

migration (Clark et al., 1982). Migrating cells show increased  $\alpha_5\beta_1$  fibronectin receptors in vivo (Clark, 1990) and in vitro (Guo et al., 1990).

Like keratinocytes from wounds, keratinocytes harvested from skin and placed into primary cell culture show activation of cell adhesion (Grinnell, 1992). This activation includes a marked increase in synthesis of fibronectin receptors (Toda et al., 1987) and  $\alpha_3\beta_1$  receptors (Klein et al., 1987). Preliminary findings suggested that this increase might result from changes in  $\beta_1$  integrin subunit processing before and after activation (Guo et al., 1991). In this study, we report that over the time course of keratinocyte activation,  $\beta_1$  integrin subunits are increasingly glycosylated and show elevated levels of cell surface expression.

## Materials and methods

### Cell culture

Human cadaver skin was obtained from the Transplant Services Center at the University of Texas Southwestern Medical Center. Epidermal cells were harvested from skin as described previously (Takashima and Grinnell, 1984) using 0.5% Dispase (Boehringer Mannheim) for 45–60 min at 37°C followed by 0.25% trypsin (GIBCO) for 10–15 min at 37°C. Harvested cells ( $5 \times 10^5/\text{cm}^2$ ) were seeded in culture flasks (Falcon) previously coated for 30 min at 37°C with 50  $\mu\text{g}/\text{ml}$  collagen (Vitrogen 100, Collagen Corp.) in Dulbecco's modified Eagle's medium (DMEM, GIBCO).

Cell cultures were maintained in a humidified incubator at 37°C with 5%  $\text{CO}_2$ . Two growth media were used for keratinocytes. Epidermal growth medium (EGM) consisted of DMEM supplemented with 10% fetal calf serum (FCS), 10% neonatal calf serum, 10 ng/ml epidermal growth factor (EGF),  $10^{-9}$  M cholera toxin, 0.4  $\mu\text{g}/\text{ml}$  hydrocortisone, 100 i.u./ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin B. MCDB medium consisted of MCDB 153 (Hazleton) supplemented with EGF, cholera toxin, hydrocortisone and antibiotics as described for EGM and 0.4% bovine pituitary extract (BPE, Clonetics) *but not serum*. Media were changed at day 1 and on alternate days thereafter. The first medium change removed non-adherent cells leaving behind >95% basal keratinocytes. The results described in this paper were collected over a period of 18 months using keratinocytes from 20–30 different patients. In general, activation of keratinocyte adhesion occurred within 4–7 days as long as the cells were viable. For unknown reasons, cells harvested from some skin specimens did not survive culture. Fibroblasts (from human foreskins) were cultured in DMEM with 20% fetal calf serum and used between the third and tenth passages.

### Radiolabeling

Keratinocytes were radiolabeled metabolically for 24 h periods (except in pulse-chase experiments) with 50  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine in the form of Trans label (ICN, >1000  $\mu\text{Ci}/\text{umol}$ ) using methionine-deficient EGM or MCDB medium. Subsequently, the cells were washed 5 times with Dulbecco's phosphate buffered saline (DPBS) (150 mM NaCl, 3 mM KCl, 6 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ ). For pulse-chase experiments, keratinocytes were radiolabeled for 4 h with 200  $\mu\text{Ci}/\text{ml}$  of [ $^{35}\text{S}$ ]methionine, washed 3 times, and then incubated with non-radioactive EGM for the times indicated.

Cell surface proteins were radiolabeled according to the method of LeBien et al. (1982). Harvested keratinocytes ( $2 \times 10^7$ ) were washed 3 times with DPBS, then resuspended in 1 ml DPBS at 4°C. Lactoperoxidase (Sigma) (200  $\mu\text{l}$ , 1 mg/ml in DPBS) was

added, followed by 20  $\mu\text{l}$  containing 2 mCi of  $\text{Na}^{125}\text{I}$  (ICN or Amersham,  $\sim 100 \mu\text{Ci}/\text{umol}$ ) and then 20  $\mu\text{l}$  of 0.12%  $\text{H}_2\text{O}_2$ . The reaction proceeded for 5 min on ice with occasional vortex mixing, at which time another 20  $\mu\text{l}$  of 0.12%  $\text{H}_2\text{O}_2$  was added and the reaction continued for 5 more minutes. The reaction was stopped by addition of 10 ml ice-cold DPBS, and the cells were washed 3 times with DPBS.

Radiolabeled cells were solubilized in 1.0 ml extraction buffer consisting of DPBS with 2% Triton X-100, 2 mM PMSF, and 10  $\mu\text{g}/\text{ml}$  leupeptin. For the experiments described in Figs 7, 8 and 10 (below) additional proteinase inhibitors 1  $\mu\text{g}/\text{ml}$  pepstatin and 10 mM EDTA also were added. After 10 min at 4°C, the extracts were centrifuged at 14,500 g (Beckman J2-21M) for 10 min, and the supernatants were flash-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until use.

### Immunoprecipitation

Immunoprecipitation of radiolabeled cell extracts ( $\sim 10^7$  cells per sample) was done as described previously (Roberts et al., 1988; Guo et al., 1991) using polyclonal rabbit anti-human fibronectin receptor antibody no. 3847 (directed predominantly against the human  $\beta_1$  subunit). Using polyclonal rather than monoclonal antibodies increased the likelihood of obtaining the full complement of  $\beta_1$  integrin receptors from the cells. Mixtures containing antibody/cell extracts and Protein A-Sepharose (Pharmacia) were centrifuged and washed 6 times with DPBS containing 1% Triton X-100, 15  $\mu\text{g}/\text{ml}$  leupeptin, and 2 mM PMSF. The pellets were then resuspended in 100  $\mu\text{l}$  of non-reducing SDS sample buffer, boiled for 4 min, and clarified by centrifugation for 5 min at 4°C (Beckman Microfuge). Samples of the supernatants were mixed with 10 ml of Budget Solve (RPI Corp.) and radioactivity measured using a scintillation counter (Beckman LS3801). Samples were adjusted with additional SDS sample buffer to obtain equal radioactivity ( $\sim 5000$  cts/min), boiled for 4 min, and then subjected to SDS-PAGE as described below.

### Endoglycosidase digestion

Endoglycosidase digestion was accomplished as described previously (Akiyama and Yamada, 1987; Akiyama et al., 1989). Immunoprecipitates were dissolved by boiling for 4 min in 10 mM sodium phosphate (pH 7.0) containing 2% SDS, 1  $\mu\text{g}/\text{ml}$  pepstatin, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM PMSF and 13  $\mu\text{M}$  EDTA. For endoglycosidase-H treatment, samples (20  $\mu\text{l}$ ) were diluted 10 times with 100 mM sodium phosphate buffer (pH 6.6) containing 0.02% azide and proteinase inhibitors as above and incubated for 24 h with 31 munits/ml endoglycosidase-H (recombinant, Boehringer-Mannheim), adding an additional 31 munits/ml of enzyme after 12 h. For endoglycosidase-F treatment, samples (8  $\mu\text{l}$ ) were diluted 5 times with 100 mM sodium phosphate buffer (pH 7) containing azide and proteinase inhibitors and incubated for 24 h at 37°C with 8 units/ml endoglycosidase-F (F/N-glycosidase, Boehringer Mannheim), adding an additional 6 units/ml enzyme after 12 h. The incubations were terminated by the addition of SDS sample buffer and samples of equal radioactivity ( $\sim 1000$  cts/min) were subjected to SDS-PAGE as described below.

### SDS-PAGE and autoradiography

SDS-PAGE was carried out according to the method of Laemmli (1970) with a 3% stacking gel and 7.5% resolving gel.  $^{14}\text{C}$ -radiolabeled proteins (Amersham) were used as size standards. Samples were subjected to electrophoresis at 40 mA for 15 min and then at 6 mA overnight. Gels were fixed in 10% ethanol, 5% acetic acid for 30 min, washed in water for 30 min, and then treated with 1 M salicylic acid (Sigma) and 2% glycerol for 30 min. Gels were dried onto filter paper at 65°C for 2 h in a gel dryer (BioRad).

Dried gels were exposed to Kodak AR X-ray film at  $-70^\circ\text{C}$  with a Kodak intensifying screen.

#### Quantification of receptor subunit expression

To compare differences in expression of the integrin subunits between different samples, autoradiographs were scanned using a scanning laser densitometer (LKB Ultrascan), and areas corresponding to the bands were quantified with a digitizing pad (Zeiss Zidas). The area of each band was divided by the total area of bands on the scanned gel and multiplied by the amount of radioactivity in the immunoprecipitate. These values were divided by total radioactivity in the cell extracts and multiplied by 100 to determine the percentage of total cell extract radioactivity in each band.

#### Reverse transcriptase-polymerase chain reaction

Amplification of cDNA fragments corresponding to the cytoplasmic domain of the human  $\beta_1$  integrin subunit was carried out using the reverse transcriptase-polymerase chain reaction technique. Total RNA preparations from human keratinocytes cultured for the time periods indicated were isolated using the guanidinium/thiocyanate/phenol-based single-step method (Chomczynski and Sacchi, 1987). A  $5\ \mu\text{g}$  sample of total RNA was hybridized to an antisense strand primer from nucleotides 2582-2601 (5' ATATTGCCCTAAAGCTACCT 3'; Argraves et al., 1987) and the first-strand cDNA was synthesized by reverse transcriptase at  $42^\circ\text{C}$ . Samples of the first-strand cDNA generated from the above reaction were used as template in polymerase chain reaction (PCR) amplification. The sense strand primer used in PCR was 5' TGGCCTTGCACTACTGCTG 3' corresponding to nucleotides 2332-2350 in the transmembrane domain of the  $\beta_1$  integrin subunit. The reaction was cycled 30 times, with denaturation for 1 min at  $94^\circ\text{C}$ , annealing for 1 min at  $60^\circ\text{C}$ , and extension for 2 min at  $72^\circ\text{C}$ . The amplified PCR product was then analyzed on a 3.5% Nusieve low-melting-point agarose gel and visualized by UV light after staining with ethidium bromide.

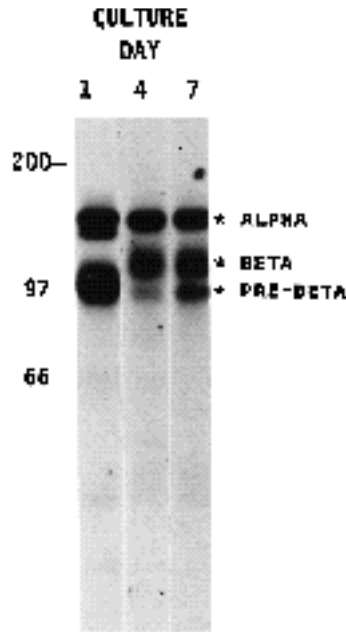
#### Attachment and spreading assay

Falcon 35 mm tissue culture dishes were coated for 10 min at  $22^\circ\text{C}$  with  $20\ \mu\text{g}/\text{ml}$  plasma fibronectin (New York Blood Center) in PBS and then counter-coated for 10 min with 1% heat-denatured bovine serum albumin as described previously (Guo et al., 1990), or the dishes were coated with collagen as described above. Keratinocytes were harvested from 4-day cultures using Dispase followed by trypsin and incubation times of 30 min and 8 min, respectively. Keratinocytes ( $10^5/\text{ml}$ ) in 2 ml of DMEM were incubated on fibronectin or collagen-coated dishes for 45 min at  $37^\circ\text{C}$ , rinsed with PBS, and fixed with 3% formaldehyde in PBS. Attachment was quantified by counting the number of attached cells in 20 separate  $0.6\ \text{mm}^2$  microscope fields. Spreading was measured by tracing the projected outline of 50 cells per dish with a computerized digitizing pad (Zeiss Zidas). Data presented are the averages  $\pm$  s.e. of duplicate samples.

## Results

#### Accumulation of keratinocyte $\beta_1$ integrin subunits after different culture periods

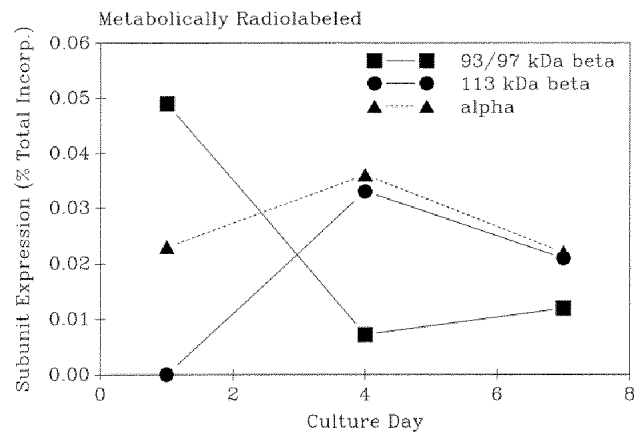
Fig. 1 shows differences between integrin receptors in keratinocytes that were cultured 1, 4 or 7 days. The cells were metabolically radiolabeled for the 24 h period prior to harvesting, and cell extracts were immunoprecipitated with polyclonal antibodies against  $\beta_1$  fibronectin receptors. Consistent with our previous observations (Guo et al., 1991), cells radiolabeled for 4 or 7 days contained a mix-



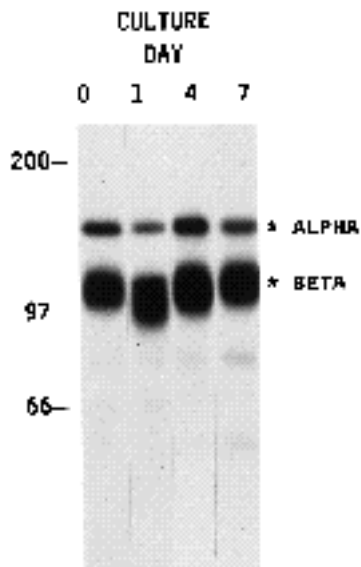
**Fig. 1.** Profile of  $\beta_1$  integrin receptors synthesized in keratinocytes cultured for different time periods. Keratinocytes metabolically radiolabeled 24 h with [ $^{35}\text{S}$ ]methionine were extracted on the culture days indicated. The extracts were immunoprecipitated with anti- $\beta_1$  antibodies and analyzed by SDS-PAGE. In cells cultured 4 days or 7 days, both  $\sim 93$  kDa (PRE-BETA) and  $\sim 113$  kDa (BETA) subunit were detected. In cells cultured 1 day,  $\beta_1$  subunits appeared to be slightly larger than pre- $\beta_1$ .

ture of  $\sim 113$  kDa beta and  $\sim 93$  kDa pre-beta (see below) subunits. In marked contrast, cells radiolabeled during the first 24 h contained only one  $\beta_1$  subunit, which was similar or slightly larger than the  $\sim 93$  kDa pre-beta subunit found in activated cells. Also, the  $\sim 140$ - $145$  kDa region contained a mixture of subunits (see Fig. 9, below).

To quantify integrin receptors, the data in Fig. 1 were analyzed by scanning densitometry and normalized for total radioactivity in the cell extracts. Fig. 2 shows that between day 1 and day 4, there was a dramatic increase in 113 kDa  $\beta_1$  subunits and decrease in the smaller  $\beta_1$  subunits. Over the same period, however, the level of subunits remained relatively constant. As will be shown below, the increase in 113 kDa  $\beta_1$  subunits was accompanied by increased cell surface expression of integrin receptors.



**Fig. 2.** Quantification of  $\beta_1$  integrin receptor synthesis. Data in Fig. 1 were quantified by scanning densitometry and normalized for radioactivity in the cell extracts. Between 1 and 4 days of culture, there was an increase in 113 kDa  $\beta_1$  subunits and a corresponding decrease in the 93/97 kDa  $\beta_1$  subunits. Over the same period, however, radiolabeling of the subunits remained relatively constant.

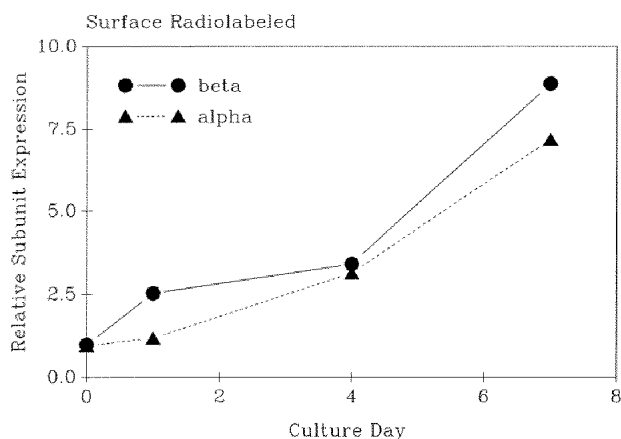


**Fig. 3.** Profile of  $\beta_1$  integrin receptors expressed on the cell surface of keratinocytes cultured for different times. Keratinocytes surface radiolabeled with  $^{125}\text{I}$  and lactoperoxidase on the culture day indicated were extracted. The extracts were immunoprecipitated with polyclonal anti- $\beta_1$  and analyzed by SDS-PAGE. In activated cells the only integrin complexes that could be detected after immunoprecipitation contained 113 kDa  $\beta_1$  subunits.

#### Cell surface expression of keratinocyte $\beta_1$ integrin subunits after different culture periods

Fig. 3 shows that when keratinocytes cultured for 4 or 7 days were surface radiolabeled, the only integrin complexes that could be detected after immunoprecipitation contained 113 kDa  $\beta_1$  subunits. The 93 kDa pre- $\beta_1$  subunits were not expressed at the cell surface, as has been reported previously for the pre- $\beta_1$  subunits of fibroblasts (Akiyama and Yamada, 1987; Hemler et al., 1987). When freshly isolated keratinocytes or cells cultured for 1 day were surface radiolabeled, the  $\beta_1$  subunits detected by immunoprecipitation appeared to be slightly smaller than the 113 kDa mature  $\beta_1$  subunit of activated cells.

To quantify integrin receptor cell surface expression, the data in Fig. 3 were analyzed by scanning densitometry and normalized for total radioactivity in the cell extracts. Fig.



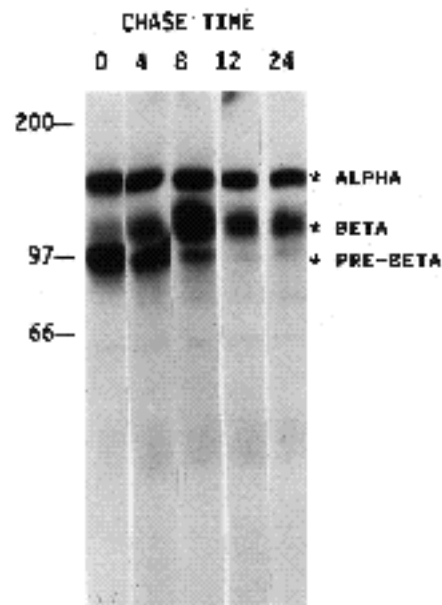
**Fig. 4.** Quantification of cell surface  $\beta_1$  integrin receptors. Data in Fig. 3 were quantified by scanning densitometry and normalized for radioactivity in the cell extracts and immunoprecipitates. Values for day 0 (freshly isolated cells) were set to 1.0. Cell surface expression of integrin receptors increased almost 10-fold in cells cultured for 7 days compared to freshly isolated cells, and the  $\alpha$  and  $\beta$  subunits increased to a similar extent.

4 shows that cell surface expression of integrin receptors increased almost 10-fold in cells cultured for 7 days compared to freshly isolated cells, and that  $\alpha$  and  $\beta$  subunits increased to a similar extent. This increase in the level of integrin receptors expressed at the cell surface correlated with the increased accumulation of mature 113 kDa  $\beta_1$  subunits in cells cultured for 4 or 7 days (Figs 1 and 2). Apparently, integrin receptors containing the smaller  $\beta_1$  subunits of pre-activated cells translocated to or were retained at the cell surface less efficiently.

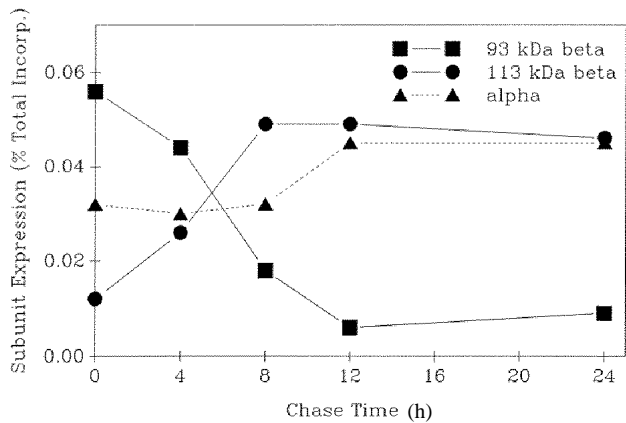
#### Processing of $\beta_1$ integrin subunits in activated keratinocytes

The presence of 93 kDa  $\beta_1$  subunits inside activated keratinocytes but not at the cell surface supported the idea that the 93 kDa component was a keratinocyte pre- $\beta_1$ . The relationship of 93 and 113 kDa components in activated cells was examined directly by pulse-chase experiments with keratinocytes that had been cultured 4 days. Fig. 5 shows the results from an experiment in which cells were radiolabeled for 4 h and then incubated in non-radioactive chase medium for up to 24 h. While primarily 93 kDa  $\beta_1$  subunits were evident at the end of the radiolabeling period, the 113 kDa  $\beta_1$  subunits became predominant by 8 h of chase conditions. At 12 and 24 h, almost all of the radiolabeled protein had been processed to the higher molecular mass form.

Fig. 6 shows the above results quantified by scanning densitometry and normalized to the amount of radioactivity in the cell extracts. Processing of 93 kDa pre- $\beta_1$  to 113



**Fig. 5.** Pulse-chase analysis of  $\beta_1$  integrin subunit processing. Keratinocytes from 4 day cultures were metabolically radiolabeled for 4 h with  $^{35}\text{S}$ methionine and then incubated in non-radioactive medium for up to 24 h. At the times indicated, the cells were extracted. The extracts were immunoprecipitated with anti- $\beta_1$  and analyzed by SDS-PAGE. Time "0" indicates the beginning of the chase period. By 8 h most of the 93 kDa subunit (PRE-BETA) had been converted to the 113 kDa subunit (BETA).



**Fig. 6.** Quantification of  $\beta_1$  integrin receptor processing. Data in Fig. 5 were quantified by scanning densitometry and normalized for radioactivity in the cell extracts. There was a precursor-product relationship between the 93 kDa and 113 kDa  $\beta_1$  subunits, and the half-time for processing was  $\sim 6$  h.

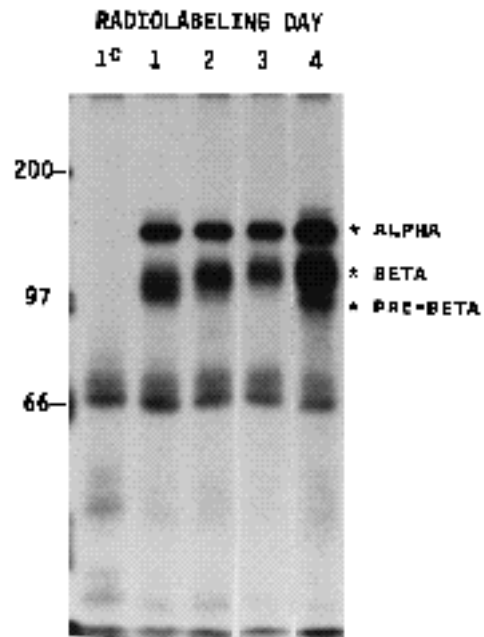
kDa mature  $\beta_1$  subunits was essentially complete by 8–12 h with a half-time of approximately 6 h, which is substantially faster than the 20 h  $\beta_1$  subunit processing time observed for fibroblasts (Akiyama and Yamada, 1987). More rapid processing of  $\beta_1$  subunits by keratinocytes compared to fibroblasts was suggested previously (Larjava et al., 1990).

#### Stability of lower molecular mass $\beta_1$ subunit after different culture periods

Long-term pulse-chase experiments were carried out to detect possible conversion of the  $\beta_1$  subunits of pre-activated keratinocytes to a higher molecular mass form. Fig. 7 shows the pattern of  $\beta_1$  integrin receptors in keratinocytes after 4 days of culture using cells that had been metabolically radiolabeled for 24 h periods during the 1st, 2nd, 3rd or 4th days of culture. (Lane 1<sup>c</sup> was immunoprecipitated with non-immune IgG.) As in Fig. 1, cells radiolabeled during the 4th culture day showed a mixture of  $\beta_1$  and pre- $\beta_1$  subunits in addition to  $\beta_1$  subunits. Cells radiolabeled during the 3rd culture day showed a  $\sim 113$  kDa  $\beta_1$  subunit but no pre- $\beta_1$ . Keratinocytes radiolabeled during the 1st or 2nd culture days contained lower molecular mass  $\beta_1$  subunits,  $\sim 106$  and  $\sim 110$  kDa, respectively. Therefore, these smaller  $\beta_1$  subunits were not processed to 113 kDa  $\beta_1$  subunits during the 48–72 h of subsequent culture even though cells developed the capacity to synthesize and accumulate the 113 kDa subunits during this time.

#### Analysis of keratinocyte integrin subunits after endoglycosidase-H treatment

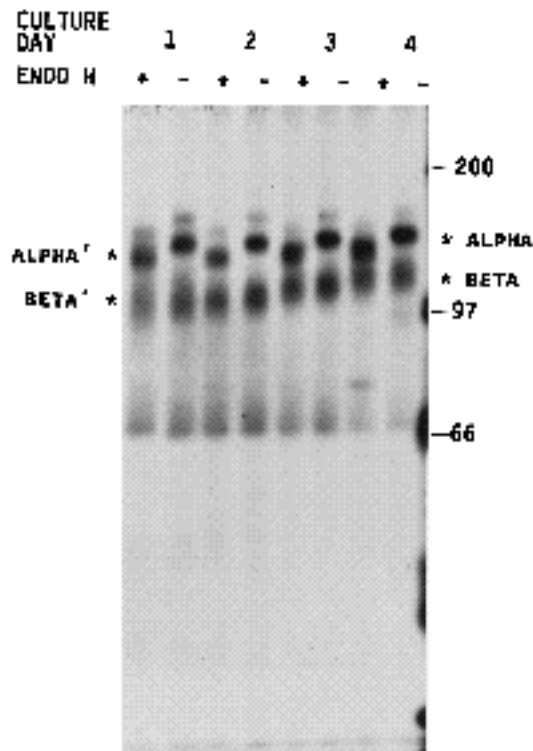
The above results suggested that the lower molecular mass  $\beta_1$  subunits in pre-activated keratinocytes, unlike 93 kDa pre- $\beta_1$ , were not precursors of the 113 kDa  $\beta_1$  subunit. To determine whether the lower molecular mass  $\beta_1$  subunits had been processed through the Golgi, we tested their sensitivity to endoglycosidase-H treatment. Fig. 8 shows results from an experiment in which cells were cultured for



**Fig. 7.** Stability of lower molecular mass  $\beta_1$  subunit after different culture periods. Keratinocytes pulsed with [<sup>35</sup>S]methionine for 24 h periods during the culture days indicated. After 4 days, the cell cultures were extracted, immunoprecipitated with anti- $\beta_1$  and analyzed by SDS-PAGE. The positions of  $\beta_1$ ,  $\beta_1$  and pre- $\beta_1$  subunits are shown for cells from day 4 cultures. Lane 1<sup>c</sup> was immunoprecipitated with non-immune IgG. Cells radiolabeled during 1st or 2nd culture days contained lower molecular mass  $\beta_1$  subunits that did not convert to the 113 kDa  $\beta_1$  subunits.

1- to 4-day periods and radiolabeled during the 24 h prior to harvesting. Each pair of lanes compares receptors after (+) and before (-) endoglycosidase-H treatment. The size of untreated  $\beta_1$  subunits ranged from  $\sim 97$  kDa in cells cultured 1 day to  $\sim 112$  kDa in cells cultured 4 days. In day 1 cultures, the  $\beta_1$  integrin subunits observed in the endoglycosidase-H-treated samples (BETA) migrated similarly to the  $\beta_1$  subunits in untreated samples. In general, regardless of the culture time, the difference in mass of the  $\beta_1$  subunits before and after endoglycosidase-H treatment was at most  $\sim 1$  kDa. This finding suggested that the subunits had been completely processed from the N-linked high-mannose form to endoglycosidase-H-resistant forms. On the other hand, most of the  $\beta_1$  subunits shifted to a lower molecular mass (ALPHA) after endoglycosidase-H treatment, consistent with previously studies on the  $\beta_1$  subunits of fibroblasts (Akiyama et al., 1989).

The endoglycosidase-H results demonstrated that the lower molecular mass  $\beta_1$  subunits in pre-activated keratinocytes were processed through the Golgi. At least two possibilities could explain the differences in molecular mass of these subunits. One was alternative splicing; at least two alternatively spliced  $\beta_1$  variants have been described (Altruda et al., 1990; Languino and Ruoslahti, 1992). The other was differences in the complex oligosaccharides added to the  $\beta_1$  subunits during processing. To distinguish between these possibilities, we searched for changes in splicing by PCR and compared receptors isolated from cul-

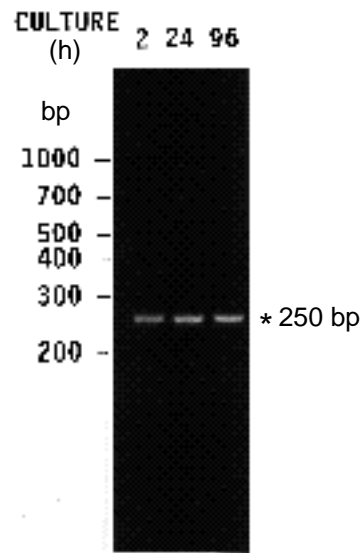


**Fig. 8.** Analysis of keratinocyte integrin subunits after endoglycosidase-H treatment. Keratinocytes were metabolically radiolabelled 24 h with [<sup>35</sup>S]methionine during the culture days indicated, and then extracted and immunoprecipitated with anti- $\alpha_5$ . Immunoprecipitates were treated with (+) or without (-) endoglycosidase-H and then analyzed by SDS-PAGE. The positions of  $\alpha_5$  and  $\beta_1$  subunits are shown for cells from day 4 cultures. The untreated  $\alpha_5$  subunits ranged from ~97 kDa in cells cultured 1 day to ~112 kDa in cells cultured 4 days, and at each time the  $\beta_1$  integrin subunit observed in the endoglycosidase-H-treated samples (BETA) migrated similarly to the  $\alpha_5$  subunits in untreated samples ( $\pm$  ~1 kDa). Most of the  $\alpha_5$  subunits shifted to a lower molecular mass (ALPHA) after endoglycosidase-H treatment.

tured keratinocytes before and after endoglycosidase-F treatment.

#### Analysis of integrin subunit alternative splicing

The only known protein structural changes in  $\alpha_5$  integrins identified to date result from alternative splicing in the cytoplasmic domain (Altruda et al., 1990; Languino and Ruoslahti, 1992). Therefore, we conducted a preliminary search for major changes in keratinocyte  $\alpha_5$  subunits by the reverse transcriptase polymerase chain reaction technique using total RNA from pre-activated and activated cells. PCR primers flanking the classical (most abundant)  $\alpha_5$  cytoplasmic domain were used to detect domains of possible altered size following reverse transcription of RNA isolated from keratinocytes. As shown in Fig. 9, samples from both pre-activated (2 h and 24 h) and activated (96 h) keratinocytes produced only a 250 bp PCR product, the predicted size of the sequence from the classical human  $\alpha_5$  cytoplasmic domain (Argaves et al., 1987), and there were no observable changes in the amount of this band.

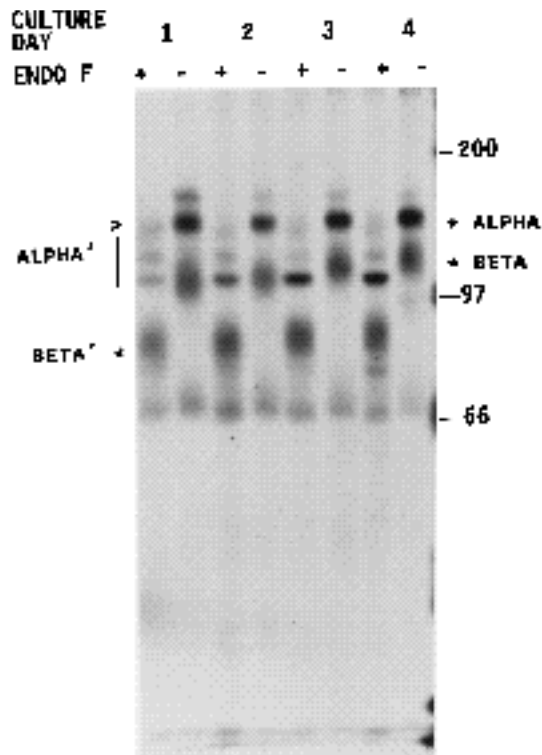


**Fig. 9.** Analysis of possible alternative splicing of keratinocyte  $\alpha_5$  integrin subunits. Total RNA samples were isolated from human keratinocytes cultured for 2, 24 or 96 h as indicated. The isolated samples were analyzed using reverse transcriptase followed by polymerase chain reaction with oligonucleotide primers located at nucleotides 2332-2350 and 2582-2601. The 250 bp fragment corresponds to the major  $\alpha_5$  integrin isoform. No minor  $\alpha_5$  integrin isoforms were detected.

#### Analysis of keratinocyte integrin subunits after endoglycosidase-F treatment

Instead of alternative splicing, changes in asparagine-linked glycosylation might have been responsible for the observed changes in  $\alpha_5$  integrin subunit size and cell surface expression. Fig. 10 shows results from an experiment analogous to that described in Fig. 8 except that each pair of lanes compares  $\alpha_5$  integrins after (+) and before (-) endoglycosidase-F treatment. After endoglycosidase-F, the  $\alpha_5$  subunits (BETA) showed little variation in molecular mass and ranged from ~84 kDa in cells from 1 day cultures to ~86 kDa in cells from in 3 or 4 days cultures. These values are close to the deduced molecular mass (86,248) of the human  $\alpha_5$  integrin subunit (Argaves et al., 1987). Therefore, the major difference in size of  $\alpha_5$  subunits observed before and after keratinocyte activation appeared to result from changes in the size or number of N-linked oligosaccharide side-chains rather than alternative splicing. Nevertheless, it should be noted that the samples from 4-day cultures contained a 76 kDa band after endoglycosidase-F treatment or endoglycosidase-H treatment (Fig. 8). The identity of this band is unknown but its size is consistent with the size of the alternatively spliced  $\alpha_5^{3v}$  subunit (Altruda et al., 1990).

Fig. 10 also shows that after endoglycosidase-F treatment, several integrin alpha subunits (ALPHA) could be resolved. After 1 day of culture, 3 different  $\alpha_5$  subunits were evident ranging from ~105 to 138 kDa. The smallest of these increased relative to the others during the 4-day culture period. In addition, a new  $\alpha_5$  subunit (>) ~146 kDa was evident beginning in the day 3 cultures. Therefore, keratinocyte activation was accompanied not only by changes



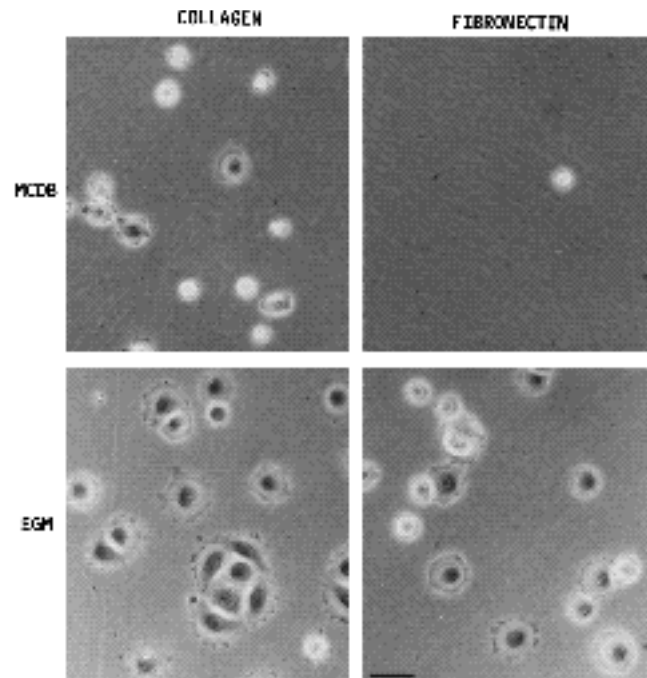
**Fig. 10.** Analysis of keratinocyte integrin subunits after endoglycosidase-F treatment. Keratinocytes were metabolically radiolabelled 24 h with [ $^{35}$ S]methionine during the culture days indicated, and then extracted and immunoprecipitated with anti- $\beta_1$ . Immunoprecipitates were treated with (+) or without (-) endoglycosidase-F and then analyzed by SDS-PAGE. The positions of  $\beta_1$  and  $\alpha_1$  subunits are shown for cells from day 4 cultures. After endoglycosidase-F, the  $\alpha_1$  subunits (BETA) showed little variation in molecular mass and ranged from ~84 kDa in 1 day cultures to ~86 kDa in 3 or 4 days cultures. Also, after endoglycosidase-F treatment, the various alpha subunits (ALPHA) could be resolved. After 1 day of cultures, 3 different subunits were evident ranging from ~105 to 138 kDa. The smallest of these increased relative to the others during the 4-day culture period. In addition, a new  $\alpha$  subunit (>) ~146 kDa was evident beginning in the day 3 cultures.

in  $\alpha_1$  subunit glycosylation but also by changes in  $\beta_1$  subunit expression.

#### *Adhesion of keratinocytes cultured in low-calcium, serum-free medium*

The changes in cell surface expression of  $\beta_1$  integrins observed over several days in primary culture could explain the activation of keratinocyte adhesion function that occurs during the same time period (Grinnell, 1992). The extracellular signal that initiates activation has yet to be determined. Since placing keratinocytes in culture exposes the cells to serum growth factors, some factor in serum might be responsible for activation. To test this idea, we studied cells cultured in MCDB medium. This serum-free, low- $\text{Ca}^{2+}$  medium supports keratinocyte proliferation and initiation of differentiation but not stratification (Hennings et al., 1980; Watt and Green, 1982; Boyce and Ham, 1983).

Keratinocytes from skin were cultured for 4 days in EGM

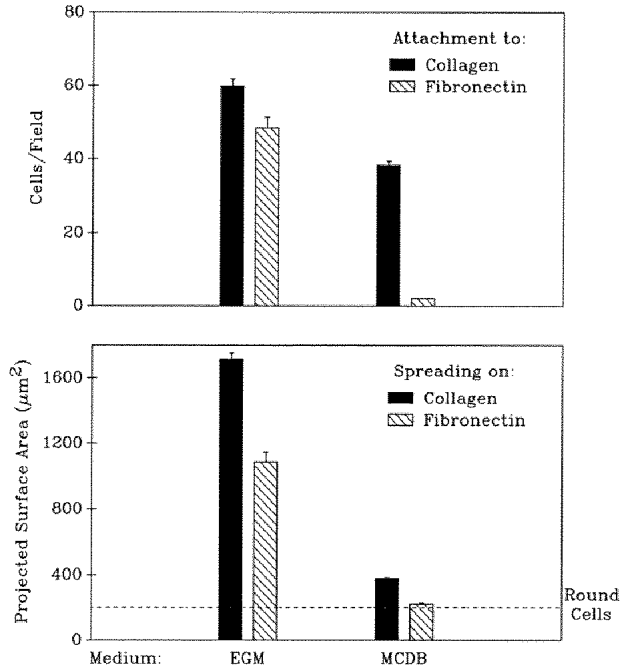


**Fig. 11.** Morphology of keratinocyte adhesion. Keratinocytes were cultured for 4 days in EGM or MCDB, harvested, and incubated for 45 min on collagen or fibronectin substrata. At the end of the incubations, the samples were fixed and photographed using a Zeiss inverted microscope. Bar, 40  $\mu\text{m}$ .

or MCDB, harvested, and tested in short-term assays (45 min) for attachment and spreading on collagen and fibronectin. Fig. 11 shows that the cells cultured in MCDB attached to collagen, but there was little spreading compared to cells cultured in EGM. Moreover, cells cultured in MCDB attached poorly to fibronectin, unlike cells cultured in EGM, which attached and spread on fibronectin. These findings were quantified by measuring attached cells per microscopic field and projected cell surface area, and the data are shown in Fig. 12. Unlike cells cultured in EGM, keratinocytes cultured in MCDB did not become activated.

In other experiments, we found that previous culture in MCDB had no adverse effects on adhesion potential. That is, changing the medium from MCDB to EGM after 4 days resulted in complete activation of keratinocyte adhesion after an additional 4 days, whereas cells kept in MCDB for 8 days remained unactivated (data not shown).

Compared to EGM medium, MCDB has a low concentration of calcium ions (0.03 mM vs. 1.8 mM) and 0.4% bovine pituitary extract but no serum. Primary keratinocyte cultures were prepared in MCDB with or without bovine pituitary extract and with high or low  $\text{Ca}^{2+}$ , but there was no activation of cell adhesiveness (data not shown). Fig. 13 shows that addition of 20% FCS to MCDB medium partially restored activation. Cells harvested from 4-day MCDB + FCS cultures attached and spread on collagen and fibronectin to a substantially greater extent than cells cultured in MCDB alone, although not as much as cells cultured in EGM. These findings suggested that activation of keratinocyte adhesion depends at least in part on exposure of the cells to a serum factor.



**Fig. 12.** Quantification of cell adhesion. Keratinocytes were cultured for 4 days in EGM or MCDB, harvested, and incubated for 45 min on collagen or fibronectin substrata. Cell attachment to collagen and fibronectin was determined by measuring the number of attached cells per microscopic field. Cell spreading was determined by measuring the projected cell surface area. Data presented are the averages + s.e. for duplicate samples.

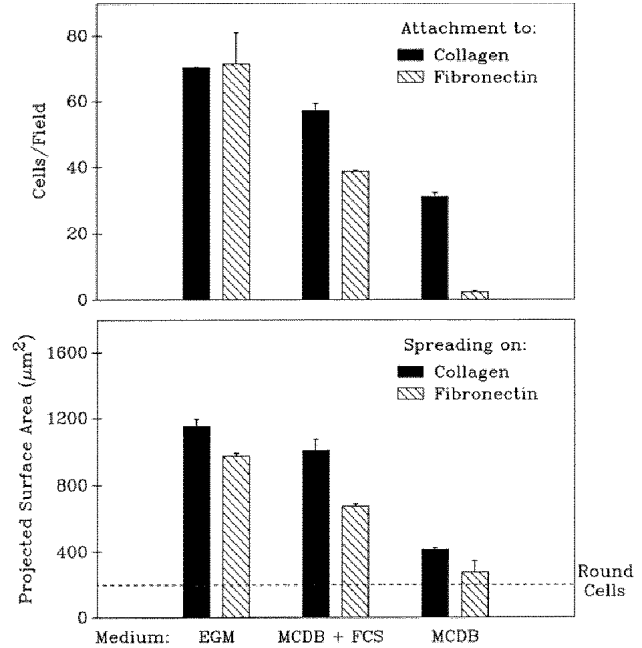
*Synthesis of β<sub>1</sub> integrin receptors by keratinocytes cultured in MCDB medium*

The results presented above show that, unlike keratinocytes cultured in EGM, keratinocytes cultured in MCDB remained unactivated. If differences in glycosylation and cell surface expression of β<sub>1</sub> subunits accounted for differences in keratinocyte adhesion activation, we would have expected keratinocytes cultured in MCDB to show lower levels of the 113 kDa β<sub>1</sub> subunit compared to cells cultured in EGM. To test this, cells cultured in MCDB or EGM for 3 days were radiolabeled metabolically, extracted, and immunoprecipitated on day 4. Fig. 14 shows that unlike cells cultured in EGM, which contained mostly 113 kDa β<sub>1</sub> subunits, cells cultured in MCDB contained primarily the 93 kDa pre-β<sub>1</sub> component.

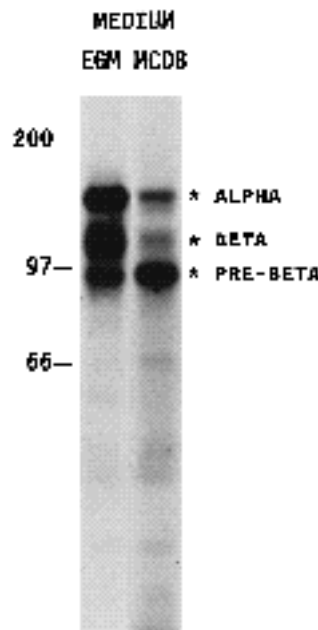
**Discussion**

Human keratinocytes harvested from skin and placed into primary cell culture show activation of adhesion at two levels: (1) specific activation of cell attachment to fibronectin and (2) general activation of cell spreading and migration on a variety of substrata including type I collagen and fibronectin (Grinnell, 1992; see Figs 11 and 12). The purpose of our studies was to analyze changes in cell adhesion receptors that could account for activation.

Consistent with previous findings (Guo et al., 1991), we observed an increase in the size of keratinocyte β<sub>1</sub> integrin



**Fig. 13.** Effect of serum on activation of cells cultured in MCDB. Keratinocytes were cultured for 4 days in EGM, MCDB + 20% FCS, or MCDB without serum, harvested, and incubated 45 min on collagen or fibronectin substrata. Cell attachment to collagen and fibronectin were determined by measuring the number of attached cells per microscopic field. Cell spreading was determined by measuring the projected cell surface area. Data presented are the averages of duplicate samples.



**Fig. 14.** Profile of β<sub>1</sub> integrin receptors synthesized by keratinocytes cultured in EGM or MCDB. Keratinocytes cultured in EGM or MCDB were metabolically radiolabelled for 24 h with [<sup>35</sup>S]methionine and extracted on the 4th day of culture. The extracts were immunoprecipitated with polyclonal anti-β<sub>5</sub> and analyzed by SDS-PAGE.

subunits during the first 4 days of cell culture. The lower molecular mass subunits were not β<sub>1</sub> subunit precursors, however. That is, subunits synthesized during the 1st two days of culture did not convert into 113 kDa β<sub>1</sub> subunits even though cells developed the capacity to synthesize and accumulate the higher form. Moreover, their resistance to



endoglycosidase-H showed that the lower molecular mass subunits had been processed through the Golgi. On the other hand, the lower molecular mass subunits did not accumulate at the cell surface as efficiently as the 113 kDa  $\alpha_1$  subunits, and along with the appearance of 113 kDa  $\alpha_1$  subunits between 4 and 7 days, there was a ~10-fold increase in the level of cell surface  $\alpha_1$  integrin receptors.

Although the smaller  $\alpha_1$  subunits in pre-activated keratinocytes were not precursors of the 113 kDa  $\alpha_1$  subunit, two sets of evidence indicated that the 93 kDa  $\alpha_1$  subunit in activated keratinocytes did correspond to a precursor form. First, pulse-chase experiments with activated keratinocytes showed a precursor-product relationship between the 93 kDa and 113 kDa components with a half-time for processing of about 6 h. Second, integrin receptors containing the 93 kDa subunit did not reach the cell surface. Previous studies showed that in fibroblasts only mature  $\alpha_1$  subunits reached the cell surface. Precursor molecules remain inside the cells (Akiyama and Yamada, 1987; Hemler et al., 1987), where they are degraded (Heino et al., 1989; De Strooper et al., 1991).

The difference between molecular masses of the  $\alpha_1$  subunits in pre-activated and activated keratinocytes could have been a consequence of alternative splicing or of differences in the sizes of oligosaccharide side-chains added to the subunits during processing. After endoglycosidase-F treatment to remove most of the N-linked oligosaccharides,  $\alpha_1$  subunits that previously ranged from 97 to 113 kDa all migrated with an apparent molecular size of 84-86 kDa, similar to that deduced from the amino acid sequence of the human  $\alpha_1$  integrin subunit (86,248 Da) (Argraves et al., 1987). Therefore, it seems likely that a single type of  $\alpha_1$  subunit with increasing degrees of glycosylation would explain the increase in the molecular mass of the  $\alpha_1$  subunits during cell culture.

Besides alternative glycosylation, alternative splicing of precursor mRNA could have resulted in changes in integrin subunit sizes. The only two forms of alternative splicing identified to date in the  $\alpha_1$  integrin subunit occur in the cytoplasmic domain. One alternative splice produces a smaller subunit (Altruda et al., 1990), and the other produces a subunit that is 27 amino acids longer (Languino and Ruoslahti, 1992). Our PCR primers spanned the commonly occurring  $\alpha_1$  cytoplasmic domain as well as the latter, larger alternative splice. We found no detectable size alteration within this domain in the  $\alpha_1$  mRNA molecules. The possibility remains that a subpopulation of  $\alpha_1$  mRNA molecules could contain a splice in the extracellular domain that has minimal effects on protein size. Also, there could be an alternative splice in the cytoplasmic domain not detected by our primers. In this regard, it is noteworthy that samples from 4-day cultures contained a new, albeit minor, 76 kDa band after endoglycosidase-F treatment or endoglycosidase-H treatment, and this size would be consistent with the size of the smaller  $\alpha_1^3$  subunit (Altruda et al., 1990).

The endoglycosidase-F experiments also permitted resolution of several subunits associated with keratinocytes  $\alpha_1$  subunits. After 1 day of culture, 3 different subunits were evident, and subsequently, the smallest of these

increased relative to the others. In addition, a new subunit appeared beginning in the day 3 cultures. Therefore, keratinocyte activation was accompanied not only by changes in  $\alpha_1$  subunit glycosylation but also by changes in subunit expression.

In trying to understand how the changes in keratinocyte integrin receptors might relate to activation of adhesion, it is important to consider that two different kinds of integrin receptor regulation have been demonstrated. In one type, changes in receptor function occur rapidly (minutes) in response to changes in receptor clustering (Detmers et al., 1987; Dustin and Springer, 1989) or conformation (Neugebauer and Reichardt, 1991; Du et al., 1991). Such a mechanism may occur in keratinocytes during differentiation since decreased adhesiveness of terminally differentiated cells occurs before loss of integrin receptors from the cell surface (Adams and Watt, 1990).

The other type of regulatory mechanism requires longer time periods (hours) and involves changes in receptor synthesis and processing, such as occurs in response to TGF $\beta$  (Roberts et al., 1988; Heino et al., 1989). In WI-38 fibroblasts, for instance, the rate of processing of  $\alpha_1$  subunits decreased from a  $t_{1/2}$  of ~10 h to ~3 h in response to TGF $\beta$ . Since there is an excess of immature  $\alpha_1$  subunits compared to subunits in fibroblasts, and selective degradation of unassociated  $\alpha_1$  precursors (De Strooper et al., 1991), the more rapid processing time in response to TGF $\beta$  was explained by increased synthesis of rate-limiting subunits (Heino et al., 1989).

Keratinocyte activation, which occurs over 4 days in culture, appears to depend on the latter type of regulatory mechanism, i.e. involving receptor synthesis and glycosylation and cell surface expression. The idea that increased cell surface expression of  $\alpha_1$  integrin receptors is responsible for apparent activation is consistent with the ability of antibodies against  $\alpha_1$  subunits to inhibit keratinocyte spreading and migration on diverse substrata such as fibronectin or type I collagen (Larjava et al., 1990; Guo et al., 1991). The precise mechanism accounting for the increase in cell surface receptors is not understood at present. Overall, there was a change in the proportion of integrin complexes that reached the cell surface but not in the total  $\alpha_1$  subunits. The observation that subunits associated with 113 kDa  $\alpha_1$  subunits were more likely to accumulate at the cell surface than subunits associated with lower molecular mass  $\alpha_1$  suggests that differential glycosylation of  $\alpha_1$  subunits may play a novel regulatory role in integrin receptor function. As already mentioned, receptors containing pre- $\alpha_1$  subunits usually remain in the cell cytoplasm (Akiyama and Yamada, 1987; Hemler et al., 1987).

How increased cell surface expression of  $\alpha_1$  integrin receptors would result in a change in cell adhesion specificity, i.e. recognition of fibronectin, is less clear. Up-regulation of specific subunits would provide the simplest explanation for the change in specificity (Toda et al., 1987). The present studies - specifically the results after endoglycosidase-F digestion - provide the first evidence for qualitative changes in subunit synthesis around the time of keratinocyte activation (i.e. day 3-4). Integrin receptors

$\alpha_3\beta_1$  and  $\alpha_5\beta_1$  have been implicated in keratinocyte adhesion to fibronectin (Carter et al., 1990; Staquet et al., 1990), but  $\alpha_5\beta_1$  was the only integrin receptor detected in binding experiments with fibronectin-Sepharose (Adams and Watt, 1990). Moreover, blocking  $\alpha_5\beta_1$  receptors with anti- $\alpha_5$  antibodies inhibited keratinocyte migration on fibronectin whereas blocking  $\alpha_3\beta_1$  receptors with anti- $\alpha_3$  subunits increased keratinocyte migration on fibronectin (Kim et al., 1992).

Turning from the change in integrin receptors during activation to the signal that triggers keratinocyte activation, one explanation we considered previously was control of activation by tissue architecture, i.e. in response to loss of contact between cells and the basement membrane. To test this possibility, keratinocyte harvested from skin were reconstituted in a tissue-like structure in vitro by allowing cells to attach to dermis with a complete basement membrane (Grinnell et al., 1987). Since cell activation still occurred under these conditions, loss of cell attachment to the basement membrane probably is not the regulatory parameter (Guo et al., 1990).

Another possibility, which we tested in the present study, was control by cell proliferation, i.e. activation in response to rapid proliferation that follows loss of epidermal integrity. To test this idea, we studied cells cultured in MCDB medium. In this medium, the cells proliferate and begin differentiation but do not form cell-cell junctions or stratify (Hennings et al., 1980; Watt and Green, 1982; Boyce and Ham, 1983). Keratinocytes cultured in MCDB medium for 4 days did not show activation of adhesion, which indicates that keratinocyte proliferation and adhesion activation are regulated separately. Partial activation of keratinocytes occurred, however, when cells were cultured in MCDB with serum added, which suggests a role for serum factors in the activation process. This finding is consistent with many studies showing that wound healing is initiated and regulated, at least in part, by polypeptide growth factors released by platelets and leukocytes at the wound site (e.g. see Ross et al., 1986; Sporn et al., 1987). In future experiments, we hope to identify the soluble factor(s) responsible for keratinocyte activation.

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