Differentiation-related changes in glycoprotein synthesis by human keratinocytes

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Human keratinocytes were cultured in media in which the Ca^{2+} concentration controlled the stage of differentiation. In media containing $< 0.1 \text{ mM-Ca}^{2+}$ keratinocytes grew as a monolayer, but in the presence of 2 mM-Ca^{2+} the cells differentiated and formed stratified colonies. Glycoproteins of both stratified and unstratified cells were radiolabelled by metabolic incorporation of radioactive precursors and by cell-surface labelling using galactose oxidase/ NaB^3H_4 . The radiolabelled keratinocytes were extracted with 0.5% Triton X-100, and the glycoproteins in both the Triton X-100-soluble and Triton X-100-insoluble fractions were analysed by polyacrylamide-gel electrophoresis in the presence of SDS. Two Triton X-100-soluble glycoproteins with high M_r values ($> 200\,000$) were major glycoproteins in stratified keratinocytes, but were present in only trace amounts in unstratified keratinocytes. Characterization of these glycoproteins by examination of the effect of tunicamycin on their synthesis and the effect of neuraminidase on their migration characteristics showed that they were cell-surface sialoglycoproteins containing *O*-glycosidically linked oligosaccharides. Analysis of the adherent cytoskeletons left after Triton X-100 extraction of stratified and unstratified keratinocytes revealed that a glycoprotein of $M_r 184\,000$ was decreased in stratified keratinocytes. Incubation of unstratified keratinocytes in high- Ca^{2+} medium resulted in a rapid modification of the glycoprotein of $M_r 184\,000$, and it is suggested that this event may be related to desmosome formation and stratification.

INTRODUCTION

In the mammalian epidermis mitosis is restricted to the basal layer, and cells that leave it undergo terminal differentiation to form in turn the spinous, granular and cornified (stratum corneum) layers. Changes in the adhesive interactions of keratinocytes during differentiation are probably important in determining their positions within the different cell layers and in regulating epidermal structure (Skerrow, 1978; Watt, 1984). There are now several lines of evidence implicating glycoconjugates in the processes of cell recognition and adhesion (Hughes & Pena, 1981; Roos, 1984), and we have identified keratinocyte glycoproteins which have a role in intercellular adhesion (Roberts & Brunt, 1985) and in cell–substratum adhesion (Roberts & Jenner, 1983). Differentiation-related changes in membrane glycoproteins in the mammalian epidermis have been detected by using fluorescein-conjugated lectins (Holt *et al.*, 1979; Brabec *et al.*, 1980; Reano *et al.*, 1982; Nemanic *et al.*, 1983; Bell & Skerrow, 1984) and blood-group-specific antibodies (Dabelsteen *et al.*, 1984). Biochemical identification of the glycoproteins involved in these changes is difficult with the intact epidermis, because of the problem of obtaining keratinocyte populations in different stages of differentiation without utilizing proteolytic digestion for dispersion of the cells. However, Rheinwald & Green (1975) have demonstrated that keratinocytes can be cultured *in vitro* in the presence of irradiated 3T3 cells, and Hennings *et al.* (1980) have shown that the differentiation of keratinocytes can be modulated by varying the Ca^{2+} concentrations of the medium. This culture system has proved very useful for investigating biochemical and morphological aspects of keratinocyte

differentiation (Hennings *et al.*, 1980; Brysk & Snider, 1982a; Watt & Green, 1982; Stanley & Yuspa, 1983; Watt, 1984; Watt *et al.*, 1984).

In the present study we have compared the glycoproteins synthesized by human keratinocytes cultured in media containing high and low Ca^{2+} concentrations. We have shown that keratinocytes induced to differentiate and stratify by culture in the presence of 2 mM-Ca^{2+} synthesized two surface sialoglycoproteins with high M_r ($> 200\,000$), which were greatly decreased in unstratified keratinocytes cultured in media containing $< 0.1 \text{ mM-Ca}^{2+}$. In addition we have shown that a glycoprotein, of $M_r 184\,000$, associated with the Triton X-100-insoluble adherent cytoskeleton from unstratified keratinocytes was modified in the early stages of Ca^{2+} -induced stratification.

EXPERIMENTAL

Materials

Tunicamycin was a gift from Dr. W. F. J. Cuthbertson, Glaxo Group Research Ltd., Greenford, Middx., U.K. L-[1- ^3H]Fucose (6.23 Ci/mmol), D-[1- ^{14}C]glucosamine hydrochloride (61 mCi/mmol), [^{14}C]methylated protein M_r markers and NaB^3H_4 (10 Ci/mmol) were supplied by Amersham International (Amersham, Bucks., U.K.). Dulbecco's modified Eagle's medium, Eagle's minimum essential medium, newborn-calf serum and 35 mm-diam. plastic dishes were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Dulbecco's modified Eagle's medium without Ca^{2+} was purchased from Imperial Laboratories (Europe) Ltd. (Salisbury, Wilts., U.K.). Foetal-calf serum was obtained from Sera-Lab Ltd.

(Crawley Down, Sussex, U.K.). Cholera toxin and 25 cm² tissue-culture flasks were purchased from Becton Dickinson (Wembley, Middx., U.K.). Epidermal growth factor, *N*-ethylmaleimide, galactose oxidase, bovine testicular hyaluronidase, cortisol, insulin, mitomycin C, neuraminidase type VI from *Clostridium perfringens*, transferrin and tri-iodothyronine were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Chelex 100 (200–400 mesh; Na⁺ form) was obtained from Bio-Rad Laboratories (Watford, Herts, U.K.).

Cell cultures

Human keratinocytes isolated from newborn foreskins were grown in the presence of 3T3 cells pretreated with mitomycin C (4 µg/ml) for 2 h (Rheinwald, 1980). The high-Ca²⁺ (2.0 mM) medium used in this study was Dulbecco's modified Eagle's medium supplemented with 5% (v/v) foetal-calf serum, cortisol (0.4 µg/ml), 0.1 nm-cholera toxin, transferrin (5 µg/ml), insulin (5 µg/ml), epidermal growth factor (10 ng/ml) and 20 pM-tri-iodothyronine (Watt & Green, 1982). The low-Ca²⁺ (<0.1 mM) medium used was either Dulbecco's modified Eagle's medium without Ca²⁺ or Eagle's minimum essential medium with the same additives as for the high-Ca²⁺ medium, except that the foetal-calf serum was depleted of Ca²⁺ by treatment with Chelex 100 resin (Brennan *et al.*, 1975). No differences in the morphology or glycoprotein patterns were detected in keratinocytes cultured in these two low-Ca²⁺ media. Cultures were grown in 35 mm culture dishes at 37 °C in a mixture of CO₂/air (1:19). First- or second-passage cultures were grown until they were 80–100% confluent, and then the 3T3 cells were removed with EDTA (Rheinwald, 1980) before radiolabelling of the keratinocytes. In some experiments tunicamycin (5 µg/ml) was added to the culture medium.

Radiolabelling of keratinocytes

Metabolic labelling with D-[1-¹⁴C]glucosamine (4 µCi/ml) or L-[1-³H]fucose (20 µCi/ml) was carried out by addition of the radioisotope to fresh medium (0.75 ml) and incubation at 37 °C for 24 h. Externally exposed glycoproteins on keratinocytes were labelled by digestion with galactose oxidase followed by reduction with NaB³H₄ as described previously (Roberts & Jenner, 1983).

Extraction of keratinocytes

Radiolabelled cells were extracted with 3 × 0.5 ml of 0.5% Triton X-100 in 50 mM-Tris/HCl (pH 7.5)/0.14 M-NaCl/10 mM-EDTA/10 mM-*N*-ethylmaleimide/1.5 mM-MgCl₂/2 mM-phenylmethanesulphonyl fluoride at 20 °C for 15 min. The residue was solubilized in electrophoresis sample buffer [2% (w/v) SDS in 0.05 M-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.001% Bromophenol Blue, 0.14 M-2-mercaptoethanol and 2 mM-phenylmethanesulphonyl fluoride] at 100 °C for 5 min. The first Triton X-100 extract was freeze-dried, extracted with acetone (2 ml) and then solubilized in electrophoresis sample buffer at 100 °C for 5 min.

Enzyme digestions

Triton X-100 extracts of keratinocytes were digested with neuraminidase (0.4 unit/ml) or hyaluronidase (0.013 unit/ml) at 37 °C for 1 h. The digests were freeze-dried, extracted with acetone (2 ml) and then

solubilized in electrophoresis sample buffer at 100 °C for 5 min.

Polyacrylamide-gel electrophoresis

Samples were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) with gel slabs containing a linear gradient of 5–15% (w/v) polyacrylamide in the separating gel and a 5% (w/v) stacking gel. Proteins were detected with Coomassie Blue R 250, and radiolabelled glycoproteins were detected by autoradiography or fluorography as described previously (Roberts & Jenner, 1983). The gels were calibrated with [¹⁴C]methylated protein *M_r* markers. Autoradiograms and fluorograms were scanned with a Kipp & Zonen densitometer.

Quantitative determinations

Protein content and radioactivity were measured as described previously (Roberts & Jenner, 1983).

RESULTS

Comparison of glycoproteins in Triton X-100 extracts from stratified and unstratified cells

Keratinocytes grown in media containing low Ca²⁺ concentrations (<0.1 mM) proliferate as a monolayer with wide intercellular spaces and no desmosomes. In contrast, when the cells are cultured in media containing high Ca²⁺ (2.0 mM), proliferation of the cells is accompanied by differentiation to yield colonies of stratified cells in which, as in intact epidermis, mitosis is restricted to the basal layer, and cells terminally differentiate as they migrate through the outer layers (Green, 1977). In this study we refer to the cells grown in high Ca²⁺ as stratified keratinocytes, and to the cells grown in low Ca²⁺ as unstratified keratinocytes. Extraction of cultured keratinocytes with 0.5% Triton X-100 removes most of the proteins and glycoproteins from the cytoplasm and plasma membranes, leaving an adherent cytoskeleton composed of the cytoskeleton and nuclear matrix adherent to the substratum (Roberts & Jenner, 1983). Comparison of the Triton X-100 extracts from stratified and unstratified keratinocytes metabolically labelled with D-[1-¹⁴C]glucosamine revealed that two glycoproteins with high *M_r* (>200 000) were major components in stratified keratinocytes, but were not detected in unstratified keratinocytes (Figs. 1a and 1c). Under these labelling conditions, approx. 22% and 15% of the radioactivity from glucosamine incorporated into keratinocytes was redistributed into galactosamine and sialic acid respectively (G. P. Roberts & J. Brunt, unpublished work).

Stratified keratinocytes also incorporated L-[1-³H]-fucose into the two high-*M_r* glycoproteins (Fig. 2a). Incubation of the Triton X-100 extract from L-[1-³H]fucose-labelled keratinocytes with neuraminidase resulted in an alteration in the migration characteristics of these glycoproteins, with the appearance of two new radiolabelled bands of lower intensity, one migrating between the two original glycoproteins and the other remaining at the origin (Fig. 2b). The reason for the decrease in intensity in the bands after neuraminidase digestion is uncertain, but it might be caused by a decrease in solubility of the glycoproteins.

Cell-surface labelling of keratinocytes with galactose oxidase/NaB³H₄ resulted in intense labelling of the

Fig. 1. Effect of tunicamycin on Triton X-100-soluble glycoproteins from stratified and unstratified keratinocytes

Keratinocytes were metabolically labelled with D-[1- 14 C]glucosamine in the presence (*b* and *d*) or absence (*a* and *c*) of tunicamycin (5 μ g/ml) in high- Ca^{2+} medium (*a* and *b*) and low- Ca^{2+} medium (*c* and *d*). Triton X-100 extracts were prepared and analysed by SDS/polyacrylamide-gel electrophoresis, and glycoproteins were detected by autoradiography. The autoradiograms were scanned as described in the text. The M_r values of protein standards run in parallel are given at the top of the scans. The arrowheads show the position of the two glycoproteins with high M_r (> 200 000), the long arrows indicate the glycoprotein of M_r 126 000, and the short arrow shows the position of the origin. The amounts of protein and radioactivity applied to the gels were: (*a*) 90 μ g, 72 000 d.p.m.; (*b*) 90 μ g, 47 000 d.p.m.; (*c*) 35 μ g, 25 000 d.p.m.; (*d*) 35 μ g, 8000 d.p.m. The time of exposure of the autoradiograms in (*c*) and (*d*) was increased to compensate for the lower amounts of protein applied to the gel.

slower-running high- M_r glycoprotein and faint labelling of the faster-running high- M_r glycoprotein on stratified cells, but no significant labelling of these glycoproteins on unstratified cells (Fig. 3, lanes 1 and 2). When this labelling procedure was combined with neuraminidase digestion of stratified keratinocytes, two intense bands were obtained, one at the origin and another migrating between the two original glycoproteins (Fig. 3, lane 3). Only trace amounts of these bands were detected when unstratified keratinocytes were subjected to the same procedure (Fig. 3, lane 4). These two glycoproteins with high M_r on stratified keratinocytes were only very faintly stained with Coomassie Blue R 250. In a previous study (Roberts & Jenner, 1983) it was shown that hyaluronic acid is synthesized by human keratinocytes and that this component migrates close to the origin under the electrophoresis conditions used in the present study. However, hyaluronic acid was mainly recovered in the Triton X-100-insoluble fraction, and the properties of the

two high- M_r glycoproteins described above are incompatible with either of these being hyaluronic acid. Confirmation of this was obtained by incubation of the Triton X-100 extracts of D-[1- 14 C]glucosamine-labelled keratinocytes with hyaluronidase, which had no effect on these two high- M_r glycoproteins. Extraction of freeze-dried Triton X-100 extracts with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.) did not remove the radioactivity from the two high- M_r bands. This eliminated the possibility that glycolipids rather than glycoproteins were responsible for these bands.

In addition to the two high- M_r glycoproteins, other less well-marked differences were detected in the Triton X-100-soluble glycoproteins from stratified and unstratified keratinocytes. Variation in the relative intensities of some of the radiolabelled bands occurred, and there was some additional fainter bands in extracts from stratified keratinocytes (Fig. 1). However, a surface glycoprotein

Fig. 2. Effect of neuraminidase on L-[1-³H]fucose-labelled glycoproteins from stratified keratinocytes

Keratinocytes were metabolically labelled with L-[1-³H]fucose in high-Ca²⁺ medium for 24 h and then extracted with 0.5% Triton X-100. The Triton X-100 extracts were either freeze-dried and analysed (a) or treated with neuraminidase for 1 h at 37 °C before being freeze-dried and analysed (b). The samples were analysed by SDS/polyacrylamide-gel electrophoresis and the glycoproteins were detected by fluorography and quantified by densitometry as described in the text. The M_r values of protein standards run in parallel are given at the top of the scans. Approx. 90 μ g of protein containing 250 000 d.p.m. was loaded on the gels. The arrowheads show the position of the two glycoproteins with high M_r (> 200 000) and the arrow shows the position of the origin.

with M_r 126 000, previously found on stratified keratinocytes and shown to be important in intercellular adhesion (Roberts & Brunt, 1985), was also detected on unstratified keratinocytes.

Effect of tunicamycin on D-[1-¹⁴C]glucosamine incorporation into keratinocytes

The nature of the carbohydrate-protein linkage in the glycoproteins synthesized by stratified and unstratified keratinocytes was examined by addition of tunicamycin

Fig. 3. Surface labelling of stratified and unstratified keratinocytes

Keratinocytes were cultured in high-Ca²⁺ medium (lanes 1 and 3) or low-Ca²⁺ medium (lanes 2 and 4) and surface labelled with galactose oxidase/NaB³H₄ (lanes 1 and 2) or neuraminidase/galactose oxidase/NaB³H₄ (lanes 3 and 4) as described in the text. The labelled keratinocytes were extracted with 0.5% Triton X-100, and the extracts were freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis. Labelled cell-surface glycoproteins were detected by fluorography. The amounts of protein and radioactivity applied to the gel were: lane 1, 70 μ g containing 550 000 d.p.m.; lane 2, 40 μ g containing 370 000 d.p.m.; lane 3, 40 μ g containing 640 000 d.p.m.; lane 4, 25 μ g containing 490 000 d.p.m. The positions and M_r values ($\times 10^{-3}$) of protein standards run in parallel are shown on the right, and the arrowheads on the left indicate the two glycoproteins with M_r > 200 000.

(5 μ g/ml) to the culture medium. This antibiotic inhibits the synthesis of dolichyl *N*-acetylglucosamine diphosphate, an intermediate required for the synthesis of *N*-glycosidically linked oligosaccharides (Struck & Lennarz, 1977). Synthesis of most of the Triton X-100-soluble glycoproteins from unstratified keratinocytes was markedly inhibited by tunicamycin (Fig. 1d). Inhibition of the synthesis of glycoproteins with M_r 60 000–200 000 by stratified keratinocytes also occurred in the presence of tunicamycin (Fig. 1b). Although tunicamycin inhibited

Fig. 4. Comparison of glycoproteins in the adherent cytoskeletons of unstratified and stratified keratinocytes

Keratinocytes were metabolically labelled with D-[1-¹⁴C]-glucosamine for 24 h in (a) low-Ca²⁺ medium or (b) high-Ca²⁺ medium. The cells were extracted with 0.5% Triton X-100 and the adherent cytoskeletons were dissolved in electrophoresis sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. Radiolabelled glycoproteins were detected by autoradiography and quantified by densitometry. The M_r values of protein standards run in parallel are given at the top of the scans. The arrowheads show the positions of the glycoproteins of M_r 184000 and 53000, and the arrow shows the position of the origin. The amounts of protein and radioactivity loaded on the gels were: (a) 100 μ g, 6000 d.p.m.; (b) 145 μ g, 24000 d.p.m.

the synthesis of glycoproteins, which produced a disperse background labelling in the upper region of the gel, the two main glycoproteins with $M_r > 200000$ were synthesized in its presence (Fig. 1b). This indicates that the oligosaccharides are linked to protein cores by O-glycosidic linkages in these two glycoproteins. Further

Fig. 5. Ca²⁺-induced changes in glycoproteins from the adherent cytoskeleton of unstratified keratinocytes

Keratinocytes were metabolically labelled with D-[1-¹⁴C]-glucosamine in low-Ca²⁺ medium for 24 h. The keratinocytes were then processed for analysis directly (lane 1) or subjected to a further incubation in high-Ca²⁺ medium without D-[1-¹⁴C]glucosamine for a further 4 h (lane 2). The cells were extracted with 0.5% Triton X-100 and the adherent cytoskeletons were dissolved in electrophoresis sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. Radiolabelled glycoproteins were detected by autoradiography. The arrowheads show the positions of the glycoproteins of M_r 184000 and 154000. Approx. 125 μ g of protein containing 11000 d.p.m. was loaded on the gel. The positions and M_r values ($\times 10^{-3}$) values of protein standards run in parallel are shown on the right of the autoradiogram.

evidence to support this conclusion was obtained by examination of the effect of alkali on these glycoproteins. Incubation of Triton X-100 extracts with 0.05 M-NaOH at 37 °C for 16 h resulted in elimination of the two glycoproteins with $M_r > 200000$.

Comparison of glycoproteins in Triton X-100-insoluble adherent cytoskeletons from stratified and unstratified keratinocytes

One of the most marked differentiation-related changes in glycoproteins associated with the adherent cytoskeletons was the decreased radiolabelling of a glycoprotein with M_r 184000 [this glycoprotein was previously reported to have an M_r of 180000 (Roberts & Jenner, 1983)] in the stratified keratinocytes (Fig. 4). When unstratified keratinocytes, which had been radio-labelled with D-[1- 14 C]glucosamine, were incubated in high- Ca^{2+} medium for 4 h, there was a decrease in the glycoprotein of M_r 184000 and an increase in a glycoprotein of M_r 154000 (Fig. 5). In this time period there is a rapid reorganization of keratin filaments and desmosome formation (Watt *et al.*, 1984). Incubation of D-[1- 14 C]glucosamine-labelled unstratified keratinocytes in low- Ca^{2+} medium without D-[1- 14 C]glucosamine for 4 h did not result in any change in the glycoprotein of M_r 184000. This eliminates the possibility that the decrease in this glycoprotein observed in the high- Ca^{2+} medium was the result of a rapid turnover rate.

Another marked difference in the glycoproteins of the adherent cytoskeletons from stratified and unstratified keratinocytes was the increased amounts of a glycoprotein with M_r 53000 in the stratified keratinocytes (Fig. 4). We have recently found that this glycoprotein is a glycosylated keratin (G. P. Roberts & J. Brunt, unpublished work).

DISCUSSION

The most marked difference in the Triton X-100-soluble glycoproteins from stratified and unstratified keratinocytes was the large amount of two high- M_r *O*-glycosylated sialoglycoproteins on the cell surface of stratified keratinocytes. These may contribute to the differentiation-related changes in lectin-binding properties of epidermal cells (Holt *et al.*, 1979; Brabec *et al.*, 1980; Reano *et al.*, 1982; Zieske & Bernstein, 1982; Nemanic *et al.*, 1983; Bell & Skerrow, 1984). We have found that these surface sialoglycoproteins do bind some lectins (G. P. Roberts & J. Brunt, unpublished work). Zieske & Bernstein (1982) used glycosidases to uncover new lectin-binding activities on epidermal cells, and concluded that epidermal differentiation was accompanied by the masking of α -D-galactose residues by addition of α -L-fucose residues. Analysis of the distribution of A, B and H blood-group antigens in human epidermis also suggested a stepwise building up of carbohydrate chains with blood-group specificities during epidermal differentiation (Dabelsteen *et al.*, 1984). It will therefore be important in future studies to establish whether these blood-group specificities are carried by the two high- M_r sialoglycoproteins on the keratinocyte surface. Carbohydrate differentiation antigens I, i and SSEA-1 on other cell types have been shown to be carried by high- M_r glycoproteins (Childs *et al.*, 1983). The significance of the increase in *O*-glycosylation in stratified keratinocytes is uncertain, but it is noteworthy that an increase in *O*-glycosylation of glycophorin, the major erythrocyte glycoprotein, occurs during the differentiation of erythrocytes (Gahmberg *et al.*, 1984).

The glycoprotein changes during Ca^{2+} -induced differentiation of mouse keratinocytes have been examined previously by Brysk & Snider (1982a,b) using ^{125}I -

labelled lectins. Although a number of differentiation-related changes were detected, an increased synthesis of high- M_r (>200000) sialoglycoproteins was not found in differentiated mouse keratinocytes. The discrepancy between these results and ours may be caused by the species difference in the keratinocytes used or by the different analytical methods employed.

In the present investigation we have shown that a prominent glycoprotein with M_r 184000 in the adherent cytoskeleton of unstratified keratinocytes is rapidly modified when the Ca^{2+} concentration is raised. It has been shown previously that increasing the concentration of Ca^{2+} rapidly induces desmosome formation and stratification in the absence of new protein or glycoprotein synthesis (Watt *et al.*, 1984). The temporal relationship of these events with the modification of the glycoprotein of M_r 184000 suggests that this glycoprotein may be involved in desmosome formation and stratification. Two possible roles are that the glycoprotein of M_r 184000 is a precursor of a desmosomal glycoprotein (possibly the glycoprotein of M_r 154000) or that modification of the glycoprotein of M_r 184000 has a permissive effect on desmosome formation and stratification. We favour the latter possibility, because it has been shown previously that the neutral proteinase Dispase cleaves the glycoproteins of M_r 184000 and 154000 (Roberts & Brunt, 1985) without breaking desmosomal contacts (Kitano & Okada, 1983). A permissive effect may function by decreasing the substrate adhesiveness of keratinocytes committed to terminal differentiation, thus allowing them to move to a suprabasal position (Watt & Green, 1982).

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