Glycoproteins and glycosaminoglycans synthesized by human keratinocytes in culture

Their role in cell–substratum adhesion

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Glycoproteins and proteoglycans synthesized by human keratinocytes in medium containing D-[1-¹⁴C]glucosamine were extracted and analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Extraction of the labelled keratinocytes with 0.5% Triton X-100 removed most of the glycoconjugates and left the cytoskeleton and nuclear residue adherent to the substratum. In addition to the cytoskeletal proteins, there was a relatively simple profile of glycoproteins and glycosaminoglycans associated with this adherent cytoskeleton. These consisted of eight glycoproteins in the mol.wt. range 99 000–232 000, five proteins in the keratin region (mol.wt. 42 000–61 000), hyaluronic acid and a sulphated glycosaminoglycan. Surface labelling of the keratinocytes with galactose oxidase (with or without neuraminidase)/KB³H₄ revealed that many of the glycoproteins were exposed on the cell surface. The importance of the glycoproteins and proteoglycans in attaching the keratinocytes to the substratum was examined by studying their expression after incubation in medium containing tunicamycin and their degradation after digestion with trypsin and hyaluronidase. These studies, together with an examination of the glycoconjugates released by sequential extraction with 0.5% Triton X-100 followed by 0.2% sodium dodecyl sulphate, revealed that the glycoprotein of mol.wt. 232 000 has an important role in mediating the attachment of keratinocytes to the substratum.

With the exception of the circulating cells in the vascular system, most eukaryotic cells exist attached to other cells, to intercellular substances such as collagen or to a basal lamina. Similarly, attachment to a substratum is a requirement for the growth of normal cells in culture, and this requirement has been termed anchorage dependence (Stoker *et al.*, 1968). In the malignant state cells tend to lose their anchorage dependence. An understanding of the molecular basis of cellular adhesion and an explanation of how this interaction contributes to the formation of tissues is a fundamental goal of many studies on developmental biology and tumour biology. There are now several lines of evidence implicating cell-surface glycoconjugates in the process of cell–cell adhesion and cell–substratum adhesion (Hughes & Pena, 1981). Extensive studies

on a major cell-surface glycoprotein called fibronectin have shown it to be one of the main agents in mediating the adhesion and spreading of fibroblasts on solid supports (Yamada & Olden, 1978). Epithelial cells differ from fibroblasts in that fibronectin does not stimulate their attachment to substratum. However, it has been shown that laminin, a glycoprotein component of basement membranes, will stimulate the attachment of a transformed line of mouse epidermal cells to plates coated with type IV collagen (Terranova *et al.*, 1980).

In the mammalian epidermis, mitosis occurs only in the innermost cells or basal layer, which are in close proximity to the basal lamina. These cells then move upwards and differentiate to form in turn the spinous, granular and cornified (stratum corneum) layers. Adhesive forces operating in the intercellular space may be of importance in regulating epidermal structure, and it is probable that these adhesive interactions change with differentiation (Skerrow,

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

1978). Furthermore, studies using lectin binding (Holt *et al.*, 1979; Brabec *et al.*, 1980) show that changes in glycoconjugate synthesis occur as epidermal cells differentiate. When skin slices were incubated in medium containing radiolabelled sugars, a complex mixture of epidermal glycoconjugates was synthesized (King *et al.*, 1980; Roberts & Marks, 1982). The investigation of the function of epidermal glycoconjugates in cellular adhesion was not easily accomplished with skin slices, and consequently the glycoconjugates synthesized by human keratinocytes cultured *in vitro* as described by Rheinwald & Green (1975) have now been examined.

In the present paper evidence is reported that a glycoprotein of apparent mol.wt. 232000 has an important role in attaching cultured keratinocytes to the substratum.

Experimental

Materials

Tunicamycin was a gift from Dr. W. F. J. Cuthbertson, Glaxo Group Research Ltd., Greenford, Middx., U.K. Dulbecco's minimum essential medium, Ca^{2+} -free Eagle's minimum essential medium, foetal-calf serum, calf serum, penicillin-streptomycin, Hepes and 30mm plastic tissue culture dishes were obtained from Flow Laboratories, Irvine, Scotland, U.K. Cholera toxin and 25cm² tissue-culture flasks were purchased from Becton Dickinson, Wembley, Middx., U.K. Cortisol, bovine testicular hyaluronidase (1.7 units/mg), protease type VI from *Streptomyces griseus*, trypsin type I from bovine pancreas, galactose oxidase (14.6 units/mg), neuraminidase type VI (2.5 units/mg) and protein A-Sepharose CL-4B were from Sigma, Poole, Dorset, U.K. Reagents for electrophoresis and general chemicals were purchased from BDH Chemicals, Poole, Dorset, U.K. L-[6-³H]-Fucose (23 Ci/mmol), D-[6-³H]glucosamine hydrochloride (22.6 Ci/mmol), D-[1-¹⁴C]glucosamine hydrochloride (61 mCi/mmol), L-[U-¹⁴C]leucine (339 mCi/mmol), [¹⁴C]methylated protein molecular-weight markers, ³⁵SO₄²⁻ and KB³H₄ (4.3 Ci/mmol) were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Osray M3 X-ray film was from Agfa-Gevaert, Dunstable, Beds., U.K., and X-Omat RP X-ray film was obtained from Kodak, Western Sales Centre, Bristol, U.K. Sheep antiserum to laminin was obtained from Dr. S. I. Katz, National Institute of Health, Bethesda, MD, U.S.A., and rabbit antiserum to human plasma fibronectin was purchased from Bethesda Research Laboratories (U.K.) Ltd., Cambridge, U.K. Protein A-bearing *Staphylococcus aureus* cells were obtained from Calbiochem-Behring Corp., Bishops Cleeve, Herts., U.K.

Cell cultures

Suspensions of trypsin-treated human epidermal cells were obtained from foreskins (donor age range 2–6 years) and cultured in the presence of irradiated mouse embryo fibroblasts by the method Rheinwald & Green (1975) as modified by Dykes *et al.* (1982). Feeder layers of fibroblasts were removed with 0.02% EDTA, and keratinocytes were subcultured by using trypsin-EDTA (Rheinwald & Green, 1975). In some experiments tunicamycin (1 mg/ml in 25 mM-NaOH) was added to the culture medium to give a final concentration of 2 µg/ml.

Radiolabelling of keratinocytes

After the keratinocytes had become 50–100% confluent, the fibroblast feeder layers were removed with EDTA. Metabolic labelling with L-[6-³H]-fucose (25 µCi/ml), D-[6-³H]glucosamine (25 µCi/ml), D-[1-¹⁴C]glucosamine (8 µCi/ml), L-[U-¹⁴C]leucine (2 µCi/ml) or ³⁵SO₄²⁻ (66 µCi/ml), either singly or in various combinations, was performed by addition of the radioisotope to fresh medium (1 ml) and incubation at 37°C for 24 h. Externally disposed glycoproteins on keratinocytes were labelled by KB³H₄ reduction of cells treated with galactose oxidase or with neuraminidase and galactose oxidase by the method of Gahmberg & Hakomori (1973) with minor modifications. The keratinocytes were first washed five times with phosphate-buffered saline (15 mM-sodium phosphate/140 mM-NaCl), pH 7.0 (4 ml), and then incubated with galactose oxidase (16 units) with or without neuraminidase (0.5 unit) in phosphate-buffered saline, pH 7.0 (0.5 ml), at 20°C for 1 h. After removal of the supernatant the cell layers were washed five times with phosphate-buffered saline, pH 7.2 (2 ml), and then reduced with 2.5 mCi of KB³H₄ in phosphate-buffered saline, pH 7.2 (0.5 ml), at 20°C for 30 min. After both labelling procedures the cell layers were washed six times with phosphate-buffered saline, pH 7.2 (4 ml), and harvested as described below.

Extraction of keratinocytes

Two different methods of extraction were used.

(a) The labelled keratinocytes were extracted three times with 0.5% Triton X-100 in phosphate-buffered saline, pH 7.4 (0.5 ml), containing 2 mM-phenylmethanesulphonyl fluoride, at 20°C for 20 min each extraction. The residue was solubilized in 0.25 ml of 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.

(b) The cells were extracted three times with 0.5% Triton X-100 as in (a) and the residue was then extracted first with 0.2% SDS (0.5 ml) at 20°C for

20 min and then with 0.25 ml of 0.2% SDS containing 0.14 M-2-mercaptoethanol at 20°C for 20 min. Before electrophoretic examination the extracts were freeze-dried, extracted with acetone (1 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 in a discontinuous buffer system (Laemmli, 1970). Gel slabs were 1.5 mm thick, with a linear gradient of 5–15% (w/v) polyacrylamide in the separating gel and a 5% (w/v) stacking gel. After electrophoresis for 18 h at constant voltage (80 V) the gels were stained with Coomassie Blue R250. Detection of ^{14}C -labelled compounds was by exposure of dried gels to Osray M3 X-ray film. Fluorography (Bonner & Laskey, 1974) with pre-sensitized X-Omat RP films (Laskey & Mills, 1975) was used to detect ^3H -labelled compounds. The gels were calibrated with [^{14}C]methylated protein molecular-weight markers, namely myosin (200 000), phosphorylase *b* (92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 300).

Radioactivity determination

Radioactivity was measured in a Rackbeta liquid-scintillation counter 12515 (LKB Wallac, Turku, Finland). The scintillation medium used contained 3.33% (w/v) 2,5-diphenyloxazole and 0.2% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene/Triton X-100 (2:1, v/v).

Enzyme digestions

Keratinocytes were digested with 0.2% trypsin in phosphate-buffered saline, pH 7.4, at 37°C for 15 min. Cell layers were digested with hyaluronidase (0.03 unit) in phosphate-buffered saline, pH 7.0 (0.25 ml), at 37°C for 1.5 h.

Isolation and characterization of glycosaminoglycans

Extracts of keratinocytes labelled with D-[6- ^3H]-glucosamine and $^{35}\text{SO}_4^{2-}$ were freeze-dried, and the residues were extracted with acetone to remove Triton X-100 or with acetone/triethylamine/acetic acid/water (17:1:1:1, by vol.) to remove SDS (Henderson *et al.*, 1979). The residues were digested with 0.25 ml of protease type VI (2 mg/ml) in 0.5 M-Tris/HCl buffer, pH 8.0, containing 2 mM- CaCl_2 and a drop of toluene. After incubation at 37°C for 24 h the digest was adjusted to 10% (w/v) trichloroacetic acid and left at 4°C for 1 h. The precipitate was removed by centrifugation at 16 000 g for 5 min and the supernatant was dialysed against water. Unlabelled hyaluronic acid, dermatan sulphate and heparin (50 μg of each) were added and

all the glycosaminoglycans were precipitated with 3 vol. of 5% (w/v) potassium acetate in ethanol at 4°C overnight. Precipitated glycosaminoglycans were collected by centrifugation and redissolved in 10 μl of water. The glycosaminoglycans were separated by electrophoresis on cellulose acetate strips in 0.05 M-phosphate buffer, pH 7.2 (Breen *et al.*, 1970), and the radioactivity in the individual glycosaminoglycans was determined as described by King (1981).

Immunoprecipitations

Immunoprecipitations were performed essentially as described by Kessler (1976). Labelled keratinocytes were extracted as described above and the 0.2%-SDS extracts were diluted with Nonidet P40 to give a final detergent concentration of 0.1% SDS/0.5% Nonidet P40 in 15 mM-Tris/HCl (pH 7.5)/0.14 M-NaCl/15 mM- MgCl_2 /2 mM-phenylmethanesulphonyl fluoride. After incubation with the appropriate undiluted antisera (10 μl) for 2 h at 20°C, the antigen-antibody complexes were precipitated by addition of protein A-bearing *Staphylococcal aureus* cells [100 μl of a 10% (w/v) suspension] or by addition of protein A-Sepharose CL-4B [50 μl of a 10% (w/v) suspension]. The samples were incubated for 1 h at 20°C and then centrifuged (16 000 g for 2 min). After the precipitates had been washed with 5 $\times$ 0.5 ml of 0.1% SDS/0.5% Nonidet P40 in 15 mM-Tris/HCl (pH 7.5)/0.14 M-NaCl/15 mM- MgCl_2 /2 mM-phenylmethanesulphonyl fluoride, the immune complexes were eluted from the pellets with electrophoresis sample buffer at 100°C for 5 min. The supernatants obtained after centrifugation were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Extraction of labelled keratinocytes with Triton X-100

Extraction of other cultured cells with non-ionic detergents such as Triton X-100 has been shown to extract most of the proteins and glycoproteins from the cytoplasm and plasma membranes, leaving the cytoskeleton and a nuclear residue adherent to the substratum (Osborn & Weber, 1977). A similar result was obtained by extracting human keratinocytes with 0.5% Triton X-100, and in the present paper the material left adherent to the substratum has been called the 'adherent cytoskeleton', since it appears to contain macromolecular components of the adhesive substance and the cytoskeleton, i.e.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of keratinocyte proteins after extraction with 0.5% Triton X-100

Human keratinocytes were incubated in medium containing L-[U- 14 C]leucine for 24 h and the cells were then extracted with 0.5% Triton X-100. The extract and the adherent cytoskeleton were analysed on SDS/polyacrylamide-gradient (5–15% w/v) slab gels as described in the Experimental section. About 50 μ g of protein was loaded on the gels. Lanes (a) and (c) show proteins in the adherent cytoskeleton, and lanes (b) and (d) show proteins in the 0.5% Triton X-100 extract. Lanes (a) and (b) were stained for protein with Coomassie Blue and lanes (c) and (d) are autoradiographs.

components of 'stick' and 'grip' (Rees *et al.*, 1977). The proteins extracted into 0.5% Triton X-100 cover a wide molecular-weight range (<14 300 to >200 000), whereas the major proteins left adherent to the substratum occur grouped together in the molecular-weight range 42 000–61 000 after electrophoresis under reducing conditions (Fig. 1). The patterns of proteins labelled by incubation with L-[U- 14 C]leucine were very similar to those revealed by staining with Coomassie Blue in both the Triton X-100 extract and the adherent cytoskeleton. However, the glycoconjugates metabolically labelled with

Fig. 2. Effect of tunicamycin and trypsin digestion on glycoconjugates associated with the adherent cytoskeletons

Keratinocytes were cultured in the presence of D-[1- 14 C]glucosamine for 24 h and then extracted with 0.5% Triton X-100. The adherent cytoskeleton was dissolved in electrophoresis sample buffer and analysed on SDS/polyacrylamide-gradient (5–15% w/v) slab gels. The labelled glycoconjugates were detected by autoradiography. Lane (a), adherent cytoskeleton of keratinocytes grown in normal medium; lane (b), keratinocytes grown in medium containing tunicamycin (2 μ g/ml); lane (c), keratinocytes grown in normal medium and then digested with 0.5% trypsin before extraction with 0.5% Triton X-100. Approx. 50 μ g of protein was applied to all the gels.

D-[1- 14 C]glucosamine differed considerably from the protein patterns. In the adherent cytoskeleton there were eight large glycoproteins, with apparent mol.wts. 232 000, 194 000, 180 000, 165 000, 148 000, 129 000, 114 000 and 99 000, and five labelled bands in the keratin region (mol.wts. 42 000–61 000) (Fig. 2). The molecular-weight values were means from seven different experiments

and, since glycoproteins bind less SDS than do proteins on a weight basis (Pitt-Rivers & Impiombato, 1968), these are only approximate values. Of the large glycoproteins, those with mol.wts. 232 000, 180 000, 148 000 and 129 000 were the most intensely labelled, and the intensities of the other bands varied in different experiments. One band, of mol.wt. 54 000, in the keratin region was consistently well labelled in all experiments, but the intensities of the other bands in this region depended on the conditions used for their detection. They were relatively intense when autoradiography was used for detection, but weak when fluorography was employed.

In addition to the glycoproteins described above, electrophoresis of the adherent cytoskeleton revealed an intensely labelled band close to the origin in the stacking gel. The radioactivity in gel slices from this area was rapidly solubilized by digestion with hyaluronidase, but not by digestion with protease type VI from *Streptomyces griseus*. On prolonged incubation (3 days at 37°C) with protease type VI a small amount of radioactivity was solubilized, and this was shown to be incorporated into proteoglycans by precipitation with cetylpyridinium chloride followed by salt fractionation as described by Saarni & Tammi (1977). The glycosaminoglycans synthesized by keratinocytes cultured in the presence of D-[6-³H]glucosamine and ³⁵SO₄²⁻ were characterized by electrophoresis on cellulose acetate. Hyaluronic acid accounted for 62% of the D-[6-³H]glucosamine in the glycosaminoglycans, and a further 30% was recovered in the area corresponding to heparan sulphate. A small amount (8%) of the D-[6-³H]glucosamine was incorporated into material migrating in the same region as chondroitin 4-sulphate, dermatan sulphate and chondroitin 6-sulphate. The identity of hyaluronic acid was confirmed by the fact that it did not contain ³⁵SO₄²⁻ and it was degraded by hyaluronidase.

A similar profile of glycoconjugates was labelled with L-[6-³H]fucose and D-[6-³H]glucosamine in both the Triton X-100 extracts and the adherent cytoskeleton, with the exceptions that the proteoglycan band was missing and labelling in the keratin region of the adherent cytoskeleton was decreased with L-[6-³H]fucose.

Effect of tunicamycin, trypsin and hyaluronidase

The importance of the glycoconjugates present in the adherent cytoskeleton in attaching the keratinocytes to the substratum was examined by investigating the effect of tunicamycin, trypsin and hyaluronidase on detachment of keratinocytes and also their effect on the glycoconjugates. When keratinocytes were cultured in the presence of tunicamycin (2 µg/ml) for up to 5 days, the keratinocytes remained attached to the substratum.

Under these conditions irradiated fibroblasts became detached during the first 24 h. The electrophoretic pattern of the adherent cytoskeleton from keratinocytes labelled with D-[1-¹⁴C]glucosamine in the presence of tunicamycin for 24 h is shown in lane (b) of Fig. 2. Labelling of the seven glycoproteins with mol.wts. in the range 99 000–194 000 was greatly decreased relative to the labelling of the glycoprotein of mol.wt. 232 000. The proteoglycans and labelled bands in the keratin region were not affected by tunicamycin.

Digestion of D-[1-¹⁴C]glucosamine-labelled keratinocytes with 0.2% trypsin at 37°C for 15 min resulted in detachment and dispersal of the cells. Extraction of these cells with 0.5% Triton X-100 followed by electrophoretic examination of the residue left from both the cells and the plastic surface resulted in the autoradiogram shown in lane (c) of Fig. 2. Whereas the labelled bands in the keratin regions were not affected by digestion with trypsin, the amounts of the glycoproteins in the mol.wt. range 99 000–232 000 were greatly decreased, and most of the proteoglycans were released from the cells.

Removal of hyaluronic acid by digestion of keratinocytes with hyaluronidase did not result in detachment of the cells. However, a sulphated glycosaminoglycan associated with the adherent cytoskeleton was resistant to hyaluronidase.

Sequential extraction of labelled keratinocytes with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol

Keratinocytes labelled with D-[1-¹⁴C]glucosamine were extracted sequentially with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol at 20°C. The adherent cytoskeleton left after extraction with 0.5% Triton X-100 started to detach from the substratum in large sheets on incubation in 0.2% SDS. The ease with which the adherent cytoskeleton detached varied in different experiments and appeared to decrease with increasing culture time. After removal of the supernatant, the sheets and remaining adherent cytoskeleton were extracted with 0.2% SDS/0.14 M-2-mercaptoethanol. This treatment solubilized the adherent cytoskeleton. An autoradiograph of the electrophoretic patterns of the three extracts is shown in Fig. 3. A complex mixture of glycoconjugates was extracted with Triton X-100, whereas the 0.2% SDS/0.14 M-2-mercaptoethanol extract contains a simpler mixture similar to that of the total adherent cytoskeleton shown previously (Fig. 2, lane a). In the 0.2%-SDS extract there was considerable enrichment of the glycoprotein of mol.wt. 232 000 relative to the other glycoproteins. This glycoprotein was not bound by anti-laminin or anti-fibronectin sera in immunoprecipitation experi-

Fig. 3. Sequential extraction of D-[1- 14 C]glucosamine-labelled keratinocytes with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol

Keratinocytes cultured in the presence of D-[1- 14 C]glucosamine for 24 h were extracted sequentially with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol as described in the Experimental section. The extracts were freeze-dried and analysed by SDS/polyacrylamide-gradient (5–15%, w/v) gel electrophoresis. The labelled glycoconjugates were detected by autoradiography. Lane (a), 0.5% Triton extract (30 μ g of protein); lane (b), 0.2% SDS extract (7 μ g of protein); lane (c), 0.2% SDS/0.14 M-2-mercaptoethanol extract (18 μ g of protein).

ments. Some glycosaminoglycan was also extracted, and a similar experiment with keratinocytes labelled with D-[6- 3 H]glucosamine and 35 SO $_4^{2-}$ revealed that some of the glycosaminoglycan was sulphated. Electrophoresis of the glycosaminoglycans on cellulose acetate revealed that hyaluronic acid (90%) was the major glycosaminoglycan, with small amounts (10%) of material with the migration characteristics of heparan sulphate.

In addition to the glycoprotein of mol.wt. 232 000 and glycosaminoglycan, the 0.2%-SDS extract contained a small amount of a glycoprotein with mol.wt. 47 000. However, this material may be an intracellular component since it was not destroyed by trypsin (Fig. 2, lane c).

In order to examine the possibility that incomplete removal of fibroblasts was contributing to the glycoconjugates found in the adherent cytoskeletons of keratinocytes, irradiated fibroblasts were metabolically labelled with D-[1- 14 C]glucosamine for 24 h. The labelled fibroblasts were extracted sequentially with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol, and the extracts were examined by SDS/polyacrylamide-gel electrophoresis and autoradiography. The 0.5%-Triton-X-100 extract contained a complex mixture of glycoconjugates, some of which corresponded to labelled bands found in the Triton X-100 extract from keratinocytes. However, the 0.2%-SDS and 0.2%-SDS/0.14 M-2-mercaptoethanol extracts from fibroblasts differed from the corresponding extracts from keratinocytes. Only a glycoprotein band of mol.wt. 221 000, together with a faint band in the glycosaminoglycan region, was found in the 0.2%-SDS extract and no bands were detected in the 0.2%-SDS/0.14 M-2-mercaptoethanol extract from fibroblasts. Furthermore, when a heavy inoculum of keratinocytes was subcultured for a short time in the absence of fibroblasts, the keratinocytes did adhere and spread on to the Petri-dish surface. These keratinocytes were labelled with D-[1- 14 C]glucosamine and subjected to sequential extraction with 0.5% Triton X-100, 0.2% SDS and 0.2% SDS/0.14 M-2-mercaptoethanol. Electrophoretic analysis of these extracts revealed that the glycoproteins synthesized were similar to those shown in Fig. 3, thus demonstrating that these glycoproteins were not products of the fibroblasts.

Surface labelling of keratinocytes with galactose oxidase/KB 3 H $_4$

Further characterization of the glycoconjugates involved in cell-substratum adhesion was performed by labelling the glycoconjugates exposed on the surface of keratinocytes with galactose oxidase/KB 3 H $_4$ either with or without neuraminidase digestion. The labelled keratinocytes were extracted with 0.5% Triton X-100 and the adherent cytoskeleton was examined by SDS/polyacrylamide-gel electrophoresis. Fluorographs of the electrophoretic patterns are shown in Fig. 4. Neuraminidase digestion increased the labelling intensity of the bands, but otherwise the electrophoretic patterns of keratinocytes labelled with and without neuraminidase digestion were similar. Comparison of the electrophoretic patterns of adherent cytoskeletons from metabolically labelled keratinocytes (Fig. 2,

Fig. 4. Surface labelling of keratinocytes with galactose oxidase/ KB^3H_4 .

Keratinocytes were surface labelled by incubation with galactose oxidase or neuraminidase and galactose oxidase, followed by reduction with KB^3H_4 , as described in the Experimental section. The labelled keratinocytes were extracted with 0.5% Triton X-100 and the adherent cytoskeletons were analysed on SDS/polyacrylamide-gradient (5–15%, w/v) slab-gels. The labelled glycoconjugates were detected by fluorography. The Figure shows the glycoconjugates of the adherent cytoskeleton after labelling with (a) galactose oxidase/ KB^3H_4 and (b) neuraminidase/galactose oxidase/ KB^3H_4 . About 20 μg of protein was loaded on the gels.

Discussion

Extraction of cultured cells with non-ionic detergents has proved useful for detecting the cytoskeletal filaments which remain firmly attached to the substratum (Osborn & Weber, 1977). It is still uncertain how the cytoskeletons are anchored to the substratum, but evidence from intact cells suggests that there is a transmembrane linkage of cytoskeletal structure to the pericellular matrix (Hynes & Destree, 1978; Singer, 1979). Rees *et al.* (1977) have studied the relationships between internal and external structures in fibroblast adhesion to substratum, and they have introduced the concept of 'grip' and 'stick' in the control of cell adhesion. Control of 'sticking' by physical forces at the outer surface is exercised through the 'grip' of the cytoskeleton. In the present study both Coomassie Blue staining and L-[U- ^{14}C]leucine uptake revealed a relatively simple pattern of proteins in the adherent cytoskeleton left after extraction of keratinocytes with Triton X-100. In addition to a protein with the mobility of actin (mol.wt. 42 000), there was a series of seven proteins in the keratin region, which were probably derived from the intermediate filaments. D-[1- ^{14}C]Glucosamine was incorporated into glycosaminoglycans (mainly hyaluronic acid, with smaller amounts of a sulphated glycosaminoglycan with the migration properties of heparin sulphate), into eight large glycoproteins (mol.wts. 232 000, 194 000, 180 000, 165 000, 148 000, 129 000, 114 000 and 99 000) and into five bands in the keratin region (mol.wts. 42 000–61 000). Surface labelling with galactose oxidase/ KB^3H_4 revealed that the glycoproteins with mol.wts. 232 000, 194 000, 148 000, 129 000 and 99 000 were located on the exterior surface of the keratinocytes. The results of trypsin digestion, tunicamycin treatment and sequential extraction with Triton X-100 and SDS indicate that the glycoprotein of mol.wt. 232 000 has an important role in attaching the keratinocytes to the substratum. Two non-collagenous glycoproteins with molecular weights in this region have been described in the basement-membrane zone of the epidermal-dermal junction. These are laminin and bullous-pemphigoid antigen (Briggaman, 1982), both of which are synthesized by keratinocytes in culture (Stanley *et al.*, 1982). Laminin consists of several subunits (mol.wts. 220 000 and 440 000) linked to each other by disulphide bonds (Timpl *et al.*, 1979) and has been shown to stimulate the adhesion of mouse epidermal cells to collagen substrates (Terranova *et al.*, 1980). However, there was no evidence of a second subunit of mol.wt. 440 000 in the present study, and in immunoprecipitation experiments the glycoprotein of mol.wt. 232 000 was not precipitated by anti-laminin sera. Stanley *et al.* (1981) have shown that the bullous-pemphigoid antigen contains disulphide-linked sub-

lane a) with those of surface-labelled keratinocytes (Fig. 4) showed that the glycoproteins with mol.wts. 232 000, 194 000, 148 000, 129 000 and 99 000 were both metabolically labelled and surface labelled. In the keratin region three bands with mol.wts. 61 000, 51 000 and 47 000 were intensely surface-labelled, but only one of them, the band with mol.wt. 51 000, was metabolically labelled with D-[1- ^{14}C]glucosamine. Two other bands, mol.wts. 75 000 and 86 000, were surface-labelled but not metabolically labelled.

units of mol.wt. approx. 220 000. Fibronectin, which has been shown to be important in fibroblast-substratum adhesion, is not synthesized in the epidermis (Fyrand, 1979), and an antiserum to fibronectin did not precipitate the glycoprotein of mol.wt. 232 000.

Proteoglycans are components of the adherent cytoskeleton, but the main glycosaminoglycan, hyaluronic acid, can be removed from the intact cells by digestion with hyaluronidase without causing detachment of the cells. This indicates that hyaluronic acid is not an essential link in anchoring keratinocytes to the substratum. In studies on the adhesion of fibroblasts, Rees *et al.* (1981) showed that hyaluronic acid was distributed quite generally over the substratum and was not concentrated at specific adhesion areas.

Incubation of keratinocytes in the presence of tunicamycin inhibited the incorporation of D-[1-¹⁴C]glucosamine into glycoproteins in the mol.wt. range 99 000–194 000 without causing the cells to detach. This indicates that the carbohydrate moieties of these glycoproteins were not directly involved in attaching the cells to the substratum. These results do not exclude the possibility that non-glycosylated forms of these glycoproteins were synthesized in the presence of tunicamycin and that these remained functional in cell-substratum adhesion. However, since these glycoproteins were only released under conditions that caused disruption of the cytoskeletal sheets, it is considered more probable that they are involved in cell-cell adhesion. Desmosomes, which are believed to play a major role in intercellular adhesion in the epidermis (Skerrow, 1978), have been isolated from bovine snout epidermis (Skerrow & Matoltsy, 1974) and shown to contain two glycoproteins of apparent mol.wts. 140 000 and 120 000. Gorbisky & Steinberg (1981) reported that the desmosomal 'cores' or intercellular regions contained glycoproteins of mol.wts. 150 000, 115 000 and 100 000. Ultrastructural examination has shown that desmosomal junctions are formed in keratinocytes cultured under the conditions used in this study (S. P. Barton, personal communication).

The incorporation of D-[1-¹⁴C]glucosamine into epidermal glycoproteins migrating in the keratin region was investigated previously (Roberts & Marks, 1982) and shown to be mainly due to glycoproteins which co-migrated with the main keratin polypeptides. In the present study the glycoprotein of mol.wt. 54 000 not only migrated in the keratin region on electrophoresis but also remained in the adherent cytoskeleton after Triton X-100 extraction and was not destroyed by trypsin digestion. The possibility that it might have a function in keratin-filament formation or organization requires investigation. Four glycoproteins, of mol.wts. 86 000, 75 000, 61 000 and 47 000, found in

the adherent cytoskeleton were surface-labelled with galactose oxidase/KB³H₄ after neuraminidase digestion, but were not detected by metabolic labelling with D-[1-¹⁴C]glucosamine of keratinocytes or irradiated fibroblasts. In view of the probability that these glycoproteins were derived from the serum in the medium, it is noteworthy that one of these glycoproteins has a similar molecular weight to epibolin, an epidermal spreading factor isolated from serum (Stenn, 1981). Epibolin is claimed to be required for epidermal cell migration *in vivo* and may be important in areas such as wound healing and epithelial tumour metastases.

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References

- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Brabec, R. K., Peters, B. P., Bernstein, I. A., Gray, R. H. & Goldstein, I. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 477–479
- Breen, M., Weinstein, H. G., Andersen, M. & Veis, A. (1970) *Anal. Biochem.* **35**, 146–159
- Briggaman, R. A. (1982) *J. Invest. Dermatol.* **78**, 1–6
- Dykes, P., Jenner, L. & Marks, R. (1982) *Arch. Dermatol. Res.* **273**, 225–231
- Fyrand, O. (1979) *Br. J. Dermatol.* **101**, 263–270
- Gahmberg, C. G. & Hakomori, S. I. (1973) *J. Biol. Chem.* **248**, 4311–4317
- Gorbisky, G. & Steinberg, M. S. (1981) *J. Cell Biol.* **90**, 243–248
- Henderson, L. E., Oroszlan, S. & Konigsberg, W. (1979) *Anal. Biochem.* **93**, 153–157
- Holt, P. J. A., Anglin, J. H. & Nordquist, R. E. (1979) *Br. J. Dermatol.* **100**, 237–245
- Hughes, R. C. & Pena, S. D. J. (1981) in *Carbohydrate Metabolism and its Disorders* (Randle, P. J., Steiner, D. F. & Whelan, W. J., eds.), pp. 363–423, Academic Press, London
- Hynes, R. O. & Destree, A. T. (1978) *Cell* **15**, 875–886
- Kessler, S. W. (1976) *J. Immunol.* **117**, 1482–1490
- King, I. A. (1981) *Biochim. Biophys. Acta* **674**, 87–95
- King, I. A., Tabiowo, A. & Williams, R. H. (1980) *Biochem. J.* **190**, 65–77
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Osborn, M. & Weber, K. (1977) *Exp. Cell Res.* **106**, 339–349
- Pitt-Rivers, R. & Impiombato, F. S. A. (1968) *Biochem. J.* **109**, 825–830
- Rees, D. A., Lloyd, C. W. & Thom, D. (1977) *Nature (London)* **267**, 124–128

- Rees, D. A., Badley, R. A., Bayley, S. A., Couchman, J. R., Smith, C. G. & Woods, A. (1981) in *Cellular Interactions* (Dingle, J. T. & Gordon, J. L., eds.), pp. 67–80, Elsevier/North-Holland, Amsterdam
- Rheinwald, J. G. & Green, H. (1975) *Cell* **6**, 331–344
- Roberts, G. P. & Marks, R. (1982) *Br. J. Dermatol.* **106**, 727
- Saarni, H. & Tammi, M. (1977) *Anal. Biochem.* **81**, 40–46
- Singer, I. I. (1979) *Cell* **16**, 675–685
- Skerrow, C. J. (1978) *Invest. Cell Pathol.* **1**, 23–37
- Skerrow, C. J. & Matoltsy, A. G. (1974) *J. Cell Biol.* **63**, 524–530
- Stanley, J. R., Hawley-Nelson, P., Yuspa, S. H., Shevach, E. M. & Katz, S. I. (1981) *Cell* **24**, 897–903
- Stanley, J. R., Hawley-Nelson, P., Yaer, M., Martin, G. R. & Katz, S. I. (1982) *J. Invest. Dermatol.* **82**, 456–459
- Stenn, K. S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6907–6911
- Stoker, M., O'Neill, C., Berryman, S. & Waxman, V. (1968) *Int. J. Cancer* **3**, 683–693
- Terranova, V. P., Rohrbach, D. H. & Martin, G. R. (1980) *Cell* **22**, 719–726
- Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M. & Martin, G. R. (1979) *J. Biol. Chem.* **254**, 9933–9937
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* **275**, 179–184