GLYCOPROTEINS AND GLYCOSAMINOGLYCANs OF CULTURED NORMAL HUMAN EPIDERMAL KERATINOCYTES

KEITH W. BROWN AND E. KENNETH PARKINSON
Cancer Research Campaign Laboratories, Department of Cancer Studies, The Medical School, University of Birmingham, Birmingham, B15 2TJ, U.K.

SUMMARY

[3H]glucosamine has been used to label metabolically keratinocyte cell-surface glycoconjugates. The major labelled bands identified on sodium dodecyl sulphate/polyacrylamide gels had apparent molecular weights of greater than 250,000, and 150,000–80,000. Most of these components were trypsin-sensitive, indicating that the label was protein-bound. Some of the labelled components were shown to be proteoglycans and the labelled glycosaminoglycans released from them by trypsin were identified as hyaluronic acid (54%), heparan sulphate (33%) and chondroitin sulphate (13%). Specific immunological methods (immunoperoxidase staining and immunoprecipitation) showed that keratinocytes produced fibronectin. Immunoperoxidase staining showed keratinocytes produce only small 'stitches' of fibronectin at cell edges; no large fibrils were seen nor any staining over or between cells.

INTRODUCTION

Mammalian cell surface glycoproteins and glycosaminoglycans are involved in many important cellular functions. Specifically, glycoproteins are involved in cell-to-cell and cell-to-substratum adhesion, cell recognition, membrane transport, hormone reception and various enzymic activities (Talmadge & Burger, 1975; Glick & Flowers, 1978; Hynes, 1979a; Hunt & Moore, 1980), whilst glycosaminoglycans, apart from being important structural components in extracellular matrices (Muir & Hardingham, 1975), can also interact with many other molecules (Lindahl & Hook, 1978; Kraemer, 1979) and may be involved in cell-adhesive properties (Kraemer, 1979). Several features of tumour cells and transformed cells could be related to alterations in surface properties (Hynes, 1979b), and in recent years many studies have been reported on transformation-induced changes of cell-surface glycoproteins (Hynes, 1979a, 1976a; Robbins & Nicolson, 1975; Vaheri, 1978; Yamada & Pouyssegur, 1978; Atkinson & Hakimi, 1980) and glycosaminoglycans (Vaheri, 1978; Kraemer, 1979; Atkinson & Hakimi, 1980). In particular, several groups have reported that loss of cell-surface fibronectin occurs in many (though certainly not all) transformed cells, and in some systems this may be correlated with tumorigenicity (Vaheri & Mosher, 1978; Yamada & Olden, 1978).

Most studies on the effect of transformation on cell-surface glycoconjugates have been carried out with fibroblasts or established cell lines. Whilst the results of these studies may be generally applicable, it is unfortunate that epithelial cells, from which
most human tumours arise, have remained relatively neglected. We have therefore used the methods of Green and his co-workers (Rheinwald, 1980) to culture human epidermal keratinocytes, and have studied their glycoproteins by metabolic labelling.

In this paper we describe our results on the glycoproteins and glycosaminoglycans of normal human keratinocytes; these results will provide the basis for future comparative studies with virally transformed keratinocytes and keratinocytes derived from skin carcinomas. To our knowledge, this is the first study of this type on cultured human keratinocytes, although conflicting reports on fibronectin synthesis have previously appeared (Chen, Maitland, Gallimore & McDougall, 1977; Peehl & Stanbridge, 1981; Alitalo et al. 1982). Laminin and several collagenous polypeptides have also been shown to be produced by keratinocytes (Alitalo et al. 1982). Some of our results have appeared previously in preliminary form (Brown, Parkinson & Gallimore, 1981).

MATERIALS AND METHODS

Cell cultures

Normal human epidermal keratinocytes were derived from foreskin and cultured by the methods of Rheinwald & Green (Rheinwald, 1980), as described previously (Parkinson & Newbold, 1980). Normal growth medium was Dulbecco’s modification of Eagle’s medium (DME), supplemented with 12% (v/v) foetal bovine serum, 0.4 μg/ml hydrocortisone, 10 μg/ml cholera toxin, 100 i.u./ml penicillin and 100 μg/ml streptomycin. For the experiments described here, cultures were initiated from frozen stocks of cells by plating out 3 × 10^6 keratinocytes with 1.5 × 10^6 lethally irradiated 3T3 cells per 9 cm dish or 1 × 10^5 keratinocytes with 0.5 × 10^6 lethally irradiated 3T3 cells per 5 cm dish. Epidermal growth factor (10 ng/ml) was added 2–3 days after initiating the cultures. Before all labelling experiments, 3T3 feeder layers were removed by treatment with EDTA and vigorous pipetting (Rheinwald, 1980). After this procedure the cultures consisted of at least 99% pure keratinocytes (Parkinson & Newbold, 1980).

Human dermal fibroblasts were cultured as described previously (Parkinson & Newbold, 1980).

Radioactive labelling

Radiochemicals: Na^{25}I (carrier-free, 100 mCi/ml), ^{35}SO_{4}^{2-} (25–40 Ci/mg), D-[6-3H]-glucosamine-HCl (20–40 Ci/mmol) and L-[35S]methionine (> 600 Ci/mmol) were all obtained from Amersham International.

Dishes (9 cm) of cultured keratinocytes were iodinated enzymically with lactoperoxidase, by the method of Hynes (1973). Keratinocytes were labelled with ^{35}SO_{4}^{2-} (5–20 μCi/ml) and/or D-[6-3H]glucosamine (5–10 μCi/ml) for 24 h in normal growth medium or in Eagle’s minimal essential medium (MEM) containing one-tenth the normal glucose concentration with the supplements as for normal growth medium. Labelling with L-[35S]methionine (10–20 μCi/ml) was carried out in MEM without methionine, with the normal supplements, for 24 h.

After labelling, cells were washed three times (on the dish) with 3 ml of cold phosphate-buffered saline (PBS; NaCl, 8 g/l; KCl, 0.2 g/l; Na_2HPO_4, 1.5 g/l and KH_2PO_4, 0.2 g/l, pH 7.3) containing 1 mM-phenylmethanesulphonylfluoride (PMSF) and then scraped from the dish with a rubber policeman. The cells were then suspended in 1 ml PBS/PMSF, transferred to a small plastic tube and pelleted by centrifugation (10 000 g for 1 min in an Eppendorf microcentrifuge). Cell pellets were either solubilized immediately in sodium dodecyl sulphate (SDS) and mercaptoethanol, or stored at −35 °C until required.

Solubilization of cell pellets and SDS/polyacrylamide gel electrophoresis

Cell pellets were dissolved in 50 mM-Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 2% (v/v)
2-mercaptoethanol and 10% (v/v) glycerol, then boiled for 2 min. Samples were analysed for protein (Geiger & Bessman, 1972) and portions containing 50 μg protein were loaded per gel lane. SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out in slab gels with a 3% (w/v) acrylamide stacking gel and linear 5% to 15% (w/v) acrylamide-gradient resolving gel. After electrophoresis, gels were stained with PAGE Blue 83 (BDH Chemicals) and then processed for fluorography (Laskey, 1980).

**Glycosaminoglycan analysis**

Keratinocytes were labelled with 35SO₄²⁻ and [3H]glucosamine for 24 h, washed in PBS and then scraped from the dishes. Cell pellets were suspended in 2 mg/ml trypsin (Difco) in PBS and incubated 1 h at 37°C. The cells were then pelleted by centrifugation (10000 g for 1 min) and the supernatant was removed and boiled for 2 min to inactivate the trypsin. Pronase (1 mg/ml) (Calbiochem) was added to the supernatant and incubated for 16 h at 37°C, after which residual polypeptides were removed by precipitation with 10% (w/v) trichloroacetic acid. Carrier glycosaminoglycans (GAGs) were added to the supernatant (0-1 mg/ml each of hyaluronic acid, chondroitin sulphate and heparin) and then this was mixed with 4 vol. of ethanol containing 5% (w/v) potassium acetate and left for 16 h at 4°C. The GAG precipitate was then collected by centrifugation, washed in ethanol and finally dissolved in water (100 μl for the GAGs from each 9 cm dish).

For enzymic digestions, 5 μl samples of GAG solutions were treated with 10 units Streptomyces hyaluronidase, or 0-1 unit chondroitinase ABC or 0-05 unit chondroitinase AC (all enzymes from Miles) in 50 μl of 50 mM-sodium acetate (pH 6-0), for 16 h at 37°C. Solutions were then boiled for 2 min to inactivate the enzymes, dried over P₂O₅ and dissolved in 5 μl H₂O. For HNO₂ treatment, 5 μl of GAG solution was mixed with 1-5 μl of 0-2M-NaNO₂/2M-CH₂COOH and incubated for at least 90 min at room temperature (Wusteman, 1979), then dried and dissolved in 5 μl of H₂O.

GAG samples (up to 5 μl) were electrophoresed on 15 cm long cellulose acetate strips in LiCl/EDTA, pH 8-4 (Schuchman & Desnick, 1981). After electrophoresis, carrier GAGs were detected by Alcian Blue staining and radiolabelled GAGs were detected by cutting the sheets into 2-5 mm strips and counting in 5 ml Fisofluor scintillation fluid (Fisons Ltd).

**Immunoperoxidase stain for fibronectin**

Keratinocytes were grown (without 3T3 feeder cells) on glass coverslips for 2-3 days, then washed in PBS and fixed in 1% (w/v) formaldehyde in PBS (freshly prepared from paraformaldehyde) for 30 min at room temperature, followed by acetone at -20°C for 2 min. Fibronectin was detected using the peroxidase/anti-peroxidase (PAP) method (Sternberger, Hardy, Cuculis & Meyer, 1970). Fixed coverslips were incubated for 30 min at 37°C in a humidified chamber with the following solutions (all dilutions in PBS): (1) 1 in 1500 rabbit anti-human plasma fibronectin (kindly donated by Dr L. B. Chen, Sidney Farber Cancer Research Institute, Boston, Mass., U.S.A.); (2) 1 in 40 swine anti-rabbit y-globulin (Dako); and (3) 1 in 40 PAP (Dako), washing extensively in PBS in between each incubation. Finally, coverslips were incubated for 5 min in 0-05% (w/v) diaminobenzidine/0-03% (w/v) H₂O₂ in PBS, washed, counterstained in haemotoxylin and then dehydrated and mounted in DPX.

**Fibronectin immunoprecipitation**

Samples of labelled culture media from [35S]methionine-labelled cells (up to 1 ml) were incubated for 2-3 h at room temperature with 10 μl of sheep anti-human plasma fibronectin (kindly supplied by Dr A. R. Bradwell, Immunodiagnostic Labs., University of Birmingham, U.K.), followed by 100 μl of pig anti-sheep γ-globulin. After incubating 16 h at room temperature, immunoprecipitates were collected by centrifugation (2000 g for 5 min), washed twice in 300 μl PBS containing 1% (v/v) Triton X-100 and once in 300 μl PBS, before solubilization in SDS/mercaptoethanol for electrophoresis.

**RESULTS**

When keratinocytes were labelled by lactoperoxidase-catalysed iodination and the radiolabelled proteins were separated on SDS/polyacrylamide gels, it could be seen...
that the major cell proteins, actin and the keratins (molecular weights 46,000 to 58,000; Sun & Green, 1978a), were extensively labelled (Fig. 1A, lanes 1, 2). Since the keratin filaments of cultured keratinocytes had been shown to be entirely cytoplasmic (Sun & Green, 1978b), this implied that the cells had been internally labelled. When cells were lysed before iodination, the labelling was greatly increased (Fig. 1A, lane 3; although the lane appears completely black in this photograph, bands corresponding to the major cellular proteins could be seen in this lane), suggesting that normally only a small proportion of the keratinocytes were permeable to the labelling reagents. In support of this, we found that about 12% of keratinocytes were permeable to Trypan Blue and internal labelling of some cells was observed by electron microscopic autoradiography.

Since lactoperoxidase-catalysed iodination was obviously not surface-specific for keratinocytes, cell-surface glycoconjugates were labelled metabolically with [3H]-glucosamine. The amount of total cell protein extracted per dish showed no decrease
Keratinocyte glycoconjugates

during the 24 h labelling period, indicating that no cell death had occurred, unlike a recently described organ-culture system for labelling epidermal glycoconjugates (King, Tabiowo & Williams, 1980). Incorporation of \(^{3}H\)glucosamine continued during the labelling period and about 40% of the radioactivity was trichloroacetic acid-precipitable at 24 h. Incorporation was improved by reducing the glucose concentration in the medium, although this did not affect the pattern of incorporation into individual glycoconjugates. Incorporation was most efficient in smaller keratinocyte colonies; larger colonies (14-day-old colonies) incorporated only 25-49% of the \(^{3}H\)glucosamine incorporated by 7-day-old colonies, therefore we normally labelled keratinocytes 7 days after initiating cultures. To determine whether the \(^{3}H\)glucosamine was metabolized to other sugars, labelled keratinocytes were extracted with trichloroacetic acid, the insoluble residues were hydrolysed in acid (2 M HCl for 3 h at 100 °C) and then the hydrolysates were run on paper and cellulose thin-layer chromatograms. A total of 80% of the radioactivity was recovered as \(^{3}H\)-glucosamine and 17% as \(^{3}H\)galactosamine, showing that there had been little metabolism of the label to other sugars. The sites of incorporation of \(^{3}H\)glucosamine

---

**Fig. 1.**

A. Lactoperoxidase-catalysed iodination of human keratinocytes. Seven-day-old keratinocyte cultures were iodinated as described in Materials and Methods and the labelled proteins separated on SDS/polyacrylamide gels. Lane 1 shows keratinocyte total proteins stained with PAGE Blue 83; lane 2 is an autoradiograph of the iodinated proteins; and lane 3 shows the iodinated proteins of cells that had been lysed by freezing and thawing prior to iodination. On the left-hand side, the positions of molecular weight markers are indicated (molecular weights ×10\(^{-3}\)).

B. \(^{3}H\)glucosamine and \(^{35}SO_4^{2-}\) labelling of human keratinocytes. Seven-day-old keratinocyte cultures were labelled with \(^{35}SO_4^{2-}\) (lanes 1-5) or \(^{3}H\)glucosamine (lanes 6-10), the labelled cell extracts were run on SDS/polyacrylamide gels, and the radiolabelled bands detected by fluorography, as described in Materials and Methods. Lanes 1, 6: keratinocytes were solubilized in SDS/mercaptoethanol. Lanes 2, 7: keratinocytes were sonicated in 50 mM-Tris- HCl (pH 6.8), containing 0.5% Triton X-100 and 1 mM-PMSF. After centrifuging at 48,000 g for 60 min, the supernatant was removed, SDS and mercaptoethanol added (2% final concn of each) and then run on the gel. Lanes 3, 8: keratinocytes extracted as in lanes 2, 7, except that the buffer contained no Triton X-100. Lanes 4, 9: keratinocytes were incubated in trypsin (Difco, 2 mg/ml in PBS) for 1 h at 37 °C, the cells were pelleted by centrifugation and the cell pellet was solubilized in SDS/mercaptoethanol and run on the gel. Lanes 5, 10: keratinocytes were incubated in ovine testicular hyaluronidase (BDH, 1 mg/ml in PBS) for 1 h at 37 °C, the cells were pelleted and then the pellets were solubilized in SDS/mercaptoethanol and run on the gel.

In this figure both the stacking gel (S) and the resolving gel (R) are shown. Positions of molecular weight markers (molecular weights ×10\(^{-3}\)) are indicated on the left-hand side.

C. Fibronectin detection in labelled culture media. Seven-day-old keratinocyte cultures (lanes 1-3) and confluent human fibroblast cultures (lanes 4-6) were labelled with \(^{35}S\)-methionine, then the labelled culture media were immunoprecipitated and the immunoprecipitates solubilized in SDS/mercaptoethanol and run on SDS/polyacrylamide gels, as described in Materials and Methods. Lanes 1, 4: total labelled culture media. Lanes 2, 5: labelled media immunoprecipitated with normal (preimmune) serum. Lanes 3, 6: labelled media immunoprecipitated with anti-fibronectin serum. Approximately equal numbers of trichloroacetic acid-insoluble counts were used for each sample. Positions of molecular weight markers (molecular weights ×10\(^{-3}\)) are indicated on the left-hand side.
were investigated by electron microscopic autoradiography. A total of 75% of the observed grains lay within 250 nm of the plasma membrane (this distance is the radius of the circle within which half of the grains would lie from a point source; Williams, 1977), indicating that the majority of the [3H]glucosamine was incorporated into, or close to, the cell surface.

[3H]glucosamine-labelled keratinocytes were solubilized in SDS and 2-mercaptoethanol and the extracts electrophoresed on SDS/polyacrylamide gels. One band was observed in the stacking gel, and in the resolving gel the major bands had apparent molecular weights (Mr) of greater than 240,000, and 150,000–80,000 (Fig. 1B, lane 6). The band at greater than 240,000 Mr, and another band at about 180,000 Mr, were soluble in dilute aqueous buffer (Fig. 1A, lane 8), indicating that they were soluble glycoproteins or perhaps weakly bound to the cell surface. Most of the other bands were only soluble in detergent-containing buffer (Fig. 1B, lane 7), strongly suggesting that they were integral membrane glycoproteins. Nearly all of the bands were digested by trypsin (Fig. 1B, lane 9), showing that the label was protein-bound.

The band in the stacking gel, and also some of the radioactivity at the top of the resolving gel, were soluble in neither dilute aqueous buffer nor detergent-containing buffer (Fig. 1B, lanes 6, 7, 8). Labelling with 35SO42− gave a smeared area at the top of the resolving gel (Fig. 1B, lane 1) with solubility and trypsin sensitivity similar to the [3H]glucosamine-labelled material in this area of the gel. The band in the stacking gel was almost completely digested by testicular hyaluronidase (Fig. 1B, lane 10) and the area at the top of the resolving gel was partially digested by this enzyme (Fig. 1B, lanes 5, 10). These results strongly suggested that the band in the stacking gel contained non-sulphated glycosaminoglycans (GAGs), i.e. hyaluronic acid, and that the area at the top of the resolving gel contained sulphated GAGs and/or proteoglycans. The solubility properties of these GAGs are similar to those of other extracellular matrix components.

To identify positively the cell-surface GAGs of human keratinocytes, a GAG extract was prepared from the material released from the cells by trypsin and electrophoresed on cellulose acetate, as described in Materials and Methods. Three [3H]glucosamine-labelled GAGs could be identified by this method. The major peak comigrated with a hyaluronic acid standard, it was not labelled significantly by 35SO42− (Fig. 2A) and it could be digested by both Streptomyces hyaluronidase (Fig. 2A) and chondroitinase ABC (Fig. 2C), but not by HNO2 (Fig. 2D), showing that it was hyaluronic acid. An area comigrating with a chondroitin sulphate standard was labelled by 35SO42− (Fig. 2A) and could be digested by chondroitinase ABC (Fig. 2C) but not by Streptomyces hyaluronidase (Fig. 2B) or HNO2 (Fig. 2D), demonstrating that it contained chondroitin sulphates. In between the hyaluronic acid and chondroitin sulphates there was an area labelled by 35SO42− (Fig. 2A), which was not digested by hyaluronidase (Fig. 2A) or chondroitinase (Fig. 2C), but was digested by HNO2 (Fig. 2D). This suggested that this area contained heparan sulphate (rather than heparin, which has an electrophoretic mobility greater than chondroitin sulphate). The relative amounts of these three GAG classes (on the basis of [3H]-glucosamine-labelling) were as follows (means±s.d. of 7 experiments): hyaluronic
**Keratinocyte glycoconjugates**

Fig. 2. Identification of keratinocyte glycosaminoglycans. Seven-day-old keratinocyte cultures were labelled with \(^{35}\)SO\(_4^{2-}\) and \([^{3}\text{H}]\)glucosamine, glycosaminoglycans were prepared and then analysed by cellulose acetate electrophoresis, and enzymic and HNO\(_2\) degradation, as described in Materials and Methods. Labelled GAGs were detected by liquid scintillation counting; the unshaded areas show \([^{3}\text{H}]\)glucosamine-labelled GAGs and the shaded areas \(^{35}\)SO\(_4^{2-}\) -labelled GAGs. A. Total GAG extract; B, after digestion with *Streptomyces* hyaluronidase; C, after digestion with chondroitinase ABC; and D, after treatment with HNO\(_2\). The position of standard GAGs (H, hyaluronic acid; C, chondroitin sulphate; HP, heparin) are shown at the top by the black bars.

Acid, 54 ± 7%; heparan sulphate, 33 ± 7%; and chondroitin sulphates, 13 ± 3%. We could not reproducibly separate chondroitin sulphates A and C from B by this electrophoretic system and the relatively small amounts of chondroitin sulphate made the interpretation of enzymic digestions difficult, but at least a part of this area was resistant to chondroitinase AC, suggesting that it contained chondroitin sulphates A, C and B.

Although no major \([^{3}\text{H}]\)glucosamine-labelled band had been seen on SDS/polyacrylamide gels in the area expected for fibronectin (200000–250000 \(M_r\); Yamada & Olden, 1978), we were interested to examine our keratinocyte cultures for fibronectin, in view of conflicting reports on fibronectin synthesis by human epidermal keratinocytes (Chen et al. 1977; Peehl & Stanbridge, 1981; Alitalo et al. 1982). When keratinocytes were seeded onto glass coverslips and stained for fibronectin
Fig. 3. Immunoperoxidase staining of fibronectin. Cells were grown on glass coverslips and then stained for fibronectin using the immunoperoxidase technique described in Materials and Methods. A and B. Human fibroblasts; and C–F, human epidermal keratinocytes. A, C, D, E: stained with anti-fibronectin serum; and B and F with normal (preimmune) serum. The black arrows in C and D indicate some of the fibronectin 'stitches' seen at the edges of keratinocytes. The open arrow in C indicates a squame detaching from the colony. Bars, 20 μm. All photographs were taken at the same magnification.
using an immunoperoxidase technique, small 'stitches' of fibronectin could be seen at the periphery of some cells (Fig. 3c, d). Only about a quarter of the cells stained positively for fibronectin; the remainder appearing essentially negative (Fig. 3e). No large fibrils of the type typical of fibroblast fibronectin (Fig. 3a) could be seen on the human keratinocytes; keratinocyte fibronectin appeared to be completely confined to cell–substratum contact areas.

To confirm that keratinocytes were synthesizing fibronectin, cultures were labelled
with $^{[35S]}$methionine and the conditioned culture medium was immunoprecipitated with anti-fibronectin serum. The labelled keratinocyte medium contained a band at about 250,000 $M_r$ (Fig. 1c), which could be specifically precipitated by anti-fibronectin serum (Fig. 1c, lane 3). Fibronectin made up a smaller proportion of the secreted proteins in keratinocytes than in fibroblasts and, also, keratinocyte fibronectin showed a slightly slower mobility on SDS/polyacrylamide gels than fibroblast fibronectin (compare Fig. 1c, lanes 1–3 with lanes 4–6). The small amounts of fibronectin synthesized by our keratinocyte cultures are unlikely to be the results of 3T3 cell or dermal fibroblast contamination, as we (Parkinson & Newbold, 1980) and others (Alitalo et al. 1982), have shown that fibroblast contamination after EDTA treatment is very low. Apart from fibronectin, the $^{[35S]}$methionine-labelled proteins secreted into the culture medium by keratinocytes, human fibroblasts and 3T3 cells show completely different patterns on SDS/polyacrylamide gels (Fig. 1c; data not shown for 3T3 cells). Also, keratinocyte cultures from foetal skin synthesize more fibronectin than those derived from other donors (K. W. Brown & E. K. Parkinson, unpublished observations). These observations are inconsistent with fibronectin being fibroblast-derived.

**DISCUSSION**

Although lactoperoxidase-catalysed iodination has proved useful in the investigation of surface proteins of many cell types (Hynes, 1976b), we have shown here that this technique is not cell-surface specific for keratinocytes, due to the presence of permeable cells in the cultures. Keratinocytes become permeable as they terminally differentiate in culture (Green, 1977) and this led to the internal labelling of some cells, as demonstrated by iodination of the keratins. Brysk & Snider (1982) have recently reported the use of lactoperoxidase-catalysed iodination to label epidermal keratinocytes, but apparently without realizing the problems involved with this cell type. With normal keratinocytes, almost all the iodinated bands they detected were in the range from 45,000–65,000 $M_r$, which strongly suggested that the keratins had been extensively labelled, therefore implying that the keratinocytes had been internally labelled. It is clear from our results that the keratinocyte proteins labelled by lactoperoxidase catalysed iodination do not represent ‘cell-surface’ proteins.

We have therefore labelled human keratinocytes metabolically with $^{[3H]}$glucosamine and we have shown that this procedure predominantly labelled cell-surface glycoconjugates. Most of the labelled components appeared to be glycoproteins and proteoglycans, since the label was released from the keratinocytes by proteases. Glycolipids were unlikely to have been labelled, because most of the glycolipids (>99%) of epidermal cells contain only glucose (Gray & Yardley, 1975). The pattern of $^{[3H]}$glucosamine-labelled components seen on SDS/polyacrylamide gels was similar to that reported by King et al. (1980), who used $^{[3H]}$glucosamine to label pig skin epidermis in organ cultures. King also showed that the major epidermal GAG synthesized by pig skin in organ culture was hyaluronic acid (King, 1981), with sulphated GAGs being relatively minor components. Our results on
Keratinocyte glycoconjugates

the [3H]glucosamine-labelled GAGs produced by human epidermal keratinocytes were similar, though hyaluronic acid made up a smaller proportion of the total GAGs than was found in organ culture (King, 1981). King found that hyaluronic acid was only synthesized efficiently by the epidermis when the dermis was present, and it appeared that dermal-epidermal contact was important rather than the production of a diffusible factor by the dermis (King, 1981). Hyaluronic acid was the major GAG produced by our keratinocyte cultures, even though we labelled in the absence of 3T3 fibroblast feeder layers, showing that no diffusible fibroblast-produced factor was essential for the production of keratinocyte hyaluronic acid. However, insoluble keratinocyte colony-stimulating factors are left attached to the substratum even after the removal of 3T3 cells with EDTA (Rheinwald, 1980), so some fibroblast-derived factors may still be important for keratinocyte GAG synthesis, as suggested by King (1981).

Little other work has been reported on epidermal glycoproteins, but it is interesting to note that the two major glycoproteins present in purified epidermal desmosomes have apparent molecular weights of about 120 000 and 150 000 (Skerrow & Matoltsy, 1974; Gorbsky & Steinberg, 1981). Since cultured keratinocytes produce desmosomes, it is possible that the major 130 000 and 150 000 M_r [3H]glucosamine-labelled glycoproteins that we have observed (Fig. 1b, lane 6), might represent desmosomal glycoproteins.

We have shown that although no major [3H]glucosamine-labelled band corresponding to fibronectin could be seen on SDS/polyacrylamide gels of labelled keratinocyte extracts, fibronectin synthesis by keratinocytes could be demonstrated by both immunoperoxidase staining of cells and immunoprecipitation of labelled fibronectin in the culture medium. This result is in agreement with a very recent report by Alitalo et al. (1982), who detected fibronectin in cultured keratinocytes by metabolic labelling and immunofluorescence, and is at variance with an earlier report by Chen et al. (1977), who found that fibronectin could not be detected in keratinocytes using indirect immunofluorescence only. However, this latter result is not surprising, in view of the low levels of fibronectin synthesis compared to fibroblasts (Fig. 3). The fibronectin on keratinocytes, when present, was detected as small stitches around the periphery of the cells, and the granular areas of staining beneath the cells reported by Alitalo et al. (1982) were absent. The latter may have been due to residual 3T3 fibronectin deposited on the culture vessel, as Alitalo et al. (1982) did not reculture the cells in the absence of 3T3 cells, as we did. Alternatively, the fibronectin distribution in isolated keratinocytes may be different from that found in the established colonies studied by Alitalo et al. (1982).

Both our results and those of Alitalo et al. (1982) are in general in agreement with the results of Peehl & Stanbridge (1981), who demonstrated keratinocyte fibronectin by immunofluorescence in keratinocyte cultures grown in medium containing levels of calcium much lower than those found in vivo. Under these culture conditions, keratinocytes formed a non-stratified monolayer and the fibronectin matrix appeared more extensive than we found in our experiments, possibly due to the different culture method used.
Our demonstration of the synthesis of both fibronectin and sulphated glycosaminoglycans by cultured epidermal keratinocytes, together with the recent report that keratinocytes can also synthesize laminin and several collagenous polypeptides (Alitalo et al. 1982), support the idea that epidermal keratinocytes are responsible for the synthesis of at least some of the components of the basement membrane in human skin (Briggman, 1981).

Since only a proportion of the keratinocytes in our cultures produced fibronectin, this might suggest that fibronectin is only produced at a specific stage of keratinocyte differentiation. This suggestion is supported by previous results, which have shown that fibronectin is not found throughout the epidermis, but is confined to the basement membrane, adjacent to the basal cells (i.e. the lamina lucida) (Stenman & Vaheri, 1978; Couchman et al. 1979; Fyrand, 1979). Other glycoconjugates are probably produced at specific stages of epidermal differentiation too, since different lectins have been shown to bind to specific layers of the epidermis (Nemanic & Elias, 1979; Brabel et al. 1980). We therefore intend, in future work, to define further at which stages of keratinocyte differentiation specific glycoconjugates are synthesized. This will be important when comparing normal keratinocytes with their transformed counterparts, as it will make possible the identification of any changes that are a direct consequence of transformation as opposed to secondary changes caused by alterations in the commitment of keratinocytes to terminal differentiation. Defective terminal differentiation has already been shown to be a characteristic of malignantly transformed keratinocytes (Rheinwald & Beckett, 1980).

The authors wish to thank Miss A. Emmerson and Mrs S. Williams for technical assistance, Mr P. Reeve for excellent assistance with electron microscope autoradiography, Miss D. Williams for typing the manuscript, Professor D. G. Harnden and Dr P. H. Gallimore for critical reading of this paper, and Dr I. King and Mr R. Williams for helpful discussions.

This work was supported by the Cancer Research Campaign.

REFERENCES


Keratinocyte glycoconjugates


(Received 11 October 1982)