Incorporation of L-[³H]Fucose and D-[³H]Glucosamine into Cell-Surface-Associated Glycoconjugates in Epidermis of Cultured Pig Skin Slices

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1. Electron microscope autoradiography indicated that L-[³H]fucose and D-[³H]-glucosamine were both incorporated into cell-surface-associated glycoconjugates in the epidermis of cultured pig skin slices. 2. Acid hydrolysis and paper chromatography of skin homogenates confirmed that there was little metabolic conversion of the labelled precursors to other sugars. 3. Epidermis was separated from dermis using CaCl₂, and was extracted with 8 M-urea/5% (w/v) sodium dodecyl sulphate and was then analysed by gel electrophoresis. The major component labelled with D-[³H]glucosamine had an apparent molecular weight in excess of 200000. This material was not labelled with L-[³H]fucose. Lower molecular-weight components were labelled to a similar extent with both L-[³H]fucose and D-[³H]glucosamine. 4. The high molecular-weight material labelled with D-[³H]glucosamine was released into the medium when the epidermal cells were dispersed with trypsin, indicating that it was either surface-associated or was extracellular. It was also labelled with D-[¹⁴C]glucuronic acid, ³⁵SO₄²⁻ and to a small extent with ¹⁴C-labelled amino acids indicating that it contained glycosaminoglycans derived from epidermal proteoglycans. This was confirmed by the fact that it was degraded by testicular hyaluronoglucosidase. It was not present in isolated membranes but was recovered in the soluble fraction from epidermal homogenates. It is therefore only very loosely bound at the cell surface or is present in the extracellular spaces. 5. Membrane-bound [³H]glycoproteins were identified after differential centrifugation of epidermal homogenates. The radioactivity profiles of membrane glycoproteins were similar whether L-[³H]fucose or D-[³H]glucosamine were used and both consisted of a major heterogeneous peak in the apparent mol.wt. range 70000–150000. [³H]-Glycoproteins in this molecular-weight range were also major components of a plasma-membrane-enriched fraction. These glycoproteins were probably bound to the membrane by hydrophobic interactions, since they were only solubilized by treatment with detergent or organic solvent. They contained terminal sialic acid residues, since they were degraded by neuraminidase.

The importance of cell-surface glycoconjugates in cell adhesion, contact inhibition and the regulation of cell growth is now generally accepted (Hughes, 1976; Glick & Flowers, 1978). There are reports of modifications to surface glycoproteins (Buck *et al.*, 1970; Warren *et al.*, 1972; Hynes, 1976), glycolipids (Hakomori & Murakami, 1968; Siddiqui & Hakomori, 1970; Brady & Fishman, 1973) and proteoglycans (Chiarugi *et al.*, 1974; Roblin *et al.*, 1975; Cohn *et al.*, 1976; Underhill & Keller, 1977)

in cultured cells whose growth and adhesive properties have been altered by transformation. Most of the studies on cultured cells have been carried out on fibroblast-like cells or cell lines derived from tumours. However, there is relatively little information about the structure and function of the surface glycoconjugates of normal epithelial cells.

In this present paper we have used L-[³H]fucose and D-[³H]glucosamine to label and to further characterize the cell-surface glycoconjugates in the epidermis of cultured pig skin slices. This procedure was developed with a view to detecting possible changes to cell surface components in small biopsy samples of diseased human epidermis.

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

We show that L-[^3H]fucose and D-[^3H]glucosamine are both incorporated into the same major integral glycoproteins present in the plasma membranes of living epidermal cells. However, D-[^3H]glucosamine is also incorporated into a peak of high molecular weight material that we consider to be extracellular rather than bound to the plasma membrane. This high molecular weight material is not glycoprotein, as suggested in a preliminary report (King & Gray, 1978) but represents extracellular proteoglycans and/or glycosaminoglycans.

Experimental

Materials

L-[1- ^3H]fucose (>1 Ci/mmol); L-[1- ^{14}C]fucose (>50 Ci/mol); D-[6- ^3H]glucosamine hydrochloride (>10 Ci/mmol); D-[1- ^{14}C]glucosamine hydrochloride (>45 Ci/mol); D-[U- ^{14}C]glucuronic acid (>50 Ci/mol); L-[U- ^{14}C]proline (>10 Ci/mol); L-U- ^{14}C -labelled amino-acid mixture (>45 Ci/g-atom of carbon) and $^{35}\text{SO}_4^{2-}$ were all obtained from The Radiochemical Centre, Amersham. SDS was from Uniscience, Cambridge CB5 8BA, U.K., and from BDH. Other reagents for electrophoresis were from BDH. Eagle's minimum essential growth medium and gentamycin were from Flow Laboratories, Irvine KA12 8NB, Scotland, U.K. Foetal-calf serum was from Gibco-Biocult, Paisley PA3 4EP, Scotland, U.K. Fungizone was from E. R. Squibb & Sons, New York, U.S.A. Trypsin (1:250) was obtained from Difco Laboratories, West Molesey, Surrey, U.K. Soya bean trypsin inhibitor, protease type VI from *Streptomyces griseus* and L-leucine-2-naphthylamide were from Sigma. Neuraminidase (500 units/ml) from *Vibrio comma* was from Boehringer. Sheep testicular hyaluronidase (>1000 i.u./mg) was from Miles Laboratories, P.O. Box 37, Stoke Poges SL2 4LY, U.K. Phosphate-buffered saline (pH 7.3) in tablet form was from Oxoid.

Organ culture

Pig ears obtained from the abattoir were stored at 4°C and were used within 4 h. After cleaning them with 5% (w/v) Cetrimide, hairs were removed with clippers and the ears were swabbed with aq. 70% ethanol. Skin slices were removed with a Castroviejo keratome (Blank *et al.*, 1961) set to cut at 0.2 mm and were trimmed to 1 cm² under aseptic conditions. These slices contained all the epidermis together with some largely acellular dermis. Skin slices were floated, dermis side down, on 1 ml of Eagle's minimum essential medium buffered with 20 mM-Hepes, pH 7.2, and containing 10% (v/v) foetal calf serum, gentamycin (5 µg/ml), fungizone (5 µg/ml) and either L-[1- ^3H]fucose (50 µCi/ml) or D-[6- ^3H]glucosamine (10 µCi/ml). For dual-labelling experiments, the medium contained D-[6- ^3H]glucosamine (10 µCi/ml) with either D-[U- ^{14}C]glucuronic

acid (10 µCi/ml), $^{35}\text{SO}_4^{2-}$ (40 µCi/ml), L-[U- ^{14}C]proline (2 µCi/ml) or L-U- ^{14}C -labelled amino-acid mixture (5 µCi/ml). Skin slices were cultured in 3 cm vented plastic Petri dishes for up to 42 h. The covered dishes were transferred to a plastic container (700 ml) that was sealed and incubated at 37°C. The gas phase was air (Caron, 1968).

Separation of epidermis from dermis

At the end of the culture period, each skin slice was washed 6 times with phosphate-buffered saline (10 ml, 30 s/wash). Two methods were used to separate epidermis from dermis. In one method, each skin slice was floated on 1 ml of 1 M-CaCl₂ in a covered Petri dish for 1 hr at 37°C. The epidermis was then easily peeled away from underlying dermis. In the second procedure each skin slice was floated on 1 ml of trypsin solution [0.2% (w/v) in phosphate-buffered saline] for 15 min at 37°C. As well as separating epidermis from dermis, trypsin dispersed the epidermal cells. The epidermis was removed and was floated on 1 ml of fresh trypsin solution for 15 min at 37°C. The epidermis and trypsin solution were transferred to a centrifuge tube which was shaken vigorously for 1 min at 15–20°C. The residue (consisting of intact stratum corneum together with attached cells) was removed and the remaining cell suspension was centrifuged (12000 g) for 30 min at 4°C. The epidermal cell pellet was washed in 1 ml of phosphate-buffered saline and the supernatants were combined.

Solubilization of epidermis in 8 M-urea/5% SDS

Whole epidermis obtained by using 1 M-CaCl₂ and epidermal fractions obtained by using trypsin were homogenized in 0.25 ml of 8 M-urea containing 5% (w/v) SDS using a tight-fitting ground-glass tissue homogenizer. The supernatant after trypsin treatment was adjusted to 8 M-urea/5% SDS by adding the solid reagents. Samples were incubated at 100°C for 10 min and after cooling were centrifuged for 30 min (12000 g) at 15–20°C to avoid the precipitation that occurs at lower temperatures.

Isolation of subcellular epidermal fractions

Three pieces of washed labelled skin were added to 5 g of unlabelled carrier pig skin (0.2 mm thick). After mincing with scissors, the tissue was chilled and was homogenized in 40 ml of 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose using a Polytron PT20 homogenizer for 30 s at speed setting 4. The homogenate was filtered through nylon bolting cloth with a hole size 0.13 mm × 0.13 mm; (John Staniar, Manchester M3 1FD, U.K.). The filtrate was centrifuged for 10 min at 4°C and 376 g_{av.} ($r_{av.}$ 8.4 cm) in the 8 × 50 ml fixed-angle rotor of an MSE 18 centrifuge. The supernatant was centrifuged for 1 h at 4°C and 113 700 g_{av.} ($r_{av.}$ 6.3 cm) in a Beckman 60Ti fixed-angle rotor. Pellets were

suspended in 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose at 10–20 mg of protein/ml. In some experiments, to check for dermal contamination, the epidermis of labelled and unlabelled skin slices were first separated from dermis using 1 M-CaCl₂ and the isolated epidermis was used for preparing subcellular fractions. For measurement of specific radioactivities, samples containing 5 mg of protein in 0.5 ml of 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose were mixed with an equal volume of 20% (w/v) trichloroacetic acid and were allowed to stand at 0°C for 1 h. Precipitated glycoproteins were collected by low-speed centrifugation, suspended in 10% (w/v) trichloroacetic acid and were dissolved using a minimum of 6 M-KOH. Protein and radioactivity were measured in both the acid-soluble and the acid-insoluble fractions.

Density gradient centrifugation

A plasma-membrane enriched fraction was isolated from the 113 700 g pellet by density-gradient centrifugation on discontinuous sucrose gradients. This method was adapted from a method utilising a zonal rotor (Gray *et al.*, 1980). Gradients were prepared at 15–20°C in cellulose nitrate tubes (3½ in × 1 in; 8.9 cm × 2.5 cm) and contained 7 ml of 2 M-sucrose, 10 ml of 1.5 M-sucrose, 10 ml of 1 M-sucrose and 10 ml of 0.5 M-sucrose. All gradient solutions contained 5 mM-Tris/HCl, pH 7.5, and gradients were used immediately after preparation. Samples (approx. 1 ml) of the 113 700 g pellet in 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose were applied to the top of each gradient, which was then centrifuged for 1 h at 4°C in a Beckman SW27 rotor at 82 500 g_{av.} (r_{av} , 11.8 cm) using the Beckman L2 65B ultracentrifuge. The material at each interface was removed with a Pasteur pipette, diluted with a large volume of water and centrifuged for 30 min at 4°C and 113 700 g. Membrane fractions were suspended in 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose. The plasma-membrane enzyme marker, amino acid-2-naphthylamidase was assayed as described previously (Gray *et al.*, 1977) using L-leucine 2-naphthylamide as substrate.

Solubilization of membrane-bound glycoproteins

Samples of the 113 700 g pellet (about 5 mg of protein/ml in 10 mM-Tris/HCl, pH 7.5) were incubated in the presence of 1 M-KCl, 10 mM-EDTA, 10 mM-2-mercaptoethanol or 1% (w/v) Triton X-100 for 30 min at room temperature and were centrifuged at 113 700 g_{av.} for 30 min at 4°C. Protein and radioactivity were measured in the supernatant and pellet fractions. The 113 700 g pellet was also treated with butan-1-ol, which has previously been shown to displace membrane-bound glycoproteins into aqueous solution (Maddy, 1966). The 113 700 g pellet, suspended as above, was vortex-mixed with

an equal volume of butan-1-ol for 1 min at room temperature. The aqueous layer was removed after low-speed centrifugation. The butanol and interfacial layers were extracted with 10 mM-Tris/, pH 7.5. The combined aqueous layers were extracted with an equal volume of butan-1-ol and were lyophilized.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in 12 cm × 0.5 cm glass tubes using the discontinuous buffer system of Ugel *et al.* (1971) modified to include 0.1% (w/v) SDS in the upper (cathode) buffer. Resolving gels (1.2 ml) usually contained a total acrylamide concentration of 7% (w/v) and a monomer/crosslinker ratio of 30:1 and were polymerized using 0.1% (w/v) potassium persulphate and *NNN'* tetramethylethylenediamine (6 µl in 20 ml of gel solution). In some experiments to resolve high-molecular-weight components the total acrylamide concentration was reduced to 6, 5 or 4% (w/v). However, the monomer/crosslinker ratio was always 30:1. Stacking gels (0.2 ml) contained a total acrylamide concentration of 3.1% (w/v) and a monomer/crosslinker ratio of 4:1 and were photopolymerized using riboflavin (5 µg/ml of gel solution). Samples of CaCl₂-separated epidermis and of trypsinized epidermal fractions in 8 M-urea/5% SDS were reduced with 10 mM-2-mercaptoethanol and were analysed in the presence of Bromophenol Blue as tracking dye. Membrane samples were solubilized using 2% (w/v) SDS and 10 mM-2-mercaptoethanol and were heated at 100°C for 10 min. The 113 700 g supernatant was lyophilized, heated with 2% (w/v) SDS and 10 mM-2-mercaptoethanol and was dialysed against a large volume of 2% SDS/10 mM-2-mercaptoethanol. Where necessary, samples were clarified by centrifugation for 30 min at 113 700 g and 4°C. Sucrose was added to a final concentration of 10% (w/v) and Bromophenol Blue was included before electrophoresis. Electrophoresis was carried out for about 1.75 h using a constant current of 2 mA/gel. Gels, containing 50 and 250 µg of protein respectively, were stained with Coomassie Brilliant Blue for protein (Fairbanks *et al.*, 1971) and with the periodic acid/Schiff reaction for glycoprotein (Zacharius *et al.*, 1969). For determination of ³H-labelled glycoproteins, gels were placed in 10% (w/v) trichloroacetic acid overnight, then were washed for at least 3 × 60 min in 10% (w/v) trichloroacetic acid and were frozen. The stacking gel, which contained no radioactivity, was removed, and the resolving gel was sliced into 2 mm sections on a bed of razor blades. Each slice was dissolved in a minimum volume of H₂O₂ (100 vols./conc. NH₄OH (19:1, v/v) at about 60°C. Apparent molecular weights were determined using ovalbumin (40 000 mol.wt.), bovine serum albumin

(68 000 mol.wt.), β -galactosidase (130 000 mol.wt.) and myosin (200 000 mol.wt.). Myosin was isolated from guinea-pig skeletal muscle using the method of Weeds & Hartley (1968).

Hydrolysis and chromatography

Three labelled skin-slices (42 h incubation) were homogenized using the Polytron P20 homogenizer in 3 ml of 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose and the homogenate was filtered through nylon bolting cloth, hole size 0.13 mm $\times$ 0.13 mm. An equal vol. of 20% (w/v) trichloroacetic acid was added and after 1 h at 0°C the precipitate was collected by low-speed centrifugation. The pellet was dissolved in a minimum of NaOH and was dialysed extensively against H₂O before hydrolysis at 100°C with 1 M-HCl under N₂ (18 h). The hydrolysate was dried over KOH and P₂O₅ and was redissolved in H₂O. Samples were analysed by descending paper chromatography (Whatman no. 1 paper) in butan-1-ol/pyridine/H₂O (6:4:3, by vol.) to separate amino sugars and in butan-1-ol/ethanol/H₂O, (10:1:2, by vol.) to separate neutral sugars. Samples were co-chromatographed with traces of [¹⁴C]fucose or [¹⁴C]glucosamine, and with sugar standards. Sugars were detected either by the AgNO₃ reagent or by the Elson-Morgan reaction (Smith, 1960). Chromatograms were cut into 1 cm-strips, eluted with 1 ml H₂O at 37°C for 3 h and the radioactivity in the eluate was measured.

Radioactivity determination

Radioactivity was measured by liquid scintillation counting using PCS scintillation fluid (Hopkin and Williams, Romford, Essex RM1 1HA, U.K.). The counting efficiency for ³H was about 35% and in dissolved gel slices was about 30% for ³H and 70–75% for ¹⁴C.

Protein determination

Protein was measured by the Hartree (1972) modification of the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Electron-microscope autoradiography

Washed skin slices were fixed in 2.5% (v/v) glutaraldehyde in 0.2 M-cacodylate/HCl buffer (pH 7.4) for 2 h, postfixed in 2% (w/v) OsO₄ for 2 h,

dehydrated in graded ethanols and were embedded in Spurr's resin. Sections (approximately 30 nm thick) were collected on nickel grids, stained with Reynolds' lead citrate (Reynolds, 1963) and uranyl acetate and were coated with carbon (5 nm thick) by evaporation. Grids held only by their edges were mounted on microscope slides using double-sided Sellotape and were coated with Ilford L4 nuclear emulsion using a nickel-chrome wire loop. After 16 days exposure at 4°C, the emulsion was developed in Kodak D19 developer for 3 min at 20°C. Photographs were taken at a magnification of $\times 3000$ and were printed to give a final magnification of $\times 7500$.

Results

Whole epidermis

In their detailed autoradiographic study of human skin, Mann *et al.* (1980) showed that L-[³H]fucose was preferentially incorporated into cell-surface-associated components by cells in all the living layers of the epidermis. We observed a similar distribution of L-[³H]fucose in pig-ear epidermis (results not shown). D-[³H]Glucosamine was also incorporated into all the living cells in the epidermis (Plate 1). Cells in the basal (Plate 1a), spinous (Plate 1b) and granular (Plate 1c) layers appeared to be labelled to about the same extent. Most (60–70%) of the silver grains in the epidermis were within two grain diameters of the plasma membrane, indicating the preferential incorporation of D-[³H]glucosamine into cell-surface components (Bennet & Leblond, 1970). Cells in the lowest two or three layers of the stratum corneum were sometimes labelled (Plate 1c). Cells higher up in the stratum corneum were never labelled.

To check that L-[³H]fucose and D-[³H]glucosamine were not being metabolized to other sugars, labelled skin slices were homogenized and treated as described in the Experimental section under 'hydrolysis and chromatography'. Most (70 and 85% respectively) of the applied radioactivity co-chromatographed with authentic L-fucose and D-glucosamine, indicating that there was minimal metabolic conversion of the added precursors. Acid-labile sialic acids were presumably destroyed by the hydrolysis procedure.

EXPLANATION OF PLATE 1

Electron microscope autoradiographs of D-[³H]glucosamine-labelled pig epidermis

Skin slices were cultured for 18 h in the presence of D-[³H]glucosamine and were washed in phosphate-buffered saline before processing for autoradiography (see the Experimental section). (a) Basal layer of epidermis; cell membrane is highly folded and consists of interdigitating microvilli (M). (b) Spinous layer; cells have large numbers of desmosomes (D) in cell membrane. (c) Granular layer; cell membrane is flat and intercellular spaces are reduced. The plasma membrane follows the line of desmosomes. S, Stratum corneum cells, mainly unlabelled; C, collagen fibres of dermis; N, nuclei. The bar represents 1 μ m.

Fig. 1. Time-course of incorporation of L-[³H]fucose and D-[³H]glucosamine into pig epidermis

Skin slices were cultured in the presence of either sugar, and were treated with 1M-CaCl₂ for 1h at 37°C. The epidermis was extracted with 8M-urea/5% (w/v) SDS at 100°C. In (a) the extracted radioactivity and protein is expressed as a percentage of the total; (b) shows the total radioactivity extracted from 1 cm² of cultured epidermis. O, L-[³H]Fucose; ●, D-[³H]glucosamine; □, protein.

Epidermal ³H-labelled glycoconjugates were analysed after separating epidermis from dermis using 1M-CaCl₂. Examination of haematoxylin/eosin-stained sections revealed no dermal contamination of the separated epidermis. The epidermis contained 60–70% of the total radioactivity in the skin slice. The epidermis was extracted with 8M-urea/5% SDS at 100°C, and insoluble material (consisting of intact and broken stratum corneum cells together with aggregates of tonofilaments) was removed by centrifugation. This procedure solubilized 95% of the total epidermal radioactivity after 4h in culture (Fig. 1a). This value decreased to 87% for L-[³H]fucose and 83% for D-[³H]glucosamine after 92h. The amount of protein extracted from the epidermis decreased markedly with increasing time in organ culture (Fig.

Fig. 2. Gel electrophoresis of 8M-urea/5% (w/v) SDS extracts from L-[³H]fucose- and D-[³H]glucosamine-labelled epidermis

Labelled epidermis (42h incubation) was obtained by using 1M-CaCl₂ and was extracted with 8M-urea/5% (w/v) SDS at 100°C. The extract was reduced with 2-mercaptoethanol and samples, containing 50 µg of protein, were analysed in 7% (w/v) polyacrylamide gels. After washing in trichloroacetic acid, the gels were frozen and sliced into 2 mm sections. Gel slices were dissolved in conc. NH₃/H₂O₂ (30%, w/v) (1:19) and radioactivity was measured. The arrow indicates the direction of electrophoresis. BPB indicates the position of the Bromophenol Blue tracking dye. O, L-[³H]Fucose; ●, D-[³H]glucosamine.

1a) and the time courses of incorporation are therefore expressed in terms of epidermal area (Fig. 1b) rather than extracted epidermal protein. With both precursors, incorporation usually continued over the first 3 days and then decreased, probably due to the death of the epidermal cells or the need for fresh medium. To obtain optimum labelling and to avoid proteolytic artefacts following cell death and lysis, cultures were maintained for a maximum of 42h in all subsequent experiments.

The epidermal extracts were fractionated by electrophoresis in 7% polyacrylamide gels, which

were then washed in 10% trichloroacetic acid and sliced. Fig. 2 compares the radioactivity profiles obtained with L-[^3H]fucose and D-[^3H]glucosamine. Both sugars were incorporated into components with a wide range of apparent molecular weights. However, one major difference was observed. A peak of high-molecular-weight material, labelled with D-[^3H]glucosamine and accounting for about 20% of the total radioactivity on the acid-washed gel after 42 h, was not significantly labelled with L-[^3H]fucose. This

material migrated in a stack of high-molecular-weight components with an electrophoretic mobility close to that of myosin in 7% (w/v) polyacrylamide gels. In an attempt to resolve further this stacked material (Ugel *et al.*, 1971), D-[^3H]glucosamine-labelled epidermis was also analysed in gels containing lower concentrations of acrylamide. However, it always migrated as a single peak of radioactivity close to the cathode (results not shown). Its molecular weight is therefore greater

Fig. 3. Gel electrophoresis of L-[^3H]fucose- and D-[^3H]glucosamine-labelled epidermis fractionated with trypsin. L-[^3H]Fucose- and D-[^3H]glucosamine-labelled epidermis (42 h) was treated with trypsin (see text) and the three epidermal fractions were extracted with 8M-urea/5% (w/v) SDS, reduced with 10mM-2 mercaptoethanol, and one-sixth of each fraction was analysed in 7% (w/v) polyacrylamide gels. (a) and (d) stratum corneum; (b) and (e) cell pellet; (c) and (f) trypsin supernatant. O, L-[^3H]Fucose; ●, D-[^3H]glucosamine. BPB, Bromophenol Blue.

than can be estimated by SDS/polyacrylamide gel electrophoresis. Components with lower apparent molecular weights were similarly labelled by both L-[^3H]fucose and D-[^3H]glucosamine (Fig. 2). Labelled components in the range of apparent mol.wts. 100 000–150 000 were prominent. ^3H -labelled glycoproteins with apparent molecular weights less than about 30 000 were not resolved, but migrated with the Bromophenol Blue tracking dye. Similar profiles were obtained when samples were analysed without prior reduction with 2-mercaptoethanol, indicating the absence of interchain disulphide linkages.

Effect of trypsin

Compared with whole epidermis (Fig. 2), the 'stratum corneum' (Fig. 3a and d) and 'cell pellet' (Fig. 3b and c) fractions both contained relatively

less ^3H -labelled glycoproteins in the range of apparent mol.wts. 100 000–150 000. Components in this range were therefore likely to be present at the epidermal cell surface. They were not detected in the 'trypsin supernatant' (Fig. 3c and f) fraction, which contained extracellular material and components released from the cell-surface, indicating that they had been degraded to low-molecular-weight peptides and glycopeptides. The peak of high-molecular-weight material labelled only with D-[^3H]glucosamine was also decreased in the 'stratum corneum' and 'cell pellet' fractions compared to whole epidermis (Fig. 2b). However, at least 70% of this material was present in the 'trypsin supernatant' fraction (Fig. 3f) indicating that it had been released either from the cell surface or the extracellular space. The electrophoretic mobility of the released material was unaltered by trypsin.

Fig. 4. Double labelling of material released by trypsin treatment of D-[^3H]glucosamine-labelled epidermis. Skin slices were cultured in the presence of D-[^3H]glucosamine together with (a) D-[^{14}C]glucuronic acid; (b) $^{35}\text{SO}_4^{2-}$; (c) L-[^{14}C]proline and (d) ^{14}C -labelled L-amino acid mixture for 18 h. The epidermis was separated from the dermis with trypsin and was treated further with trypsin before shaking to give a suspension of epidermal cells. After centrifugation the trypsin supernatant fraction was incubated with 2% (w/v) SDS/10 mM-mercaptoethanol at 100°C , and samples were analysed in 7% (w/v) polyacrylamide gels. ●, D-[^3H]Glucosamine; ○, D-[^{14}C]glucuronic acid, $^{35}\text{SO}_4^{2-}$, L-[^{14}C]proline or ^{14}C -labelled L-amino acids, BPB, Bromophenol Blue.

The radioactivity profile of material labelled with D-[^3H]glucosamine closely matched that of material labelled with [^{14}C]glucuronic acid (Fig. 4a) and with $^{35}\text{SO}_4^{2-}$ (Fig. 4b). Since D-glucuronic acid is a major constituent of glycosaminoglycans and since $^{35}\text{SO}_4^{2-}$ is incorporated mainly into sulphated glyco-

Fig. 5. Effect of hyaluronoglucosidase on the high-molecular-weight material released by trypsin

The trypsin supernatant fraction prepared from D-[^3H]glucosamine-labelled epidermis (18h) was treated with soya bean trypsin inhibitor (2 mg/ml) and then with (b) hyaluronoglucosidase (1 mg/ml) or (c) Pronase (1 mg/ml) for 1 h at 37°C. The control (a) received no enzyme. The digestion was stopped by adding 2% (w/v) SDS/10 mM-2-mercaptoethanol and heating at 100°C for 1 min. Samples were analysed in 7% (w/v) polyacrylamide gels. BPB, Bromophenol Blue.

saminoglycans (Barker *et al.*, 1964), this high molecular weight material released by trypsin is likely to contain glycosaminoglycans derived from epidermal proteoglycans. To check that it did not represent collagen-like proteins, rich in proline and hydroxyproline, the incorporation of [^{14}C]proline was also examined (Fig. 4c). Most of the [^{14}C]proline-labelled material released by trypsin was present as low-molecular-weight components that were well separated from the high molecular weight material labelled with D-[^3H]glucosamine. A similar profile was obtained with ^{14}C -labelled amino acids (Fig. 4d).

Further evidence that this peak of high molecular weight material was glycosaminoglycan rather than glycoprotein was provided by the effect of testicular hyaluronoglucosidase, which digests hyaluronic acid and chondroitin sulphates mainly to tetrasaccharides (Meyer, 1971). The high molecular weight material was completely degraded by hyaluronoglucosidase (Fig. 5b) but resisted digestion with pronase (Fig. 5c).

Membrane-bound ^3H -labelled glycoconjugates

To identify which labelled components were membrane bound, labelled skin slices were mixed with unlabelled slices as carrier and the tissue was homogenized in 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose. The filtered homogenate was fractionated by differential centrifugation as described in the Experimental section. Table 1 compares the specific radioactivity of each fraction after precipitation with 10% trichloroacetic acid. The relative specific radioactivities of each fraction were similar from L-[^3H]fucose- and D-[^3H]glucosamine-labelled epidermis. The 113 700 g_{av} . pellet, consisting mainly of epidermal membranes, was enriched about 1.8-fold in ^3H -labelled glycoprotein compared to the starting homogenate. Most (93 and 94% respectively) of the total L-[^3H]fucose and D-[^3H]glucosamine in this fraction was acid-insoluble compared to only 40 and 51% in the 113 700 g_{av} . supernatant fraction.

Greater than 90% of the ^3H -labelled glycoprotein in the 113 700 g_{av} . pellet was solubilized with 2% SDS/10 mM-mercaptoethanol at 100°C. The complex ^3H -labelled glycoprotein composition was similar for L-[^3H]fucose- (Fig. 6a) and D-[^3H]glucosamine labelled membranes (Fig. 6b). The precise radioactivity profiles varied somewhat due to minor variations in electrophoretic migration and in gel slicing. Some ^3H -labelled glycoprotein (usually 5% or less) was not fully disaggregated and remained at the top of the gel. The major ^3H -labelled glycoproteins migrated in the range of apparent mol.wts. 70 000–150 000. Within this range at least three, and sometimes four, peaks, apparent mol.wts. $137\,000 \pm 10\,000$, $113\,000 \pm 8000$, $93\,000 \pm 7000$ and $74\,000 \pm 6000$ (means $\pm$ s.d. for six experi-

ments) were distinguished. ³H-labelled glycoprotein peaks with apparent mol.wts. of 61000, 52000, 43000 and 35000 were rather more variable but were usually only minor components. ³H-labelled

glycoproteins with mol.wts. less than about 30000 were not resolved but migrated as a single band at the bottom of the gel. These various components were also present when the 113 700 *g*_{av.} pellet was

Table 1. Subcellular fractionation of ³H-labelled epidermis

³H-labelled skin slices were mixed with unlabelled tissue, homogenized and fractionated by differential centrifugation (see the Experimental section). Samples were precipitated with 10% (w/v) trichloroacetic acid at 0°C. Values are the means (± s.d.) from three separate experiments. The percentage of protein or radioactivity in each fraction that was precipitated with trichloroacetic acid is given in brackets. The total acid-insoluble protein in the filtered homogenate was 174 ± 34 mg. The total acid-insoluble radioactivity was 3.76 × 10⁶ ± 0.76 × 10⁶ c.p.m. for L-[³H]fucose-labelled and 1.49 × 10⁶ ± 0.3 × 10⁶ c.p.m. for D-[³H]glucosamine-labelled skin slices.

Fraction	Percentage of total protein	Percentage of total radioactivity		Specific radioactivity (c.p.m./μg of protein)	
		L-[³ H]Fucose	D-[³ H]Glucosamine	L-[³ H]Fucose	D-[³ H]Glucosamine
Homogenate	100 (86)	100 (58)	100 (65)	18.8 ± 4.8	8.2 ± 2.4
376 <i>g</i> _{av.} pellet	37 ± 7 (95)	21 ± 3 (80)	20 ± 3 (82)	13.9 ± 3.3	6.7 ± 1.6
113 700 <i>g</i> _{av.} pellet	18 ± 4 (95)	38 ± 1 (94)	30 ± 4 (93)	34.0 ± 10.0	13.9 ± 2.5
113 700 <i>g</i> _{av.} supernatant	48 ± 4 (80)	41 ± 3 (40)	50 ± 2 (51)	12.9 ± 5.9	6.8 ± 3.3

Fig. 6. ³H-labelled glycoprotein compositions of the 113 700 *g*_{av.} pellet and amino-acid 2-naphthylamidase-enriched membranes isolated from labelled epidermis

The membrane fractions were solubilized in 2% (w/v) SDS/10mM-2-mercaptoethanol at 100°C, and were centrifuged at 113 700 *g*_{av.} for 30 min. Samples of the supernatant, containing 500 μg (a and b) or 250 μg (c and d), of protein, were analysed in 7% (w/v) polyacrylamide gels. (a) and (b), 113 700 *g*_{av.} pellet; (c) and (d) amino-acid 2-naphthylamidase-enriched fraction. O, L-[³H]Fucose; ●, D-[³H]glucosamine. BPB, Bromophenol Blue.

Table 2. *Fractionation of ^3H -labelled epidermal membranes by sucrose-density-gradient centrifugation*

The 113 700 g_{av} . pellet from ^3H -labelled skin slices was fractionated on discontinuous sucrose-density gradients (see the Experimental section). Interfacial fractions and a sample of the original pellet were diluted with water, collected by high-speed centrifugation and were assayed for amino acid 2-naphthylamidase activity. Specific radioactivities were measured without trichloroacetic acid precipitation. Values are the means ($\pm$ S.D.) from five separate experiments.

Fraction	Amino-acid 2-naphthylamidase activity (fluorescence units/ 15 min per μg of protein)	Specific radioactivity (c.p.m./ μg of protein)	
		L-[^3H]Fucose	D-[^3H]Glucosamine
113 700 g_{av} . pellet	10.3 $\pm$ 3.5	51.9 $\pm$ 16.7	21.2 $\pm$ 5.7
0.5/1.0 M-Sucrose	29.3 $\pm$ 6.7	119.7 $\pm$ 19.2	42.2 $\pm$ 2.5
1.0/1.5 M-Sucrose	14.5 $\pm$ 5.4	63.4 $\pm$ 17.7	31.2 $\pm$ 4.6
1.5/2.0 M-Sucrose	7.1 $\pm$ 3.7	23.9 $\pm$ 9.4	14.2 $\pm$ 3.7

Table 3. *Solubilization of membrane-bound epidermal ^3H -labelled glycoproteins*

The 113 700 g_{av} . pellet (about 5 mg of protein/ml) from ^3H -labelled skin slices was incubated for 30 min at 22°C in 10 mM-Tris/HCl, pH 7.5, containing the indicated reagents and were centrifuged at 113 700 g_{av} . for 30 min. Controls received no additions. Solubilization with butan-1-ol was carried out as described in the Experimental section.

Reagent	Percentage of protein solubilized	Percentage of radioactivity solubilized	
		L-[^3H]Fucose	D-[^3H]Glucosamine
	20	15	16
1 M-KCl	35	21	22
10 mM-EDTA	12	13	15
10 mM-2-Mercaptoethanol	21	18	16
1% Triton X-100	40	68	71
Butan-1-ol	25	46	44

prepared using CaCl_2 -separated epidermis rather than skin slices, indicating that there was minimal contamination with dermal ^3H -labelled glycoproteins.

The 113 700 g_{av} . pellet was fractionated on discontinuous sucrose-density gradients. A vesicular membrane fraction, banding at the 0.5/1.0 M-sucrose interface, was enriched in amino-acid 2-naphthylamidase, 5'-nucleotidase and phosphodiesterase activities and was derived mainly from the epidermal cell plasma membrane (Gray *et al.*, 1980). This fraction was enriched in ^3H -labelled glycoprotein (Table 2). Compared to the 113 700 g_{av} . pellet, the specific radioactivities of the L-[^3H]fucose- and D-[^3H]glucosamine-labelled glycoproteins in the plasma membrane fraction were increased by factors of 2.3 and 2.0 respectively. Amino-acid 2-naphthylamidase activity was enriched 2.9-fold in the same membrane fraction. The ^3H -labelled glycoprotein composition of the plasma-membrane fractions (Fig. 6c and d) was similar to that of the 113 700 g_{av} . pellet in that the major ^3H -labelled glycoproteins migrated as a broad peak in the range of apparent mol.wt. 70 000–150 000.

The conditions required to solubilize these membrane-bound ^3H -labelled glycoproteins were investi-

gated to determine whether they were peripheral or integral components of the plasma membrane (Singer & Nicolson, 1972). Treatment with 1 M-KCl, 10 mM-EDTA and 10 mM-2-mercaptoethanol did not release significant amounts of ^3H -labelled glycoprotein from the 113 700 g_{av} . pellet (Table 3). The ^3H -labelled glycoproteins were therefore not bound to the membranes solely by salt linkages, Ca^{2+} salt bridges or disulphide bonds. Triton X-100 (1%) solubilized about 70% of the total membrane-bound ^3H -labelled glycoprotein. A 5-fold increase in Triton X-100 concentration solubilized only an additional 5–10% of the total ^3H -labelled glycoprotein. Treatment with butan-1-ol rendered at most about 45% of membrane-bound ^3H -labelled glycoprotein water-soluble. This value could not be significantly increased when the protein concentration (0.5–10 mg/ml), temperature (0–37°C) or salt concentration (0–1 M-KCl) was varied. The butan-1-ol-solubilized ^3H -labelled glycoproteins remained aggregated in the aqueous phase, since they were eluted at the void volume during gel-permeation chromatography on Sephadex G-200 (results not shown). Butan-1-ol treatment preferentially solubilized the major ^3H -labelled glycoproteins (range of apparent mol.wts. 70 000–150 000) present in the

Fig. 7. Effect of neuraminidase on membrane-bound ^3H -labelled glycoproteins solubilized by butan-1-ol treatment

The 113 700 g_{av} . pellet from labelled epidermis was treated with butan-1-ol (see the Experimental section) and the aqueous phase was concentrated by lyophilization. One-half of each sample was incubated with neuraminidase [5 units in 50 mM-sodium acetate (pH 5.5)/0.9% (w/v) NaCl/0.1% (w/v) CaCl_2] at 37°C for 1 h. Controls received no enzyme. The enzyme was inactivated by adding 2% (w/v) SDS/10 mM-mercaptoethanol and heating at 100°C. Identical sample volumes were analysed in 7% (w/v) polyacrylamide gels. ○, □, L- ^3H]Fucose; ●, ■, D- ^3H]glucosamine; ○, ●, control; □, ■, neuraminidase-treated. BPB, Bromophenol Blue.

membrane fractions (Fig. 7). These glycoproteins contained labelled terminal sialic acid residues since they were degraded by neuraminidase (Fig. 7b). Neuraminidase removed 40–50% of the total radioactivity from D- ^3H]glucosamine-labelled glycoproteins that had been solubilized by butan-1-ol treatment. L- ^3H]Fucose-labelled glycoproteins were not degraded after identical neuraminidase treatment, confirming the specificity of the enzyme action (Fig. 7a). Removal of sialic acid did not significantly affect the electrophoretic mobility of ^3H -labelled glycoproteins labelled with either sugar.

Soluble epidermal ^3H -labelled glycoproteins

Acid-insoluble ^3H -labelled glycoproteins accounted for 41 and 50% respectively of the total L- ^3H]fucose- and D- ^3H]glucosamine-labelled glycoproteins in the homogenate. The radioactivity profiles of the soluble fractions were more variable than those of the membrane-bound ^3H -labelled glycoproteins. This is probably due to proteolytic degradation during dialysis. However, the high-molecular-weight material labelled only with D- ^3H]glucosamine was always present in this soluble epidermal fraction providing it was heated with SDS prior to dialysis. It was also present in the 113 700 g_{av} . supernatant prepared from CaCl_2 -separated epidermis rather than skin slices, confirming that it was mainly epidermal in origin.

Discussion

Autoradiography indicated that a large proportion of the L- ^3H]fucose and D- ^3H]glucosamine incorporated into cultured pig epidermis was associated with the cell surface (Plate 1). The surface glycoconjugates of eukaryotic cells are present as glycoproteins, glycolipids and proteoglycans. Since epidermal glycolipids contain mainly glucose (Gray & Yardley, 1975), they are unlikely to be labelled with either sugar. In the present work we have been able to distinguish two distinct types of surface-associated glycoconjugate synthesized in cultured epidermis. These are (a) glycoproteins strongly bound to the plasma membrane, and (b) high molecular weight proteoglycans and/or glycosaminoglycans present either at the surface of, or between, the cells but which are not membrane bound. The latter are referred to as extracellular.

The ^3H -labelled glycoproteins recovered in particulate fractions (Table 1) accounted for 59 and 50% respectively of the total L- ^3H]fucose- and D- ^3H]glucosamine-labelled glycoproteins. The major ^3H -labelled glycoproteins in all particulate fractions migrated as a complex peak (range of apparent mol. wts. 70 000–150 000), and ^3H -labelled glycoproteins in this range were enriched in isolated plasma-membrane vesicles (Table 2 and Fig. 6) indicating that they were derived mainly from plasma membranes. The major ^3H -labelled glycoproteins labelled with either sugar had similar electrophoretic mobilities, subcellular distribution and solubilization properties. It thus seems likely that both fucose and glucosamine were incorporated into the same individual glycoprotein molecules in the plasma membrane. These glycoproteins contained terminal sialic acid residues since they were degraded by neuraminidase (Fig. 7). However, sialic acid probably only accounted for a small proportion of the weight of the glycoproteins, since its removal had no apparent effect on electro-

phoretic mobility. Fucose is also usually present at the termini of glycoprotein sugar sequences (Coffey *et al.*, 1964). The major glycoprotein molecules are likely to contain terminal sialic acid and fucose residues in a similar ratio, since the radioactivity profiles were always similar. Solubilization of the major membrane-bound glycoproteins was only achieved by treatment with solvents or detergents that disrupt the structure of the membrane (Table 3). They are therefore likely to be integral membrane components and are probably bound to the membrane by hydrophobic interactions (Singer & Nicolson, 1972). Their partial hydrophobic character is supported by the observation that the ^3H -labelled glycoproteins solubilized by butan-1-ol treatment were aggregated in aqueous solution.

This identification of plasma-membrane glycoprotein by metabolic labelling is in partial agreement with other analyses of desmosomal plasma-membrane fractions (Skerrow & Matoltsy, 1974*a,b*; Drochmanns *et al.*, 1978). We have examined pig epidermal plasma membrane fractions that contain no desmosomes and identified a major band of glycoproteins in the range of apparent mol.wts. 90 000–120 000 using the periodic acid/Schiff's reaction (Gray *et al.*, 1980). The greater heterogeneity of plasma membrane glycoproteins demonstrated by metabolic labelling may reflect differences in their turnover rates in culture.

The effect of trypsin provided further evidence that ^3H -labelled glycoproteins in the range of apparent mol.wts. 100 000–150 000 were present in the surface membranes of the epidermal cells. Compared to lower molecular weight glycoproteins in the epidermis, these components were preferentially digested releasing low molecular weight glycopeptides into solution. However, a high molecular weight component labelled only with D- ^3H glucosamine was also released with no apparent change in electrophoretic mobility. The possibility that this material leaks from the interior of trypsinized cells rather than being released from the cell surface is unlikely for several reasons. Firstly, the trypsinized cells appeared morphologically intact by electron microscopy. Secondly, 70–80% of the cells were viable as judged by their non-penetration by 0.5% nigrosine (I. A. King, unpublished work). Release of most of this high molecular weight material from the cell interior would require considerably more cell damage. Thirdly, other components were not present in the cell supernatant fraction, as would be expected if significant leakage had occurred. This high molecular weight material was not detected in any membrane fractions but it was recovered in the soluble epidermal fraction. It may be only weakly bound at the cell surface, perhaps in association with other surface components, or it may be present in the extracellular spaces.

In a preliminary report we suggested that this material was a glycoprotein since it remained on the gels after several washes with trichloroacetic acid (King & Gray, 1978). However, our present results indicate that it contains proteoglycans and/or glycosaminoglycans. Thus it is resistant to trypsin and Pronase but is degraded by hyaluronoglucosidase (Fig. 6). It is labelled both by ^{14}C -glucuronic acid and by $^{35}\text{SO}_4^{2-}$, and to a small extent by ^{14}C -labelled amino acids (Fig. 4). Several other possibilities have been considered. It is unlikely to be fibronectin-like (Yamada & Olden, 1978), since it is not susceptible to proteinases (Hynes, 1973), is not cross-linked by disulphide bonds (Keski-Oja *et al.*, 1977) and is not labelled by L- ^3H fucose (Sear *et al.*, 1977). Also, immunohistochemical studies have not revealed any fibronectin in the epidermis (Stenman & Vaheri, 1978). It is unlikely to be collagen-like, since the main monosaccharides present in collagen are glucose and galactose (Butler, 1978). Also it was not enriched in ^{14}C proline (Fig. 4c). Extracellular collagen has been shown to be synthesized in cultured epidermis (Stenn *et al.*, 1979) but this would probably be degraded during the trypsin separation procedure.

Further characterization of this extracellular material will require its isolation in sufficient amounts for chemical analysis. Nevertheless the metabolic labelling procedures characterized in this paper should allow an investigation of possible alterations in the structure and metabolism of surface glycoconjugates in diseased human epidermis (King *et al.*, 1979).

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