Concanavalin (ConA)-reactive Human Epidermal Glycoproteins

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The possibility that glycoconjugates play a role in epidermal differentiation has stimulated attempts at their biochemical identification. We have examined the Concanavalin (ConA)-binding glycoproteins present: in whole human epidermis, epidermal cells obtained by trypsinization of human skin, non dissociated layers (the most differentiated ones) remaining after preparation of these cells and human epidermal cell cultures. ^{125}I-ConA was applied to NP-40 soluble molecules separated by SDS-PAGE. ConA labelling of the whole epidermal extract visualized numerous bands. That of trypsinized cells differed in the intensity of some bands and the absence of others. However most of the latter bands could be identified in the most differentiated layers. These glycoproteins were absent or poorly labelled in the cultures of keratinocytes. The restriction of some ConA-binding glycoproteins to the most differentiated epidermal layers, suggests that these molecules are involved in, or can be considered as markers of the human epidermal differentiative process. *Key words: Human epidermis; Glycoproteins; Lectins; Differentiation.* (Received March 6, 1984.)

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Recent works have shown that some cellular constituents may be considered as markers of the various stages of human epidermal cell differentiation. Among them, are high MW keratin subunits recognized by monoclonal and polyclonal antibodies (1, 2); the basal cell layer cytoplasmic antigen, found in basal cells, the upper cytoplasmic antigen expressed only by more mature keratinocytes (3, 4) and cell surface antigens such as the basement zone antigen which is also specific for basal cells (5). Experimental evidence suggests that the cell surface plays a major role in diverse cellular functions: cell surface glycoconjugates, cell membrane proteins and submembraneous fibrillar structures are widely involved in these processes. Lectins, by their binding to specific sugar residues, have proved to be a useful tool in the study of glycans associated with cell surfaces (6). Previous studies by fluorescence microscopy have demonstrated that the expression of membrane receptors for lectins by human epidermal cells may change according to the degree of epidermal differentiation (7, 8). Such studies do not reveal how many different macromolecules are stained nor their nature. Recently, epidermal glycoproteins of rodent epidermal cells have been analyzed biochemically by the application of iodinated lectins to molecules separated by SDS-PAGE (9, 10).

Nevertheless, no such reports have been made with regard to human epidermis. The present study was conducted in order to characterize ConA-binding glycoproteins present in substrates corresponding to various stages of human epidermal differentiation: whole epidermis, the most differentiated layers, and epidermal cells from the deeper layers. In parallel ConA-binding glycoproteins in epidermal cell cultures were investigated.

Our results show that some ConA-reactive glycoproteins are only present in the most differentiated layers of the epidermis and might be considered as markers of human epidermal differentiation.

MATERIALS AND METHODS

Epidermal cell culture

Human epidermal cell cultures were prepared from breast skin removed during plastic surgery. After storage overnight in Hank's solution lacking Ca^{2+} and Mg^{2+} (HBSS) containing antibiotics (400 µg/ml streptomycin, 400 U/ml penicillin, 1 µg/ml fungizone), skin slices were prepared with a keratotome and epidermis was separated from dermis by incubation in trypsin (GIBCO, 0.25 % in HBSS) for 1 h 30 min at 37°C. Epidermis was removed and placed in HBSS containing 10 % fetal calf serum (FCS). A cell suspension was prepared by agitation of the epidermal sheets, then filtered through cheesecloth, washed once in complete medium (Dulbecco's modified Eagle medium (GIBCO) containing 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin 0.25 µg/ml fungizone, non essential amino acids (GIBCO) and 10 mM Hepes pH 7.0) and counted. Cells were then seeded at $2 \times 10^5/cm^2$ in culture dishes (Corning) previously coated with Type I rabbit skin collagen. Cultures were maintained at 37°C in 5 % CO_2.

Tissue extraction

For biochemical studies of whole epidermis, dermo-epidermal separation was achieved by heat treatment (11). Pieces of skin were rinsed in phosphate buffered saline (PBS) plunged into EDTA 0.5 M pH 7.5 at 60°C for 1 min, and the epidermis removed. Epidermis was subjected to 2 min homogenization with a "Polytron" at 4°C in extraction buffer (0.05 M Tris HCl pH 7.5, 0.15 M NaCl, 0.5 % Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 5 mM NEM) followed by 30 strokes in a teflon/glass homogenizer. After centrifugation (57000 g, 4°C, 15 min) the supernate was collected and stored at −70°C. Typically, 0.4 mg of protein was solubilized/cm² of epidermis. In some experiments, cells put into suspension by trypsinization as described above, were extracted as were the non-dissociated remains of epidermal sheets, corresponding to the most differentiated layers. For cell cultures, the cell layer was rinsed with PBS and the cells scraped into extraction buffer at 4°C. After the teflon/glass homogenization, the homogenate was briefly sonicated.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed on slab gels using the system of Laemmli (12), modified by the use of a 5–15 % gradient of polyacrylamide. 300 µg of protein were dissolved in 50 µl SDS-sample buffer, and heated to 100°C for 1.5 min. Gels were stained with Coomassie blue R250, 0.25 % in methanol : acetic acid : H_2O (50 : 7 : 43) and destained in methanol : acetic acid : H_2O (30 : 7 : 43).

Iodination of lectins and labeling of gels

Concanavalin A (ConA) (EY Laboratories, San Mateo, CA, USA) was dissolved in buffer A (0.05 M Tris, HCl pH 7.0, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1 % NaN_3) and iodinated by the method of Burridge (13) as described by Brysk (9). Specific activity of iodinated ConA was typically in the range of 10^8–10^9 cpm/mg. Iodinated lectin was examined by SDS-PAGE; single radioactive bands corresponding to lectin sub-units were resolved.

Destained polyacrylamide gels were processed with 10^7 cpm of lectin as described by Brysk (9). Specificity of lectin binding was controlled by exposure of some gels to the relevant inhibitory sugar 1-0-methyl-∝-D-glucopyranoside (at 0.2 M) before and during contact with the ^{125}I-lectin; gels were dried and exposed to Kodak X-Omat AR film in the presence of an intensifying screen.

Fluorescence microscopy

Cultures were rinsed in PBS, fixed in 2 % formaldehyde, washed with buffer A then flooded with FITC-ConA diluted to 25–150 µg protein/ml. After 1 h, cultures were washed, mounted under coverslips and viewed with a fluorescence microscope.

In control FITC-ConA conjugate was incubated in a 0.2 M solution of 1-0-methyl-∝-D-glucopyrano-side.

Electron microscopy

After control in light microscopy the sections were fixed by 1 % osmium tetroxide dehydrated and included in epoxy medium. Ultrathin sections were observed without supplementary contrast using a Hitachi HU 12A microscope under 50 kV. As controls, the same sections were secondarily contrasted and observed under 80 kV.
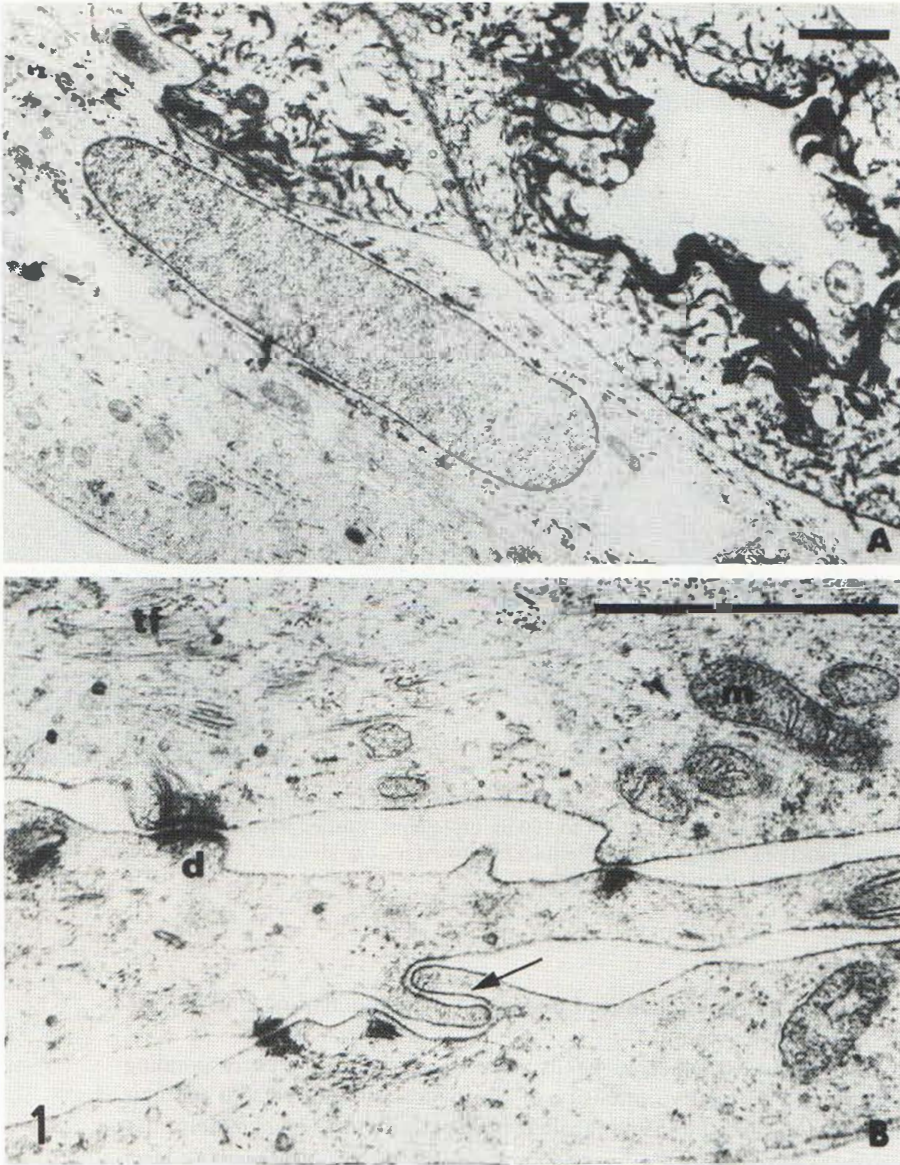
Fig. 1. Electron micrographs illustrating the pattern of epidermal cell culture organization at days 13. Stratified multilayers can be observed (A). Original magnification: ×12 000. Some typical structures of keratinocytes: desmosomes (*d*) between adjacent cells, tonofilaments (*tf*) dispersed in the cytoplasm and interdigitations (→) of the membranes can be seen by higher magnification (B): ×40 000. (Bar: 1 μm.)

RESULTS

Glycoproteins of whole epidermis

As expected, SDS-PAGE revealed that the NP-40 extraction buffer effectively solubilizes numerous proteins from whole epidermis (Fig. 3 *e*). The major proteinaceous components
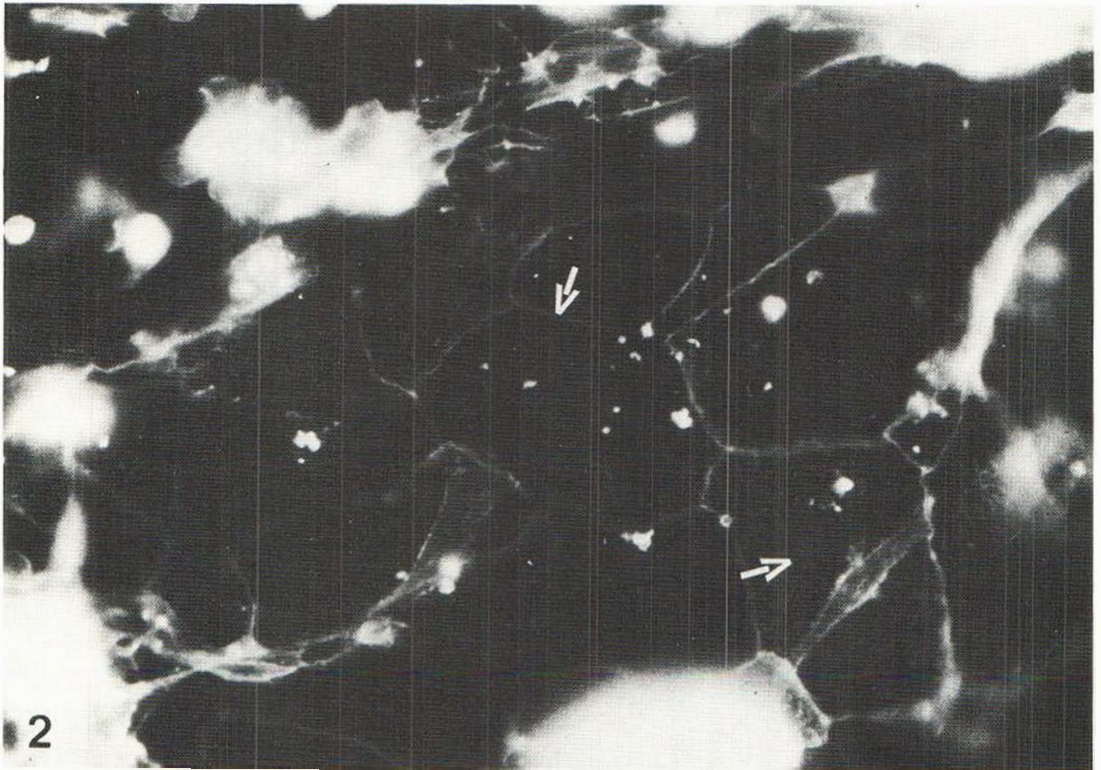
Fig. 2. Fluorescence microscopy of epidermal cell culture using ConA-FITC. Cultures were fixed in 2% formalde-
hyde. washed. flooded with the lectin-FITC diluted to 100 µg protein/ml, washed and viewed with a fluorescence
microscope. Intercellular junctions strongly react. Vermicular structures are identified by ConA at the surface of
some cells (white arrows). Original magnification: ×400.

of epidermis, the keratins, are not solubilized under these conditions and thus do not
overload the gel in the 67–40 kD region.

After labelling with ^{125}I-ConA, numerous bands were visualized on the corresponding
autoradiogram (Fig. 3 e'). The relative intensity of the bands was not related to their
protein content: in fact, the most intensely labelled bands were in the higher molecular
weight (MW) range (>70 kD), where Coomassie staining was the least intense. Control
incubation with inhibitory sugar verified the specific nature of the labelling procedure (not
shown).

Glycoproteins of epidermal cells after trypsinization and in culture

Under our conditions of culture, a confluent layer of cells is established within a few days
and stratified, keratinizing cultures are obtained by 10 days. By electron microscopy some
typical structures of keratinocytes are observed (Fig. 1). Surface receptors for ConA are
expressed by epidermal cells (Fig. 2).

The protein (Coomassie blue stained) composition of whole epidermis and preparations
of cells are essentially the same (Fig. 3 a–e). Only the most differentiated tissue prepara-
tion shows notable differences, in that proteins of lower MW (<68 kD) are particularly
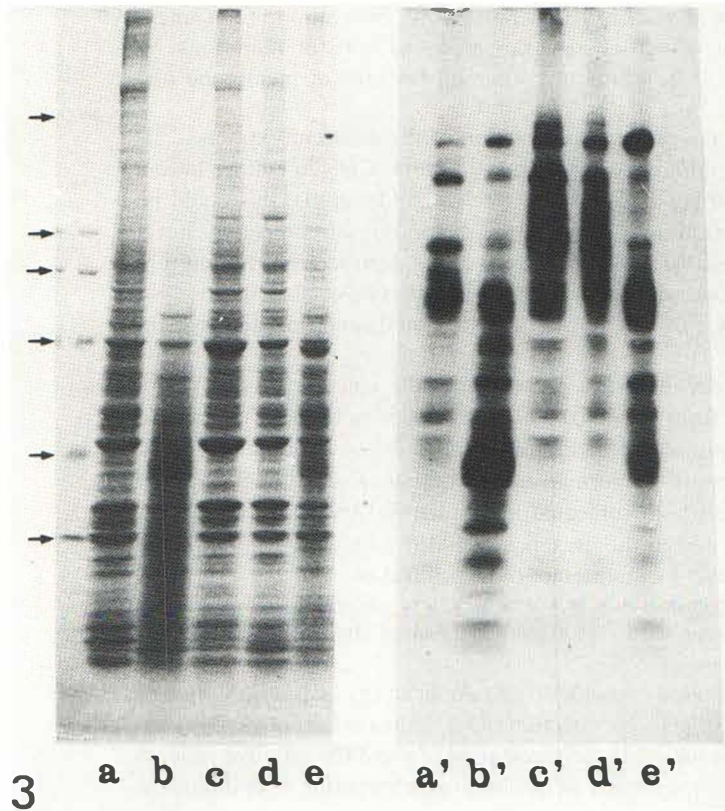abundant (Fig. 3 b).

Fig. 3. SDS-PAGE of extracts of (*a*). trypsinized cells; (*b*). the most differentiated layers: (*c*). (*d*). cells after 4 and 13 days in culture respectively: (*e*). whole epidermis: (*a'*)–(*e'*), autoradiogram obtained after ^{125}I-ConA labelling of the same gel. → Apparent MW and migration positions of MW markers: myosin, 200 kD; β-galactosidase. 116 kD, phosphorylase B, 97 kD; bovine serum albumin. 68 kD; ovalbumin. 44 kD; carbonic anhydrase, 29 kD.

Differences are much more apparent after labelling of these preparations with ^{125}I-ConA. Generally, the trypsinized cells lack several discrete and diffuse bands of lower MW which are present in the whole epidermis (Fig. 3 *a'* and 3 *e'*). However, these same bands are present in the most differentiated tissue preparation (some in increased proportions), and it is the higher MW material that is absent or shows reduced labelling (Fig. 3 *b'* and 3 *e'*). Cells in cultures for 4 or 13 days show a particularly heavy labelling around the 90–160 kD range which is not present in whole epidermis (Fig. 3 *c'*, *d'*). These cultures apparently do not re-express the lower MW glycoproteins which are absent from freshly trypsinized cells.

DISCUSSION

The extraction procedure effectively solubilized a large number of ConA-reactive glycoproteins from the tissues studied. Potentially, both cell surface and non-surface components were extracted, but as lectin staining on skin sections labels mainly surface or intercellular components (7, 8, 10), these presumably are the major contributors to the labelling seen on gels. Concerning the proteins extracted from the most differentiated tissue, our findings agree with already published results (14), with respect to the preponderance of lower MW material. The work of Brysk (9, 10) on lectin labelling of rat epidermal glycoproteins is difficult to compare directly with our present study because of slight differences in tissue preparation and gel electrophoresis. However, one common

feature is the multiplicity of bands marked by ^{125}I-ConA. This observation is not unexpected, because ConA is capable of recognizing the mannose core of numerous N-asparagine-linked oligosaccharides (15), which are common features of membrane and secreted glycoproteins.

Despite the overall similarity of the protein composition of the different extracts, ^{125}I-ConA labelling revealed numerous differences in glycan structure. Cells in culture have a particularly abundant expression of glycoproteins between 90 and 160 kD; this could be due to a modification of glycosylation in culture, or the expression of completely novel glycoproteins. In addition, the possible adherence of proteins derived from the culture medium must also be taken into consideration. The work of Brysk et al. (10) has also demonstrated differences between ConA labelling of heat-separated epidermis and trypsin-released cells from rat epidermis.

Because several lower MW glycoproteins are present in whole epidermis and trypsin-generated upper layers, but absent from trypsinized cells of deeper cell layers, we suggest that these proteins are characteristic of the highly differentiated layer, and can thus be considered as glycoprotein markers of epidermal differentiation. Even though stratum corneum and deeper layer cells were both subjected to an identical trypsinization procedure and were subsequently processed in the presence of PMSF, a trypsin inhibitor, the observed difference could conceivably be an artefact of trypsinization. However, the *non*-reappearance of the lower MW glycoproteins in culture, where complete expression of differentiative characteristics is rarely seen (16) argues in favor of the proposed hypothesis.

Our results demonstrate the resolution, specificity and applicability of the technique of detection of glycoproteins on SDS-gels by reaction with iodinated lectins. Work in progress exploiting this technique is aimed at the elucidation of the differentiative process of human epidermis *in situ* and *in vitro*, as well as its abnormal expression in pathological conditions.

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