Onset of expression of peanut lectin-binding glycoproteins is correlated with stratification of keratinocytes during human epidermal development

in vivo and in vitro

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Summary

During gestation the epidermis develops from a single layer of ectoderm into a layer of keratinocytes overlaid by a layer of periderm; this is followed by a progressive increase in the number of layers of keratinocytes, until finally the distinct granular and cornified layers characteristic of mature epidermis are formed. As part of our investigation into the function of the peanut lectin-binding glycoproteins of cultured human keratinocytes, we have examined their expression at different stages of human epidermal development. We found that the onset of expression of the glycoproteins coincided with the transition from a two- to a three-layered epidermis, both in vivo and in organ culture. In adult epidermis, the patterns of binding of peanut lectin and Limax flavus lectin are complementary, with peanut binding more strongly to suprabasal keratinocytes and Limax flavus lectin binding more strongly to cells in the basal layer. We found that the complementary pattern of binding of the two lectins was established at, or shortly after, the onset of stratification and retained throughout development. In contrast, expression by keratinocytes of involucrin, a protein precursor of the cornified envelope, occurred after stratification had begun. Finally, we identified the peanut lectin-binding glycoproteins of adult epidermis by immunoblotting with an antiserum raised against the glycoproteins of cultured neonatal keratinocytes. In conclusion, expression of the peanut lectin-binding glycoproteins is an early event in epidermal development, and this would be consistent with a role for the glycoproteins in stratification.

Key words: keratinocytes, epidermal development, differentiation, stratification, lectins, glycoproteins.

Introduction

The epidermis consists of multiple layers of keratinocytes: proliferation is confined to the basal layer, attached to the basement membrane, and terminal differentiation occurs as the cells move upwards through the suprabasal layers to the tissue surface (reviewed by Matoltsy, 1986). The correlation between keratinocyte position and stage of terminal differentiation is important for normal epidermal function, since keratinocytes in the outermost layers have aggregated keratin filaments and an insoluble subplasmalummal protein envelope (the cornified envelope) that protect cells in the underlying living layers from desiccation and mechanical damage. In epidermal neoplasia the spatial relationship between proliferating and terminally differentiating cells is perturbed. The mechanism by which terminally differentiating keratinocytes move upwards out of the basal layer is therefore of considerable interest.

Studies with cultured human keratinocytes have shown that migration out of the basal layer is not a prerequisite for initiation of terminal differentiation, but rather a consequence (Watt and Green, 1982). As part of the programme of terminal differentiation the adhesive affinity of the cells for the culture substratum and for one another is reduced (Watt, 1984). The search for molecules that might play a role in the changes in cell–cell adhesiveness led us to investigate the lectin binding glycoproteins of cultured keratinocytes, and, in particular, the glycoproteins that bind peanut lectin (PNA, Arachis hypogaea agglutinin; carbohydrate specificity: β-D-Gal(1→3)-β-D-GalNAc; β-D-galactosyl).
PNA binds more strongly to suprabasal than basal keratinocytes in adult epidermis and stratified cultures, and also shows specificity for terminally differentiating keratinocytes in low-calcium monolayers (Watt, 1983). The PNA-binding glycoproteins of cultured human keratinocytes are two heavily glycosylated proteins that migrate as diffuse bands of 250 and 110K (K=10^3 M_2) during polyacrylamide gel electrophoresis. Unlike PNA, an antisera raised against the glycoproteins (anti-PNA-gp) binds to both basal and suprabasal keratinocytes, suggesting that some epitope(s) of the glycoproteins is expressed prior to terminal differentiation (Morrison et al. 1988). Recent evidence suggests that the increase in PNA binding above the basal layer is due to loss of terminal sialic acid residues from the 250K glycoprotein.

A sialic acid-specific lectin, Limax flavus agglutinin (LFA), binds more strongly to basal than to suprabasal epidermal keratinocytes and recognises the same 250K band that binds PNA and anti-PNA-gp (Keeble and Watt, unpublished).

There is indirect evidence of a role for the PNA-binding glycoproteins in stratification. Fisher and Holbrook (1987) have found that expression of PNA-binding sites coincides with the onset of stratification during epidermal development in vitro and in organ culture; and irregular staining with PNA has been reported in epidermis affected by the blistering disorder epidermolysis bullosa simplex (Fine and Griffith, 1985). In keratinocyte cultures, the PNA-binding glycoproteins are present on cell surface microvilli and become concentrated at the boundaries between cells during calcium-induced stratification (Morrison et al. 1988; Keeble and Watt, unpublished). Finally, a strain of human keratinocytes that are unable to stratify or terminally differentiate fail to bind PNA, but do stain with anti-PNA-gp (Adams and Watt, 1988) and LFA (Keeble and Watt, unpublished).

The aim of the experiments described in this report was to investigate the onset of expression of the PNA-binding glycoproteins during the development of human epidermis. We have studied the timing and location of expression of binding sites for LFA, PNA and anti-PNA-gp during epidermal development in vitro and in organ culture. We have also used immunoblotting with anti-PNA-gp to compare the glycoproteins of adult epidermis with those of cultured neonatal keratinocytes. The different stages of development were assessed by epidermal ultrastructure and expression of involucrin, the major protein precursor of the cornified envelope (Simon and Green, 1984). Our results show that the onset of expression of the PNA-binding glycoproteins coincided approximately with the transition from a two- to a three-layered epidermis, both in vitro and in vitro, and that the patterns of LFA and PNA binding were the same as in adult epidermis. The significance of these findings is discussed.

Materials and methods

Source of epidermis and fixation procedures
Skin samples were taken from morphologically normal human foetuses obtained at elective terminations of pregnancy. The estimated gestational age (EGA) of the foetuses was determined using standard criteria (Streeter, 1921; Nishimura and Yamasaki, 1968). For histological examination, the number of cases at each gestational age was: 3, 7 weeks; 2, 8 weeks; 3, 10 weeks; 4, 11 weeks; 1, 12 weeks; 5, 13 weeks; 2, 14 weeks. Autopsy samples of skin were also taken from a stillbirth (40 weeks EGA) and a 2-week-old neonate. Adult breast skin was taken from the margins of surgical pathology specimens, well away from any abnormal areas.

Each skin sample was divided into three. One part was dusted in maize starch, snap-frozen and stored in liquid nitrogen; one part was fixed in methacarn (a mixture of methanol, chloroform and acetic acid, 6:3:1, by vol.) for 1 h and then paraffin-embedded; and one part was fixed in formal saline for 1 h and then paraffin-embedded. In preliminary experiments the same staining patterns were observed in all three types of preparation; methacarn fixation was selected for further experiments, because it gave optimal histological preservation.

Organ culture
Embryonic skin was cultured as previously described (Fisher and Holbrook, 1987). Briefly, skin was obtained from the trunk of human embryos, in accordance with American DHEW regulations, from the Central Laboratory for Human Embryology, University of Washington. Estimated gestational age was determined by crown-rump length, foot length, and menstrual age. Two 20 mm^2 pieces of skin were arranged dermis-side-down on sterile HA-type Milipore filters (Millipore Corp., Bedford, MA), 0.45 μm pore size, in each culture dish. The filters were supported at the air-medium interface by wire mesh screens (Falcon) in Falcon 3037 Organ Tissue Culture Dishes (Becton, Dickinson and Co., Oxnard, CA). The culture medium consisted of Dulbecco's modified Eagle's medium (Flow Labs, McClean, VA) supplemented with 10% foetal calf serum (FCS), 100 μg ml^(-1) streptomycin, 100 units ml^(-1) penicillin, 300 μg ml^(-1) ascorbate, and 0.37% sodium bicarbonate.

In the present study two specimens of 54 days and 57 days EGA were used. Duplicate samples were taken at each time point (0, 1 and 2 days of culture) for each type of fixation (Carnoy's and 1/2 strength Karnovsky's fixatives). Carnoy's fixed material was embedded in paraffin for immunohistochemical studies and Karnovsky's fixed tissue was embedded in Epon. Methylene Blue-stained 1 μm sections of the Epon-embedded material were prepared for confirmation of the state of stratification of the embryonic epidermis.

Antibodies and lectins
PNA-peroxidase was purchased from Vector Laboratories (Bretton, Peterborough) and LFA-peroxidase was obtained from E-Y Laboratories (San Mateo, CA). Peroxidase-conjugated goat anti-rabbit IgG was supplied by ICN Biomedicals Ltd, High Wycombe, Bucks. The preparation and properties of rabbit anti-involucrin (Dover and Watt, 1987) and rabbit anti-PNA-gp (Morrison et al. 1988) have been described previously.

Preparation and staining of sections
PNA-peroxidase (1 mg ml^(-1)), diluted 1:75–1:100, and LFA-peroxidase (1 mg ml^(-1)), diluted 1:150, were applied to the sections for 1 h, washed thoroughly with phosphate-buffered saline (PBS) and the reaction sites were visualised with diaminobenzidine. Anti-PNA-gp, diluted 1:1000–1:5000, and anti-involucrin, diluted 1:500–1:1000, were applied for 1 h, washed thoroughly in PBS and incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:50). After further washing, the reaction sites were visualised with di-
Fig. 3. Histological sections of foetal epidermis. A–D, 8 weeks EGA; E–H, 12 weeks EGA; I–L, 22 weeks EGA; M–N, neonatal (2 weeks); O–P, term (40 weeks EGA). A,E,I,M, PNA; B,F,J,N, LFA; C,G,K,O, anti-PNA-gp; D,H,L,P, anti-involucrin. Bars: A–L,O,P, 50 μm; M,N, 100 μm.
Fig. 4. Histological sections of organ culture of foetal skin (57 days EGA) after 2 days in culture. A. Stained with Methylene Blue; B. parallel section stained with anti-PNA-gp. Bar, 50μm.
aminobenzidine. Sections were counter-stained with haematoxylin. In some cases, primary antibody binding was visualised using a biotinylated anti-rabbit second antibody, followed by an avidin-biotinylated peroxidase complex (Vector Laboratories or Dako Ltd), as previously described (Hsu et al. 1981; Fisher and Holbrook, 1987).

PNA and LFA staining could be inhibited by 0.2M-galactose and sialic acid (N-acetyl neuraminic acid), respectively. The control for the rabbit antisera was non-immune serum.

**Electron microscopy**

For ultrastructural examination, selected skin samples were fixed in 4 % glutaraldehyde, post-fixed in 1 % buffered osmium tetroxide, dehydrated and embedded in Araldite. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and examined in an AEI Corinth electron microscope.

**Western blotting**

The epidermis was separated from the dermis of adult human skin by two cycles of heating to 55 °C for 1 min and cooling to 4 °C in PBS, and then solubilised in sample buffer. Newborn foreskin keratinocytes were cultured with a 3T3 feeder layer as described previously (Morrison et al. 1988) and extracted in sample buffer. Samples were electrophoresed on 5 % polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose paper at 50 V overnight at 4 °C, as described previously (Towbin et al. 1979; Morrison et al. 1988). After transfer the nitrocellulose was incubated for 6–8 h at room temperature in 0.05 % Tween 20 in 0.5 M-sodium chloride, 20 mM-Tris–HCl, pH 7.5 (TBST) containing 10 % low-fat milk powder, to block nonspecific binding. The nitrocellulose was incubated with anti-PNA-gp diluted 1:1000 with DMEM+10% newborn calf serum overnight at 4 °C, washed in TBST, 4x5 min, and then incubated with 0.1 µCi ml⁻¹ of ¹²⁵I-labelled protein A (specific activity >30 mCi mg⁻¹, Amersham International, Amersham, England) for 1 h, washed 4x10 min in TBST, air-dried, and then exposed to Hyperfilm (Amersham International).

**Results**

**The different stages of epidermal development**

The different stages of human epidermal development have been described in detail elsewhere (Holbrook, 1983) and will only be summarised briefly here (Fig. 1). The primitive epidermis is established at about 1 week of gestation when the ectoderm and endoderm become defined in the inner cell mass of the blastocyst: at this stage it consists of a single layer of ectoderm. After approximately 1 month the epidermis consists of two layers: the outermost layer, the periderm, is an embryonic cell layer that is eventually sloughed off in utero and the inner layer is a layer of keratinocytes.

During the end of the second month of gestation (approximately 60–70 days EGA), proliferation of keratinocytes in the basal layer gives rise to a second layer of keratinocytes, intermediate in position between the basal layer and the periderm (Fig. 2). Two or three more layers of keratinocytes are added from 3-6 months' gestation. During the sixth month, distinct granular and cornified layers, characteristic of mature epidermis, can be distinguished and the periderm sloughs off. At term the foetal epidermis is almost indistinguishable in appearance from adult epidermis.

**Time-course of expression of the PNA-binding glycoproteins and involucrin in vivo**

Fig. 3 illustrates the staining patterns observed for PNA, LFA, anti-PNA-gp and anti-involucrin at different stages of gestation. In 7- to 8-week-old foetal epidermis (2 cell layers thick), no positive staining with any of the lectins or antisera was observed (Fig. 3A–D), although LFA stained the dermis strongly (Fig. 3B). By 11 weeks a second keratinocyte layer had formed between the basal layer and the periderm, and PNA, LFA and anti-PNA-gp

**Glycoproteins in developing epidermis**
all stained the epidermis (Fig. 3E–G). It is difficult to judge the relative intensity of staining in the different layers at this stage, because the basal cells are more tightly packed than the suprabasal cells. However, PNA appeared to stain the suprabasal layers more strongly than the basal layer (Fig. 3E) while LFA staining appeared strongest in the basal layer (Fig. 3F). Anti-PNA-gp stained all the epidermal layers with the same intensity (Fig. 3G). Involucrin was detected only in occasional cells of the periderm (Fig. 3H).

From 12 weeks onwards, as the number of keratinocyte layers increased, PNA bound more strongly to suprabasal than basal keratinocytes (Fig. 3I); LFA bound more strongly to basal keratinocytes (Fig. 3J) and anti-PNA-gp bound equally to all cell layers (Fig. 3K). Involucrin was detected in the periderm and the outermost layer of keratinocytes (Fig. 3L).

At the time when distinct granular and cornified layers appeared and the periderm was lost (approximately 6 months' gestation) the pattern of expression of the glycoproteins and involucrin was the same as in adult epidermis. Fig. 3M–P shows the staining of term and neonatal skin. PNA bound more strongly to the suprabasal than basal keratinocytes (Fig. 3M), whereas LFA bound most strongly to the basal layer (Fig. 3N). Anti-PNA-gp stained all the living cell layers (Fig. 3O) and anti-involucrin bound to cells in the upper spinous and granular layers (Fig. 3P).

Stratification of foetal epidermis in organ culture
The staining of sections of foetal epidermis (Fig. 4) showed that the onset of binding of PNA, LFA and anti-PNA-gp coincided with the onset of stratification, when a second layer of keratinocytes appeared between the basal layer and the periderm. We investigated whether the onset of staining was the same in organ cultures of foetal skin: 54- or 57-day-old foetal skin was cultured for 1 or 2 days, then fixed, embedded and sectioned. After 1 day, stratification had begun, resulting in two to three layers of keratinocytes underneath the periderm by 2 days in culture. As in vivo, the cells did not bind LFA, PNA or anti-PNA-gp prior to stratification, but did when stratification was initiated. The same patterns of staining were observed in organ culture and in vitro (Fig. 4 and results not shown).

Characterisation of the PNA-binding glycoproteins in adult epidermis
The PNA-binding glycoproteins of cultured human neonatal keratinocytes migrate as two diffuse bands of 250 and 110K on polyacrylamide gels (Morrison et al. 1988). Since anti-PNA-gp detects glycoproteins of different mobility in a range of epithelial cell lines (Keeble and Watt, unpublished), we investigated the nature of the glycoproteins in adult epidermis (Fig. 5). Previously, of the two bands expressed in culture, only the 250K band was detected in immunoblots (Morrison et al. 1988). However, using a more sensitive detection method, we could also resolve the 110K glycoprotein (Fig. 5, track 1). In adult epidermis, two diffuse bands were observed, one of which comigrated with the 110K band of keratinocytes, while the other migrated at about 200K (Fig. 5, track 2). In addition, material migrating close to the dye front was abundant in extracts of adult epidermis, but barely detectable in cultured cells.

Discussion
We have shown that the onset of binding of PNA, LFA and anti-PNA-gp coincided with the onset of stratification (12 weeks EGA) and that the complementary patterns of lectin binding noted in adult epidermis were established at, or shortly after, this time. Involucrin expression was restricted to occasional cells in the periderm at the time of stratification, and was expressed in the outermost layers of keratinocytes from approximately 22 weeks onwards, as observed by Holbrook et al. (1987).

Fisher and Holbrook (1987) previously demonstrated that PNA binding to developing epidermis correlated with stratification in vivo and in vitro. It was demonstrated that neuraminidase would expose PNA binding sites on basal cells of stratified, but not on basal cells of unstratified, epidermis. These results suggested that the PNA binding sites are present, but masked (presumably by terminal sialic acid residues), on basal cells of stratified epidermis, and that these sites are missing from the...
basal cells of unstratified epidermis. It was unclear from these studies whether the de novo appearance of PNA binding sites was accompanied by the appearance of the protein moiety carrying them. The correlation of anti-PNA-gp and PNA binding during stratification that we have now observed suggests that both the protein and carbohydrate moieties are expressed de novo. Their appearance during stratification lends further credence to the concept that the basal cell layer undergoes a significant differentiative event at this time (Fisher and Holbrook, 1987).

Immunoblots of cultured neonatal keratinocytes showed that anti-PNA-gp recognised bands at 250K and 110K, as previously demonstrated by immunoprecipitation (Morrison et al. 1988). Extracts of adult epidermis contained one band that comigrated with the 110K glycoprotein; another that migrated slightly ahead of the 250K band; and additional lower molecular weight material. It seems likely that the different mobilities of the high molecular weight bands isolated from epidermis and cultured keratinocytes reflect differences in glycosylation. Which, if any, of the adult epidermal bands binds LFA remains to be determined.

What is the function of the PNA-binding glycoproteins? Expression of a PNA-binding glycoprotein is known to be an early marker of epidermal development in Xenopus (Slack, 1985). In humans the onset of expression of the glycoproteins coincides with initiation of stratification, and precedes changes in keratin expression (Fisher and Holbrook, 1987), initiation of filaggrin (Dale et al. 1985) and involucrin synthesis (Holbrook 1983). Furthermore, the onset of expression of the PNA-binding glycoproteins precedes changes in a range of carbohydrate antigens on the surface of epidermal keratinocytes (Dabelsteen et al. 1986). Expression so early in human development is consistent with a role for the glycoproteins in stratification. However, direct evidence is lacking at present and it is possible that the glycoproteins fulfil some other role in the development and maintenance of epidermal function.

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References


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