

Patterned acquisition of skin barrier function during development

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SUMMARY

Skin barrier function is conferred by the outer layer of epidermis, the stratum corneum, and is essential for terrestrial life. Quantitative trans-epidermal water loss assays show that barrier forms late in embryogenesis, permitting the foetus to survive a terrestrial environment at birth. Using qualitative in situ assays for skin permeability, we show that barrier forms in a patterned manner late in mouse gestation. Barrier forms at specific epidermal sites, then spreads around the embryo as a moving front. The moving front of permeability change is accompanied by multiple changes in the outer, stratum corneum-precursor cells. We use the permeability assays to show that final stages of cornified envelope assembly are coordinated with initial stages of barrier formation. Hence the whole-mount permeability assays record

developmental acquisition of a known, essential component of the adult barrier. We demonstrate the authenticity of the whole-mount assays after maternal glucocorticoid therapy (known to accelerate barrier formation) and in additional species including the rat where barrier formation is well characterized by TEWL assay (Aszterbaum, M., Menon, G. K., Feingold, K. R. and Williams, M. L. *Pediatr. Res.* 31, 308-317). The demonstration of patterned barrier formation in other species suggests patterned change as the universal mode of embryonic barrier acquisition. These results highlight the importance of patterning as a mode of epidermal maturation during development.

Key words: Epidermal patterning, Skin barrier, Cornified envelope, Loricrin, Glucocorticoid, Mouse

INTRODUCTION

The epidermal permeability barrier prevents desiccation, protects against infection and poisoning from the environment and is essential for terrestrial life. Barrier function is defective in a variety of skin diseases (reviewed Roop, 1995) and in premature infants (Hammarlund and Sedin, 1979; Wilson, 1980). Experimental disruption of embryonic barrier formation results in neonatal lethality in transgenic mice (Imakado et al., 1995).

Barrier function is conferred by the outer layer of epidermis, the stratum corneum (Blank, 1953; Kligman, 1964), which consists of dead, keratin-filled cells embedded in a lipid matrix (reviewed Elias and Menon, 1991; Downing, 1992; Roop, 1995). Stratum corneum is formed from granular layer keratinocytes during terminal differentiation of normal adult epidermis. As keratinocytes terminally differentiate, they flatten and the intracellular contents are degraded. Lipid-containing lamellar bodies fuse with the plasma membrane and disperse their contents extracellularly. A tough, insoluble cornified envelope is assembled by sequential incorporation of precursor proteins (e.g. involucrin, small proline-rich proteins (SPRs) and loricrin), followed by covalent attachment of extracellular lipid. Keratohyalin granules, the distinguishing feature of granular cells, disperse and release the protein filaggrin, which promotes aggregation and packing of keratin filaments to form a dense cell matrix (reviewed Hohl and Roop, 1993; Dale et al., 1994;

Holbrook, 1994; Simon, 1994; Steinert and Marekov, 1997 and refs. within). The resultant structure has been likened to 'bricks and mortar' (Elias and Menon, 1991), with cornified cells forming the bricks and extracellular lipid the mortar.

Barrier function is usually measured by transepidermal water loss (TEWL) assay. Quantitative TEWL assay shows that barrier forms rapidly between days 19 and 21 of the 22 day rat gestation (Aszterbaum et al., 1992), and between weeks 30 and 33 of the 40 week human gestation (Hammarlund and Sedin, 1979; Wilson, 1980). Identification of the time that barrier forms permits analysis of correlative changes in epidermal morphology. As expected, barrier acquisition correlates with stratum corneum development. Aszterbaum et al. (1992) showed increased stratum corneum thickness together with specific changes in intercellular lipid content and structure in the rat over the period of barrier acquisition, while Evans and Rutter (1986) showed that stratum corneum is well developed after barrier institution at week 34 of human gestation.

Epidermal development (Sengel, 1976; Holbrook and Odland, 1980; Sengel, 1990) and gene induction are patterned (Byrne et al., 1994) rather than uniform during the early stages of embryogenesis. It is possible that patterned gene induction and differentiative change is a consequence of the need to coordinate developmental events in epidermal cells that are spread over a large surface area. If so, we predict that patterned change should occur during late gestation, when the epidermal

surface area is further expanded. We tested this hypothesis by examining embryonic barrier institution *in situ*.

We developed qualitative, whole-mount assays for skin permeability and show that the assays measure a first stage in barrier formation. We use the assays to demonstrate that barrier formation is indeed highly patterned during development. Barrier forms first at distinct epidermal sites then spreads across the epidermis as a moving front. Discovery of the moving front means that precise morphological correlates to barrier acquisition can be identified. We demonstrate that late stages of cornified envelope assembly accompany movement of the front. Hence the whole-mount permeability assays record developmental acquisition of a known, essential component of the adult barrier.

We demonstrate the authenticity and utility of our assays by monitoring barrier formation after hormonal treatment known to accelerate foetal barrier development. Our assays demonstrate the predicted acceleration and, additionally, reveal pattern distortion. We find that late stages of cornified envelope assembly accompany the accelerated front despite the relative immaturity of the epidermis. Finally, we demonstrate patterned skin barrier acquisition in additional species. Hence patterned change is probably a ubiquitous mode of epidermal differentiative change during mammalian development.

MATERIALS AND METHODS

Embryos

Mice were time-mated within a 5 hour mating window (permeability assays, TEWL and EM studies) or a 2 hour mating window (cornified envelope, glucocorticoid experiments) and the mid-point of the mating window designated gestational age zero. Mice used in Fig. 3 were derived from random matings within a 4 day period and categorized according to gross morphology and barrier status. ICR, MFI and CDI mice were used in permeability assays and ICR strain mice were used for other procedures. Sprague-Dawley rats were time-mated within a 12 hour mating window and the mid-point of the window designated zero. Time-mated New Zealand white rabbits were obtained from Charles River and gestational age was calculated from observed mating times.

Estimated gestational age (EGA) was calculated from the time designated zero. For example, 16 days/5 hours after time zero was termed E16/5 or 16/5 days EGA. Animals were killed by terminal anaesthesia by inhalation.

Transepidermal water loss (TEWL) assay

Gravimetric TEWL assay (Nolte et al., 1993; Hanley et al., 1996a) was performed on skin samples excised from embryo flank. Samples sealed on the dermal and lateral surfaces were weighed every 30 minutes for 5 hours at ambient temperature with constant humidity using a Cahn balance (accurate to 0.001 mg). The surface area of skin samples was determined using an area measurement computer program (S. Bagley, personal communication), following photography and image capture. TEWL was calculated as milligrams water loss per square millimetre of epidermis per hour ($\text{mg H}_2\text{O}\cdot\text{mm}^{-2}\cdot\text{hour}^{-1}$).

Skin permeability assay

Assay 1 depends on barrier-dependent access of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) to untreated skin. At low pH skin contains abundant endogenous β -galactosidase activity (Byrne, unpublished), which cleaves X-gal to produce a coloured precipitate. Unfixed, untreated, freshly isolated embryos were rinsed in phosphate-buffered saline (PBS) and immersed in standard X-gal

reaction mix (Bonnerol and Nicolas, 1994), except that the pH was adjusted to 4.5. Incubation was at 30°C until colour develops (2 hours to overnight).

Assay 2 modifies skin to permit barrier-dependent penetration by histological dyes. The basis of this skin modification is unknown but is likely to involve extraction of polar lipid (Wertz and Downing, 1987). Unfixed, untreated embryos were incubated for 1-5 minutes in methanol and rinsed in PBS, followed by incubation in 0.5% hematoxylin or 0.1% toluidine blue.

After staining, embryos were embedded in agarose and photographed using a Zeiss Stemi SV11 microscope with transmitted and surface illumination. Scanned images were processed with Adobe Photoshop and agarose background was removed.

Assays 1 and 2 were compared and gave the same staining pattern. Comparisons are performed on sagittal sides of a single embryo to eliminate variation arising from differences in developmental stage. As assays 1 and 2 give the same staining patterns, we can infer that they measure the same skin change.

For histological analysis and protein/envelope preparation, a dorsal-ventral skin strip was cut into the shape of a comb (see Fig. 5a) and samples excised from between the teeth prior to permeability staining. Permeability status of the samples was determined by staining the comb after sample removal; i.e. samples for histological analysis were never subjected to permeability staining.

Western analysis

Epidermal-dermal heat separations were as described (Hohl et al., 1991). Protein was isolated from epidermal samples as described (Yuspa et al., 1989) and equal amounts were fractionated on 5-15% gradient gels. Western analysis was by standard technique (Ausubel et al., 1995). Affinity-purified anti-filaggrin and anti-loricrin rabbit polyclonal peptide antibodies (Yuspa et al., 1989) were gifts from Dr Stuart Yuspa and Dr Elaine Fuchs, respectively. In addition, a similar anti-filaggrin antibody was synthesized by the Zymed Corporation (CA, USA) and used after affinity purification.

Electron microscopy and immunoelectron microscopy

Samples for light and electron microscopy were fixed immediately after removal from untreated embryos. Light microscopy was performed on 1 μm sections prepared for standard electron microscopy and stained with toluidine blue. Standard electron microscopy was performed on samples fixed in half-strength Karnovsky's fixative and osmium tetroxide. For lipid visualization, ruthenium tetroxide was used as the secondary fixative (Swartzendruber et al., 1989). Immunoelectron microscopy was performed on paraformaldehyde-fixed, LR White-embedded material as described (Newman and Hobot, 1987; Robertson et al., 1992) using a 15 nm gold-conjugated goat anti-rabbit IgG H+L chain secondary antibody (BioCell International, UK).

Cornified envelope preparations

Skin samples were excised, epidermis separated from dermis as above and cornified envelopes (Michel et al., 1988) prepared as described (Hohl, 1991). Briefly, skin samples were incubated for 10 minutes in cornified envelope extraction buffer (Hohl et al., 1991) at 96°C, cornified envelopes were collected by centrifugation and the extraction repeated. Envelopes were resuspended in extraction buffer and analyzed by phase-contrast microscopy (Zeiss, Axiophot microscope). Subsequently they were categorized as rigid or fragile and the relative proportions of each calculated.

Glucocorticoid treatment

Virgin ICR mice were time-mated and injected intramuscularly with 1 $\text{mg}\cdot\text{kg}^{-1}$ body mass β -methasone (Sigma) in 6% ethanol or with vehicle (6% ethanol) alone 3 days prior to killing and on subsequent days following the regimes below.

Regime 1: injection at E14/1, E15/1 and killed at E16/1

Regime 2: injection at E13/8, E14/8 and E15/8 and killed at E16/1.

RESULTS

Skin barrier forms at 16 days EGA in mouse embryos

Skin barrier formation has been monitored during human and rat embryogenesis using an evaporimeter. Evaporimeters measure water vapour pressure at the skin surface (Nilsson, 1977). The evaporimeter necessitates larger skin samples than can be obtained from mouse embryos. Therefore, we used a modified gravimetric transepidermal water loss (TEWL) assay (Nolte et al., 1993; Hanley et al., 1996a), which is compatible with the small mouse embryo, to demonstrate skin barrier formation at day 16 of the 19 day mouse gestation (Fig. 1). High TEWL values at E14/5 and E15/5 indicate absence of skin barrier. The sudden fall in TEWL at E16/5 represents barrier institution and is comparable to the sudden decreases in TEWL observed in rats and humans during late gestation (Aszterbaum et al., 1992; Hammarlund and Sedin, 1979; Wilson, 1980). The apparent lack of barrier improvement after E17 (Fig. 1) is an artifact of gravimetric TEWL assay, which is insensitive at low TEWL levels (Hanley et al., 1996a), and contrasts with the work of Aszterbaum et al. (1992) who reported continued slight improvement of barrier until, and shortly after, birth.

Our finding that barrier forms at E16 is consistent with the observation that stratum corneum is first detected at E16 in the mouse (Hanson, 1947) and shows that barrier forms 2-3 days prior to birth in both mice and rats (Aszterbaum et al., 1992).

Patterned permeability change also occurs at E16

Since skin development is regional (Sengel, 1990; Byrne et al., 1994), we sought ways to monitor barrier formation *in situ*. We developed novel whole-mount skin permeability assays (methods) that rely on either penetration of the epidermis by a

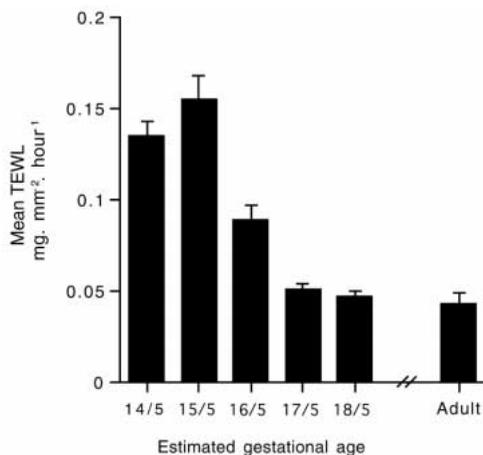


Fig. 1. Skin barrier forms at 16 days gestational age. High transepidermal water loss (TEWL) measurement at 14/5 and 15/5 days gestational age indicate lack of barrier. At E16/5 TEWL values fall, indicating formation of barrier. By E17/5 and E18/5 TEWL values have fallen to levels that are indistinguishable from the adult values (however, see Aszterbaum et al. (1992) for perinatal barrier change). Birth occurs at 19 days in this strain of mice (ICR). TEWL values represent the mean of five data sets. The increase in TEWL between E14 and E15 is not statistically significant. Error bars \pm s.e.m.

reagent producing a coloured reaction in the skin, or modify the skin to permit penetration by histological stains. The two assays demonstrate the same pattern. The assays yield negative results (absence of staining) on adult skin provided the barrier is intact (C. Byrne, unpublished data).

Application of these assays to whole mouse embryos shows that skin permeability changes dramatically at day 16 of gestation (Fig. 2). Until E16 skin gives uniformly positive reaction to the assays (Fig. 2Ai). During E16 impermeable regions appear at specific sites (Fig. 2Aii-vii). Eventually the whole embryo becomes impermeable (Fig. 2Aviii), with eyelids, eartips and a ventral neck region becoming impermeable last (Fig. 2B). Skin gives a uniformly negative reaction from E17.0 to birth and thereafter (data not shown).

As E16 is also the gestational age at which barrier forms (Fig. 1), we propose that the permeability assays indicate barrier formation. If so, then murine barrier formation is highly patterned.

Permeability assays indicate the first step in barrier formation

The patterned permeability change is identical on sagittal sides of a mouse embryo. Therefore we compared TEWL and permeability assays on skin fragments dissected from sagittal sides of the same embryo (Fig. 3). The results show that patterned permeability change corresponds to a distinct, initial step in the institution of barrier.

TEWL was calculated for dorsal (negative by permeability assay), ventral (positive by permeability assay) and mid (permeable-impermeable mixture) flank skin from a 'transitional' embryo (Fig. 3). E16 ventral (permeable) skin gave TEWL values that were statistically indistinguishable from E14 and E15 (pre-barrier) skin. However, E16 dorsal (impermeable) skin gave significantly reduced TEWL values. TEWL values indicated a 30-40% gain in barrier function compared to final (E17) barrier levels. The results demonstrate conversion of skin from pre-barrier status to possession of partial barrier as the skin changes its permeability pattern. Therefore the permeability assays indicate the first stage towards acquisition of complete barrier function.

This confirms our hypothesis that barrier formation is patterned during embryogenesis. Barrier forms at specific sites on the mouse embryo (asterisk in Fig. 2ii, iii, v, vi). These 'initiation sites' activate in a defined sequence, with dorsal initiation sites first, followed by sites on the tail and head, then limbs. Barrier function spreads from initiation sites across the epidermis as a moving front converging at dorsal and ventral midlines (Fig. 2Avi, B).

The permeability assays indicate barrier formation with greater precision than was previously possible with TEWL assays alone. Consequently, we are able to identify exact morphological changes accompanying barrier acquisition.

Morphological reorganization accompanies barrier formation

16 day embryonic mouse epidermis has stratified and differentiated (Fig. 4A) but lacks a typical stratum corneum (Fig. 4B), despite positive results with our assays for barrier function. We sought the morphological basis for this observation. Skin fractions were dissected from a transitional embryo using a comb device that records sample permeability

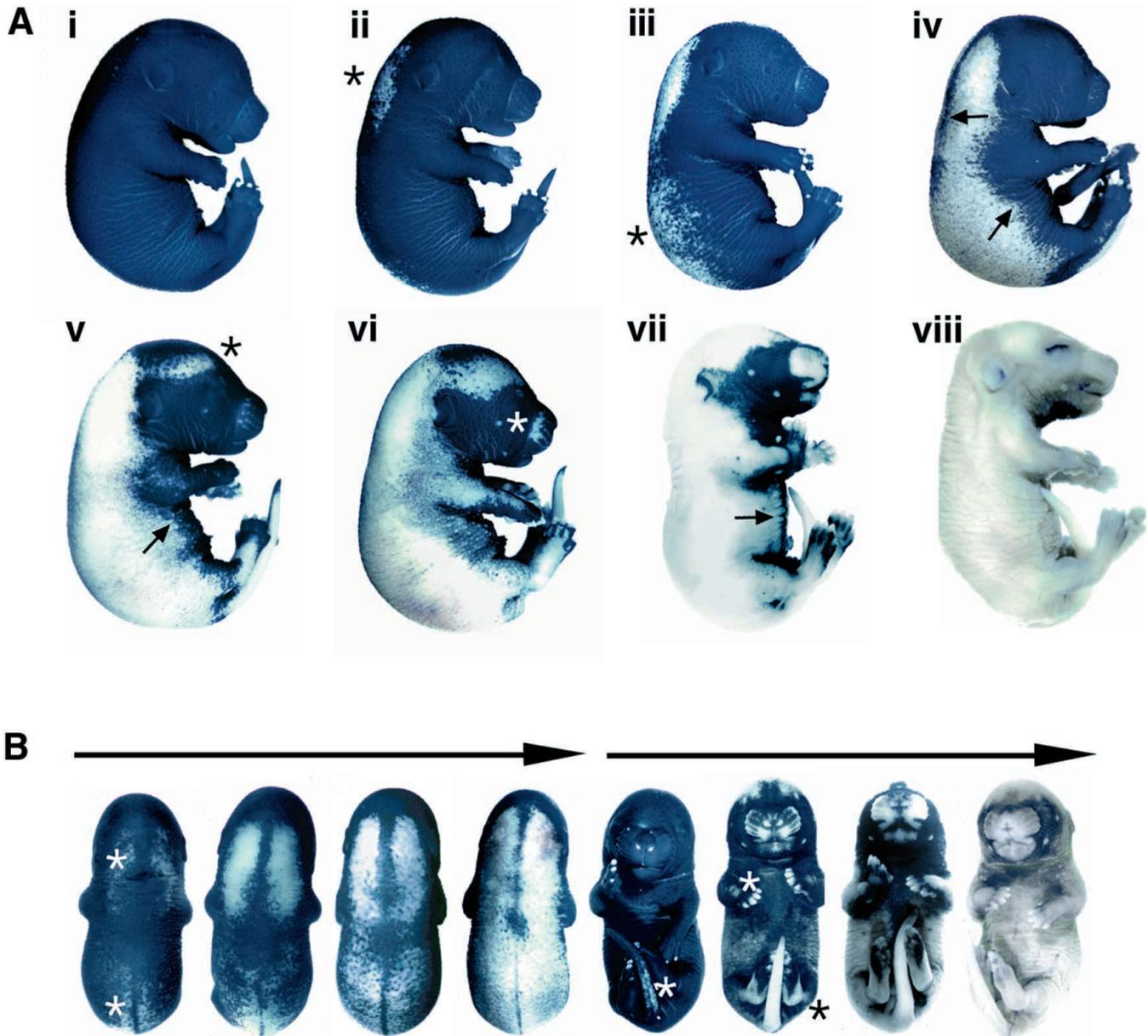


Fig. 2. Skin permeability changes in a patterned manner at embryonic day 16; (Ai-viii) increasing embryonic age. Specific skin sites (initiation sites) acquire impermeable characteristics (asterisk in ii, iii, v and vi), then a moving front of impermeability spreads across the animal's body (arrows). (B) Dorsal and ventral views of embryos showing progress of the front (arrows) and evidence of additional late initiation sites on the ventral region (asterisks). Approximate average gestational ages for each barrier stage in ICR strain mice are: Ai, <E16; Av, E16/11; Aviii, >E17.

while permitting morphological analysis of untreated/unstained skin (Materials and Methods, Fig. 5A). The only detectable variation across the permeability change was the status of the upper layer of cells, which eventually form the stratum corneum (Fig. 5B, arrowheads). Cells from most ventral skin fractions (permeable skin) showed scattered keratohyalin granules and lacked detectable cornified envelope (Fig. 5Bi). Cells from permeable epidermis flanking the change differed in that the keratohyalin granules had accumulated in arrays and there was sometimes a discernable cornified envelope (Fig. 5Bii). Following the transition to impermeable skin (Fig. 5Biii), this upper stratum corneum precursor layer of cells altered dramatically. The cornified envelope became

very prominent, some of the granules dispersed and the cells were flattened. The major histological hallmark of impermeable skin is transition of the stratum corneum precursor cells to a dark (electron-dense) flattened appearance. The most dorsal (impermeable) skin often had several layers of these dark flattened cells (Fig. 5Biv). Therefore we demonstrate (i) the difference between permeable/impermeable epidermis (Fig. 5C,D) and (ii) a gradient of stratum corneum development from dorsal to ventral flank epidermis.

Flanking each cell are accumulations of lipid derived from lamellar bodies that have fused with the plasma membrane and dispersed their contents. The intercellular lipid appears

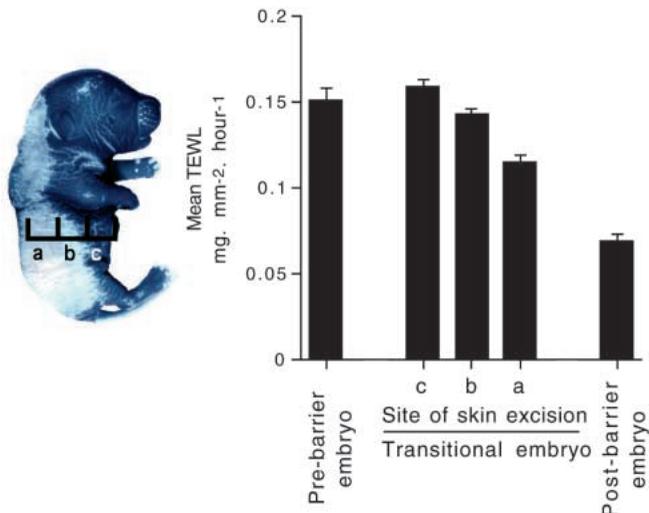


Fig. 3. Patterned permeability change corresponds to skin barrier formation. Skin was excised from body sites a, b and c on an embryo undergoing patterned permeability change (transitional embryo), and on skin from embryos before permeability change (pre embryos, E14-16) and after completion of permeability change (post embryos, E17). TEWL was assayed gravimetrically on sites a (impermeable skin), b (permeable/impermeable) and c (permeable skin). TEWL was highest on ventral (c site) skin where values were statistically indistinguishable from pre-barrier control embryos. In contrast, TEWL values show statistically significant ($P < 0.004$) reduction on dorsal (a site) skin, demonstrating that the patterned change corresponds to the start of barrier formation. Sample site b, containing the front of the change, showed intermediate TEWL values as expected. TEWL values represent the mean of six data sets. Error bars \pm s.e.m.

disorganized in both types of epidermis (Fig. 5E,F), as previously reported for embryonic epidermis (Aszterbaum et al., 1992). It is not yet organized into parallel lamellar units characteristic of adult stratum corneum (Elias and Menon, 1991; Downing, 1992) in either permeable or impermeable epidermis. It is likely that such reorganization will correlate with further acquisition of barrier function (Aszterbaum et al., 1992).

In summary, morphological analysis reveals changes in the cornified envelope, cell matrix and status of keratohyalin-associated granules as the epidermis acquires barrier function (Fig. 5C,D). Also, a gradient of epidermal change was observed, suggesting that the specific step of barrier acquisition may not be the only epidermal developmental event that shows patterning. Instead, we suspect that patterning is linked to the process of terminal differentiation itself and is revealed by our barrier assays because they illustrate a specific, single step in the process.

The first stage of barrier acquisition precedes production of active filaggrin

During terminal differentiation, the protein profilaggrin is released from keratohyalin granules and processed through multiple steps, from its phosphorylated 500 kDa precursor form to a 26 kDa dephosphorylated form, filaggrin (Dale et al., 1994). Filaggrin participates in cross-linking keratins to form the insoluble matrix of the cornified cell. The dramatic

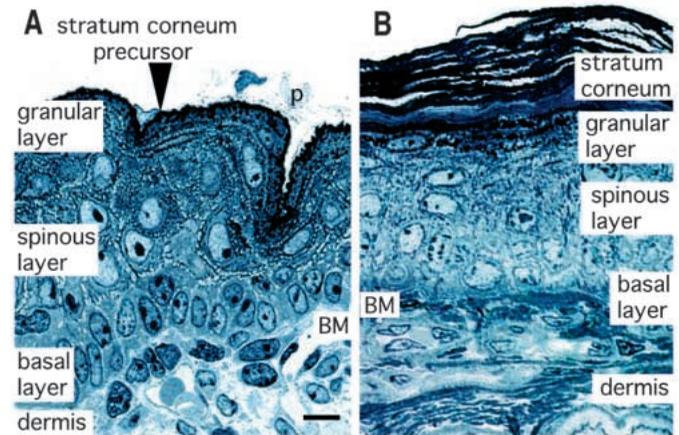


Fig. 4. Comparison of embryonic (E16) and adult skin. Light micrograph of E16 skin (A) shows absence of the prominent stratum corneum characteristic of adult epidermis (B) but a well-developed granular layer. The stratum corneum forms from the upper granular layer cells (arrowhead). Periderm (p) is a transient, embryonic cell layer forming an interface between the embryo and the amniotic fluid. BM, basement membrane. Bar, 6.5 μ m.

alteration in the opacity of the cells suggested that changes to the matrix may be responsible for the change in function. Also, the appearance of profilaggrin-containing granules (PF or type I keratohyalin granule; Dale et al., 1994) alters as the moving front crosses the skin (Fig. 5C,D).

However, although increased profilaggrin abundance accompanies the change to impermeability, there is no specific change at the permeability transition point (Fig. 6A). The fully processed, active form of filaggrin is never detected until well after the permeability change (Fig. 6A). Filaggrin cannot be detected even after massive overloading of gels (data not shown), although it is readily detected by day 17 of gestation (Fig. 6A; Bickenbach et al., 1995). Therefore cross-linking of keratins by filaggrin is unlikely to be responsible for the sudden permeability change. These results are consistent with the finding that there is only partial gain in barrier function as the front crosses the skin.

Movement of loricrin to the cornified envelope accompanies barrier acquisition

Morphological data suggest that the cornified envelope matures as permeability changes (Fig. 5). During cornified envelope formation specific proteins are sequentially attached to the cell envelope by isopeptide cross-linking. The major cornified envelope protein, loricrin (Mehrel et al., 1990; Hohl and Roop, 1993), is amongst those last incorporated (Steven and Steinert, 1994). Loricrin abundance does not change significantly following changes in skin permeability (Fig. 6B) so we asked if, instead, its intracellular location alters as occurs during adult terminal differentiation (Steven et al., 1990).

We show (Fig. 7A-C) a dramatic and sudden relocalization of loricrin as skin permeability status changes. Prior to the transition loricrin localizes to a subset of keratohyalin granules (L-granules, Steven et al., 1990; or type III granules, Manabe et al., 1991; Fig. 7A, arrowhead). Concurrent with the change loricrin moves to the intracellular side of the cornified envelope (Fig. 7B, arrowhead). The abrupt nature of the transition is

illustrated in Fig. 7C where adjacent cells show different localizations. Since loricrin is believed to be one of the last components incorporated into the cornified envelope (Steven and Steinert, 1994; Steinert and Marekov, 1997), it is possible that cornified envelope maturation correlates with acquisition of barrier function.

Cornified envelopes adopt a mature or rigid morphology as the barrier front crosses the skin

Cornified envelopes can be isolated intact from epidermis (Sun and Green, 1976). Envelopes isolated after sequential removal of adult stratum corneum layers adopt two distinct morphologies (Michel et al., 1988; reviewed Simon, 1994). Smooth, polygonal, 'rigid' envelopes derive from the outer layers of stratum corneum and irregular, crumpled 'fragile' envelopes derive from the inner stratum corneum near the granular layer. Rigid envelopes are proposed to possess lipid covalently attached to the extracellular side of the membrane (Michel et al., 1988; Lazo et al., 1995).

During cornified envelope formation, specific proteins are attached sequentially to the cell envelope by transglutaminase-mediated, isopeptide cross-linking. Major constituents include involucrin and loricrin (Steinert and Marekov, 1997 and references within). Involucrin is believed to be one of the first components incorporated and may form a scaffold for addition of subsequent proteins. Interestingly, fragile envelopes isolated from the inner stratum corneum are enriched with involucrin whereas rigid envelopes from outer stratum corneum contain increased loricrin (75% of total protein mass) and relatively less involucrin (Steinert and Marekov, 1997). Since we showed loricrin membrane localization in tandem with barrier front movement (Fig. 7), we asked if mature or 'rigid' envelopes were being formed as the front crosses the epidermis.

Cornified envelopes were isolated from transitional embryo epidermal samples (Fig. 8). We detect low numbers of envelopes in the most permeable, pre-barrier epidermis, with envelope numbers increasing dramatically in dorsal fractions. Pre-barrier epidermis contains exclusively or predominantly fragile envelopes (Fig. 8A, ventral, DEF fractions). The ratio of the two types changes suddenly as the front crosses the epidermis (Fig. 8A, dorsal, AB fractions), with rigid envelopes becoming the major form. Hence, cornified envelope maturation coincides precisely with first-step barrier acquisition.

Hormonal treatment that accelerates barrier formation also accelerates permeability pattern change and cornified envelope formation

Prenatal maternal glucocorticoid therapy decreases mortality and morbidity in the preterm human infant and has been reported to

accelerate skin barrier formation (reviewed Ballard, 1986). Glucocorticoid-induced barrier acceleration has been demonstrated and analyzed in the rat (Aszterbaum et al., 1993; Okah et al., 1995; Hanley et al., 1996a, 1997). We sought to verify our assays as a measure of barrier formation by applying them to embryos after maternal glucocorticoid treatment. We find that the whole-mount permeability assays demonstrate predicted barrier acceleration after glucocorticoid treatment (Fig. 9). Barrier pattern is dramatically accelerated despite expected growth retardation (~10 hours acceleration, ~10% reduction in weight; see Sicard and Werner, 1992 for expected growth retardation; Fig. 9A,B). However, there are pattern distortions and widened fronts (Fig. 9B, compare i and iii, see discussion).

Comparison of hormone- and vehicle-treated epidermis does not reveal significant difference by light microscopy (not

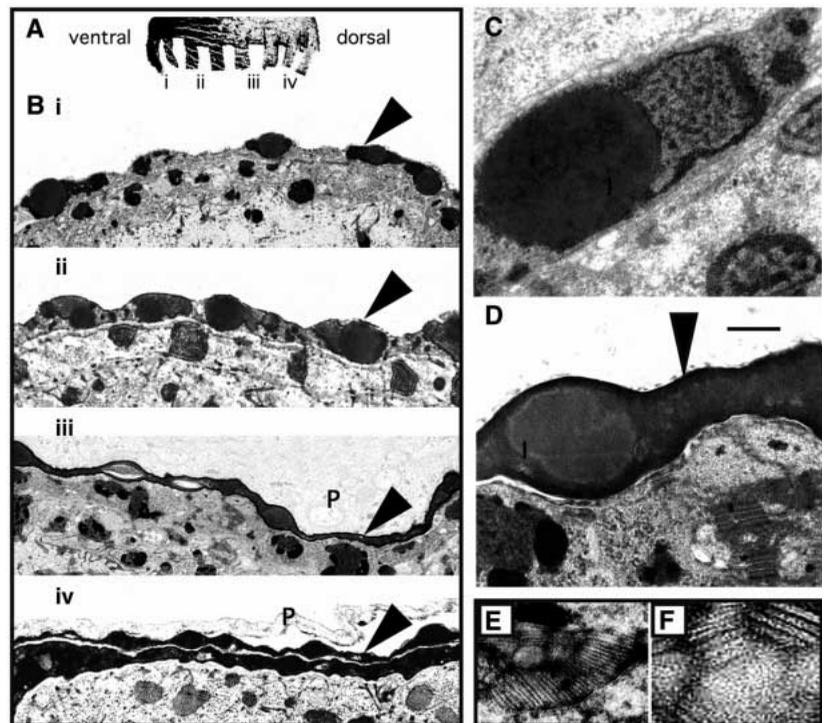


Fig. 5. Change in epidermal structure as skin barrier forms. (A) Flank skin strip (comb) used for assessing permeability status of samples (i-iv) removed for electron microscopy before permeability staining; dark stain indicates permeable, ventral skin. (B) There is a gradient of developmental change from most ventral (i) to dorsal (iv) skin in the upper cells of the granular layer (arrowheads) which will form the stratum corneum. Samples (i) and (ii) (permeable skin) lack a cornified envelope and possess prominent keratohyalin granules aligned in arrays in (ii). Samples (iii) and (iv) (impermeable skin) have distinct cornified envelopes and flattened, electron-dense stained cells. There are several layers of these flattened cells in iv. (C,D) Comparison of the morphological changes across the permeability/impermeability discontinuity. Note type I (PF, profilaggrin containing) and other keratohyalin granules in C (permeable skin) and retention of type I granules and loss of additional keratohyalin granules and prominent envelope (arrowhead) in D (impermeable skin). The type I (profilaggrin-containing) keratohyalin granules are present in both types of skin; however, a core of differentially stained material appears in post-barrier epidermis (D). The arrowhead shows the prominent cornified envelope. (E,F) Lipid lamellar units appear randomly oriented in the intercellular spaces and fused lamellar bodies of permeable (E) and impermeable (F) skin. Absence of periderm in Bi and ii is an artifact of EM specimen preparation. Scale bars: B, 180 nm; C,D, 1500 nm; E,F, 180 nm.

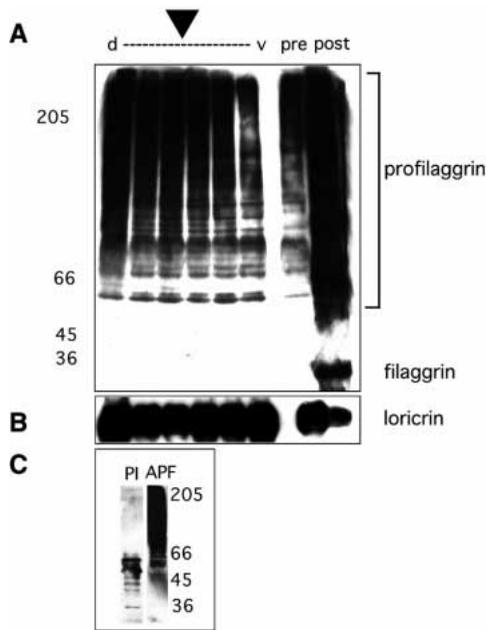


Fig. 6. Profilaggrin, filaggrin and loricrin expression in embryonic epidermis as barrier forms. (A) Western analysis of flank epidermal protein fractions showing increased abundance of partially processed forms of profilaggrin in dorsal epidermal fraction. There is no major change in profilaggrin processing as permeability changes. Filaggrin is detected in post-barrier (17 day) epidermis only, i.e. long after the permeability change. The arrowhead shows the transition between impermeable and permeable epidermis. (B) Loricrin is expressed abundantly in all epidermal fractions. (C) Preimmune serum (PI) detects epidermal proteins that are absent after affinity purification of the anti-profilaggrin antibody (APF). Both tracks contain pre-barrier epidermal protein. d, dorsal; v, ventral; pre, pre-barrier; post, post-barrier (17 day) epidermis. Molecular mass standards: $M_r \times 10^{-3}$.

shown). However, the glucocorticoid-mediated acceleration is obvious after ultrastructural comparison of hormone-treated and untreated epidermis. This is dramatically illustrated by examination of upper, stratum corneum precursor cells from an extreme dorsal region on a hormone-treated embryo (E16/2, a gestational age that would typically have no, or very little, permeability pattern; Fig. 2). Cells have a characteristic post-barrier morphology (Fig. 9C) whereas epidermis from an identical position on age-matched, vehicle-treated controls not only lacks any trace of cornification but is so immature that it still contains abundant, unfused lamellar bodies (arrowheads, Fig. 9D). Permeable, pre-barrier ventral epidermis from hormone-treated embryos (Fig. 9E) lacks any trace of cornification, as does position-matched ventral control epidermis (Fig. 9F).

However, examination of epidermis closer to the induced front shows that the accelerated development is abnormal. Epidermis adopts a heterogenous mixture of differentiation states. In post-front epidermis from treated embryos, we again detect membrane-localized loricrin (Fig. 9G-I) while, in pre-front epidermis, localization is exclusively granular (Fig. 9J). However, membrane localization now is uncoupled from the other cellular changes typically accompanying front movement. Membrane-localized loricrin is found in cells that retain morphology typical of pre-barrier epidermis (Fig. 9G,H)

as well as cells that adopt the typical post-front morphology (Fig. 9I). Cells with membrane-localized loricrin often retain granules (Fig. 9G,H) and remain unflattened (Fig. 9G). Another anomalous feature of hormone-induced post-barrier epidermis is simultaneous granule and membrane-localized loricrin (Fig. 9G-I; arrowheads). These results identify loricrin movement to the membrane, rather than the additional cohort of cellular changes that we previously noted accompanying the front, as a marker for barrier front movement. This suggests, again, that the important feature of the initial step in barrier institution is assembly of the cornified envelope. When we examine cornified envelope morphology, we find that rigid envelope formation again accompanies the barrier front (Fig. 9K), despite the immaturity and abnormality of the epidermis.

Our demonstration of glucocorticoid-mediated pattern acceleration in the mouse authenticates our permeability assays as a measure of barrier development and demonstrates the usefulness of the assays. The forced formation of barrier on premature epidermis is accompanied by the same changes (rigid cornified envelope formation, loricrin membrane localization) as we proposed to be important during normal barrier formation.

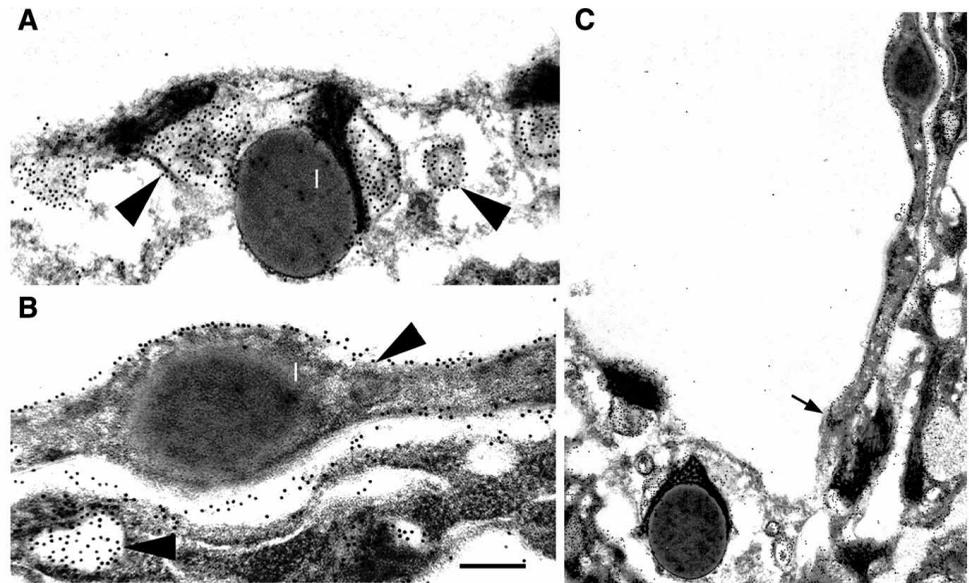
Patterned barrier acquisition in other species

To assess the likelihood of patterning being a universal mode of barrier acquisition in mammals, we applied our assays to further species. In addition, as Aszterbaum et al., (1992) had identified the gestational age when barrier forms in the rat by TEWL assay, we were able to verify our assays in a second species. The assays revealed that patterned change occurs in rats at precisely 19 days gestation as we predicted (Fig. 10; note that gestational age estimates differ, 19 days using our procedure (methods) is equivalent to 20 days estimated gestation age by the method of Aszterbaum et al., 1992).

We found that skin barrier forms in a patterned manner in mice, rats and rabbits; however, the detail differs. The similar shapes of the rat and mouse embryo permit comparison of patterning. Mouse and rat embryos have identical initiation sites, but they are activated in a different order (Fig. 10A), resulting in pattern differences. For example, the mouse embryo activates anterior and posterior dorsal sites (sites x and y, respectively, Fig. 10A), followed by a site on the head (site z). However, the rat embryo activates the anterior dorsal site (site x, Fig. 10A), the head site (site z), then the posterior dorsal site (site y). Barrier formation in the rat is completed by convergence of fronts (Fig. 10B) as in the mouse.

Having authenticated the permeability assays as a measure of barrier, we then applied them to a previously uncharacterized species. We show that barrier forms at day 25 of the 30 day rabbit gestation and that barrier formation is again patterned (Fig. 10C). The different shape of the rabbit embryo complicates interpretation of pattern differences. However, there are similarities with rodent embryos, including the sequential activation of initiation sites. The first initiation site to be activated is on the head and is probably analogous to activation site z in rodents. Sites analogous to the x and y sites are activated later. The rabbit embryo differs from rodents in having additional activation sites (asterisks, Fig. 10C) but, like rodents, there is a moving front and persistence of permeable skin on eartips and eyelids. This latter condition is exacerbated on the long eartips of the rabbit embryo (inset, Fig. 10C).

Fig. 7. Movement of loricrin to the cornified envelope during barrier formation. (A) Loricrin localizes to type III or L-granules in uncornified cells (arrowhead). I denotes type I (profilaggrin containing) granules. (B) Loricrin redistributes to the membrane in cornified cells (arrowhead), note the granular localization in the lower uncornified cell (arrowhead) as found in adult skin (Steven et al., 1990). Type I granules are still present but have acquired a differentially stained core. (C) Epidermis from the junction between uncornified and cornified cells showing abrupt relocation of loricrin as cells change to the cornified morphology of post-barrier epidermis (Fig. 5D). The arrow denotes the transition point. Absence of periderm is an artifact of EM specimen preparation. Bar in A, 420 nm; B, 350 nm; C, 700 nm.



DISCUSSION

We report in situ assays for skin barrier function that demonstrate barrier institution during murine embryogenesis. We show that barrier formation is highly patterned and describe epidermal ‘initiation sites’, which acquire barrier function first. Moving fronts of barrier acquisition emanate from initiation sites. We have authenticated the assays by comparison with established barrier assay and by use after maternal glucocorticoid therapy (known to accelerate barrier formation) and in additional species including the rat, where barrier formation is well characterized (Aszterbaum et al., 1992, 1993).

As the barrier front passes across the skin, there is reorganization of the upper layer of the epidermis, the layer that eventually forms the stratum corneum. We demonstrate cornified envelope maturation accompanying the front by two criteria. First, envelopes adopt the ‘rigid’ morphology typical of differentiated squames (Michel et al., 1988) and second, loricrin, a major component of cornified envelope and one of the last proteins incorporated (Steven and Steinert, 1994), relocates from keratohyalin L-granules to the cell membrane. Therefore acquisition of barrier function is coordinated precisely with maturation of the cornified envelope. After hormone-induced formation of barrier in premature epidermis, we find an accelerated appearance of these markers of cornified envelope maturation despite the immature and aberrant nature of the treated epidermis. Our work is consistent with the idea that an intact cornified envelope is essential for acquisition of barrier function and correlates with the identification of skin

Fig. 8. Cornified envelope morphology changes as the barrier front crosses the epidermis. (A) Epidermis from three transitional embryos was divided into six fractions (a-f). Fraction a represents the most dorsal (post-barrier front) epidermis and fraction f represents most ventral (pre-barrier front) epidermis. The position of the barrier front is marked with an arrow. Envelopes were isolated and classified as rigid (B) or fragile (C). The proportion of rigid envelopes rises dramatically at the transition point and approaches that found in the adult epidermis (A).

diseases with a compromised cornified envelope (Huber et al., 1995; Russell et al., 1995; Maestrini et al., 1996).

The mature stratum corneum has been likened to ‘bricks and mortar’ (Elias, 1983) and there is considerable evidence that the extracellular lipid ‘mortar’ provides barrier function (reviewed Feingold, 1991). However, we find abundant extracellular lipid before barrier formation and no detectable

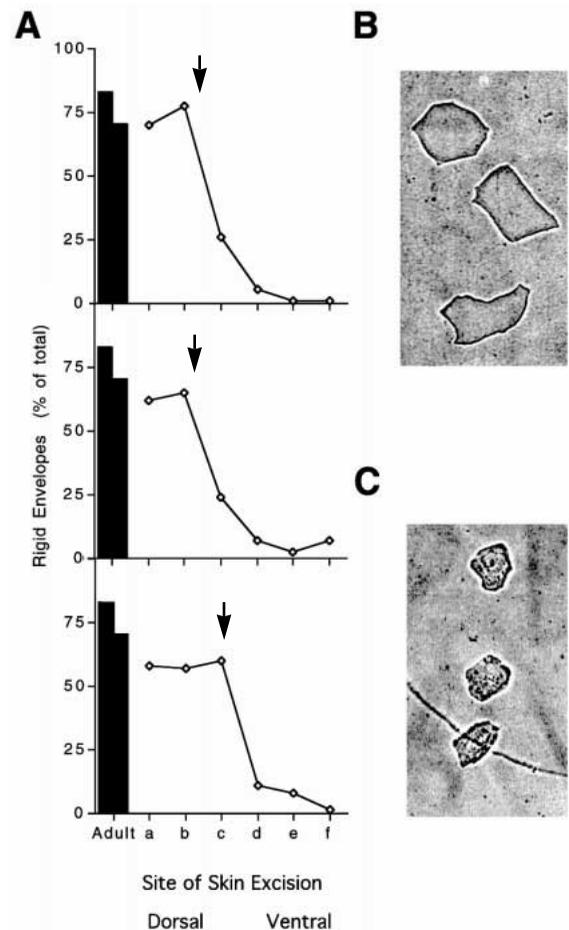


Fig. 9. Glucocorticoid treatment produces accelerated permeability pattern. (A) Mean % barrier coverage was assessed on E16/2 mice subjected to vehicle (control group) or two different regimes of β -methasone injection (group 1 and group 2, see Materials and Methods). Mean % barrier coverage represents the mean of three data sets (controls) or two data sets (treated). Barrier coverage was dramatically accelerated in glucocorticoid-treated groups. Error bars \pm s.e.m. (B) Barrier pattern is compared on representative embryos from glucocorticoid-treated (Bi; E16/2) and vehicle-treated groups (Bii; E16/2). An older (E16/11) untreated embryo (Biii) is included to show the pattern distortion in the glucocorticoid-treated embryo (asterisks), also note the wider abdominal front. (C) Stratum corneum precursor cells in glucocorticoid-treated epidermis (from a dorsal region distant from the front) show a typical post-barrier morphology (bracket) whereas precursor cells from position-matched, vehicle-treated epidermis (D) are very immature (arrowheads show abundant, unfused lamellar bodies). Ventral, position-matched precursor cells from treated (E) and untreated (F) epidermis have typical pre-front morphology. (G-I) Post-barrier epidermis close to the front shows a range of morphologies, arrowheads show lorincrin localization. (G) Lorincrin is membrane-localized, despite the pre-front morphology, note absence of flattening, retention of vesicles, dual vesicular and membrane-localization of lorincrin. (H) Dual vesicular and membrane-localization of lorincrin, however note retention of vesicles. (I) Lorincrin is membrane-localized and the cells have post-barrier morphology. (J) Pre-front glucocorticoid-treated precursor cells show typical pre-front morphology and strict granular localization of lorincrin, as do all vehicle-treated cells from this age group (not shown). (K) Conversion of cornified envelopes to a mature, 'rigid' phenotype correlates with front movement (arrow shows the dorsal position of the widened abdominal front in hormone-treated epidermis). Disaggregation of periderm (p) is an artifact of EM specimen preparation. Bar, 500 nm in C,D,G-I; 2-8 μ m E,F.

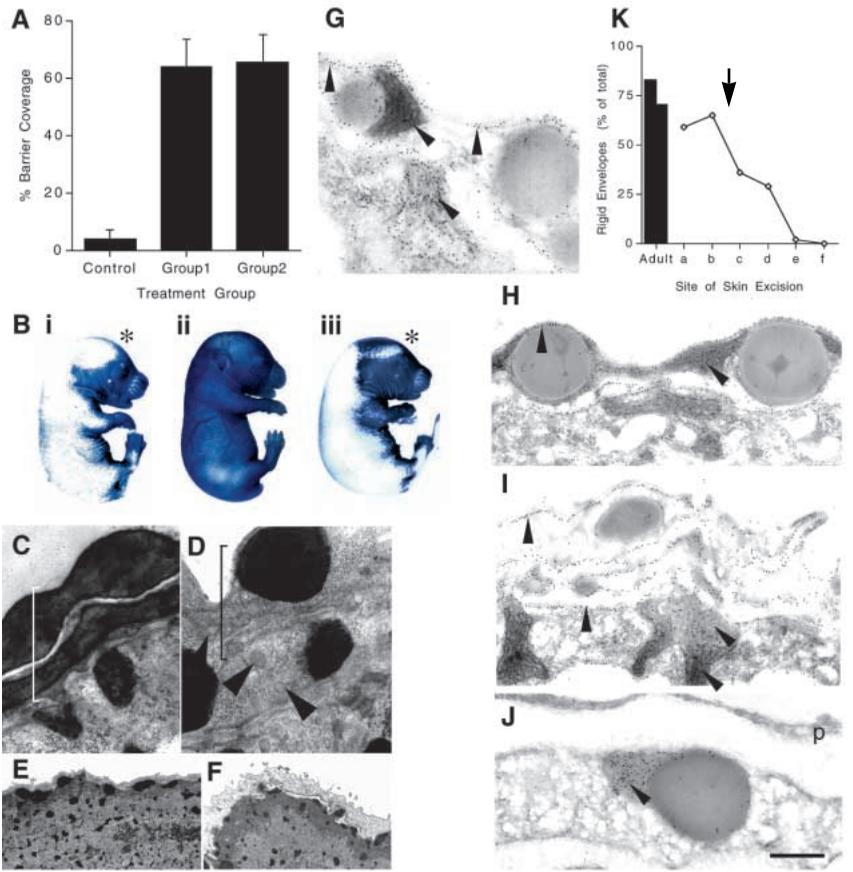
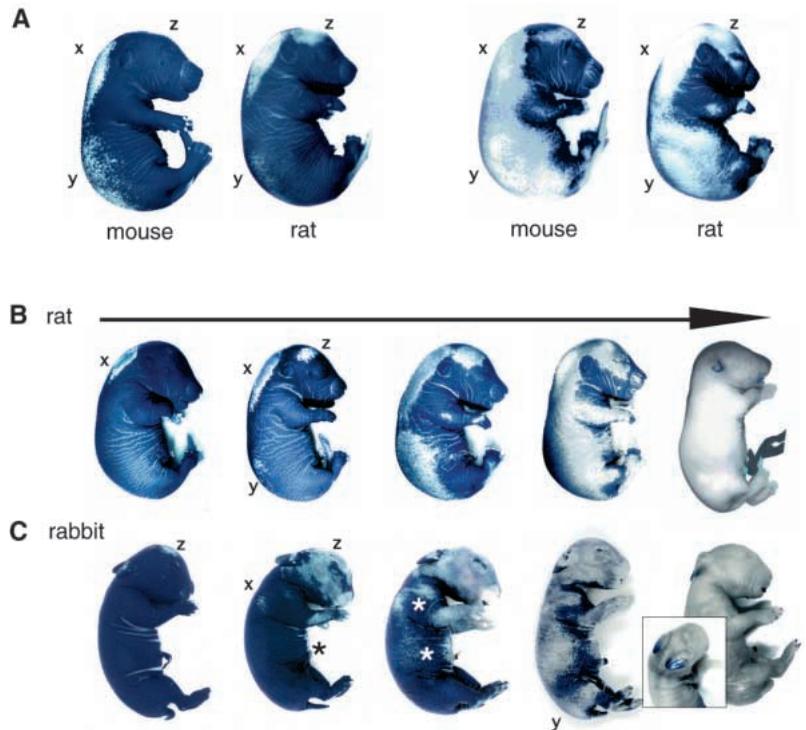


Fig. 10. Patterned epidermal permeability change in mice (A), rats (A, B), and rabbits (C). Barrier forms at E16 of mouse gestation, E19 of rat gestation (note differences in estimation of gestation age by different authors, see text) and E25 of rabbit gestation. The initiation sites are activated in a different order. (A) Mouse embryos activate initiation sites x, y then z. Rat embryos show a different pattern because dorsal initiation sites are activated in the order x, z then y. (B) Completion of front movement in rat embryos. (C) Rabbit embryos first activate an initiation site on the head (probably analogous to z) then additional initiation sites (limbs, ventral region, a dorsal site similar to x). The different shapes of the embryos impose pattern differences, as illustrated by the retention of permeable epidermis on the lengthened ears of the rabbit embryo long after completion of the permeability change (inset).



change in the abundance or organization of extracellular lipid lamellar units as the barrier front crosses the skin. An explanation of this apparent anomaly is provided by our demonstration that 'rigid' or mature cornified envelope formation accompanies barrier front movement. Extracellular lipid is covalently bound to the cornified envelope in mature stratum corneum (Swartzendruber et al., 1987; Wertz and Downing, 1987; Lazo et al., 1995) and it has been proposed that rigid envelopes possess covalently attached lipid (Michel et al., 1988). Hence, assembly of keratinocyte 'bricks' may be rate limiting for acquisition of functional stratum corneum in embryonic epidermis because envelope assembly is a prerequisite for lipid attachment.

The permeability assays reveal the first stage, only, in barrier formation. This is consistent with our demonstration that the initial stage in barrier acquisition precedes production of mature filaggrin and dissolution of profilaggrin-containing granules. Filaggrin-mediated cross-linking of the keratins is an important part of cornified cell formation and production of mature filaggrin (Fig. 6) correlates with an improved barrier at E17. Also, the immature stratum corneum that confers partial barrier function at E16 differs markedly from mature stratum corneum. Adult stratum corneum contains many layers of cornified cells, rather than the single layer formed in tandem with initial barrier acquisition (Fig. 4). The extracellular lipid of adult stratum corneum is organized into parallel arrays of lamellar units. Conversion of extracellular lipid from the disorganized structures reported here in the mouse, to an adult-like morphology correlates with full barrier acquisition in the rat (Aszterbaum et al., 1992).

This study shows that barrier forms in stages during embryogenesis and that, from a single embryo, it is possible to isolate epidermal samples at different stages in terminal differentiation and barrier development. Our demonstration of multistep barrier development is compatible with previous studies of late gestational skin development (Bichenbach et al., 1995) and with the demonstration of sequential, multistep assembly of the cornified envelope during adult terminal differentiation (Steinert and Marekov, 1995, 1997; Ishida-Yamamoto et al., 1996).

During this study, we authenticated novel *in situ* barrier assays suitable for whole-mount analysis and we have shown that the assay staining patterns mark initial stages in barrier formation in embryonic skin. A similar histological approach to barrier assay uses lanthanum tracers (Hayward, 1983) followed by skin sectioning to assess lanthanum penetration. Both assays record change in embryonic skin permeability to exogenous substances. However, assay 2 requires modification of the epidermis by methanol to permit barrier-dependent access of histological dyes. Although the precise nature of the modification is unknown, it is probable that it involves extraction of polar lipid. Methanol treatment does not affect permeability status of the mature, lamellar-based barrier (this work). However methanol treatment is necessary for dye entry into E14-15 embryonic epidermis (C. Byrne, unpublished), which lacks any trace of lamellar bodies or intercellular units and has very high TEWL. Therefore embryonic epidermis must contain polar lipid unassociated with the lamellar system which, though ineffectual as a barrier to water, prevents entry of other substances.

Patterning is characteristic of earlier epidermal

developmental change and has been demonstrated both morphologically and with molecular markers. However, the pattern associated with barrier institution bears no resemblance to patterns associated with earlier forms of skin development (Byrne et al., 1994). At E15 epidermal cells have stratified and express keratin markers of terminal differentiation but the gene induction patterns differ from the barrier institution pattern. For example, there is no induction gradient down the dorsal-ventral flank. However, by E16 the granular cells of flank epidermis are in different stages of terminal differentiation. Dorsal cells are most differentiated and ventral cells least i.e. there is now a gradient of development.

Terminal differentiation leads to barrier formation. Our assays reveal (i) the stage during terminal differentiation when barrier forms and (ii) associated patterning. However, the morphological data strongly suggest preexistence of pattern. We propose that the pattern revealed by barrier assay was initiated when cells entered the programme of terminal differentiation. If so, then terminal differentiation would commence at the initiation sites.

We have not established what distinguishes initiation site epidermis from surrounding epidermis, but are currently investigating the possibility that initiation sites have properties of classical organizers. Initiation sites have not as yet been detected in earlier forms of epidermal patterning, so it is not known if they represent a general means of promoting epidermal developmental change.

Epidermal cells are spread over a large surface area yet must coordinately change during development. Epidermal patterning reflects non-simultaneous change, which strongly suggests environmental influence on epidermal development. It is well established that epidermal development is regulated through epithelial-mesenchymal interaction (Sengel, 1976; Fisher, 1994). Heterogeneity of the underlying mesenchymally derived dermis could account for much of the patterning. If so, then the difference between initiation site and other epidermis lies in the underlying dermis. The existence of a moving barrier front suggests that change is transmitted from the initiation site by diffusion of a substance, or by cell-cell contact. These hypotheses are the subject of further experimentation.

In this study, we demonstrate acceleration of barrier pattern in the mouse after maternal glucocorticoid treatment. Glucocorticoid treatment in tandem with surfactant therapy (administration of pulmonary surfactants directly into the trachea) is used to prevent and treat respiratory distress syndrome in premature infants. Glucocorticoid therapy accelerates lung epithelial lamellar body maturation and epithelial surfactant production. It coincidentally accelerates skin barrier formation. Glucocorticoid acceleration has been demonstrated in the rat (Azsterbaum et al., 1993; Okah et al., 1995; Hanley et al., 1996a, 1997) and correlative acceleration of lamellar body/stratum corneum maturation and lipid biochemistry noted (Azsterbaum et al., 1993).

By comparison of accelerated and vehicle-treated epidermis, we show that glucocorticoid treatment accelerates multiple features of epidermal development and not simply maturation of the epidermal lamellar body secretory system. However, accelerated epidermis has unusual features including an uncoupling of loricrin-membrane localization and barrier formation from morphological changes (cell-flattening, granule dispersal and cell-matrix changes) normally

accompanying the front. Hence analysis of barrier front in the perturbed epidermis of hormone-treated animals strengthens the correlation between cornified envelope maturation and initiation of barrier formation.

The glucocorticoid-accelerated pattern in the mouse is distorted. The head (z) initiation site appears to have been prematurely activated compared to dorsal initiation sites (x, y; Fig. 9b), suggesting temporal compression of initiation site activation as well as an overall acceleration (ie. we suggest that the z site is activated relatively earlier). Change in initiation site activation is proposed (see below) as the source of interspecies pattern differences.

We use pharmacological levels of glucocorticoids to mimic previously noted accelerations. Therefore the biological significance of the acceleration is unknown. However, we do demonstrate the susceptibility of late gestational murine skin development to hormonal control. Also, interestingly, an upregulation of glucocorticoid receptors has been noted in fetal rat epidermis during the late gestational period preceding barrier formation (Kitraki et al., 1997) suggesting that glucocorticoids play a role in late gestational skin development. Steroid hormone influence on skin development is likely. In vivo there is a sex difference in barrier development (Hanley et al., 1996b), presumably due to effects of other steroid hormones (testosterone and estrogen) on skin development. Also, sex steroid affects on late gestational skin development have been demonstrated in vitro (Hanley et al., 1996b).

Patterned barrier formation was demonstrated in other species, suggesting that it is a ubiquitous mode of late gestation developmental change in mammals. This is important because, if patterning occurs in additional species, it is likely to be present in humans. Infants born before 30 weeks gestation lack a skin barrier and hence can suffer from fluid imbalance, infection and heat loss (Vernon et al., 1990; Cartledge and Rutter, 1992). Using the same rationale that we successfully applied to the rat, we predict that our assays will reveal barrier formation between 30 and 33 weeks gestation. Demonstration of pattern will have clinical relevance.

We use the permeability assays to demonstrate barrier formation at E16 of mouse gestation, E19 of rat gestation and E25 of rabbit gestation. The rodent results were predicted following identification of barrier formation by TEWL assay (this study; Aszterbaum et al., 1992); however, barrier formation had not been reported previously in the rabbit. Temporal determination of barrier formation in rabbit illustrates the utility of these assays.

Barrier formation is patterned in the three species studied. Similarly shaped rat and mouse embryos have identical 'initiation sites'. Comparison of rat and mouse embryos shows how interspecies pattern variation can arise by difference in the order of initiation site activation. Rabbit embryo barrier formation precedes via sequential activation of initiation sites and moving fronts. Direct comparison between rabbit and rodent embryos is complicated by differences in body shape. However, rabbits appear to activate similar initiation sites in a new order and to activate novel initiation sites.

Late gestation embryos differ markedly in shape and size. Consequently, the distance barrier fronts must travel varies. The inability of moving fronts to travel long distances or to converge in sufficient time may necessitate different sequences

of initiation site activation or activation of further initiation sites. Thus, although we find pattern difference in other species, the underlying mechanism of barrier propagation appears to be identical.

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