Barrier Formation in the Human Fetus is Patterned

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We recently demonstrated patterned stratum corneum maturation and skin barrier formation during fetal development in rodents and rabbit. The presence of skin patterning in these mammals led us to predict patterned barrier formation during human infant development. Here we extend our mammalian study and demonstrate patterned stratum corneum development and skin barrier formation in the pre-term human infant. Surprisingly, we show initiation of human barrier regionally as early as 20–24 wk gestational age (22–26 wk menstrual age), bringing barrier formation close to the time of periderm disaggregation. We use the mouse model to show that patterns of periderm disaggregation mirrors barrier formation. Periderm disaggregation follows and recapitulates barrier pattern, suggesting a relationship between the processes. This work reveals regional patterning in skin maturation and barrier formation in the human infant and demonstrates that initiation of human skin barrier formation *in utero* coincides with the current lower limit of viability of the pre-term infant. ***Key words: development/epidermis/follicles/periderm. J Invest Dermatol 113:1106–1113, 1999***

The skin permeability barrier is essential for terrestrial life and forms during late gestation (reviewed in Roop, 1995; Williams *et al*, 1998). Human infants born before 30-32 wk gestation can suffer from problems related to barrier deficiency including desiccation (Lorenz *et al*, 1982), thermoregulatory problems (Harpin and Rutter, 1982; Hammarlund *et al*, 1986), microbial infection (Leyden, 1982; Askin, 1995; Rowen *et al*, 1995), and accidental poisoning (reviewed in Nachman and Esterly, 1971; Goutieres and Alcardi, 1977; Wester and Maibach, 1982). The skin barrier is conferred by the stratum corneum and skin barrier development correlates with stratum corneum development (Evans and Rutter, 1986; Azsterbaum *et al*, 1992; Hardman *et al*, 1998).

Human skin development from single-layered surface ectoderm to multilayered keratinized epidermis has been well documented (reviewed in Breathnach, 1975; Holbrook and Odland, 1980; Dale *et al*, 1985; Foster *et al*, 1988; Holbrook, 1994) and "keratinization" or stratum corneum formation reported at 22 wk in epidermis of head/scalp and palmar/plantar skin (Holbrook and Odland, 1975) and by 25 wk in the interfollicular epidermis of the remainder of the body (Foster *et al*, 1988). A transitory fetal layer, the periderm, interfaces between developing epidermis and amniotic fluid (Hashimoto *et al*, 1966; Hoyes, 1968). Periderm differentiates in tandem with epidermal development and is sloughed into amniotic fluid at about 24–25 wk, as the skin keratinizes (Holbrook and Odland, 1975; Foster *et al*, 1988). Skin barrier activity has been reported at 34 wk (Evans and Rutter, 1986). A recent study of ultra-low birth weight infants, however, identified 30 wk post-conception age as a point where fetal barrier assumes functionality of adult barrier (Kalia *et al*, 1998).

Recently, we developed whole-body assays for skin permeability and verified them as a measure of barrier by comparison with transepidermal water loss (TEWL) assay, a standard barrier assay (Nolte *et al*, 1993). When applied to developing rodent and rabbit fetuses the new assays showed that skin barrier forms in a patterned manner late in fetal development (Hardman *et al*, 1998). Barrier forms at epidermal initiation sites, then spreads around the body as moving fronts converging on ventral and dorsal midlines (**Fig 1**). Initiation sites and moving fronts were identified in mouse, rat, and rabbit. As a consequence of these studies we suggest that patterned barrier acquisition may be widespread among mammals. If so, we predict patterned barrier formation in the human infant.

Our previous studies of developing rodent skin showed that barrier forms in stages (Hardman *et al*, 1998). The skin permeability assays used in these studies demonstrate a skin change that correlates with maturation of cornified envelopes and an initial fall in TEWL. The assays measure an early stage in barrier formation, whereas other forms of barrier assay (e.g. transepidermal water assay by evaporimeter; Nilsson, 1977) measure a late stage of barrier formation. The skin permeability assays, therefore, provide a new tool for precise *in situ* identification of initiation of barrier formation in the human infant, a finding that could have significant clinical relevance.

In this study we extend our analysis of whole-body barrier formation in mammals and use the new assays to (i) ask whether skin barrier formation is patterned in the human infant; (ii) find the precise stage in human skin development correlating with initiation of barrier formation; and (iii) investigate the pattern of periderm disaggregation and its temporal relationship to barrier formation.

MATERIALS AND METHODS

Animal fetal material ICR mice were time-mated within a 2 h mating window and the mid-point of the window designated gestational age 0. Sprague–Dawley rats were time mated within a 12 h mating window and

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Abbreviations: EGA, estimated gestational age; TEWL, transepidermal water loss.

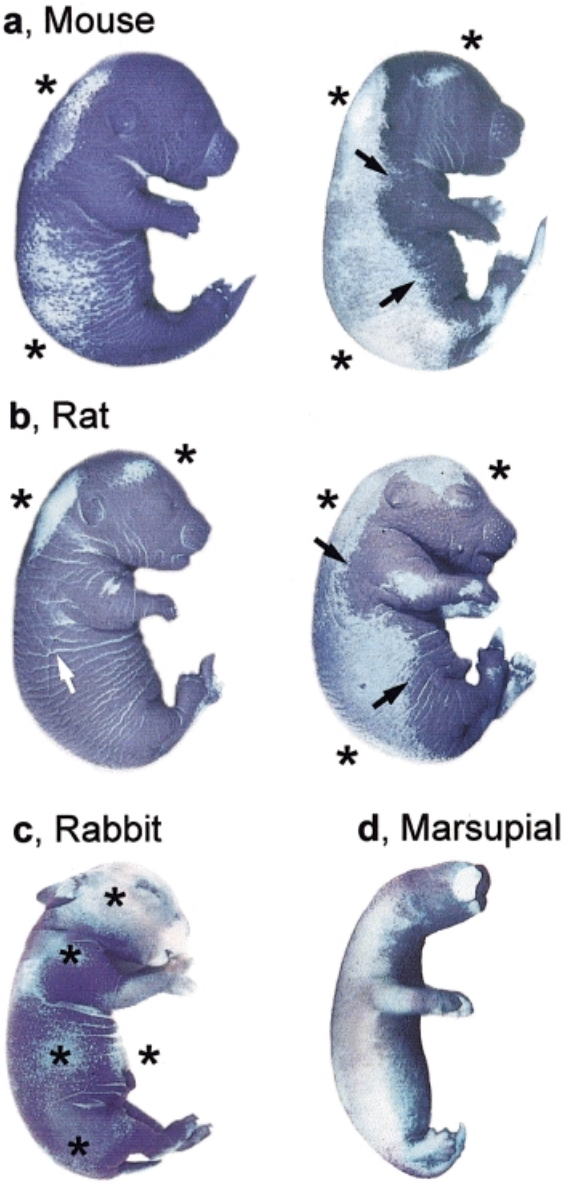

Figure 1. Barrier formation is conserved. (*a*) Mouse, approximate gestation ages E16/4, E16/12; (*b*) rat, approximate gestation ages E19/8, E19/16; (*c*) rabbit, gestation age E25; (*d*) marsupial possum, *M. domestica*, day of birth. Mechanism of barrier formation appears conserved in four mammalian species with use of initiation sites (*asterisks*) and moving fronts (*arrows*). Barrier activity demonstrated by permeability assay depends on dye exclusion. White skin possesses the barrier, blue skin lacks the barrier. Obvious linear streaking (e.g., white arrow in *b*) is due to failure of the dye to penetrate cutaneous vaginations associated with dermatoglyphic patterning of the skin surface.

the mid-point of the window designated 0. Pouch young marsupial *Monodelphis domestica* were killed at defined times after birth (birth occurs at 13.5 d). Estimated gestational age (EGA) was calculated from the time designated 0. For example, 16 d 5 h after time 0 was called E16/5 or 16/5 d EGA.

Human fetal material Human fetal ages are EGA unless otherwise specifically indicated. EGA = menstrual age minus 14 d. Human skin samples (minimum 1 cm in one dimension) were taken post mortem from spontaneously aborted infants of 17–30 wk EGA (19 infants) or supplied by Medical Research Council Tissue Bank, London (five infants, source of circumferential skin strips). Significantly growth retarded or macerated infants, or those who died more than 3 h after birth, were excluded from the study. EGA was calculated from multiple parameters including last menstrual period, crown–rump length, crown–heel length, foot length, head circumference, and organ weights. Research with human material was performed according to the Polkinghorne Code of Practice, U.K., after approval of protocols by the local Research Ethics Committee.

Skin permeability assay Skin permeability assay was performed using the novel dye exclusion technique described in detail in Hardman *et al* (1998). Briefly, embryonic skin was incubated for 1–5 min in methanol, rinsed in phosphate-buffered saline, followed by incubation in 0.5% hematoxylin or 0.1% Toluidine Blue, as described (Hardman *et al*, 1998). After extensive destaining dye penetration reveals barrier status. White skin has barrier, stained skin lacks barrier. In some instances, where the skin invaginates due to dermatoglyphic patterning, artifactual white streaks appear (e.g., **Fig 1**). The new assay has been validated previously by comparison with standard methods for barrier assay, e.g., TEWL assay (Hardman *et al*, 1998). For histologic analysis samples were excised from skin prior to permeability staining. The permeability status of the samples was determined by staining the surrounding skin after sample removal, i.e., samples for histologic analysis were never subjected to permeability assay. Disaggregating periderm is visualized on post-barrier animals after staining as above. Periderm stains transiently with applied dyes and can be visualized against epidermis which is resistant to dye penetration due to barrier activity.

Histologic analysis Samples for light and electron microscopy were fixed in half strength Karnovsky's fixative and osmium tetroxide using standard techniques. Samples were visualized with a Philips 400 transmission electron microscope at 80 kV.

RESULTS

Patterned barrier formation in mammals We recently demonstrated barrier formation in rodents and rabbit (Hardman *et al*, 1998). Barrier forms at distinct epidermal sites, called initiation sites (asterisks, **Fig 1*a–c***), then spreads around the fetus as moving fronts that converge ventrally and dorsally. Although the three species showed patterned permeability change (presence of initiation sites and moving fronts) the detail differed. Comparison of the similarly shaped rodent fetuses showed that mice and rats used identical initiation sites; however, they activate sites in a different temporal order. The larger rabbit fetus used additional initiation sites but shape and size differences complicate comparison with rodent. Hence, it was proposed that patterning was probably a generally mode of barrier acquisition in mammals.

We sought to verify this proposal by examining barrier formation in an evolutionarily distant mammalian species. Here we demonstrate barrier formation in a marsupial possum, *M. domestica* (**Fig 1*d***). *Monodelphis domestica* is born after 13.5 d gestation and, like all marsupials, completes late stages of development, including skin development, *ex utero* (Armstrong and Ferguson, 1995, and references within). We show patterned barrier formation on the day of birth (**Fig 1*d***). Significantly, barrier forms via moving fronts as in other mammals. Demonstration of pattern in a marsupial lends considerable weight to our proposal that skin matures in mammals by a common mechanism. Therefore, we looked for patterned change in the human infant.

Barrier formation in the human infant Ethical considerations preclude whole-body analysis of barrier formation in the late gestation human infant. Therefore, skin samples were collected during post mortem examination from separate sites (abdominal midline, dorsal head, neck, chest, and lateral torso skin) on infants of 17–30 wk EGA and tested for barrier activity using the new dye permeability assays. Infants who had survived for more than a few hours post-birth were excluded from the study as previous researchers have demonstrated accelerated skin and barrier development upon air contact (Harpin and Rutter, 1983; Evans and Rutter, 1986; Hanley *et al*, 1997; Kalia *et al*, 1998; Williams and Feingold, 1998). Barrier differed in a site-dependent manner. Detailed results are presented for abdominal midline and dorsal head (scalp) sites as they represent extremes of developmental rates from areas sampled. Results are summarized in **Table I**.

Abdominal midline Epidermis from abdominal midline at 17 wk gestational age tested negatively by permeability assay (**Fig 2*a***). By 18/19 wk, however, follicular epidermis had clear barrier activity

that increased in prominence by 21 wk (white streaks, *arrows*; **Fig 2*b*, *c***). Sectioning revealed that the white streaks correspond to keratinization confined to the hair canal (**Fig 2*n*, *o***; *arrow*). This has been previously reported in section analysis of fetal skin at 22 wk EGA (Holbrook and Odland, 1980). Additionally, the follicular plug expelled to the epidermal surface (Hardy, 1992) provides a physical barrier (**Fig 2*p*, *q***; *arrow*) that contributes to the white streak.

Between 22 and 24 wk abdominal epidermis undergoes a transition from negative to positive barrier activity. At 22 wk barrier is provided by follicles but is also present in the vicinity of some of the follicles (**Fig 2*d***, *asterisks*). By 23 wk barrier has moved out from some of the hair follicles appearing to join in places (**Fig 2*e***), suggesting that follicular epidermis can act as initiation sites in human. This differs markedly from the primary mode of barrier initiation in other mammals (**Fig 1**). Several skin samples, however, show sharp changes in interfollicular barrier that resemble the animal moving fronts (e.g., **Fig 2*e***, *arrows*, also see below). Therefore, it is not clear from the analysis of small skin samples whether barrier forms in abdominal skin by movement from follicles or as a result of a moving front superimposed over the follicular barrier. By 24 wk barrier formation appears complete (**Fig 2*f***). Infants of 25 wk or greater always test positively for barrier at this site (**Fig 2*g***).

Table I. Barrier formation and keratinization are temporally linked during human fetal development

Gestational age	Barrier		Keratinization	
	Abdomen	Head	Abdomen	Head
17	–	–	–	–
19	–	±	–	±
21	–	+	–	+
22	±	+	–	++
23	±	+	±	++
24	+	+	+	++
28	+	+	++	+++
30	+	+	++	+++

Head/scalp From 17 to 19 wk head epidermis is heterogeneous, with interfollicular skin yet to form barrier and follicular skin testing positively for barrier (**Fig 2*h–j***) as in abdominal skin. Head skin, however, matures much more rapidly than abdominal skin (cf. 19 and 21 wk samples – **Fig 2*j*,*k*** with ***b***, ***c***). Detection of barrier this early in development was unexpected. Emanation of barrier from follicles, noted in abdominal skin at 22 wk, is much more prominent in head epidermis at 19 wk (**Fig 2*j***, *asterisks*). This may be due to higher scalp follicle density. Epidermis from the dorsal side of the head at 21 wk or later tests uniformly positive for barrier (**Fig 2*k–m***). Accumulating vernix caseosa (Agorastos *et al*, 1988 and references within) on the epidermal surface appears as a purple remnant increasing in prominence in later samples (**Fig 2*k–m***).

Our finding that head epidermis forms barrier before abdominal epidermis correlates precisely with a previous report of accelerated epidermal keratinization in head (e.g., Holbrook and Odland, 1980). Human barrier formation, however, has never been reported this early in development.

Barrier formation summary Analysis of barrier formation using new permeability/dye exclusion assays demonstrates the following. (i) Interfollicular barrier first forms at approximately 20–21 wk on head epidermis and 23–24 wk on abdominal epidermis. This places initiation of barrier formation earlier than suspected. Our results, however, correlate well with previous reports describing timing of keratinization in skin (e.g., Holbrook and Odland, 1980) and a report of change in fetal skin water permeability between 18 and 22 wk (Parmley and Seeds, 1970). (ii) Barrier formation is distinctly regional in the human infant. (iii) Human barrier could develop by two mechanisms. Barrier arises first from follicular epidermis but could complete either by convergence from follicular sites or front movement, as in other mammals.

Follicular emanation or moving fronts? Although examination of small skin samples demonstrated that barrier first forms in follicular epidermis it was unclear whether full barrier is achieved by convergence from follicular sites or whether fronts cross the epidermis, as in other mammals. Several samples contained

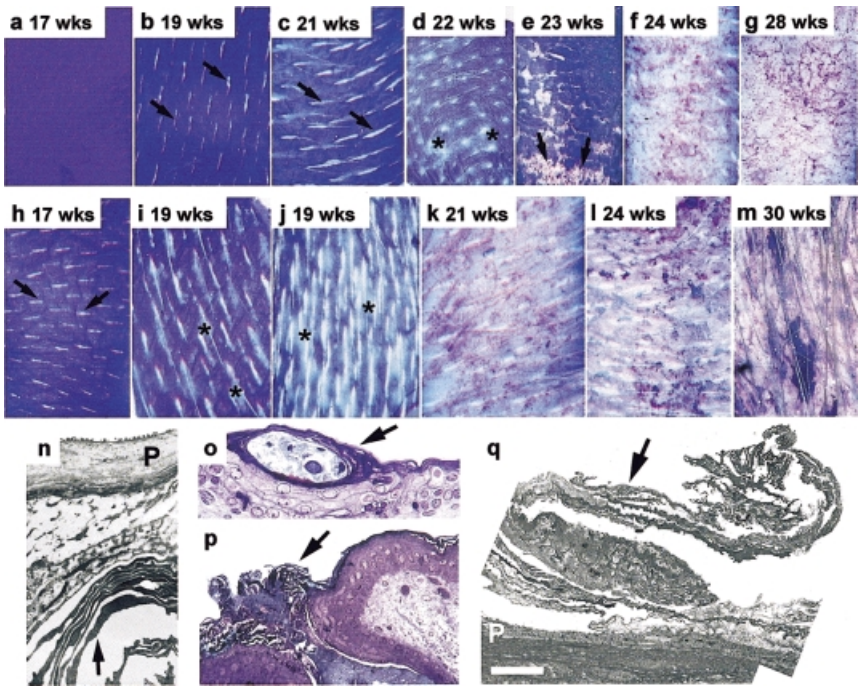

Figure 2. Formation of human permeability barrier. Samples from the abdomen (*a–g*) and head (*h–m*) were subjected to permeability assay, blue signifies lack of barrier and white barrier presence. The barrier is initially detected in follicular canals or hair shafts at 19 wk in the abdominal site and at 17 wk in the scalp (*arrows b, h*). Barrier emanates from the follicles at 22 wk (abdomen, *asterisks d*) and 19 wk (scalp, *i, j, asterisks*). Completion of barrier formation appears to be by front movement in the abdomen (*e, arrows*) or emanation of barrier from follicles in the scalp (*j*). By 24 wk in the abdominal skin (*f, g*) and 21 wk in the scalp (*k–m*) the entire surface has barrier activity. Prior to interfollicular keratinization there is prominent keratinization within the hair canal (*n, o, arrows*) producing the white streaks (*b, c, h, i*) Note the retention of periderm (P) on interfollicular epidermis overlying the follicle canal (*n*). Exudate of keratinized material from the hair canal (*p*) remains attached to epidermal surface (*q*) and contributes to dye exclusion. Note that surrounding epidermis is immature and retains periderm (P, *q*). *Bar* = 3 μm in *n, q* electron micrographs; *bar* = 9 μm in *o, p*, Toluidine Blue stained 1 μm light micrographs.

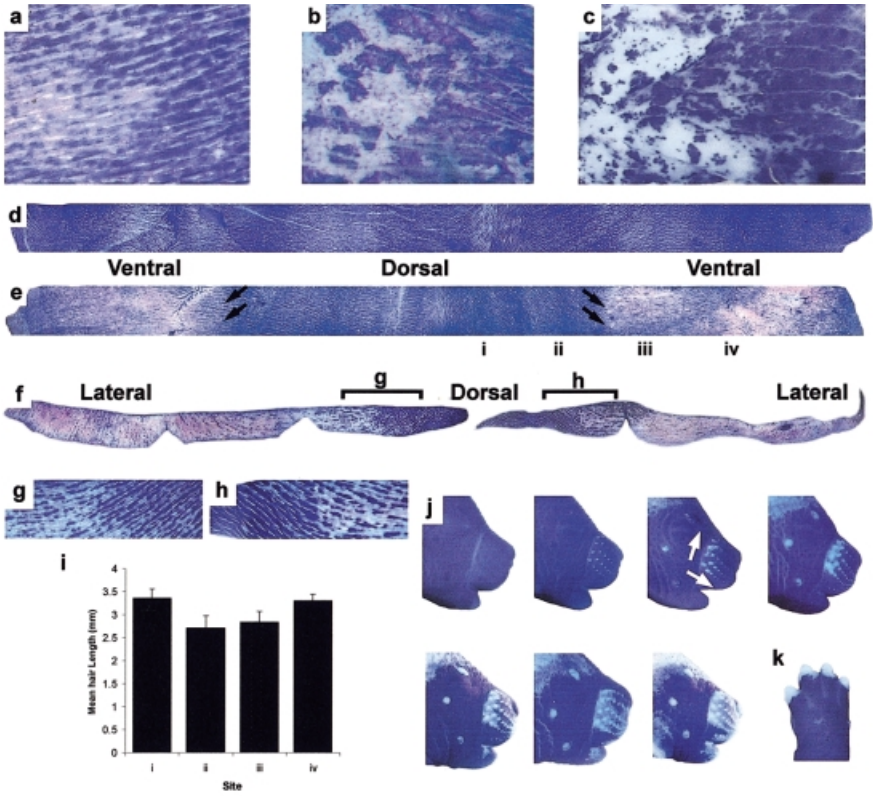

Figure 3. Modes of barrier formation in human and mouse are comparable. Post-mortem skin samples were subjected to permeability assay; blue signifies lack of barrier and white barrier presence. (*a*) Barrier front on lateral torso skin from a 21 wk human infant. Follicles have keratinized providing a barrier and, within an area preceding the front, interfollicular epidermis has also formed a barrier. (*b*) Barrier front from neck regions of 22 wk infant is very similar to front from 16 d mouse skin (*c*). (*d*, *e*) Circumferential skin strip from the torso of 19/20 wk (*d*) and 21 wk (*e*) human infant. Distinct barrier positive regions on the ventrolateral regions in the older infant (*arrows*, *e*) resemble skin generated by movement of front from initiation sites. Barrier also forms regionally on skin strips encompassing ear to dorsal scalp regions (*f*) and moving fronts (*g*, *h*) are apparent. Barrier forms first in the ear region then moves dorsally. Maturity of follicles in torso skin strip from (*e*) was assessed by plucking hairs from areas i–iv in (*e*) and measuring hair length (*i*). Small variation in follicle maturity does not reflect the pattern of barrier acquisition. Error bars, mean ± SEM, n=3. (*j*) Profile of barrier formation from precociously developing vibrissae follicles in mouse. Vibrissae follicles show a pattern of increasing maturing (*arrows* point down maturation gradient). (*k*) Nails can also act as initiation sites.

fronts (**Fig 3*a*, *b***) that superimpose barrier over the islands of barrier radiating from follicles (**Fig 3*a***, torso skin), or appear independent of follicles (**Fig 3*b***, neck skin). At high magnification the follicle-independent front is identical to the animal fronts (cf. **Fig 3*b*, *c***), indicating that barrier can form via fronts in human. The barrier fronts appear at different body locations at different gestational ages suggesting that they are moving across the epidermal sheets, as in other mammals.

Although whole-body analysis is not permissible it was possible to obtain a limited number of circumferential torso samples (**Fig 3*d*, *e***). Analysis of these samples demonstrates the regional nature of barrier formation in the human. Barrier forms first at ventral–lateral sites on torso (*arrows*, **Fig 3*e***). The regional nature of barrier formation is confirmed by analysis of skin strips encompassing ear to ear dorsal scalp samples (**Fig 3*f***). Patterned barrier formation is apparent across the scalp with barrier initiating at lateral locations (near the ears) and fronts (**Fig 3*g*, *h***) appearing to converge dorsally.

The circumferential torso samples were used to analyse the two proposed methods of barrier formation. Follicles across the human dorsoventral torso region are of approximately equivalent maturity, as demonstrated by presence of hairs of approximately equal length (**Fig 3*i***). Follicular epidermis has barrier activity uniformly around the body (**Fig 3*d***). Later a region similar to an animal initiation site appears on the ventral side of the infant (**Fig 3*e***). Interfollicular epidermis acquires barrier activity only within this region. Therefore, we conclude that in humans barrier arises from follicles, and also via larger initiation sites and moving fronts as in other mammals.

We were particularly intrigued by the apparent difference between barrier formation in human and other species. Barrier is propagated across the body primarily via moving fronts in rodents, rabbit, and the marsupial possum, *Monodelphis*. Humans are the only species so far examined where barrier initiates at follicles. Closer examination of the animal models, however, shows that animal follicles, at certain stages of development, can also act as initiation regions.

Developing vibrissae follicles in rodents clearly act as barrier initiation sites (**Fig 3*j***) whereas the body or pelage follicles do not function as initiation sites (**Fig 3*j***). A primary difference between vibrissae and pelage follicles in rodents is the degree of development relative to interfollicular differentiation. Vibrissae follicles develop precociously, appearing 2–3 d earlier than pelage follicles (Van Exan and Hardy, 1980). In addition, vibrissae follicles demonstrate a hierarchy of maturation with the most mature follicles displaying most prominent barrier formation (arrows show decreasing maturity, **Fig 3*j***). Other skin appendages, such as nails, can also initiate barrier formation prior to subsequent propagation by moving fronts (**Fig 3*k***). Therefore, in species such as humans where follicle development is advanced relative to interfollicular differentiation, the follicles can act as initiators. It is possible that in regions such as the head/scalp where follicular density is high and/or follicular maturation is accelerated, the movement of barrier from follicles may be the primary mode of barrier formation.

Morphologic changes accompanying human barrier formation The close temporal correlation between barrier initiation and previous reports of fetal keratinization (Holbrook and Odland, 1980) were gratifying as we have previously demonstrated that initiation of barrier formation correlates with conversion of the outer, stratum corneum cells precursor cell layer to an electron-dense morphology, an early step in keratinization (Hardman *et al*, 1988). In addition, demonstration of barrier initiation as early as 20–24 wk EGA means that barrier is forming near the reported time of periderm release (Holbrook and Odland, 1975). This suggests a very close temporal relationship between the processes. We examined human fetal samples at the ultrastructural level to define the relationship between barrier formation, keratinization and periderm release. Again, detailed results are presented for abdominal midline and dorsal head sites (**Table I**).

Abdominal midline Changes in keratinization mirror the permeability changes between 18 and 30 wk. At the cellular level pre-barrier abdominal epidermis (19 wk EGA) is thin, lacks interfollicular cornification (**Fig 4*a***) and has intact blebbed periderm with clear microvilli protruding from the outer surface (**Fig 4*b***). Periderm decreases in thickness up to 23 wk (**Fig 4*c*–*e***), and the

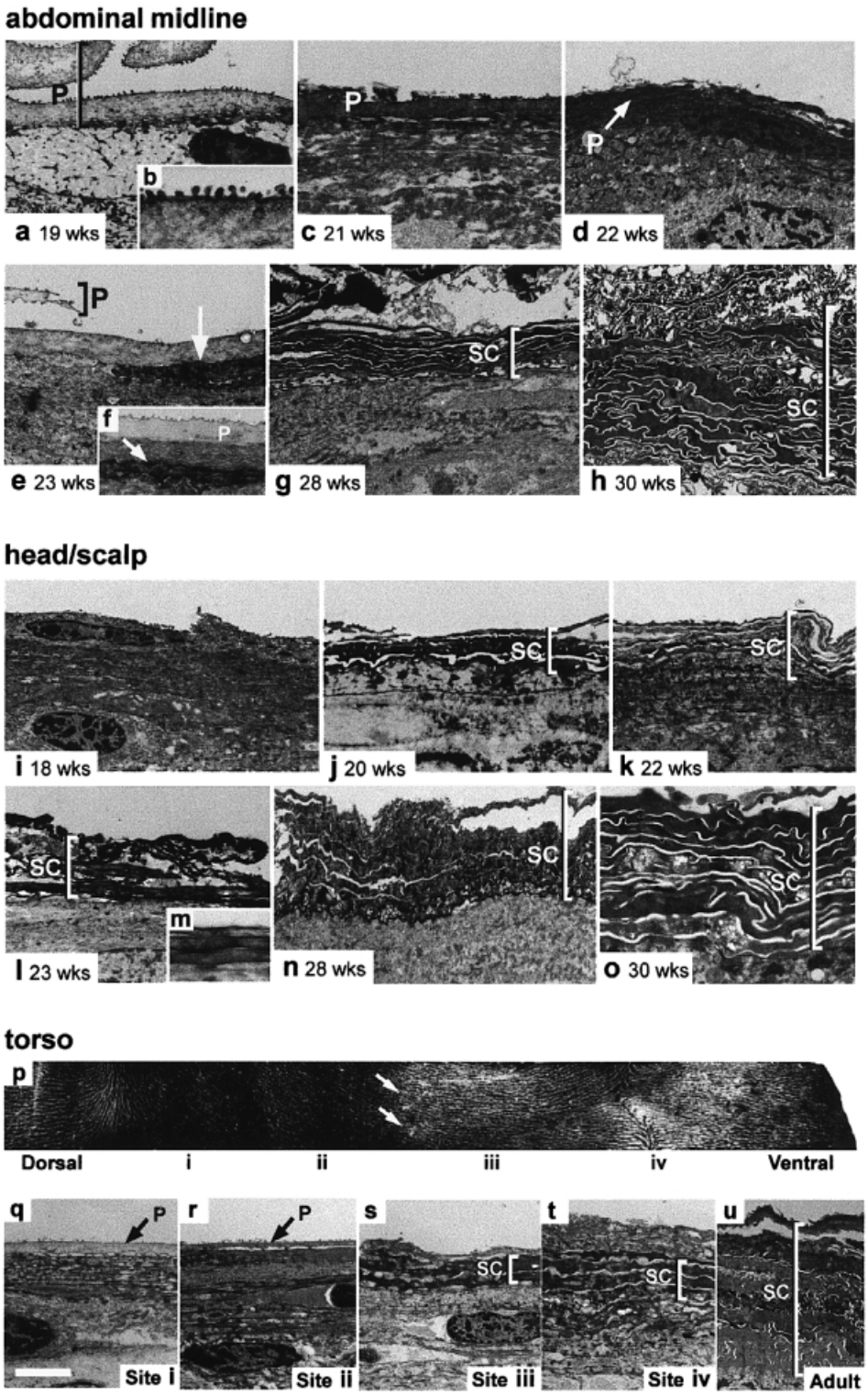

Figure 4. Temporal and spatial pattern of keratinization and periderm release at different skin sites in humans. Ultrastructural analysis by electron microscopy shows that abdominal skin (*a–h*) displays progressive degradation and loss of periderm coincident with increasing keratinization. (*a*) At 19 wk periderm is prominent and blebbed, (*b*) microvilli indicate relative immaturity. Periderm appears to thin between 21 and 22 wk (*c*, *d*) and is regionally disaggregating by 23 wk (*e*). By 23 wk single cells below the surface (*arrows*, *e*, *f*) display the electron-dense phenotype that indicates early stages of keratinization. In mice this correlates with formation of the barrier. By 28 wk skin is well-keratinized (*brackets*, *g*, *h*). Skin at this period always tests positive for barrier activity. (*i*, *j*) Scalp skin matures more rapidly than abdominal skin correlating with accelerated barrier formation. At 18 wk (*i*) interfollicular epidermis is clearly unkeratinized and periderm appears partially degraded or regionally absent. By 20 wk there is clear evidence of keratinization (*brackets*, *j–o*) and periderm is absent. (*m*) Newly formed stratum corneum precursor cells have an electron-dense keratinized morphology. Regional variation in keratinization and periderm status correlates with the barrier pattern on dorsoventral torso skin strip from a 21 wk infant (*p*). Epidermis was sampled from areas i–iv (*q–t*). Periderm is present in pre-barrier interfollicular epidermis (*q*, *r*) but absent from behind the barrier front (*arrows*, *p*). Interfollicular epidermal samples from regions with the barrier (*s*, *t*) show prominent stratum corneum (*brackets*) that have not yet achieved the number of layers or morphology of adult stratum corneum (*u*). *Bar* = 3 μm in *a*, *c–e*, *g–l*, *n*, *o*: 0.9 μm in *b*: 2.3 μm in *f*: 0.8 μm in *m*: 3.3 μm in *q–t*: 5.4 μm in *u*. P, periderm and SC, stratum corneum.

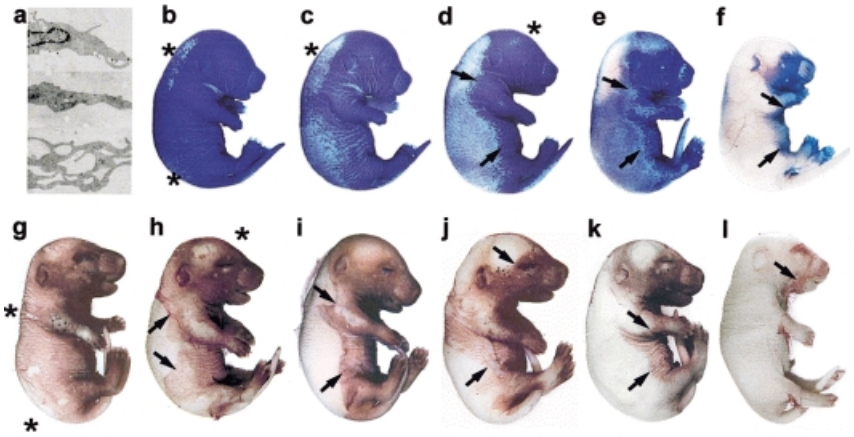

Figure 5. Periderm loss recapitulates barrier acquisition pattern. (*a*) Analysis of disaggregating material identifies it as periderm. (*b–f*) Pattern of barrier formation by permeability assay, increasing gestation age. (*g–l*) Demonstration of periderm disaggregation in fetuses of increasing gestation age. Periderm is indicated by red staining (see text, *Materials and Methods*). Between 17 and 18 d EGA periderm dissociates at dorsal sites (*g*, *h*, *asterisks*) that correspond to the barrier initiation sites used approximately 12 h earlier (see *b*, *c*). Periderm then disaggregates from the epidermis in a pattern that resembles barrier acquisition pattern (*h–l*). Arrows show sites of periderm disaggregation.

microvilli noticeably reduce in number (**Fig 4*f***). At 23 wk the periderm is regionally detaching (**Fig 4*e***), and absent by 28 wk (**Fig 4*g***).

Abdominal interfollicular skin undergoes keratinization between weeks 22 and 23. A single layer of electron-dense cornified cells appears at 23 wk (**Fig 4*e***; *arrow*). By 28 wk up to seven cornified cells are present (**Fig 4*g***) and by 30 wk the epidermis has a prominent stratum corneum (**Fig 4*h***).

Head/scalp site At 17/19 wk head skin lacks interfollicular keratinization and the periderm appears to be partially degraded (**Fig 4*i***). Follicular epidermis is cornified. By 20 wk skin from the head site is entirely covered by dense, flattened cells with prominent cornified envelopes (**Fig 4*j***). The number of keratinized cells at the epidermal surface in head skin continues to increase (**Fig 4*k*, *l***), reaching approximately three–five layers by 23 wk and eight layers by 30 wk (**Fig 4*m*** and ***o***, respectively).

Morphologic variation in a single specimen We find that barrier formation is patterned and note a temporal relationship between barrier formation and keratinization. If so, there should be spatial variation in keratinization in a single sample in the vicinity of a front. We examined a dorsoventral 21 wk fetal trunk sample that is transitional (i.e., barrier has formed in the ventral regions and is absent in dorsal regions **Fig 4*p***). Skin from interfollicular areas are negative by barrier assay, shows presence of periderm and lacks keratinization (**Fig 4*q*, *r***) whereas on the barrier side of the front interfollicular skin is keratinized and periderm absent (**Fig 4*s*, *t***).

Morphologic change—summary Ultrastructural analysis by electron microscopy revealed (i) keratinization in human epidermis is distinctly regional. (ii) The timing of keratinization, approximately 19–20 wk EGA in head epidermis and 23–24 wk EGA in abdomen, correlates extremely well with that of barrier formation and agrees very well with previous reports (Holbrook and Odland, 1980). (iii) Keratinization, barrier formation, and periderm release occur in a closely linked temporal sequence during human fetal development.

Periderm disaggregation We have demonstrated that barrier formation initiates in the human earlier than expected and is occurring at around the time of periderm loss. In the human infant we find that periderm disappears between 20 and 24 wk gestation (**Fig 4**), in agreement with previous reports (Hoyes, 1968; Holbrook and Odland, 1975; Foster *et al*, 1988). The close relationship between barrier formation and periderm loss suggests that the processes may be linked in humans.

To test this hypothesis we returned to the mouse to perform whole-body analysis. We had observed a thin layer of material detaching from freshly isolated postbarrier fetuses in a similar pattern to barrier formation. Ultrastructural analysis by electron microscopy confirmed that the material was periderm (**Fig 5*a***). The layer, a single cell in thickness, displays the characteristic morphology of keratinized periderm. It shows signs of degradation including loss of nuclei and organelles, and membrane thickening. To permit wholemount recording of periderm release fetuses of varying gestational age were subjected to staining (see *Materials and Methods*) to permit visualization of periderm. Periderm appears as a thin covering on the surface of the epidermis (**Fig 5**). At 17 d EGA, when the entire body surface tests positively for barrier, the thin layer detaches from the epidermal surface.

The pattern of barrier acquisition (**Fig 5*b–f***) is mirrored by the pattern of periderm disaggregation (**Fig 5*g–l***), although periderm loss occurs after barrier formation. Initially, periderm is lost from dorsal initiation sites where barrier first forms (**Fig 5*g*, *h***, *asterisks* compare with ***b***, ***c***, *asterisks*). Periderm then sheds around the body following the front of barrier acquisition (**Fig 5*h–l***, *arrows*). The pattern of periderm loss is not as consistent as barrier acquisition, instead periderm is lost in patches. Where periderm detaches it affects the surrounding periderm, pulling it away in somewhat unpredictable strips. The overall pattern of periderm loss, however, is very similar to the pattern of barrier acquisition and, more importantly, periderm disaggregates at a constant time interval (20–24 h in mouse) after initiation of barrier formation.

DISCUSSION

In this study we used permeability assays that measure an early stage in barrier formation to show that barrier forms regionally in the human infant between 20 and 24 wk gestational age (22–26 wk menstrual age). We have shown that barrier forms in a patterned manner in a variety of mammals, including human. Unexpectedly, we find a second mechanism for barrier formation in human. Barrier forms at developing hair follicles and, in addition, formation occurs via initiation sites and moving fronts as in other mammals. A reappraisal of rodent barrier development shows that rodent follicles of specific developmental status can also act as initiation sites.

Our current model for late gestation epidermal development predicts that epidermis enters late stages of terminal differentiation first at epidermal initiation sites in response to an, as yet, unknown signal. A wave or moving front then emanates from the initiation sites, instructing cells to precede with terminal differentiation. We show that rodent vibrissae follicles, but not pelage follicles, can also act as initiation sites. Vibrissae follicle development is advanced relative to pelage follicle development and it seems likely that it is the stage of follicle development relative to epidermal development that determines whether a follicle can act as an initiation site. Hence, we propose that there is a window of time during skin development where follicles can initiate terminal differentiation. Human follicles act as initiation sites because they develop relatively early during gestation compared with rodent, rabbit, and marsupial pelage follicles. Demonstration of accelerated terminal differentiation in the vicinity of human follicles correlates well with a recent report of accelerated maturation of follicular epidermis indicated by filaggrin staining (Lee *et al*, 1999).

It is not yet understood what distinguishes initiation sites from surrounding epidermis. The discovery that follicles (at certain stages of development) can act as initiation sites for barrier development suggests conservation between molecular processes mediating follicle development and initiation site activation. As follicle development is well characterized at the molecular level it should be possible to identify and test candidate molecules for initiation site activation.

The signal from initiation sites appears to be propagated across the epidermis (or underlying mesenchyme) either by diffusion or cell–cell contact. It is possible that this method of barrier formation had been adapted to ''fit'' different species. The increased epidermal surface area in larger animals necessitates a compensatory increase in the number of initiation sites. Many of these extra initiation sites appear at lateral and ventral locations resulting in complex patterns (e.g., rabbit, **Fig 1**). In humans we find that follicles can act as initiation sites. In the large human fetus this mechanism may have evolved as a means to achieve barrier formation over the entire body surface within a reasonable time.

In the mouse we find reorganization of the upper layer of epidermis coincident with barrier formation. We have shown that the early stages of barrier formation correlate with the formation of a flattened electron-dense cell and maturation of the cornified envelope (Hardman *et al*, 1998). We show in this work that human barrier acquisition also correlates with the formation of an electron-dense, flattened cellular phenotype in the upper epidermal layer, demonstrating conservation between species and explaining the very close agreement between our work on barrier initiation and previous studies of human skin keratinization (Holbrook and Odland, 1980). We also demonstrate that by 30 wk the skin from all sites is well keratinized. Thirty weeks has been designated as a ''milestone'' in skin development in a recent study (Kalia *et al*, 1998) as it marks the stage when fetal epidermis acquires barrier capabilities comparable with adult.

Amniotic fluid changes composition at about 25 wk gestation in humans due to the accumulation of urine from the newly functional kidneys (reviewed in Brace, 1997). The coincidence between change in amniotic fluid composition and skin keratinization has been noted (Parmley and Seeds, 1970) and the suggestion made that keratinization is essential for protection from amniotic fluid during late gestation. We provide direct evidence that barrier forms during this period when amniotic fluid composition changes.

The discovery that barrier formation initiates at 20–24 wk gestation in humans means that barrier is forming close to the time of periderm disaggregation (Holbrook and Odland, 1975). Periderm is proposed to have an absorptive or secretory role during much of pregnancy (Parmley and Seeds, 1970; Holbrook and Odland, 1975; Serri *et al*, 1976; Verma *et al*, 1976; Boneko and Merker, 1988). If the periderm's role is interactive then development of barrier would make the periderm redundant. We show that this redundant skin layer disaggregates soon after barrier acquisition in humans and mouse.

Correlation between periderm disaggregation and barrier formation in human is consistent with a recent report showing that periderm cornification precedes epidermal cornification, and is followed by periderm regression (Akiyama *et al*, 1999). Periderm differentiation preceding barrier formation may permit cornification in an aqueous environment by altering water transport to underlying epidermis, as water contact inhibits barrier formation in fetal epidermis (Hanley *et al*, 1997).

Although we show that murine periderm disaggregates soon after barrier acquisition, this does not occur in the rat (Hoath *et al*, 1993; Wickett *et al*, 1993; Hardman and Byrne, unpublished observations) where periderm persists postnatally. The reason for the difference in rodents is unclear. Human and rat newborn skin is highly hydrophobic (Wickett *et al*, 1993; Koah *et al*, 1994) and is considered important in heat and water balance in the newborn. Hydrophobicity is conferred by vernix caseosa in humans and postnatally persisting periderm in rats. Hence, postnatal hydrophobicity in mice may be conferred by extruded lipid and periderm remnant as in human vernix. Significantly, following periderm disaggregation in mouse underlying skin is water repellent indicating hydrophobicity (Hardman and Byrne, unpublished observations). These observations show that mice may be a useful model for late gestational human skin changes.

Murine periderm loss occurs in a patterned manner that recapitulates barrier formation pattern. This implies that a skin developmental gradient persists after barrier formation. We have demonstrated previously that a skin developmental gradient exists well before barrier formation (Hardman *et al*, 1998; Marshall *et al*, submitted) so that cells across the gradient exhibit a range of stages of terminal differentiation. Hence, ''barrier pattern'' is produced when assaying for specific events associated with late terminal differentiation. These events include gene induction (Marshall *et al*, submitted), keratinization, barrier formation and periderm release.

Our demonstration of murine periderm disaggregation at E17 suggests that a previous report describing periderm granules on epidermis at E17 and E18 (Chang *et al*, 1986) represents material persisting after periderm release.

Previous studies used an evaporimeter to measure barrier. The evaporimeter (Nilsson, 1977) measures differences in water vapour pressure at the skin surface, a direct measure of TEWL. Evaporimeter studies report skin barrier in humans at 34 wk (Evans and Rutter, 1986) and 28–30 wk (Hammarlund and Sedin, 1979; Wilson and Maibach, 1980). The skin permeability assays used in this study measure an early stage in barrier formation and demonstrate regional barrier formation initiating at 20–24 wk gestation (Hardman *et al*, 1998). We have shown in the rodent model that TEWL values associated with highly hydrated skin from early stages of barrier formation are too high to be measured using a conventional evaporimeter (Hardman and Byrne, unpublished) suggesting that evaporimeters could not report early steps in human barrier formation. Additionally, the fine pattern detail present on the human skin could not be characterized using an evaporimeter probe (Hammarlund *et al*, 1977).

Ethical considerations may preclude determination of the exact pattern of barrier formation over the entire human fetus. The data reported here and future data collections, however, will have clinical relevance and may affect treatment of premature infants. For example, dorsal torso skin and ventral midline skin forms barrier relatively late in gestation and should be avoided as a site for attaching adhesive tape to hold tubes and monitoring devices in the extremely premature infant. Tape removal has been shown to lead to significant skin disruption (Lund *et al*, 1997).

The limit of pre-term viability has been extended over the last decade due to adoption of perinatal steroid hormone regimens and use of surfactant to address respiratory distress syndrome (Allen *et al*, 1993; Sanders *et al*, 1995; Cooke, 1996). Survival from 20 to 21 wk gestational age (22–23 wk menstrual age) is now possible; however, such infants must have severely compromised skin barrier. We demonstrate in this study that skin barrier formation has initiated regionally in these infants. Further elucidation of pattern is critical for the management of this expanding infant population.

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