Formation of the Epidermal Calcium Gradient Coincides with Key Milestones of Barrier Ontogenesis in the Rodent

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The epidermal permeability barrier forms late in gestation, coincident with decreased lipid synthesis, increased lipid processing, and development of a mature, multilayered stratum corneum. Prior studies have shown that changes in the epidermal Ca^{++} gradient *in vivo* regulate lamellar body secretion and lipid synthesis, and modulations in extracellular Ca^{++} *in vitro* also regulate keratinocyte differentiation. We asked here whether a Ca^{++} gradient forms in fetal epidermis *in utero,* and whether its emergence correlates with key developmental milestones of barrier formation and stratum corneum development. Using either ion precipitation or proton induced X-ray emission analysis of fetal mouse and rat skin, we showed that a Ca^{++} gradient is not present at gestational days 16–18, prior to barrier formation, and that a gradient forms coincident with the emergence of barrier competence (day 19, mouse; day 20, rat) prior to birth. These results are consistent with a role for Ca^{++} in the regulation of key metabolic events leading to barrier formation. Whether the calcium gradient is formed actively or passively remains to be determined. ***Key words: epidermis/permeability barrier/protein induced X-ray emission (PIXE)/transepidermal water loss. J Invest Dermatol 110:399–404, 1998***

The epidermis displays a characteristic calcium (Ca^{++}) gradient, shown both by ion capture cytochemistry and by biophysical methods (Menon *et al*, 1985; Forslind, 1987). Ca^{++} is sparse in the basal and spinous layers, increasing to the highest levels in the granular layer (SG) of untreated epidermis, and declining again in the stratum corneum (SC). With acute barrier disruption, Ca^{++} levels decrease in the outer epidermis, due to displacement of this ion outward through the SC (Menon *et al*, 1992a; Mao-Qiang *et al*, 1997). The Ca^{++} gradient in turn is restored over 24 h in parallel with barrier recovery, but it does not return under occlusion (Menon *et al*, 1992a), just as the barrier fails to normalize under these conditions (Grubauer *et al*, 1989). Moreover, the Ca^{++} gradient is abnormal in other, more sustained or chronic forms of barrier disruption, i.e., in essential fatty acid deficiency, repeated lovastatin treatment, and psoriasis (Menon and Elias, 1991; Menon *et al*, 1994a) with increased levels of Ca^{++} present in all epidermal cell layers. Occlusion in these models normalizes the Ca^{++} gradient in parallel with partial restoration of the barrier (Menon *et al*, 1994a). Finally, the epidermal Ca^{++} gradient is thought to regulate not only barrier homeostasis, but also keratinocyte/epidermal differentiation (e.g., Hennings *et al*, 1983; Yuspa *et al*, 1989).

Permeability barrier requirements regulate several metabolic processes in the underlying nucleated layers of the epidermis (Feingold, 1991; Elias, 1996). These responses include the synthesis of all three major classes of SC lipids, the generation and secretion of epidermal lamellar bodies (LB), and epidermal DNA synthesis. The link between these responses and the barrier is demonstrated by experiments that show that artificial restoration of the barrier by occlusion with vapor-impermeable, but not vapor-permeable wraps, immediately blocks, wholly or in part, the various metabolic responses detailed above after acute barrier perturbation (Grubauer *et al*, 1989; Feingold, 1991). Yet, transepidermal water loss is not the signal for these metabolic responses, because the barrier recovers normally when perturbed skin sites are immersed in isotonic, hypertonic, or hypotonic solutions (Lee *et al*, 1992); however, when a mixture of ions, particularly Ca^{++} and K^{+}, is added to the solution, recovery is blocked to an extent comparable with occlusion (Lee *et al*, 1992, 1994). Moreover, exogenous exposure to these ions also downregulates the expected increase in the activity of HMGCoA reductase, the rate limiting enzyme in cholesterol synthesis, following barrier perturbation (Lee *et al*, 1992). Ca^{++} accounts for many of the inhibitory effects on barrier recovery, as shown by the reversal of inhibition of barrier recovery by cotreatment with inhibitors of either L-type calcium channels or calmodulin (Lee *et al*, 1992, 1994). Finally, using high frequency sonophoresis to alter Ca^{++} levels in the SG, we showed that lamellar body secretion occurs in response to decreases in extracellular Ca^{++} , without alterations in barrier function (Menon *et al*, 1994b).

The permeability barrier develops relatively late in fetal development. In rats, the barrier is still incompetent on day 19, with partial formation on day 20 and mature function by day 21 (gestation is on day 22) (Aszterbaum *et al*, 1992). In fetal mice, a multilayered SC develops between days 17 and 19 (gestation is between days 19 and 20; see references in Hanley *et al*, 1997a). Epidermal lipid synthesis peaks prior to barrier formation, declining as the barrier is formed (Hurt *et al*, 1995), whereas the lipid processing enzymes, steroid sulfatase and β-glucocerebrosidase, increase in parallel with barrier formation (Hanley *et al*, 1997b, c). Likewise, markers of epidermal terminal differentiation,

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Abbreviations: PIXE, proton induced X-ray emission; SC, stratum corneum; SG, stratum granulosum.

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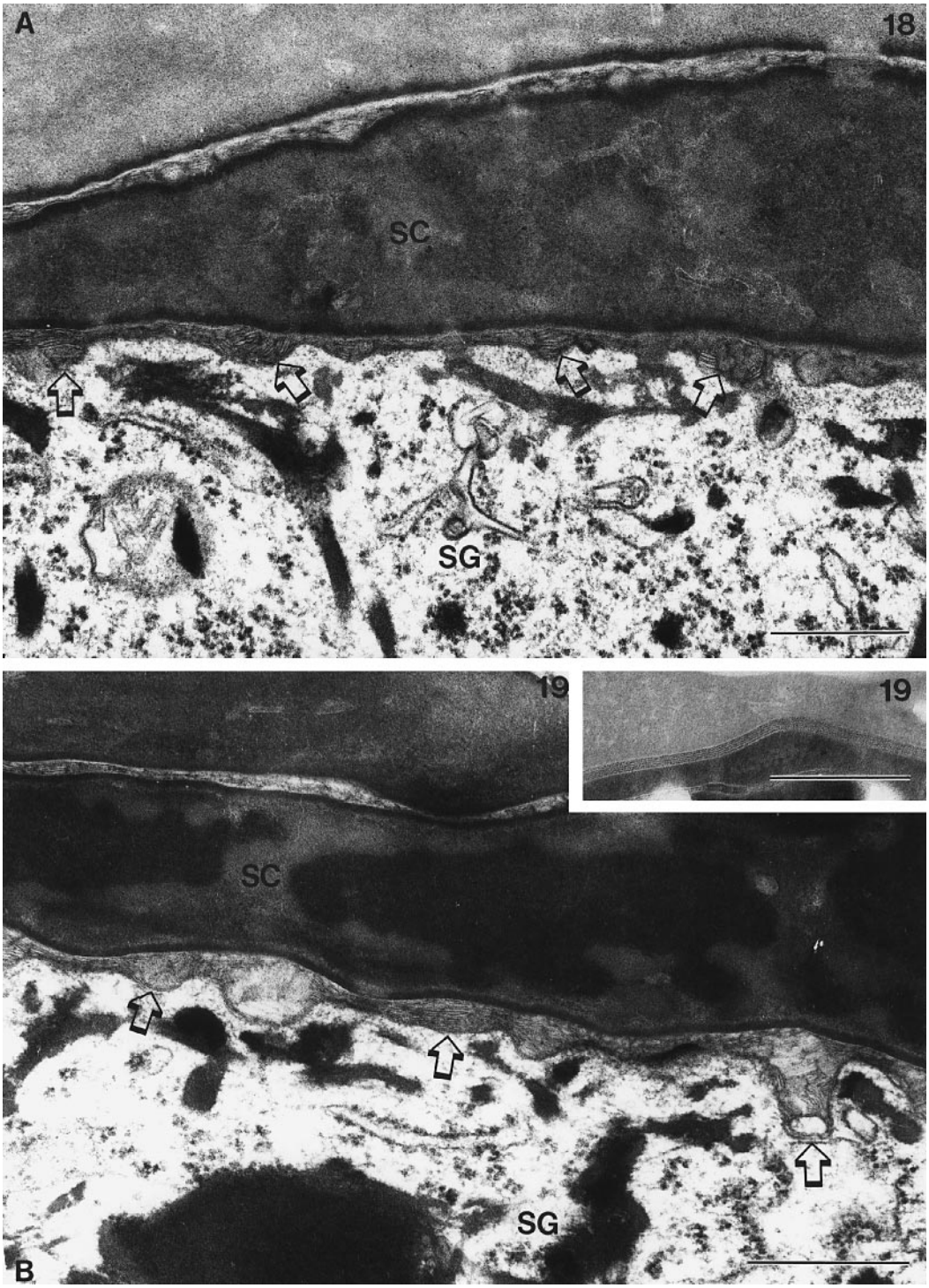

Figure 1. Electron micrograph of 18 and 19 d old fetal mouse epidermis shows accelerated LB secretion. SG cytosol contains few LB, but the SG–SC interface is engorged (*open arrows*). After day 17–18, mature extracellular lamellae are present throughout the SC interstices (*B*, insert). Ruthenium tetroxide postfixation. *Scale bars*, 0.33 μm.

such as involucrin and profilaggrin, peak later, coincident with barrier formation (Bickenbach *et al*, 1995).

Here, we asked first whether the epidermal Ca^{++} gradient forms i*n utero,* despite being bathed in an isotonic milieu; second, whether changes in the Ca^{++} gradient correspond with, and account for, some or all of the developmental milestones described above. Our results show that: (i) a Ca^{++} gradient forms i*n utero,* coincident with barrier ontogenesis; (ii) the Ca^{++} gradient is virtually absent in fetal rodent epidermis at a time when lipid synthesis is high, and lamellar body secretion appears accelerated; and (iii), conversely, a Ca^{++} gradient forms late in gestation, coincident with a decline in lipid synthesis, and in parallel with the generation of a multilayered SC and a functional barrier.

MATERIALS AND METHODS

Animals Timed pregnant (plug date = day 0) Sprague-Dawley rats and SwissWebster mice were obtained from Simonsen Laboratories (Gilroy, CA). Maternal rats were anesthetized on gestational days 19, 20, and 21, and fetuses delivered prematurely by Cesarean section, whereas maternal mice were treated similarly on days 17, 18, 19, and 20. In addition, postnatal and adult epidermis were assessed, as indicated in the text and figure legends.

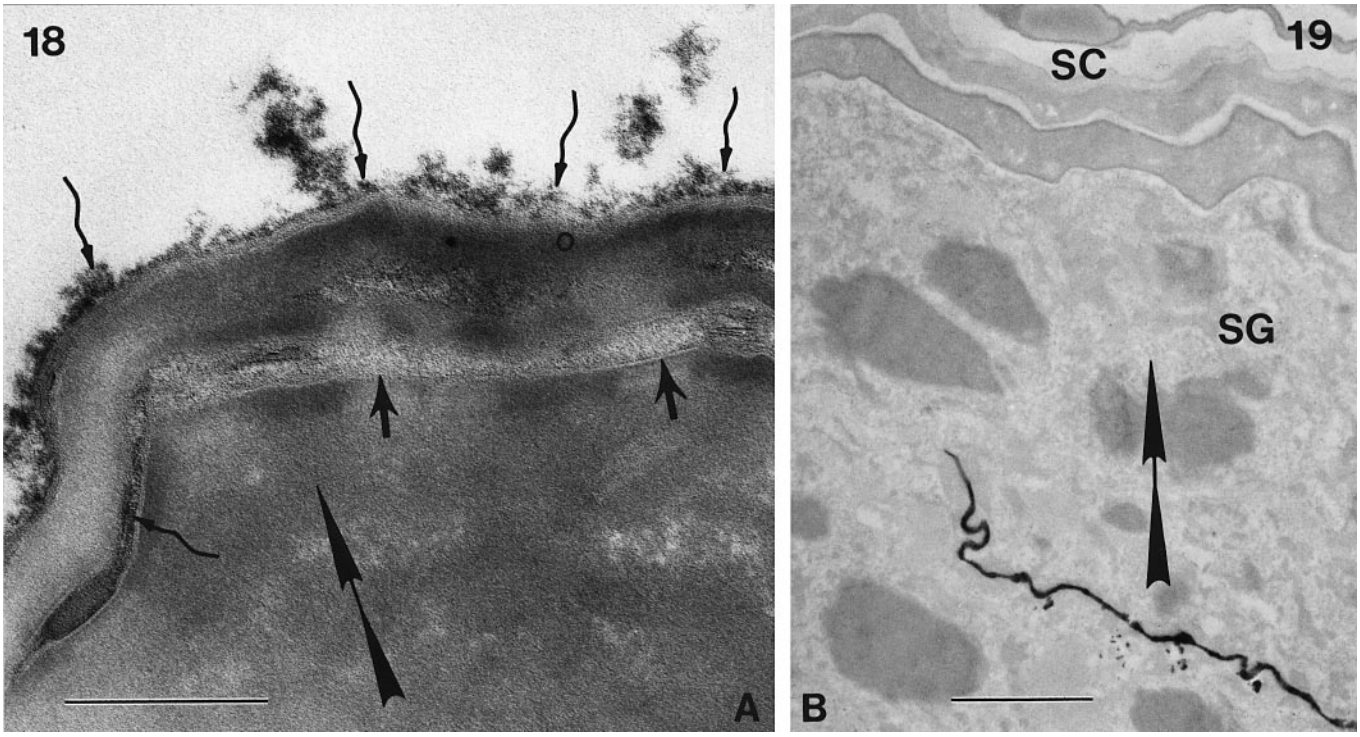

Figure 2. Lanthanum still permeates on 17–18 d old, but not 19 d old fetal mouse epidermis. Direction of lanthanum nitrate perfusion is indicated by *large arrows*. *Small arrows* depict lanthanum in extracellular spaces of SC, and *wavy arrows* show lanthanum that has passed through to the outer surface of SC. Osmium tetroxide postfixation. *Scale bars*, 0.33 μm.

Lanthanum perfusion We employed lanthanum perfusion, as described previously (Elias *et al*, 1981; Hanley *et al*, 1996), to delineate the time course of barrier development in fetal mouse skin, because fetal mice are too small for routine transepidermal water loss measurements. Briefly, skin samples are incubated for 1 h in a 1:1 solution of 8% lanthanum nitrate (Electron Microscopy Sciences, Ft. Washington, PA) in 0.05 M Tris buffer and Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer) at room temperature. Samples are then washed in cacodylate buffer, fixed further in half strength Karnovsky's fixative overnight at 4°C, and transferred to ethanol solutions for dehydration and embedding.

Ion capture cytochemistry Ion capture cytochemistry was performed on fetal rat and mouse samples, as described in prior publications (e.g., Menon *et al*, 1985). Briefly, the primary fixative contains 2% paraformaldehyde, 2.5% glutaraldehyde, 0.09 M potassium oxalate, 0.04 M sucrose, adjusted to pH 7.4. Samples are fixed overnight at 4°C, and then postfixed in 1% osmium tetroxide, containing 2% potassium pyroantimonate, pH 7.4 for 2 h at 4°C in the dark. Tissue samples are then washed in alkalinized distilled water (pH 10), and transferred to ethanol solutions for dehydration and embedding.

Electron microscopy Fetal rat and mouse skin samples were fixed in half strength Karnovsky's fixative, postfixed in both osmium tetroxide and reduced ruthenium tetroxide (Hou *et al*, 1991), and embedded in an epoxy mixture. Ultra-thin sections, with and without additional contrasting (lead citrate/uranyl acetate), were examined in a Zeiss 10 A electron microscope operated at 60 kV.

Proton induced X-ray emission (PIXE) PIXE studies were performed using a modification of the methods of Bunse *et al* (1991). Four millimeter squared slice skin samples were collected from 18, 19, and 21 d old fetuses and from 1 or 2 d old pups and adult rats. Samples were frozen in liquid propane, transferred to liquid nitrogen, and stored at −50°C. After 30 μM sections were cut, they were transferred to nylon foils and freeze-dried for 12 h at −80°C. Samples were analyzed by microbeam particle induced X-ray emission, with beam currents of up to 900 pA, beam spatial resolution of 2–3 μm, and a beam energy of up to 3 MeV. X-rays were detected with a Si (Li) detector that subtended a solid angle of ≈100 msr. The detector was located at an angle of 135 with respect to the incident beam. Charge was collected in a biased Faraday cup located behind the sample. X-rays were recorded in list mode along with coincident beam spatial coordinates arising from scanning the beam electrostatically over the sample in a point by point raster mode.

Data were reduced off-line so that X-ray spectra from subregions could be extracted from each irradiated region. X-ray spectra were analyzed with the PIXE spectrum fitting code (Antolak and Bench, 1994). A series of thin film calibration standards containing CA2+ were used to measure the efficiency of the X-ray detection system. To normalize the X-ray yields for variation in target thickness in order to obtain concentration data, scanning transmission ion microscopy was used to measure tissue projected densities. Each sample was measured in triplicate. Data are presented as the mean ± SD.

RESULTS

Barrier function in rodents develops late in fetal development Prior studies in the fetal rat have shown that the appearance of mature lamellar unit structures in a multilayered SC corresponds with the appearance of a competent barrier by day 21, measured both by the ability of the SC to exclude lanthanum nitrate (Hanley *et al*, 1996) and by a decrease in transepidermal water loss levels comparable with postnatal skin (Aszterbaum *et al*, 1992). Because rat epidermis fails to generate a prominent Ca^{++} gradient (see below), we examined epidermal development in the fetal mouse, which displays a prominent Ca^{++} gradient. A distinct SC first appeared at day 17, and between days 17 and 18 the extracellular spaces at the SG–SC interface became engorged with secreted lamellar body contents (**Fig 1*A***). In contrast, the cytosol of the outermost granular cell was largely devoid of lamellar bodies, consistent with accelerated formation and secretion of these organelles. Mature lamellar membrane unit structures first appeared in a patchy distribution in the extracellular spaces of the SC by day 17–18 (**Fig 1*B***). Finally, abundant lamellar bodies again were present in the cytosol after day 18 (not shown; see Hanley *et al*, 1997a). These studies show that fetal murine epidermis develops morphologic features of a competent barrier 1 or 2 d prior to birth, as does fetal rat epidermis.

To determine whether these morphologic landmarks indicate the development of a competent barrier in the fetal mouse, we next examined the permeation of an electron-dense, water-soluble tracer, lanthanum nitrate, in the epidermis of fetal mice between days 15 and 19 of gestation. Whereas prior to day 17–18 (i.e., days 15–17) tracer freely permeated throughout the entire epidermis (**Fig 2*A***), after day 17–18 tracer was excluded from both the SG–SC interface and the extracellular spaces of the SC (**Fig 2*B***). These studies show that a competent barrier emerges in parallel with structural markers of SC development in fetal mouse skin, i.e., between days 17 and 18.

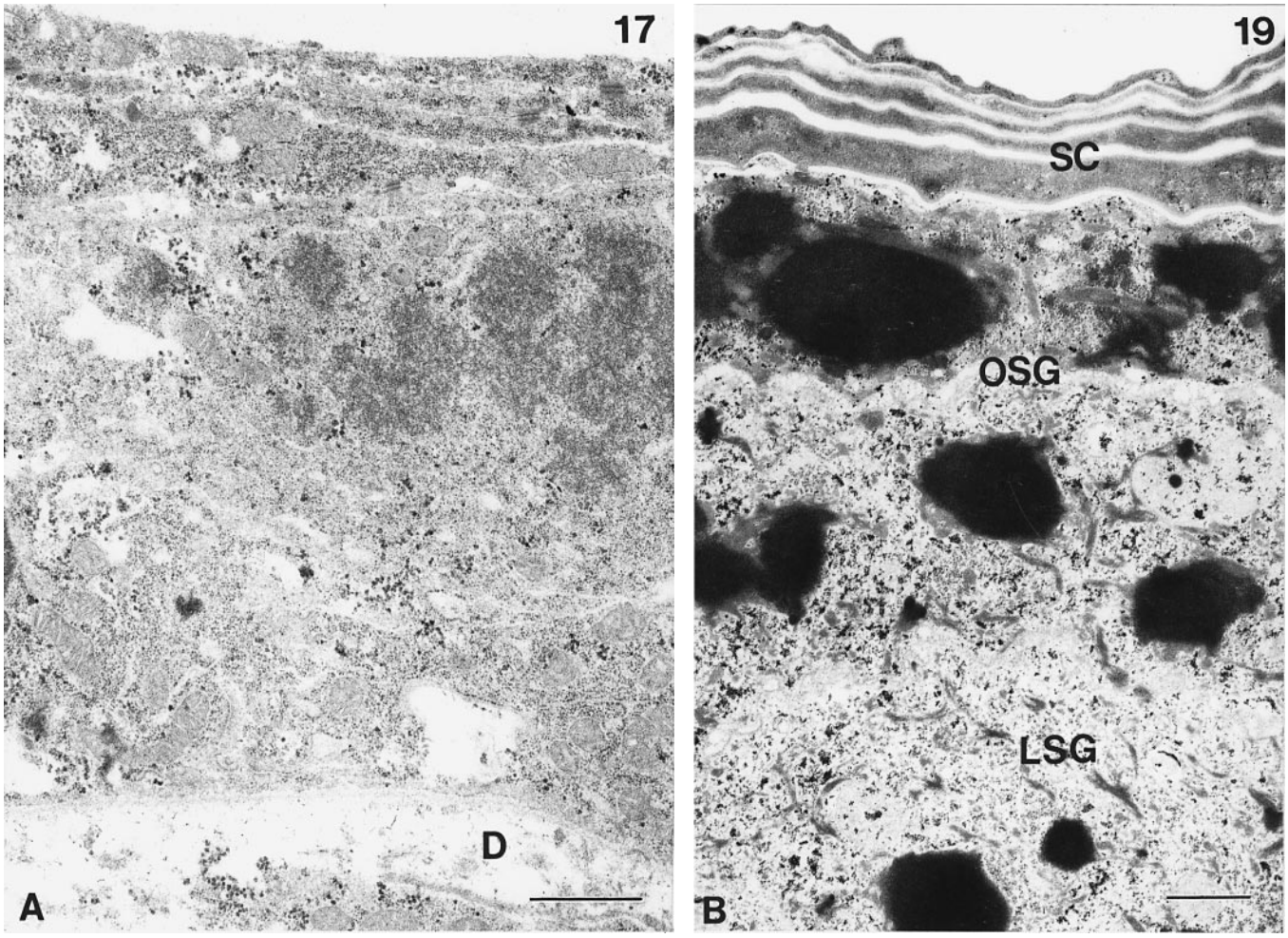

Figure 3. Ion capture cytochemistry depicts a Ca^{++} gradient in 19 d old, but not 17 d old fetal mouse epidermis. (*A*) Day 17 epidermis displays incompletely developed SC, with evenly dispersed Ca^{++} precipitates in all epidermal layers, and high levels in the dermis (D). (*B*) In contrast, day 19 epidermis shows a distinct Ca^{++} gradient, with the highest Ca^{++} levels in the outer SG, lower levels in subjacent layers and SC. Osmium tetroxide postfixation. *Scale bars*, 0.33 μm.

Both cytochemistry and PIXE show emergence of the calcium gradient in parallel with barrier development We next asked whether the above-described, sequential changes in morphologic markers of barrier formation might be linked to changes in the epidermal Ca^{++} gradient (**Figs 3, 4**). Whereas the neonatal mouse exhibits a prominent Ca^{++} gradient on ultrastructural cytochemistry (**Fig 3*B***), the neonatal rat displays much lower, absolute levels of Ca^{++} precipitates in the epidermis (not shown), making it difficult to evaluate changes in the Ca^{++} gradient in relation to fetal barrier development by ultrastructural cytochemistry. As seen in **Fig 3(*A*)**, the neonatal mouse displays no calcium gradient at days 16 and 17, a partial Ca^{++} gradient at day 18 (not shown), and a prominent Ca^{++} gradient at day 19 (**Fig 3*B***).

The appearance of the latter is comparable with neonatal murine epidermis (Menon *et al*, 1985); i.e., accumulation of extra- and intracellular Ca^{++} in the SG, and low visible levels in the SC and lower epidermal levels.

Because the rat exhibits a less distinct Ca^{++} gradient than the mouse when examined by ultrastructural cytochemistry, we utilized an alternative, more sensitive and quantitative technique, PIXE, to measure changes in the epidermal Ca^{++} gradient during fetal rat development (**Fig 4**). Moreover, our prior studies have shown good correlation between ion capture cytochemistry and PIXE when both were applied to the same samples (Mao-Qiang *et al*, 1997). Despite the low absolute levels of Ca^{++} in the epidermis (for comparison, peak Ca^{2+} levels in human epidermis are ~400–500 mg per hg), an epidermal Ca^{++} gradient appears late in gestation (between days 20 and 21), coincident with barrier development *in utero*. Moreover, gradient formation is due to both a progressive decrease in Ca^{++} in the lower epidermis, and increased Ca^{++} levels in the outer epidermis. These results show, in two different species, and by two complementary techniques, that a Ca^{++} gradient appears in fetal epidermis in parallel with barrier development.

DISCUSSION

These studies show that an epidermal Ca^{++} gradient develops late in gestation, parallel to the emergence of a competent permeability barrier. The epidermal Ca^{++} gradient, first recognized by ultrastructural cytochemistry (Menon *et al*, 1985), has also been demonstrated by several quantitative methods in adult human and murine epidermis (Forslind, 1987. In our most recent study, ion capture cytochemistry and PIXE provided very similar results (Mao-Qiang *et al*, 1997), further validating the application of either one or both methods to experimental systems. Because a prominent gradient is present in postnatal murine epidermis (Menon *et al*, 1985), we utilized cytochemistry for the studies in fetal murine skin, and PIXE (a more sensitive and quantitative method) for studies in fetal rat skin, where the gradient is less prominent. Regardless of the method of barrier disruption, prior studies have shown the Ca^{++} gradient is lost (Menon *et al*, 1992a; Mao-Qiang *et al*, 1997), reappearing over 24 h in parallel with barrier recovery.

The reappearance of the Ca^{++} gradient during barrier recovery following acute insults, and the gradual appearance of a Ca^{++} gradient during fetal development, appear to be analogous in several respects. In postnatal epidermis, acute barrier disruption depletes the Ca^{++} gradient, provoking the *en masse* secretion of preformed lamellar bodies

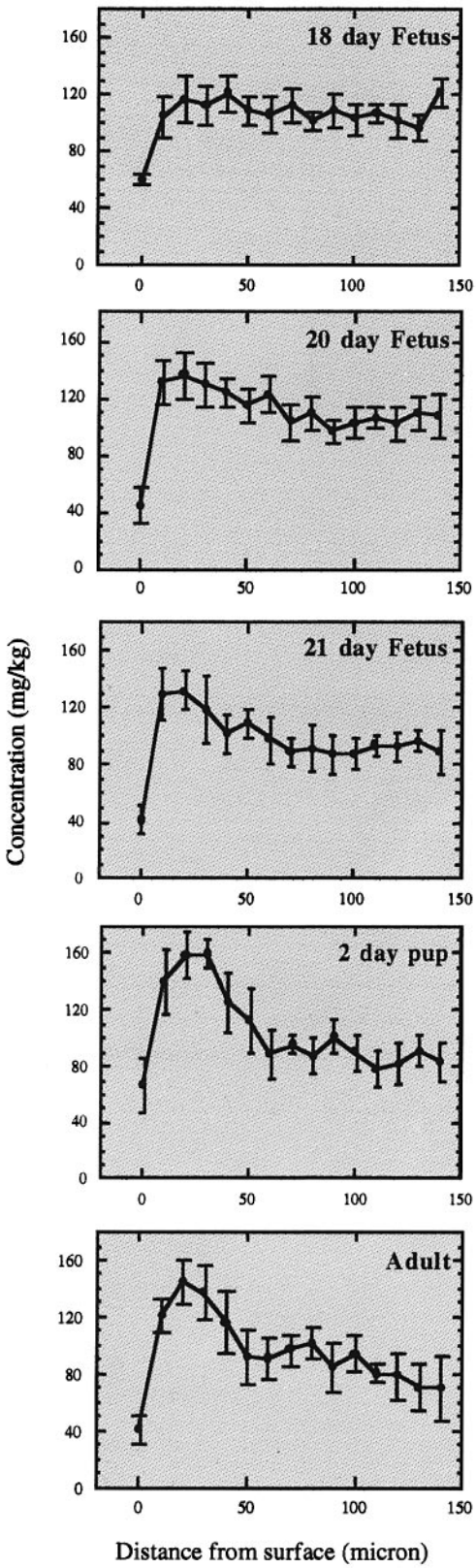

Figure 4. *PIXE* shows development of the Ca^{++} gradient in rat epidermis late in fetal development. A distinct gradient is present as early as day 20, with further acceleration thereafter. Data are mean ± SD for three separate recordings from three different samples.

from the outermost SG cell (Menon *et al*, 1992a, b). Shortly thereafter, cholesterol and fatty acid synthesis increase; and nascent lamellar bodies are generated and secreted in an accelerated fashion (reviewed in

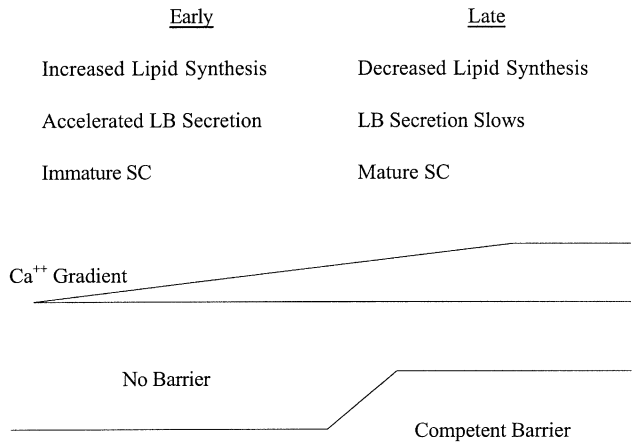

Figure 5. Potential relationship of Ca^{++} gradient to milestones of barrier development.

Feingold, 1991; Elias, 1996). In parallel with the reappearance of the Ca^{++} gradient, both lipid synthesis and lamellar body secretion slow down. A similar, if not identical relationship appears to pertain during fetal barrier development. We also showed previously that lipid synthesis peaks prior to barrier formation (Hurt *et al*, 1995), at a time when no demonstrable gradient is present (these studies), and synthesis rates decline as the barrier is formed. Moreover, we showed here a potential relationship between the status of the Ca^{++} gradient and the cytosolic pool of lamellar bodies, i.e., secretion appears to accelerate prior to barrier formation, whereas large numbers of lamellar bodies reappear coincident with gradient and barrier formation, implying slowed secretion rates. The declining rates of lipid accumulation in the SC late in barrier development (Aszterbaum *et al*, 1992), in the face of maintenance secretion rates, can be explained by the continued expansion and retention of the SC compartment i*n utero*.

Because artificial restoration of the barrier with a vapor-impermeable wrap blocks reformation of the Ca^{++} gradient (Menon *et al*, 1992a), it seems likely that the gradient forms passively as ions are trapped under a competent barrier, which would minimize net water movement and prevent escape of ions. Yet, in both of the fetal rodent models, a Ca^{++} gradient forms despite exposure of the outer epidermis to an isotonic milieu, where water movement should be minimal, suggesting a role for active mechanism(s) in Ca^{++} gradient formation. Moreover, an active rather than a passive mechanism is suggested by the PIXE data shown here, which show a reduction in Ca^{++} levels in the lower epidermis, rather than a progressive accumulation of Ca^{++} in the outer epidermis during development. Alternatively, the Ca^{++} gradient could form passively as Ca^{++} is sequestered by calcium binding proteins, recently demonstrated to be present in amniotic fluid (Hitomi *et al*, 1996). At this point, direct evidence for either active or passive mechanisms in epidermal ion gradient formation is lacking.

Finally, fetal barrier development can be divided conceptually into two overlapping stages (**Fig 5**). During the initial phases of barrier development, epidermal cholesterol, fatty acid, and ceramide synthesis peak, and lamellar bodies form and are rapidly secreted (shown previously for the fetal rat and here for the fetal mouse). At this stage, epidermal differentiation is still rudimentary, mature lamellae are not present in the SC interstices, enzymes related to lamellar processing are expressed at low levels, and a competent barrier is not yet present. In contrast, during the later stage of barrier development, lipid synthesis slows down, and lamellar body formation and secretion decrease to normal (i.e., maintenance) levels. Simultaneously, epidermal differentiation proceeds (Bickenbach *et al*, 1995), mature extracellular lamellae form, expression of processing enzymes increases, and a competent barrier emerges. Prior studies have shown that lamellar body secretion accelerates in low Ca^{++}, whereas physiologic Ca^{++} inhibits cholesterol synthesis and lamellar body secretion. Moreover, a large number of *in vitro* studies have shown that several key steps in the terminal epidermal differentiation program require a high extracellular Ca^{++}

level (Hennings *et al*, 1983; Yuspa *et al*, 1989). Because the above-described processes are regulated by changes in ambient Ca^{++} concentrations, it is tempting to link each of them to the observed changes in the Ca^{++} gradient (**Fig 5**); however, other experimental approaches will be required to determine which of these developmental milestones are regulated directly by changes in the Ca^{++} gradient.

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