Additional studies have examined the accuracy of self-reported family history. In a cohort of 276 consecutive American CM patients, melanoma in relatives was histologically confirmed in 27 of the 31 subjects (87%) who had initially reported a family history for CM.[1] In an Australian study, Aitken *et al* showed that about 40% of self-reported melanomas in familial settings were incorrect. In that study, people affected by CM tended to over-report melanoma in their family members compared with other cancers involving the breast, colon, and pancreas (Aitken *et al*, 1996).

In this study, 2.5% of 589 CM patients had a confirmed family history of melanoma in first-degree relatives. Overall, familial CM represents a small fraction of the melanoma burden. If we consider the emerging increase in the melanoma incidence rate in western countries, however, the prevalence of familial melanoma clustering is becoming considerable. The identification of genes, host, and environmental factors involved in these aggregations may help to elucidate the etiology of both familial and sporadic CM, and have a significant impact on prevention and clinical activities.

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Origin of the Corneocyte Lipid Envelope (CLE): Observations in Harlequin Ichthyosis and Cultured Human Keratinocytes

To the Editor:

As cells of the outer stratum granulosum (SG) transform into corneocytes, they generate a rigid, chemically resistant, ≈15 nm thick peripheral envelope, the cornified envelope (CE) (Matoltsy, 1976; Steven and Steinert, 1994). CE formation begins with the deposition initially of involucrin (Steinert and Marekov, 1997), followed by the subjacent, catalytic cross-linking of several other peptide precursors (Steinert and Marekov, 1995, 1997), by the keratinocyte-specific, Ca^{++} dependent enzyme, transglutaminase 1 (TG1) (Parenteau *et al*, 1986; Michel and Démarchez, 1988; Polakowska *et al*, 1992).

A novel hydrophobic envelope, which is assumed to play an important role as either a scaffold for the extracellular lamellae (Downing, 1992) or in corneocyte cohesion (Wertz *et al*, 1989b), lies external to the CE (Farbman, 1966; Matoltsy, 1976; Elias *et al*, 1977). This structure is enriched in ω-hydroxyceramides (ω-OH Cer) (Wertz and Downing, 1986, 1987), linked covalently by ester bonds to glutamine/glutamate residues within the outer CE (Wertz *et al*, 1989a; Downing, 1992; Marekov and Steinert, 1998). Whereas the corneocyte lipid envelope (CLE) persists following exhaustive, organic-solvent treatment (Swartzendruber *et al*, 1987; Wertz *et al*, 1989a), the extracellular lamellar membrane system is removed readily by either organic solvents or detergents (Elias *et al*, 1977; Wertz *et al*, 1989a).

The CLE is presumed to originate from epidermal lamellar bodies (LB), because: (a) both are enriched in acylglucosylCer, with an ω-esterified N-acyl group (Wertz *et al*, 1984; Freinkel and Traczyk, 1985; Grayson *et al*, 1985); and (b) the composition of acylglucosylCer in LB is comparable with ω-OH Cer in the CLE. Accordingly, it has been proposed that secretion of acylglucosylCer is followed by multistep extracellular processing, including: (1) deglucosylation by β-glucocerebrosidase (Holleran *et al*, 1992, 1993; Takagi *et al*, 1999); (2) ω-esterase/ω-lipase-directed hydrolysis of acylCer to ω-OHCer (Sando *et al*, 1997); followed by (3) covalent attachment to the CE, perhaps by transglutaminase 1 (TG1) (Nemes *et al*, 1999). Yet, CLE lipids could originate from the limiting membrane of the LB, rather than its contents; however, fusion of the LB limiting membrane with the apical plasma membrane of the outermost SG cell would allow covalent attachment of Cer to CE proteins by TG1 (Nemes *et al*, 1999). The issue of the origin of the CLE can be approached through models where cornification occurs, independent of normal LB generation; e.g., does a CLE occur in cultured human keratinocytes (CHK), which cornify in Ca^{++}-containing media (Pillai *et al*, 1988), while generating few, if any, LB? Likewise, does a CLE occur in the rare, often lethal, autosomal recessive disorder, Harlequin ichthyosis (HI), a plate-like SC forms (Williams and LeBoit, 1996), with few or absent LB (Buxman *et al*, 1979; Fleck *et al*, 1989; Dale *et al*, 1990; Milner *et al*, 1992; Hashimoto *et al*, 1993)? But, in order to assess the issue of the CLE's origin, it is necessary to employ procedures that allow its optimal morphologic assessment, coupled with lipid biochemical analysis. Whereas standard ultrastructural procedures portray the CLE as an electron-lucent monolayer (Swartzendruber *et al*, 1987; Wertz *et al*, 1989a), with opposing CLEs forming a lucent-dense-lucent pattern between adjacent corneocytes (Swartzendruber *et al*, 1987; Wertz *et al*, 1989b), treatment with the dipolar organic solvent, pyridine, yields electron-dense-lucent-

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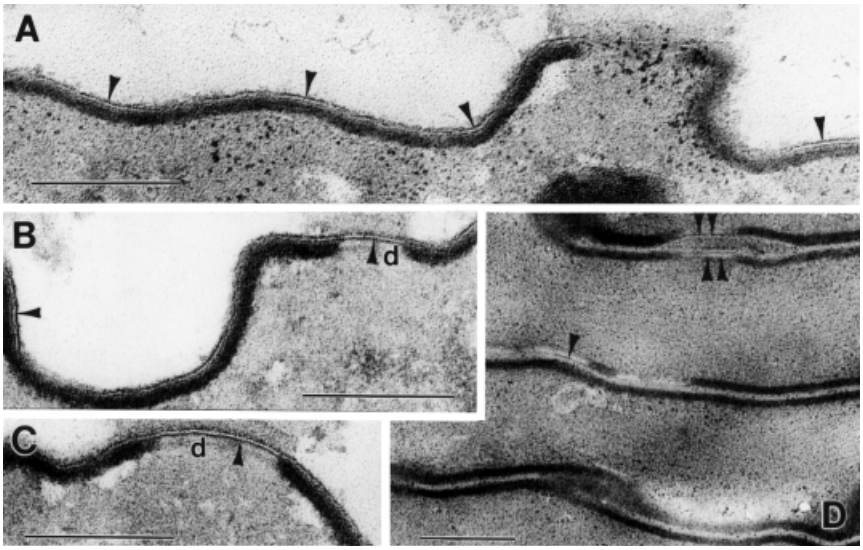

Figure 1. The CLE appears as a bilayer structure in pyridine-treated murine stratum corneum. (*A–C*) Murine SC treated for 2 (*A*) and 16 (*B, C*) h with pyridine. The CLE is more visible in samples treated for the longer period (*B, C*: *arrowheads*), and at sites of desmosomes (d) embedded in the CE. (*D*) Murine stratum corneum treated for 16 h with chloroform:methanol (Wertz *et al*, 1989a). The CLE is only seen occasionally, and when visible, unilamellar images predominate (*single arrowhead*), although occasional dense-lucent-dense images can be found (*double arrowheads*). *Scale bar*: 0.2 μm.

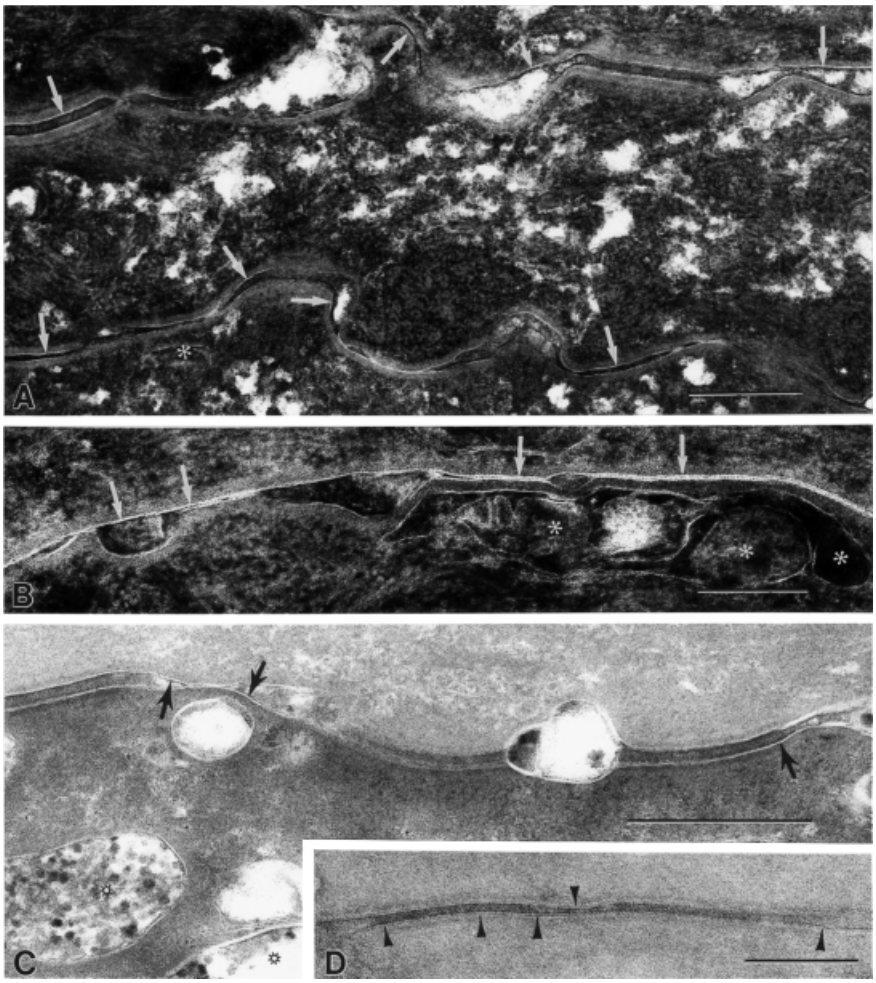

Figure 2. A CLE is present around corneocytes in harlequin ichthyosis. Few or no LB were present in these subjects. No extracellular membranes are seen; vesicular structures (*A–C*, *asterisks*) persist in the corneocyte cytosol, and a prominent CLE is present around corneocytes in all HI subjects (*A–C*, *arrows*). Following pyridine treatment of aldehyde/osmium tetroxide fixed samples, the dense-lucent-dense structure of the CLE can be seen (*D*, *arrows*). (*A–C*) Ruthenium tetroxide postfixation. *Scale bar*: 0.2 μm.

dense images of the CLE in which a distinct, electron-dense inner leaflet can be seen contiguous to the CE (Elias *et al*, 1977). With the pyridine method, we found that a typical CLE is generated around corneocytes in both HI and in differentiated CHK, suggesting that LB contents are not a likely source of CLE precursors.

Samples were available from four infants with typical HI (Williams and LeBoit, 1996), with an absence or virtual absence of LB on electron microscopy. Subject #1 (B.S.) was a full-term, still-born male. Subject #2 (E.K.) was born with a typical HI phenotype, survived, and evolved into a severe ichthyosiform erythroderma. His TG1 levels were normal, and LB were absent on electron microscopy (Choate *et al*, 1998). Subject #3 (B.B.) was a 24-wk-old still-born fetus. Subject #4 (B.R.) was a full-term infant, who died shortly after birth. Biopsy samples were obtained from the back and buttocks from all infants while they were still alive, or within 2 h of death. Full thickness skin biopsies also were

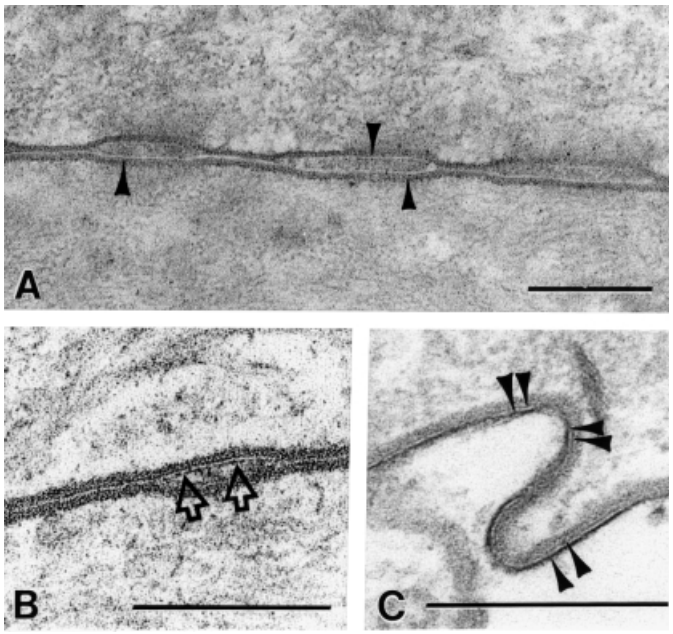

Figure 3. A CLE is present around corneocytes in postconfluent human keratinocyte cultures. (*A*, *B*) Non-pyridine-treated cultures reveal a continuous electron-lucent gap and an electron-dense external leaflet around corneocytes (*A*, CLE gap, *solid arrows*; *B*, CLE external leaflet, *open arrows*). (*C*) Pyridine treatment of parallel cultures reveals the CLE to comprise a dense-lucent-dense structure (*solid arrows*). (*A–C*) Osmium tetroxide postfixation. *Scale bar*: 0.2 μm.

obtained from the flanks of 6–8-wk-old male hairless mice (Charles River).

Human keratinocytes (CHK) were isolated from newborn human foreskins, and grown to confluence in Dulbecco's modified essential medium (DMEM) with 5% fetal calf serum. Second passage keratinocytes were grown to 90–100% confluence in keratinocyte growth medium (KGM) (0.07 mM Ca^{2+}), and transferred to complete DMEM and Ham F-12 (2:1, vol/vol) in 1.2 mM Ca^{++}, supplemented with 10% fetal calf serum, 10 μg insulin per ml, and 0.4 mg hydrocortisone per ml for 7–10 d. Under these conditions, keratinocytes express abundant CE and other protein markers of terminal differentiation (Pillai *et al*, 1988).

HI samples were prefixed in Karnovsky's fixative, either before or after immersion in absolute pyridine for 2 h or overnight at room temperature (Elias *et al*, 1977). Twenty-day-old cultures (14 d postconfluent) were washed two times with phosphate-buffered saline, once with 0.1 M cacodylate buffer, and fixed *in situ* with 2 ml glutaraldehyde solution for 2 h at room temperature. Samples were postfixed in either 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, pH 7.3, or in ruthenium tetroxide (RuO_4), as described previously (Madison *et al*, 1987; Hou *et al*, 1991). RuO_4 postfixation allows visualization of the CLE in relation to the extracellular lamellae, while OsO_4, which does not capture images of the extracellular lamellae, instead provides unobscured images of the CLE. Ultrathin sections were examined both with and without further contrasting in a Zeiss 10 A electron microscope.

Hairless mouse SC sheets were prepared by incubation with 0.5% trypsin in phosphate-buffered saline (Grayson and Elias, 1982), cut into small pieces, and unbound lipids were extracted with absolute pyridine (Elias *et al*, 1977) or chloroform:methanol (Wertz *et al*, 1989a). To obtain CLE lipids, the residual SC samples (i.e., following lipid extractions) then were incubated with 1N NaOH in 90% methanol at 60°C for 1 h and extracted/fractionated, as described previously (Wertz *et al*, 1989a).

Individual lipid species were separated by high-performance thin layer chromatography (HPTLC), as described previously (Haratake *et al*, 1997). Lipid bands were visualized with cupric acetate-phosphoric acid reagent and charred at 160°C for 15 min, followed by quantitation by scanning densitometry (Camag) set to 650 nm *versus* authentic standards (hydroxy- and nonhydroxy-Cer, cholesterol, glycerol dipalmitate, glycerol tripalmitate, cholesterol palmitate) (Sigma, St. Louis, MO).

Structure of the CLE in normal stratum corneum Whereas chloroform:methanol-treated samples commonly demonstrated a distinct, electron-lucent gap, external to the CE demarcated by a single, electron-dense leaflet, pyridine-treated samples (12–16 h) revealed not only the electron-dense external leaflet, but also an additional, electron-dense domain contiguous to the CE (**Fig 1*D*** *versus* **1*A–C***). This electron-dense-lucent-dense CLE structure could be seen most readily at sites of desmosomal attachment (**Fig 1*B***, ***C***; d = desmosomes). We next compared lipid extracts and residual lipids following either pyridine or chloroform:methanol treatment. Pyridine not only extracted nearly identical levels of unbound SC lipids (i.e., 344 *vs* 347 μg per mg dry SC), but also left equivalent amounts of covalently bound Cer (i.e., 8.7 *vs* 6.2 μg per mg dry SC in pyridine *versus* chloroform:methanol-treated tissues, respectively; data from three combined samples). Thus, the CLE structures observed with pyridine cannot be explained by differences in either extracted or residual lipids.

CLE in HI Although typical LB were absent/nearly absent from the granular cell cytosol of all four HI patients, vesicular structures were present in abundance in the SG cytosol (not shown). Consistent with the absence of LB contents, all four patients displayed an absence of extracellular lamellar bilayers with RuO_4 postfixation. Yet, despite the absence of secreted LB contents, all four HI subjects demonstrated a CLE surrounding all corneocytes (**Fig 2*A–C***). Moreover, this structure was comparable with the CLE in normal epidermis, since pyridine-extracted, HI material again revealed an electron-dense-lucent-dense structure external to the corneocyte CE (**Fig 2*D***, *arrowheads*; cf. **Fig 1*A***, ***B***). Thus, a CLE forms around HI corneocytes, despite a virtual absence of LB contents.

CLE in differentiated CHK CHK, grown to 7–10 d postconfluence in 1.2 mM Ca^{++}, express markers of epidermal terminal differentiation, and a prominent CE, but few or no LB (Pillai *et al*, 1988). Moreover, no mature extracellular lamellar membrane structures were seen with RuO_4 postfixation (not shown). Yet, a continuous CLE occured around corneocytes, comparable in structure and dimensions with the CLE in intact SC (**Fig 3*A***, ***B***; cf. **Fig 1*A***, ***B***). Moreover, pyridine treatment again reveals an electron-dense-lucent-dense CLE around corneocytes (**Fig 3*C***). Finally, biochemical studies confirm that substantial, covalently bound ω-OH Cer is present in these cultures; i.e., base hydrolysis after exhaustive solvent extraction yielded 0.149 μg ± 0.018 (mean ± SEM; n = 6) of ω-OH Cer/mg dry tissue. Thus, a CLE occurs around cultured corneocytes in the virtual absence of detectable LB.

During epidermal terminal differentiation, the phospholipid-enriched plasma membrane abruptly disappears coincident with the appearance of the CE and an externally associated ω-OH Cer-enriched, membrane structure, the CLE (Wertz and Downing, 1987). Corneocytes are further surrounded by an extracellular lamellar membrane system, which derives primarily from the metabolized lipid contents of LB (Elias and Menon, 1991). GlucosylCer comprise a major portion of LB contents (Freinkel and Traczyk, 1985; Grayson *et al*, 1985), of which a substantial proportion are unique ω-hydroxylated N-acylated species (Wertz *et al*, 1984). Following apical transport and fusion with the plasma membrane, a portion of the LB GlcCer pool is deglucosylated, forming Cer, critical constituents of the extracellular lamellar membrane system (Holleran *et al*, 1993). It is generally assumed that a portion of the LB-derived, acylGlcCer contents is diverted toward CLE production, a process that would require de-

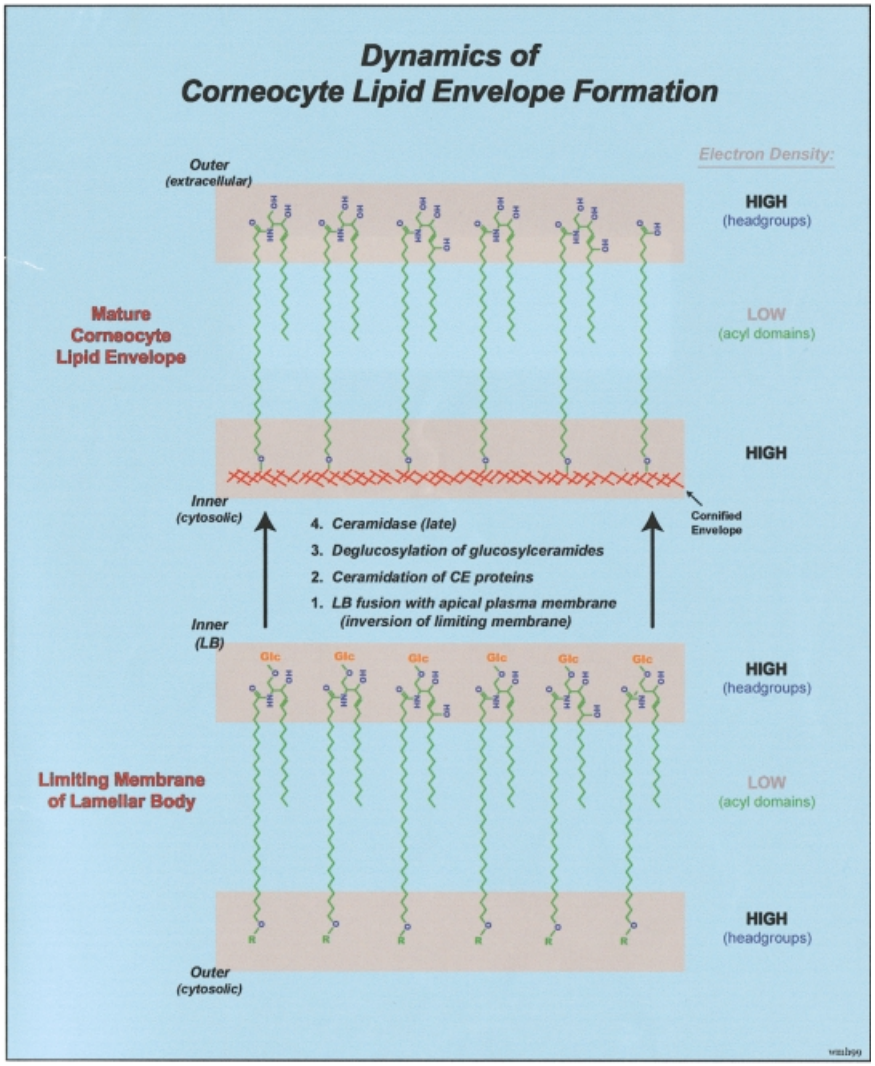

Figure 4. Membrane events during epidermal terminal differentiation. Schematic model of changes in membrane lipid composition/localization that occur during CLE formation, including: (1) fusion of the limiting membrane of the lamellar body with the plasma membrane of the apical stratum granulosum cell (*lower panel*); (2) covalent attachment of Cer and fatty acids to CE proteins (red) (*upper panel*); and (3) removal of glucose moieties (Glc) from GlcCer, resulting in the dense-lucent-dense structure of the CLE. Shaded areas (gray) indicate areas of high electron density; i.e., electron-rich headgroup domains (blue), with acyl domains (green) of low electron density.

esterification (Sando *et al*, 1997), de-glucosylation (Doering *et al*, 1999a), and covalent attachment to the external surface of the CE; i.e., ceramidation (**Fig 4**). The attachment of ω-OH Cer to the CE could be carried out by TG1 (Nemes *et al*, 1999), which is anchored into the plasma membrane at the level of the SG (Chakravarty and Rice, 1989; Phillips *et al*, 1993). In this location, TG1 is situated appropriately to catalyze the *in situ* attachment of ω-OH Cer to peptides in the outer portion of the CE (Nemes *et al*, 1999). Because a normal amount of bound Cer (i.e., as GlcCer) occurs in the SC of Gaucher epidermis (Doering *et al*, 1999a, 1999b; Uchida *et al*, 1999), ω-OH-dependent ceramidation proceeds with glucosylated Cer substrate(s). Finally, because the CLE contains not only ω-OH Cer, but also ω-OH fatty acids (Wertz and Downing, 1986, 1987), some *in situ* degradation of ω-OH Cer by ceramidase must occur following deglucosylation (**Fig 4**).

To address still unresolved questions about the origin of the CLE, we utilized an alternate tissue preparation method, based upon pyridine treatment (Elias *et al*, 1977). Using the polar solvent, pyridine, we confirmed that the CLE *in situ* possesses a modified bilayer structure (Elias *et al*, 1977). Visualization of the inner leaflet of this bilayer structure (**Fig 4**) becomes possible because pyridine either focally solubilizes portions of the outer CE, or reveals sites of incomplete protein ceramidation/transesterification. Using the pyridine method, we assessed whether LB contents are the obligate source of CLE precursors in two models where replete LB occur rarely. Indeed, a CLE is present both in HI and in postconfluent CHK, suggesting that processing of ω-OH Cer through an intact LB secretory pathway is not a prerequisite for CLE formation (Madison *et al*, 1987). The CLE could originate entirely or in part from the limiting membrane of LB, rather than its internal contents (**Fig 4**). If present in HI or CHK, where LB contents are absent, the LB and its limiting membrane would not be recognizable by electron microscopy. Theoretically, ω-OH glucosylCer also could be delivered either constitutively; e.g., from the Golgi apparatus to the cell surface, or as a result of sphingomyelin (SM) hydrolysis within the plasma membrane. Pertinently, the plasma membrane of granular cells is highly enriched in SM (Gray *et al*, 1978), and with the degradation of the plasma membrane that accompanies terminal differentiation, its entire SM pool potentially becomes available for CLE formation. Normal human and hairless mouse epidermis, however, do not generate ω-OH-N-acyl sphingomyelin (Uchida *et al*, 2000), effectively excluding plasma membrane-derived SM as a source of the ω-OH Cer in the CLE. In summary, these studies show that the CLE originates from sources other than LB contents, suggesting instead that an alternate mechanism delivers ω-OH Cer not only in HI and CHK, but also in normal SC.

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