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. 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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REFERENCES

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) (Matatly, 1976; Steven and Steinitz, 1994). CE formation begins with the deposition initially of involucrin (Steinert and Marek, 1997), followed by the subjacent, catalytic cross-linking of several other peptide precursors (Steinert and Marek, 1995, 1997), by the keratinocyte-specific, Ca2+ dependent enzyme, transglutaminase 1 (TG1) (Parenteau et al, 1986; Michel and Démarèche, 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; Matatly, 1976; Elias et al, 1977). This structure is enriched in ω-hydroxyacylceramides (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; Marek and Steinitz, 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 acylglycosylceramide with an ω-esterified N-acyl group (Wertz et al, 1984; Freinkel and Traczyk, 1985; Grayson et al, 1985); and (b) the composition of acylglycosylCer in LB is comparable with ω-OH Cer in the CLE. Accordingly, it has been proposed that secretion of acylglycosylCer is followed by multistep extracellular processing, including: (1) deglucosylation by β-glycerecerbrosidase (Holleran et al, 1992, 1993; Takagi et al, 1999); (2) ω-esterase/ω-lipase-mediated hydrolysis of acylCer to ω-OH Cer (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 coexist in Ca2+-containing media (Pilla 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-
dense images of the CLE in which a distinct, electron-dense inner leaflet can be seen contiguous to the CE (Elías 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 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, arrow). Following pyridine treatment of aldehyde/osmium tetroxide fixed samples, the dense-lucent-dense structure of the CLE can be seen (D, arrow). (A–C) Ruthenium tetroxide post-fixation. Scale bar: 0.2 μm.
phosphoric acid reagent and charred at 160°C for 15 min, followed by quantitation by scanning densitometry (Camag) set to 630 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 1D versus 1A–C). This electron-dense-lucent—dense CLE structure could be seen most readily at sites of desmosomal attachment (Fig 1B, 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 RuO₄ postfixation. Yet, despite the absence of secreted LB contents, all four HI subjects demonstrated a CLE surrounding all corneocytes (Fig 2A–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 2D, arrowheads; cf. Fig 1A, 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 RuO₄ postfixation (not shown). Yet, a continuous CLE occurred around corneocytes, comparable in structure and dimensions with the CLE in intact SC (Fig 3A, B; cf. Fig 1A, B). Moreover, pyridine treatment again reveals an electron-dense—lucent—dense CLE around corneocytes (Fig 3C). 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 ± 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-acetylated species (Wertz et al., 1984). Following apical transport and fusion with the plasma membrane, a portion of the LB GlCer pool is deglucosylated, forming Cer, critical constituents of the extracellular lamellar membrane system (Hollera et al., 1993). It is generally assumed that a portion of the LB-derived, acylCer contents is diverted toward CLE production, a process that would require de-
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 \( \omega \)-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 \( \omega \)-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), \( \omega \)-OH–dependent ceramidation proceeds with glucosylated Cer substrate(s). Finally, because the CLE contains not only \( \omega \)-OH Cer, but also \( \omega \)-OH fatty acids (Wertz and Downing, 1986, 1987), some in situ degradation of \( \omega \)-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 locally solubilizes portions of the outer CE, or reveals sites of incomplete protein ceramidization/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 \( \omega \)-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, \( \omega \)-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) hydrolisis 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 \( \omega \)-OH-N-acyl sphingomyelin (Uchida et al., 2000), effectively excluding plasma membrane-derived SM as a source of the \( \omega \)-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 \( \omega \)-OH Cer not only in HI and CHK, but also in normal SC.

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REFERENCES


Steinitz PM, Marek VN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isopeptide cross-linked components of the human epidermal cornified cell envelope. J Biol Chem 270:17702–17711, 1995


