

# Cellular Changes that Accompany Shedding of Human Corneocytes

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Corneocyte desquamation has been ascribed to the following: 1) proteolytic degradation of corneodesmosomes (CDs); 2) disorganization of extracellular lamellar bilayers; and/or 3) “swell-shrinkage-slough” from hydration/dehydration. To address the cellular basis for normal exfoliation, we compared changes in lamellar bilayer architecture and CD structure in D-Squame strips from the first versus fifth stripping (“outer” vs. “mid”-stratum corneum (SC), respectively) from nine normal adult forearms. Strippings were either processed for standard electron microscopy (EM) or for ruthenium-, or osmium-tetroxide vapor fixation, followed by immediate epoxy embedment, an artifact-free protocol, which, to our knowledge, is previously unreported. CDs are largely intact in the mid-SC, but replaced by electron-dense (hydrophilic) clefts (lacunae) that expand laterally, splitting lamellar arrays in the outer SC. Some undegraded desmoglein 1/desmocollin 1 redistribute uniformly into corneocyte envelopes (CEs) in the outer SC (shown by proteomics, Z-stack confocal imaging, and immunoEM). CEs then thicken, likely facilitating exfoliation by increasing corneocyte rigidity. In vapor-fixed images, hydration only altered the volume of the extracellular compartment, expanding lacunae, further separating membrane arrays. During dehydration, air replaced water, maintaining the expanded extracellular compartment. Hydration also provoked degradation of membranes by activating contiguous acidic ceramidase activity. Together, these studies identify several parallel mechanisms that orchestrate exfoliation from the surface of normal human skin.

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## INTRODUCTION

In normal human skin, the lower layers of the stratum corneum (SC; stratum compactum) are highly cohesive, a characteristic important for permeability barrier function, antimicrobial defense, and SC integrity (resistance to shear

forces) (Elias and Choi, 2005; Cork *et al.*, 2006; Elias, 2007). SC cohesion can be partly attributed to an epidermis-unique, junctional structure, the corneodesmosome (CD), which replaces desmosomes in the SC. As corneocytes migrate apically, CDs are degraded by extracellular kallikreins (serine proteases), as well as by cysteine and aspartate proteases (Brattsand *et al.*, 2005; Ovaere *et al.*, 2009; Zeeuwen *et al.*, 2009; Rawlings, 2010), in a pH-dependent manner (Hachem *et al.*, 2003, 2005; Gunathilake *et al.*, 2009), yielding the less-compact stratum disjunctum. The putative biochemical correlates of this sequence include the initial proteolysis of an SC-unique protein, corneodesmosin (Lundstrom *et al.*, 1994; Jonca *et al.*, 2011), followed by the sequential proteolysis of other CD constituent proteins, including two e-cadherins, desmoglein 1 (DSG1) and desmocollin 1 (DSC1; Lundstrom and Egelrud, 1990; Caubet *et al.*, 2004). Yet, morphological, biochemical, and immunofluorescence studies suggest that CDs, with the possible exception of a subgroup of peripheral CDs (Chapman and Walsh, 1990; Haftek *et al.*, 2006; Ishida-Yamamoto *et al.*, 2011), are largely degraded before corneocytes arrive in the outer SC (Rawlings *et al.*, 1994). Which further distal alterations in CDs or other cohesion factors (see below) accomplish normal exfoliation is one of the key questions addressed in this study.

Although alterations in CDs clearly represent a key, but relatively early, desquamatory mechanism, disorganization,

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Abbreviations: CC, cellular compartment; CD, corneodesmosome; CDase, ceramidase isoform; CE, cornified envelope; CLE, corneocyte lipid envelope; DSC1, desmocollin 1; DSG1, desmoglein 1; ECC, extracellular compartment; EM, electron microscopy; Os-I, osmium tetroxide immersion; Os-V, osmium tetroxide vapor postfixation; Ru-I, ruthenium tetroxide immersion; Ru-V, ruthenium vapor tetroxide postfixation; SC, stratum corneum; SSS, swell-shrinkage-slough

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or degradation of lamellar bilayers could also facilitate desquamation. Although lamellar bilayers reportedly persist unaltered within the outer SC (Madison *et al.*, 1987), inherited disorders of lipid metabolism that are associated with aberrant desquamation (ichthyosis) exhibit altered bilayer composition and structure (Williams and Elias, 1987; Elias *et al.*, 2008, 2010). Changes in lipid composition across the SC, particularly in cholesterol sulfate content, have been proposed to regulate desquamation (Epstein *et al.*, 1981; Long *et al.*, 1985; Rehfeld *et al.*, 1988). Moreover, mild organic solvent and/or detergent treatment of SC generates individual corneocytes (Chapman *et al.*, 1991; Shukuwa and Kligman, 1997). Because two ceramidase isoforms (Cdase), acidic Cdase and alkaline Cdase, are expressed in the SC (Wertz and Downing, 1990; Houben *et al.*, 2006), either or both could account for the substantial amounts of sphingosine and  $\omega$ -hydroxy fatty acids that are present in the SC (Uchida and Holleran, 2008). Whether an attack by ceramidases on lamellar bilayers could degrade lamellar membranes, further facilitating exfoliation, is a second question addressed in this study.

Finally, it has been proposed (but not yet shown) that abrupt changes in surface area:volume ratios, which follow hydration (e.g., with bathing), could induce volume shifts in SC subcellular compartments, which are sufficient to provoke exfoliation by physical forces ("swell-shrinkage-slough," SSS; Williams, 1992; Williams and Elias, 1993; Elias *et al.*, 2010). Whether hydration induces changes in SC subcellular compartments that could contribute to shedding is a third issue addressed in this study.

Yet, it has been difficult, if not impossible, to address the mechanisms that lead to normal corneocyte detachment, because the SC is unusually susceptible to artifacts that are provoked during fixation and embedding for light and electron microscopy (EM). One such distortion is lipid extraction during tissue processing, which yields the "normal basket-weave" appearance of SC on light microscopy, images that relegated the SC to functional irrelevance for decades. In frozen sections, the SC instead was subsequently revealed to be exquisitely organized into vertical, interlocking columns of polyhedral cells (Christophers and Kligman, 1964; Menton and Eisen, 1971; Mackenzie, 1975), with SC membrane domains enriched in hydrophobic lipids (Elias and Friend, 1975; Elias *et al.*, 1977, 1983; Grayson and Elias, 1982), which are organized into extracellular lamellar bilayers that extend throughout the SC interstices (Hou *et al.*, 1991). Ruthenium tetroxide (RuO<sub>4</sub>) immersion (Ru-I) postfixation, developed to examine these domains in epoxy-embedded tissue sections (Madison *et al.*, 1987; Hou *et al.*, 1991), not only revealed that extracellular domains replete with lamellar bilayers (Madison *et al.*, 1987), but also that Ru-I postfixation preserves lamellar bilayer dimensions with fidelity (Hou *et al.*, 1991). Yet, Ru-I and osmium tetroxide immersion (Os-I) postfixation are routinely preceded by aldehyde pre-fixation, which also produces draconian, hydration-induced alterations in the volume relationships of the cellular (CCs) and extracellular (ECCs) compartments. Cognizant of these drawbacks, we developed an OsO<sub>4</sub> and

RuO<sub>4</sub> vapor-phase fixation protocol (Os-V and Ru-V, respectively), followed by immediate epoxy embedding, which obviates the need for both pre-fixation, and immersion in solvents, nullifying the dehydration and extraction artifacts that accompany standard tissue processing. Our results show that exfoliation from the skin surface is orchestrated by multiple, often subtle, processes that operate in parallel within the outer SC.

## RESULTS

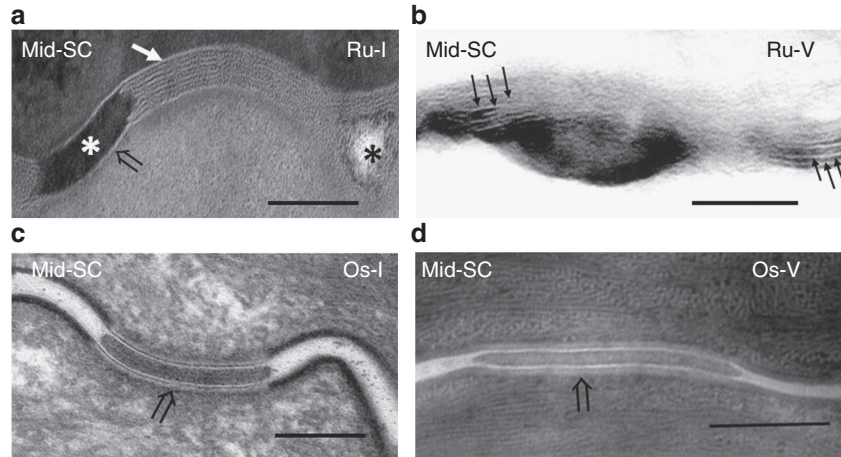
### Comparison of SC structure in immersion versus vapor-fixation protocols

We initially assessed the efficacy and potential utility of vapor fixation by comparing the ultrastructure of biopsies and/or strippings from nine normal human subjects (Supplementary Table S1 online), which were processed in parallel by standard immersion methods and the vapor-fixation protocols. Ru-I revealed multilayered lamellar bilayers that were also well preserved and readily visualized in Ru-V-fixed samples (Figure 1b vs. a, arrows). Although there were frequent extraction artifacts (empty, electron-lucent spaces) in Ru-I samples, such extraction artifacts were not observed in Ru-V-fixed samples (Figure 1b vs. a, asterisks), suggesting that Ru-V fixation does not disturb SC volume relationships. Yet, owing to its strong oxidant characteristics, neither Ru-I nor Ru-V reliably preserved cornified envelopes (CE) or CDs (Figure 1). Thus, Ru-V fixation can be used to visualize the extracellular lamellar bilayers, and both Ru-V and Os-V preserve volume relationships in the SC (Supplementary Table S2 online).

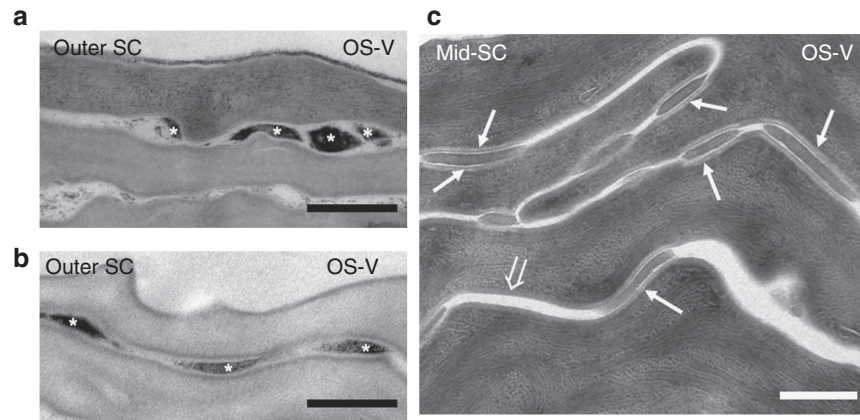
Immersion fixation with OsO<sub>4</sub> (Os-I), particularly if preceded by pyridine treatment, provides pristine images of CEs, CDs, and the corneocyte lipid envelope (CLE; Figure 1c; Elias *et al.*, 1979, 2010; Behne *et al.*, 2000), but lamellar bilayers cannot be visualized. As with Ru-I, electron-lucent loci, which likely represent extraction artifacts, occur throughout the extracellular matrix in Os-I-treated samples (universally shown in prior published studies) but not in Os-V-treated samples (Figure 2c). Although Os-V postfixation, similar to Os-I, preserves the CLE, lamellar bilayers again could not be visualized (Figure 1d). Both CDs and CEs appeared less electron-dense than in parallel Os-I postfixed tissues (Figure 1d vs. c). Yet, similar to Ru-V, Os-V fixation appears to preserve ECC dimensions (Figure 2c, open arrows). Thus, either Ru-V or Os-V could be deployed for delineation of volume relationships in the SC (Supplementary Table S2 online).

### Volume of extracellular compartment in normal and hydrated SC

The unique susceptibility of the SC to dehydration and extraction artifacts during immersion fixation, postfixation, and embedding has impeded prior attempts to assess changes in the volume of the CC and ECC. Hence, we next deployed Os-V and Ru-V fixation to visualize and quantitate changes in the ECC during transition from the mid- to the outer SC in multiple ( $\geq 40$ ), randomly selected micrographs from at least 10 micrographs of the nine normal subjects. Under normal



**Figure 1. Preservation of stratum corneum structures by vapor fixation.** Parallel tape strippings and full-thickness skin biopsies were treated with either ruthenium tetroxide, or osmium tetroxide vapor fixation (Ru-V and Os-V; **b** and **d**, arrows), or ruthenium tetroxide or osmium tetroxide immersion post-fixation (Ru-I and Os-I, respectively; **a** and **c**, arrows). Arrows indicate lamellar bilayers, open arrows indicate corneodesmosomes (CD), and asterisk indicates extraction artifact. Scale bar = 100 nm.



**Figure 2. Corneodesmosomes (CDs) are not present in the outer stratum corneum (SC).** Tape strippings from the outer SC (**a** and **b**) and mid-SC (**c**). CDs are still present in the mid-SC (**c**, arrows), but transform into lacunae (**a** and **b**, asterisks) in the outer SC. Scale bar, **a-c** = 200 nm.

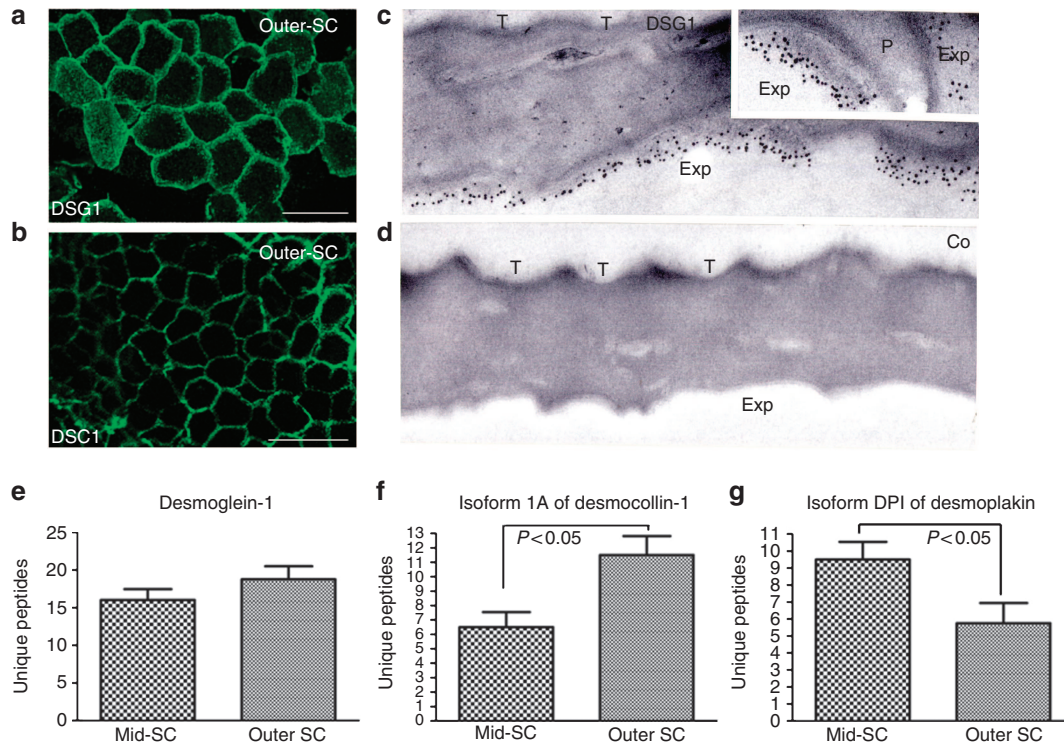
(basal) conditions, the volume contribution of the ECC was ~12% in the mid-SC, increasing to ~17% in the outer SC, an increase that did not achieve statistical significance (Supplementary Table S3 online;  $P < 0.1$ ).

SSS of corneocytes with hydration, followed by drying, could provoke shedding of individual cells, because abrupt shifts in water content could stress cell-to-cell contacts (Williams, 1992; Williams and Elias, 1993; Elias *et al.*, 2010). Therefore, we next examined the SSS hypothesis by quantitative analysis of Os-V samples (simultaneously visualized with Ru-V), before, immediately after, and 45 minutes and 2 hours after hydration. Hydration expanded the ECC in both the mid-SC (from  $11.8 \pm 0.9\%$  before immersion to  $20.2 \pm 1.4\%$  immediately after immersion (Supplementary Fig. S1a online and Supplementary Table S3 online;  $P < 0.001$ )), and in the outer SC (from  $17.4 \pm 1.3\%$  before immersion to  $20.0 \pm 1.6\%$  immediately after immersion (Supplementary Fig. S1a online)). Yet, ECC volume remained

unchanged at 45 minutes and 2 hours post hydration, because air appeared to replace water as the latter evaporated from the ECC (Figure 5, asterisks). Changes in the volume of individual corneocytes were assessed quantitatively as corneocyte area/corneocyte length (see Materials and Methods). Notably, the volume contribution of the CC did not change before or after hydration in either the mid-SC or outer SC (Supplementary Fig. S1b online). Together, these results show, first, that SSS expands the ECC in both the mid-SC and outer SC, maintaining hydration-induced separation of adjacent corneocytes, and, second, that the CC does not change with hydration of either the mid-SC or outer SC.

#### Distal consequences of the CD degradation

To assess the structural and biochemical alterations that accompany normal shedding, we next assessed changes in CD and CE structure in the mid-SC and outer SC, in samples exposed to Os-I postfixation, which optimally preserves both



**Figure 3. Despite prior loss of corneodesmosomes (CDs), desmoglein 1 (DSG1) and desmocollin 1 (DSC1) persist in the outer stratum corneum (SC), and are distributed uniformly in the cell periphery.** Frozen sections (6  $\mu\text{m}$ ) immunostained as in Materials and Methods (Supplementary Material online). (a, b) Confocal immunofluorescence images show persistence of DSG1 and DSC1 in the outer SC peripheral membranes. Z-stack of 11 images over 2.5  $\mu\text{m}$  show DSG1 distributed uniformly along basal membranes (not shown). (c) Immunoelectron microscopy (IEM) for DSG1 shows uniform distribution of immunogold labeling of cornified envelopes (CEs) along basal and peripheral membranes (c, insert) of corneocytes. Co, control; Exp, exposed; P, periphery; T, tape. (d) Negative control for IEM with omission of primary antibody. (e-g) Proteomic analysis of three CD proteins (DSG1, DSC1, and desmoplakin) in the mid- and outer SC. Scale bar: a = 50  $\mu\text{m}$ , b = 80  $\mu\text{m}$ , c (and insert), d = 100 nm.

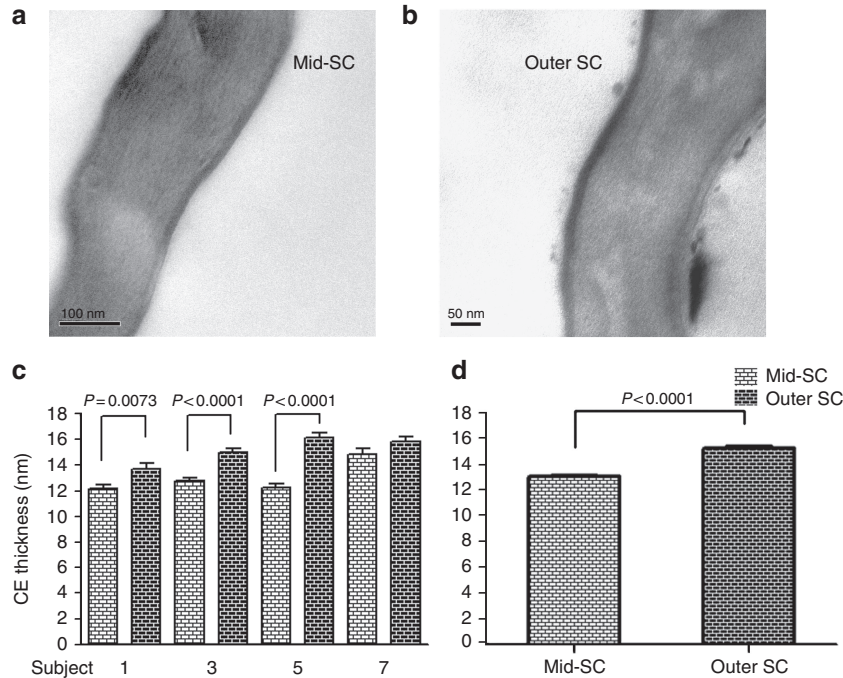
CEs and CDs (Supplementary Table S1 online). As noted above, although CDs still appeared largely intact in the mid-SC, all CDs lost their characteristic plug-like structure in the outer SC. Despite the widely accepted view that a subgroup of CS persists up to the skin surface (Hafték *et al.*, 2006; Naoe *et al.*, 2010; Ishida-Yamamoto *et al.*, 2011), we found no remaining intact CDs in the outer SC of any of our seven human subjects. Instead, CDs appeared to be replaced by lacunar (lenticular) dilatations filled with amorphous, electron-dense material (Figures 1 and 2). Because the spacing between these dilatations corresponds to intervals between adjacent CDs, these dilatations likely represent sites of prior CD degradation (Menon and Elias, 1997; Hafték *et al.*, 2006). These lacunae then expand in the outer SC, splitting lamellar bilayers into single or double lamellar arrays (see below).

Despite the apparent loss of CD structures during progression from the mid-SC to the outer SC, confocal immunofluorescence images show that both DSG1 and DSC1 continue to exhibit both a granular “dot-like” distribution and uniform CE labeling in the outer SC (Figure 3a and b). Although it has been proposed that these proteins concentrate in residual (intact) CDs in the cell periphery (Hafték *et al.*, 2006; Naoe *et al.*, 2010; Ishida-Yamamoto *et al.*, 2011), the uniform redistribution of much of these proteins to CEs was confirmed in Z-stack images of both DSG1- and DSC1-

labeled samples (Figure 3a), and by immunoEM (Figure 3c and d (negative controls showed no immunolabeling)). Moreover, DSG1 immunogold labeling revealed diffuse labeling of CEs; i.e., there was no increase at the cell periphery that would indicate concentration of this protein in “peripheral” CDs (Figure 3c, insert). Thus, a decrease in corneocyte vertical dimensions in the outer SC, as well as retention of some undegraded DSG1/DSC1 in CD-derived lacunae, likely account for the previously described “peripheral localization” of these proteins.

Proteomic analysis of samples also suggests that DSG1 and DSC1 are largely redistributed from CDs to CEs. Although the unique peptide isoform of desmoplakin declined in the outer SC compared with the mid-SC (Figure 3e), the DSG1 and DSC1 (isoform 1A) content of CEs increased significantly in the outer SC (Figure 3c and d), whereas the content of other CE proteins (e.g., loricrin, keratins 1/10, small proline-rich proteins, transglutaminase 5, and DSC3) did not change in samples from the mid-SC to the outer SC. These results, coupled with the immunofluorescent and immunoEM studies, suggest that some DSG1/DSC1 remains in CD-derived lacunae, but some is released intact from CDs and then incorporated into CEs.

If DSG1 and DSC1 are (re)incorporated into CEs, one would expect that CE dimensions would expand accordingly.



**Figure 4. Increased thickness of cornified envelopes (CEs) during transit from the mid-stratum corneum (SC) to the outer SC.** Os-V fixation of the mid-SC and outer SC from four subjects, with quantification of CE width in perpendicular (cross) sections of randomized, coded micrographs, as described in Materials and Methods (Supplementary Material online). (c) Individual samples, (d) pooled data. Scale bar, **a** and **b** = 50 nm.

Hence, we next measured changes in CE dimensions in perpendicular sections during the transition from the mid-SC to the outer SC. Consistent with the redistribution of a pool of DSG1 and DSC1 to CEs, and the ongoing cross-linking of CE precursors that continues across the entire SC (Michel *et al.*, 1988; Kalinin *et al.*, 2002; Eckert *et al.*, 2005a, 2005b), CE thickness increased significantly (by ~15%) as corneocytes transitioned from the mid-SC to the outer SC (Figure 4a and b). Together, these results suggest that, contrary to current dogma, all CDs are degraded before arrival in the outer SC, but that some DSG1/DSC1 remains in lacunar domains, whereas some is incorporated into CEs.

#### SSS provokes further changes in the lamellar bilayer structure

To assess changes in lamellar bilayer organization and substructure during transition from the mid-SC to the outer SC, we used Ru-V fixation. Under basal (non-hydrated) conditions, lamellar bilayers remain largely intact in the mid-SC (Figure 5c; cf, Figure 1b). As CD-derived lacunae expand laterally during transition to the outer SC (*vide supra*), these non-lamellar domains split lamellar multilayers into single or double lamellar arrays (Figure 5b).

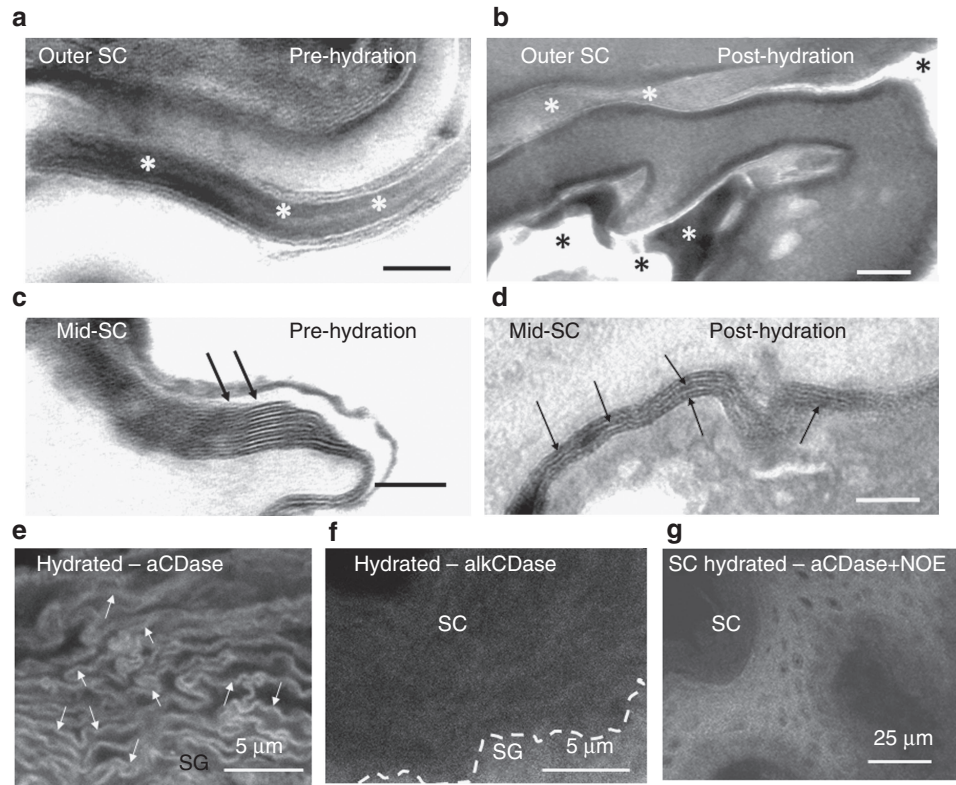
We next assessed whether SSS provokes further alterations in lamellar bilayer substructure. Whereas lamellar bilayers in the mid-SC appeared largely unaltered before immersion, small punctate discontinuities appeared in lamellar bilayers immediately after immersion, and these alterations persisted 45 minutes and 2 hours post immersion (Figure 5d, 2 hours post hydration). Thus, hydration not only disorganizes (splits)

lamellar bilayer arrays, but it also appears to provoke degradation of individual lamellae.

Hydration is required to activate various types of hydrolytic enzyme activity, including two CDases present in the SC (Wertz and Downing, 1990; Houben *et al.*, 2006). Ceramidases are the only hydrolases that are known to partially degrade ceramides into sphingoid bases and fatty acids (Uchida and Holleran, 2008). To evaluate whether activation of either CDase could account for the hydration-induced lamellar bilayer degradation, we developed a zymographic assay (which, to our knowledge, is previously unreported) that allows visualization and localization of ceramidase activity under either acidic (pH 4.5) or neutral-to-alkaline (pH 7.8) conditions (Figure 5e–g). Both acidic and alkaline activities peak in the stratum granulosum, and persist into the SC. Yet, although alkaline activity declines in the outer SC (Figure 5f), acidic activity not only persists throughout the SC, but also concentrates at the periphery of the corneocytes (Figure 5e). Sections pretreated with the pan-ceramidase inhibitor, N-oleoylethanolamine, greatly reduced both enzyme activities (Figure 5g), verifying that enzyme activity in SC membrane domains can be attributed to acidic ceramidase. Together, these results show that hydration accelerates lamellar bilayer disorganization and provokes bilayer degradation by acidic ceramidase in the outer SC.

#### DISCUSSION

We deploy here an artifact-free vapor-fixation protocol (that, to our knowledge, is previously unreported) to address the basis for shedding from the surface of normal human SC.



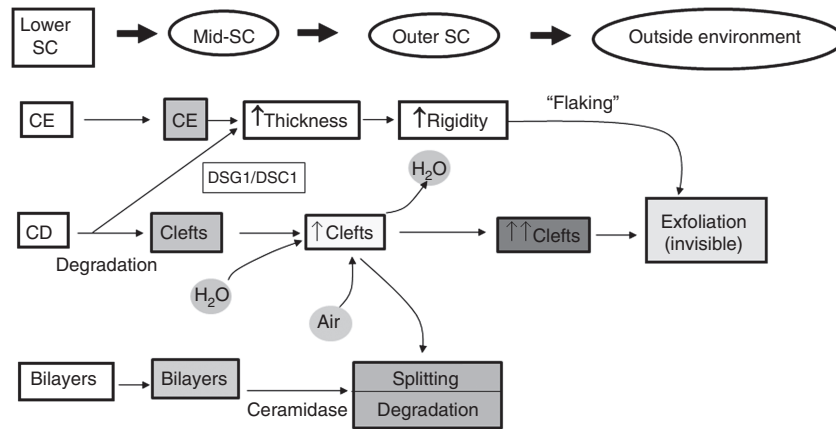
**Figure 5. Splitting and degradation of lamellar membranes between mid-stratum corneum (SC) and outer SC.** (a and b) Cleft formation (lacunae) and horizontal membrane splitting (a and b, asterisks); further expansion of clefts, and replacement of water by air, 2 hours after water immersion (b, asterisks). (c and d) Intact lamellar bilayers in the mid-SC before immersion (c, black arrows), with appearance of punctate membrane discontinuities that persist at 2 hours after immersion (d, red arrows). (e–g) Confocal microscopy of *in situ* zymography for localization of alkaline and acid ceramidase activity. (e) Membrane localization of acidic ceramidase (aCDase; pH 4.5) in the outer SC (arrows). (f) Low levels of alkaline ceramidase (alkCDase; pH 7.6) activity in the SC. (g) Reduced enzyme activity in the SC after preincubations with a pan-ceramidase inhibitor, N-oleoylethanolamine (NOE). (a–d), Ru-V fixation. Scale bar: a–d = 0.25  $\mu\text{m}$ ; e and f = 5  $\mu\text{m}$ ; g = 25  $\mu\text{m}$ .

Standard methods of fixation and embedding produce dehydration and extraction artifacts that shrink and detach corneocytes, even though Ru-I postfixation preserves lamellar bilayer arrays (Hou *et al.*, 1991; Swartzendruber *et al.*, 1995). Although we show here that vapor fixation preserves extracellular dimensions and SC structures, without inducing artifactual cell separation, Ru-V, similar to Ru-I, is highly reactive, oxidizing protein-enriched structures, such as CDs and CEs, whereas in contrast Os-I (but not Os-V) postfixation appears to preserve these structures (Supplementary Table S2 online). We deployed these vapor-phase protocols (which, to our knowledge, are previously unreported) in conjunction with ultrastructural, immunofluorescent, proteomic, and zymographic methods, to evaluate the basis for normal exfoliation, comparing tape strippings from the outermost and mid-SC. As the first stripping removed a variable number of cell layers, we concentrated on changes in the first versus fifth stripping.

Although corneocytes become less cohesive as they progress through the SC (e.g., King *et al.*, 1979), how individual corneocytes desquamate from the cell surface is less clear. Serine proteases, particularly kallikreins 5 and 7, are known effectors of desquamation (Egelrud, 2000; Brattsand

*et al.*, 2005; Rawlings, 2010). Accordingly, desquamation accelerates at the higher-than-normal pH of inflammatory dermatoses, threatening the cohesion of SC (Hachem *et al.*, 2003, 2005, 2006a; Cork *et al.*, 2006; Haftek *et al.*, 2006; Voegeli *et al.*, 2009). Conversely, desquamation is delayed in parallel with retained CDs and restricted enzyme activity, in x-linked ichthyosis (Elias *et al.*, 2004), where the pH of SC is lower than normal (Ohman and Vahlquist, 1998). Our observations confirm that CD degradation is an important and likely required antecedent for desquamation, but apparently, certain still-later events provoke distal shedding, because all CDs are degraded before corneocytes reach the outer SC. Importantly, we did not find a persistent pool of “peripheral CD” in the outer SC, as described by others (Chapman and Walsh, 1990; Naoe *et al.*, 2010; Ishida-Yamamoto *et al.*, 2011). What has been called CDs instead comprise lacunar domains that appear to occupy sites formerly occupied by CDs (Menon and Elias, 1997; Haftek *et al.*, 2006). These lacunae then expand laterally, splitting lamellar bilayers, a process that we show here accelerates following hydration.

Such expansion of lacunar domains, separating lamellar bilayers, likely also facilitates distal shedding (Figure 6). Thus,



**Figure 6. Basis for normal exfoliation: summary of observations.** CD, corneodesmosome; CE, cornified envelope; DSC1, desmocollin 1; DSG1, desmoglein 1; SC, stratum corneum.

the ECC of the SC expands with hydration and contracts as it loses water. Although the absolute volume of the CC does not change with hydration, its relative volume declines as the ECC correspondingly expands. Moreover, as water evaporates following SSS, it appears to be replaced by air, maintaining the expanded volume of the ECC in the outer SC. The expansile characteristics of SC extracellular domains have been noted previously (Nemanic and Elias, 1980; Rawlings, 2010). Together, this sequence likely sustains gaps between adjacent corneocytes, thereby facilitating intercellular detachment (Figure 6).

The observation that the volume of the CC does not change with hydration refutes a commonly held view that it is the cellular (rather than the extracellular) compartment that changes with hydration/dehydration (reviewed in Rawlings *et al.*, 1994). The inability of corneocytes in the mid- and outer SC to retain water could be because of the presence of ceramidase activities in the SC (Wertz and Downing, 1990; Houben *et al.*, 2006), which attack the  $\omega$ -hydroxyceramide monolayer that forms the CLE (Downing, 1992; Uchida and Holleran, 2008). Although corneocytes in the lower SC display a replete CLE that likely retains hygroscopic substances, ceramidase-mediated hydrolysis of the CLE should render corneocytes unable to retain hygroscopic molecules, allowing water to move in and out of corneocytes. Thus, the ECC becomes the expansile compartment in the mid-SC to outer SC. Although these results validate the SSS hypothesis, the underlying processes do not operate by “stretch and strain” because of the corneocyte swelling and shrinkage, but rather by physical separation of adjacent corneocytes, coupled with disorganization and degradation (see below) of lamellar bilayers.

CD degradation likely contributes to shedding by another previously undescribed mechanism. Our immunofluorescent, immunoEM, and proteomic results suggest that much of the proteolytic attack on CD may be directed at proteins, other than DSG1 and DSC1, such as corneodesmosin and desmoplakin, and that their degradation likely accounts for CD degradation. In contrast to *in vitro* studies (Caubet *et al.*,

2004), little or no DSG1 and DSC1 appear to be degraded *in vivo*. Instead, the proteomic, immunofluorescent, immunoEM, and ultrastructural data suggest that proteolysis of CDs releases abundant, full-length protein that is then both retained in lacunar domains and partially redistributed uniformly within CEs of the outer SC. (It is worth noting that detachment of full-length proteins from the CE is not unprecedented, as filaggrin also is released intact from the CE, before its proteolytic degradation (Scott and Harding, 1986)). Comparable immunofluorescent images have been interpreted by others to indicate that DSG1 and DSC1 are retained within a pool of residual, peripheral CDs that persist in the outer SC (Naoe *et al.*, 2010; Ishida-Yamamoto *et al.*, 2011). Yet, we did not observe any residual CDs in the outer SC in either immersion or vapor-fixed samples. Moreover, our Z-stack images show that the apparent peripheral localization of CD constituent proteins likely represents an optical illusion due to the progressive flattening of corneocytes. Furthermore, the subsequent (re)incorporation of DSG1, DSC1, and likely other CE precursor peptides into the CE also accounts, at least in part, for the uniform increase in thickness of CEs in the outer SC, which is consistent with the known persistence of CE cross-linking activity in the SC (Michel *et al.*, 1988; Koch *et al.*, 2000; Kalinin *et al.*, 2002; Segre, 2003; Schmuth *et al.*, 2004; Candi *et al.*, 2005; Eckert *et al.*, 2005a, 2005b). Pertinently, both DSG1 and DSC1 possess potential TG1 binding sites (Haftek *et al.*, 1991; Robinson *et al.*, 1997). CE thickening would not only increase mechanical resistance, but likely also increase envelope rigidity, thereby facilitating individual cell separation (“flaking”) immediately before shedding of individual corneocytes (Figure 6).

We also observed changes in extracellular lamellar bilayer organization and substructure that likely contribute to exfoliation. Electron spin resonance (Yagi *et al.*, 2007), Fourier transform infrared (Berthaud and Boncheva, 2011), and scanning EM (Lopez *et al.*, 2000) show that lamellar bilayer organization changes in the outer SC, attributed (incorrectly) to the imbibement of sebaceous lipids. We

observed here instead splitting of multilayered stacks of lamellar bilayers into single- or double-membrane arrays, which likely account for these previously observed changes. Disorganization (splitting) occurs even under basal (non-hydrated) conditions, but it is further accentuated by SSS. Yet, hydration provoked further alterations in the lamellar bilayer structure; i.e., punctate discontinuities in individual membranes consistent with membrane degradation, which could further facilitate shedding (Figure 6). Indubitably, enzymatic activity increases with hydration, because hydrolases require an aqueous environment for optimal activity. We show here that acidic ceramidase co-localizes to sites where lamellar bilayers show focal degradation. Notably, activities of another key family of hydrolases, i.e., serine proteases, declines in the outer SC (Hachem *et al.*, 2006b, 2010; Gunathilake *et al.*, 2009).

## MATERIALS AND METHODS

### Immersion (standard) and vapor postfixation

For vapor fixation, portions of D-Squames were suspended immediately after stripping above either 0.5% aqueous RuO<sub>4</sub> (Polysciences no. 18253) or 4% aqueous OsO<sub>4</sub> (EMS no. 19190) in Eppendorf tubes under an exhaust hood at room temperature, with no light exposure, with care being taken to avoid any contact with the fluid phase. Although Ru-V postfixation was terminated after 2 hours, Os-V exposure was maintained for 16 hours. D-Squame pieces were then removed from the tubes, dried under the exhaust hood for 15 minutes to remove residual RuO<sub>4</sub>/OsO<sub>4</sub> vapor, and then cut into still smaller pieces (≤0.5 mm diameter), followed by immersion in a freshly prepared epoxy mixture for 1 hour. Individual pieces were then stacked together and oriented horizontally in rubber molds. Parallel portions of each stripping were processed by standard immersion methods, including prefixation in glutaraldehyde, postfixation in OsO<sub>4</sub> or RuO<sub>4</sub>, followed by solvent dehydration and epoxy embedding (Hou *et al.*, 1991). All EM samples were polymerized at 80 °C overnight, before ultrathin sectioning and viewing under a Zeiss 10A electron microscope, operated at 60 KV.

### Quantitative morphology

Measurements of CE dimensions were taken from perpendicular (cross) sections in 10 separate, randomly obtained images from each stripping from four different male Asian subjects (Supplementary Table S1 online), using the Gatan Bioscan with DigitalMicrograph software (Gatan, Pleasanton, CA). The volume contribution of the CCs versus ECCs was determined in Ru-V samples by a stereological (morphometric) point-count procedure, which allows quantitative reconstruction of the three-dimensional structure from measurements of two-dimensional images (Wassermann *et al.*, 1967). The volume contribution of the ECC (V<sub>ECC</sub>) versus the CC (V<sub>CC</sub>) was determined by randomly superimposing an array of points over images, and quantifying the number (N) of "hits" that overlie each compartment (>10 randomly selected micrographs from each tape-stripped sample, from each subject (Supplementary Table S1 online)). Points that fell in areas of ambiguous or potentially artifactual (empty) spaces were excluded, except in post-hydration samples, in which empty spaces appeared at extracellular sites previously occupied by saline. The volume contribution of the ECC was calculated as a percentage of total SC, derived as

$N_{ECC}/(N_{ECC} + N_{CC}) \times 100$ . A second quantitative procedure was also used to evaluate the impact of hydration/dehydration on compartment volumes in strippings from the same four subjects. Changes in the volume of ECC and CC before and after immersion were normalized to the cumulative length of cellular borders, determined planimetrically by Map Wheel (Scalex, Carlsbad, CA). Please see Supplemental Material online for more Materials and Methods.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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