Hyaluronan–CD44 Interaction Stimulates Keratinocyte Differentiation, Lamellar Body Formation/Secretion, and Permeability Barrier Homeostasis

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In this study we investigated whether hyaluronan (HA)–CD44 interaction influences epidermal structure and function. Our data show that CD44 deficiency is accompanied by reduction in HA staining in CD44 knockout (k/o) mouse skin leading to a marked thinning of epidermis *versus* wild-type mouse skin. A significant delay in the early barrier recovery (following acute barrier disruption) occurs in CD44 k/o *versus* wild-type mouse skin. To assess the basis for these alterations in CD44 k/o mouse epidermis, we determined that differentiation markers are greatly reduced in the epidermis of CD44 k/o *versus* wild-type mice, while conversely HA binding to CD44 triggers differentiation in cultured human keratinocytes. CD44 downregulation (using CD44 small interfering RNAs) also inhibits HA-mediated keratinocyte differentiation. Slower barrier recovery in CD44 k/o mice could be further attributed to reduced lamellar body formation, loss of apical polarization of LB secretion, and downregulation of cholesterol synthesis. Accordingly, HA–CD44 binding stimulates both LB formation and secretion. Together, these observations demonstrate new roles for HA–CD44 interaction in regulating both epidermal differentiation and lipid synthesis/secretion, which in turn influence permeability barrier homeostasis. HA–CD44 signaling could comprise a novel approach to treat skin disorders characterized by abnormalities in differentiation, lipid synthesis, and/or barrier function.

Journal of Investigative Dermatology (2006) **126,** 1356–1365. doi:10.1038/sj.jid.5700260; published online 23 March 2006

INTRODUCTION

Hyaluronan (HA), the major glycosaminoglycan of extracellular matrix component serves not only as a primary constituent of connective tissue extracellular matrices but also as a bio-regulatory molecule (Toole, 1991; Lee and Spicer, 2000). Many studies indicate that HA is abundant in stratified squamous epithelia, including the epidermis of skin, and that it is involved in epidermal functions and integrity (Lee and Spicer, 2000; Maytin *et al.*, 2004). However, the mechanisms by which HA stimulates keratinocyte activities and regulates tissue integrity are not understood. HA is often anchored to CD44, a ubiquitous, abundant and functionally important surface receptor that displays HA-binding site(s) (Underhill, 1992). Several lines of evidence indicate that CD44 selects its unique downstream effectors and coordinates intracellular signaling pathways to initiate a concomitant onset of multiple cellular functions (reviewed in Turley *et al.*, 2002). The binding of HA to CD44-expressing cells also stimulates intracellular Ca^{2+} mobilization, a prerequisite for the onset of a variety of biological activities (Singleton and Bourguignon, 2002; Bourguignon *et al.*, 2004a; Singleton and Bourguignon, 2004). A recent study shows that HA–CD44 interaction with Rac1-PKNγ plays a pivotal role in PLCγ1-regulated Ca^{2+} signaling as well as cortactin-cytoskeleton function required for cell–cell adhesion and differentiation in keratinocytes (Bourguignon *et al.*, 2004a). These observations are consistent with a previous report showing that transgenic mice expressing an antisense construct to CD44 in their skin fail to accumulate HA in epidermis and display abnormal keratinocyte proliferation (Kaya *et al.*, 1997). Thus, CD44 appears to participate in keratinocyte function and normal epidermal physiology.

The major function of the epidermis is to provide a barrier between the external environment and the organism (Madison, 2003). To fulfill this function keratinocytes undergo a

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Abbreviations: CHK, cultured human keratinocyte; HA, hyaluronan; K-10, cytokeratin 10; k/o, knockout; LB, lamellar body; SC, stratum corneum; SG, stratum granulosum; siRNA, small interfering RNA; TEWL, transepidermal water loss

Received 15 September 2005; revised 24 January 2006; accepted 26 January 2006; published online 23 March 2006

complex pathway of differentiation, which culminates in keratinocyte cornification and in the formation of extracellular, lipid-enriched lamellar membranes in the stratum corneum (SC) (Elias, 1996; Madison, 2003). These lipid-enriched membranes are primarily responsible for the permeability barrier to water and electrolyte transit. Organization of the lamellar matrix regulates normal cornification (Elias, 1996), which is characterized by extensive cross-linking of loricrin, involucrin, and other structural proteins by transglutaminase to form the cornified envelope. The cornified envelope serves as a scaffold for the deposition of the lamellar membranes, but is also essential for the normal mechanical strength of the skin. Thus, the SC can be thought of consisting of two interdependent compartments that are formed during keratinocyte differentiation, the corneocytes (i.e., the ''bricks'') and the extracellular lipid-enriched membranous matrix (i.e., the ''mortar'') (Elias, 1996). Despite its functional importance, the regulation of keratinocyte differentiation and the factors that allow for the coordinate formation of the corneocytes and extracellular lipid lamellar membranes during keratinocyte differentiation are largely unknown.

The lipid-enriched extracellular lamellar membranes are derived from the exocytosis of lamellar bodies (LBs) from stratum granulosum (SG) cells (Elias, 1996; Madison, 2003). In order for the SG cells to form LBs, three families of lipids, specifically cholesterol, phospholipids, and glucosylceramides, must be generated at sufficient levels in SG cells (Feingold, 1991). If these lipids are not synthesized in the appropriate molar ratios abnormal LBs are formed and a functional barrier is not formed (Elias, 1996). The epidermis is a very active site of lipid synthesis, and inhibition of cholesterol, fatty acid, ceramide, or glucosylcermide synthesis blocks LB formation and perturbs permeability barrier homeostasis (Feingold, 1991; Elias, 1996).

In the present, manuscript we report that CD44 deficiency in CD44 knockout (k/o) mouse skin leads to abnormal epidermal structure and function. In assessing the basis of these epidermal alterations, we have found that HA-mediated CD44 signaling regulates keratinocyte differentiation, cholesterol synthesis, and LB formation/secretion required for normal SC structure and epidermal barrier function.

RESULTS

CD44 k/o mice display abnormalities in epidermal structure attributable to decreased proliferation and differentiation

To assess the role of HA–CD44 interaction in epidermal structure and function, we first analyze the expression and localization of CD44 and its ligand (HA) in the epidermis of CD44 k/o *versus* wild-type mice. Both CD44 (Figure 1c) and HA (Figure 1e) are present in all of the nucleated layers of wild-type mice epidermis (Figure 1a), displaying a distinctive pattern of membrane staining. In contrast, no CD44 (Figure 1d) and very little endogenous HA (Figure 1f) can be detected in the epidermis of CD44 k/o mouse skin (Figure 1b). Although CD44 k/o mice do not display macroscopic abnormalities, both histologic sections (Figure 1a–f) and ultrastructural observations (Figure 1g *vs* h) demonstrate substantial alterations, including a marked thinning of the epidermis (Table 1 for quantitation data). Epidermal thinning can be attributed largely to loss of the rete ridges, resulting in flattening of the epidermal–dermal interface (Figure 1g *vs* h).

In order to ascertain the basis for epidermal thinning in CD44 k/o mice, we next compared epidermal proliferation in CD44 k/o *versus* wild-type mice. Our results indicate that epidermal proliferation (shown by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) (Figure 1i *vs* j); and Table 1 for quantitation data) is decreased, as reported previously (Kaya *et al.*, 1997). Therefore, reduced epidermal proliferation could contribute to the epidermal thinning in CD44 k/o mice.

Permeability barrier homeostasis is abnormal in CD44 k/o mice

To address whether the CD44-linked abnormalities in epidermal structure result in functional consequences, we next analyzed permeability barrier recovery kinetics in CD44 k/o *versus* wild-type mice. Two unrelated methods of acute disruption, sequential cellophane tape stripping (Figure 2a) and acetone wipes (Figure 2b), were utilized to raise transepidermal water loss (TEWL) levels until rates exceeded $>5\ mg/cm^2$ per hour ($Nm = 0.2\ mg/cm^2$ per hour). Although the basal TEWL, SC, hydration and surface pH were comparable in CD44 k/o and wild-type mice (data not shown), a significant delay in barrier recovery kinetics occurred in CD44 k/o mice as compared to the wild-type mice, independent of the method of disruption (Figure 2a and b). Interestingly, the most significant divergence in recovery kinetics was apparent at the earliest time point assessed; that is at 1 hour, and by 6 hours after acute disruption, recovery rates normalized in the CD44 k/o mice. These findings show that lack of CD44 in epidermis results in a transit abnormality in permeability barrier homeostasis.

Aberrant HA–CD44-mediated signaling of keratinocyte differentiation could contribute to the permeability barrier abnormality in CD44 k/o mice

Normal permeability barrier homeostasis requires the differentiation-dependent generation of corneocytes embedded in a lipid-enriched extracellular matrix forming the SC. Therefore, in the next group of studies, we assessed epidermal differentiation in CD44 k/o *versus* wild-type mice. As shown in Figure 3a–f, the expression of involucrin (Figures 3a *vs* b), profilaggrin (Figure 3c *vs* d), and cytokeratin 10 (K-10) (Figure 3e *vs* f) was impaired in CD44 k/o mouse skin, indicating a decrease in epidermal differentiation, which would inevitably result in the formation of effete corneocytes.

To assess whether HA–CD44 interaction directly regulates keratinocyte differentiation, we next investigated HA–CD44 signaling of differentiation in normal cultured human keratinocytes (CHK). Addition of HA to CHK grown in low Ca^{2+} (0.03 mM Ca^{2+}) increased the expression of keratinocyte differentiation markers, including involucrin (precursor for the cornified envelope) (Figure 3ga, lanes 1 and 2), profilaggrin (Figure 3gb, lanes 1 and 2), and K-10 (Figure 3gc, lanes 1 and 2). Since pretreatment of keratinocytes with rat anti-CD44 antibody (Figure 3ga–c, lane 3) (but not normal rat

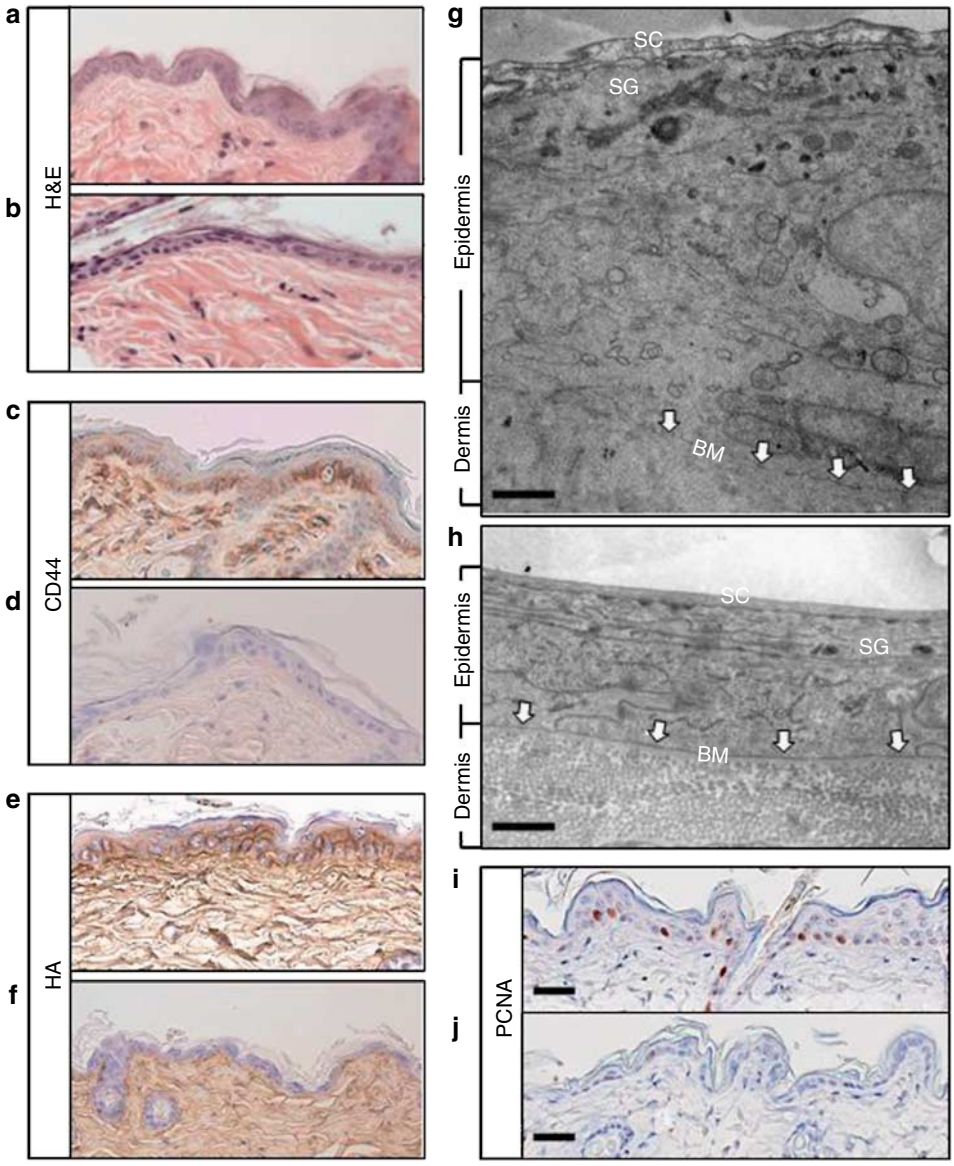

Figure 1. Immunohistochemical and ultrastructural analyses of wild-type (CD44 +/+) and CD44 k/o (CD44−/−) mouse epidermis. Skin biopsies were obtained from the back of the mice. (**a**–**f**) Light microscopic analyses of the wild-type (CD44 +/+) (**a**) *versus* CD44 k/o (**b**) mouse epidermis using hematoxylin and eosin staining; Detection of CD44 staining in the wild-type (CD44 +/+) (**c**) *versus* CD44 k/o (CD44−/−) (**d**) mouse epidermis using anti-CD44-mediated immunoperoxidase staining; and detection of HA staining in the wild-type (CD44 +/+) (**e**) *versus* CD44 k/o (CD44−/−) (**f**) mouse epidermis using HA-binding protein-mediated immunoperoxidase staining (bars: 20 μm). (**g** and **h**) Electron microscopic analyses of the wild-type (CD44 +/+) (**g**) *versus* CD44 k/o (CD44−/−) (**h**) mouse epidermis. (Note epidermal attenuation, loss of rete ridges, and thin SC in CD44 k/o mice. SC: stratum corneum; SG: stratum granulosum; BM: basement membranes (white arrows); OsO4 post-fixation; bars: 1.0 μm). (**i** and **j**) Detection of a proliferation marker in wild-type (CD44 +/+) (**i**) and CD44 k/o (CD44−/−) (**j**) mouse epidermis using anti-PCNA-mediated immunoperoxidase staining (bars: 20 μm).

IgG) (Figure 3ga–c, lane 4), largely reduced HA-stimulated upregulation of these differentiation markers (Figure 3ga, lane 3; Figure 3gb, lane 3; Figure 3gc, lane 3), the HA effects on keratinocyte differentiation appear to be CD44 specific.

Transfection of mammalian cells with synthetic, small interfering RNAs (siRNAs 21–23 nucleotide in length) can be employed to specifically suppress expression of endogenous and heterologous genes by RNA interference (Montgomery, 2004; Schutze, 2004). In order to further confirm that CD44 is needed for HA-stimulated keratinocyte differentiation, we next transfected CHK with a specific siRNA sequence targeting CD44. As shown in Figure 3h and i, this siRNA successfully suppressed CD44 expression in CHK (Figure 3ha, lane 2). In control experiments, CHK were treated with transfection reagents containing scrambled sequences and no CD44 downregulation was observed (Figure 3ha, lane 1). Since other cellular proteins, such as actin, continued to be expressed at comparable levels in the CD44siRNA-treated cells (Figure 3hb, lane 2) as well as in control cells containing scrambled sequences (Figure 3hb, lane 1), we conclude that

Table 1. Measurement of epidermal thickness and PCNA-positive cells in wild-type (CD44+/+) *versus* CD44 k/o (CD44−/−) mouse skin

Animals	Epidermal thickness (μm)[1]	PCNA-positive cells per mm[3]
Wild type (CD44+/+) (n=5)	15±2 (100%)	82±4 (100%)
CD44 k/o (CD44−/−) (n=5)	7±1 (47%)[2]	33±2 (40%)[4]

[1]Data are presented as mean±SEM. The total length of the epidermis analyzed varied between 8 and 15 mm for each sample. At least five measurements of epidermal thickness were made per specimen.
[2]Significant different ($P<0.001$) as compared with CD44 wild-type (CD44+/+) samples.
[3]Data are presented as mean number of PCNA-positive cells ±SEM per mm. The total length of the epidermis analyzed varied between 10 and 16 mm for each sample.
[4]Significant different ($P<0.001$) as compared with CD44 wild-type (CD44+/+) samples.

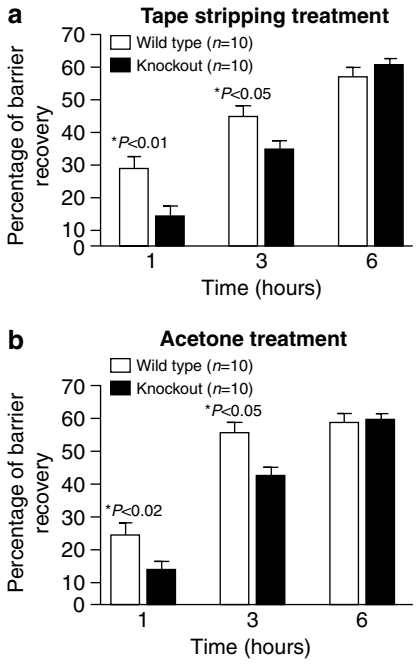

Figure 2. Analyses of barrier recovery (a and b) in wild-type (CD44+/+) and CD44 k/o (CD44−/−) mouse epidermis. (**a** and **b**) TEWL in wild-type (CD44+/+) and CD44 k/o (CD44−/−) mouse epidermis was measured after 1, 3, and 6 hours after tape stripping (**a**) and acetone treatment (**b**). The degree to which recovery of the barrier after these two treatments in wild-type (CD44+/+) *versus* CD44 k/o (CD44−/−) mouse epidermis is achieved at these time points is recorded (The error bars enclosed mean±SD).

the selective downregulation of CD44 expression by CD44siRNAs is target specific. Further analyses indicated that HA-induced stimulation of differentiation marker expression (e.g., involucrin, profilaggrin, and K-10) was greatly inhibited in CHK transfected with CD44siRNA (Figure 3ia, lanes 3 and 4; Figure 3ib, lanes 3 and 4; Figure 3ic, lanes 3 and 4), as compared to expression of these differentiation markers in CHK treated with siRNA with scrambled sequences (Figure 3ia, lanes 1 and 2; Figure 3ib, lanes 1 and 2; Figure 3ic, lanes 1 and 2). Together, these findings show that CD44 is directly involved in HA-mediated stimulation of keratinocyte differentiation. Moreover, defective HA/CD44-dependent differentiation could provide one mechanism that explains the defect in permeability barrier homeostasis in CD44 k/o mouse skin.

Permeability barrier abnormality in CD44 k/o mice can be further attributed to defects in epidermal lipid synthesis, LB formation, and LB dispersion/secretion

Epidermal *de novo* lipid synthesis (including cholesterol synthesis) is required to provide the bulk lipids that form the hydrophobic, extracellular lamellar membrane systems (Feingold, 1991). To analyze whether alterations of lipid synthesis or secretion contribute to the permeability barrier abnormality in CD44 k/o mice, we next assessed epidermal fatty acid and cholesterol synthesis in CD44 k/o mouse epidermis. Our results showed that both total cholesterol (Figure 4aa) and total non-saponifiable lipid synthesis (Figure 4ab) were markedly impaired in CD44 k/o mice as compared to wild-type mice (Figure 4a (a and b)). In contrast, synthesis of other lipid components of the permeability barrier: that is, saponifiable lipids (fatty acids) (Figure 4ac) and sphingolipids (e.g., glucosylceramides and ceramides) remain normal in CD44 k/o mouse epidermis (data not shown). In addition, we have observed that HA promotes cholesterol synthesis in CHK (Figure 4b (a and b)). Most importantly, downregulation of CD44 by transfecting CHK with CD44siRNA effectively inhibited HA/CD44-mediated cholesterol synthesis (Figure 4bc). Finally, pretreatment of CHK with anti-CD44 antibody also blocked HA-mediated cholesterol synthesis (data not shown). Together, these findings show that HA–CD44 interaction is closely involved in cholesterol synthesis in keratinocytes, and that impaired cholesterol synthesis could contribute to the delay in barrier recovery in CD44 k/o mouse epidermis.

Epidermal lipid synthesis provides the bulk lipids that are packaged into epidermal LBs prior to their secretion, forming the lipid-enriched extracellular matrix of the barrier (Feingold, 1991). Therefore, we next asked whether the defect in cholesterol synthesis impacts LB formation and secretion in CD44 k/o mouse epidermis, leading to epidermal barrier dysfunction. Under basal conditions (without prior barrier disruption), the density of epidermal LB in the cytosol of SG cells was markedly reduced in the cytosol of CD44 k/o keratinocytes, as compared to their density in wild-type mouse epidermis (Figure 5b vs a; see Table 2 for quantitation

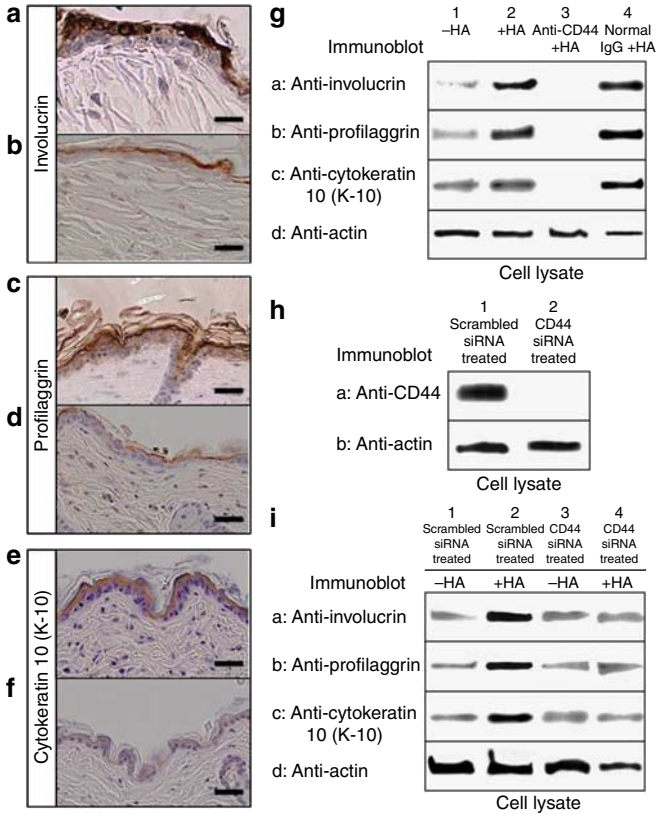

Figure 3. Analyses of differentiation marker expression in mouse epidermis (a–f) and CHK (g, h, and i). (**a**–**f**) Detection of differentiation markers in wild-type (CD44 + / +) and CD44 k/o (CD44 − / −) mouse epidermis (using the skin biopsies obtained from the back of the mice). (**a** and **b**) Anti-involucrin-mediated immunoperoxidase staining of the wild-type (CD44 + / +) (**a**) *versus* CD44 k/o (CD44 − / −) and (**b**) mouse epidermis; (**c** and **d**) Antiprofilaggrin-mediated immunoperoxidase staining of the wild-type (CD44 + / +) (**c**) *versus* CD44 k/o (CD44 − / −) and (**d**) mouse epidermis; (e and f): Anti-K-10-mediated immunoperoxidase staining of the wild-type (CD44 + / +) (**e**) *versus* CD44 k/o (CD44 − / −) and (**f**) mouse epidermis (Bars: 20 μm). (**g**) Expression of differentiation markers (involucrin, profilaggrin, and K-10) in CHK. Keratinocytes (untreated or pretreated with rat anti-CD44 IgG or normal rat IgG) were incubated with HA (50 μg/ml) at 37°C for 36 hours. Subsequently, cells were solubilized by 1% Nonidet P-40 (NP-40) buffer. Cell lysates (isolated from keratinocytes treated with no HA (lane 1) or with HA (lane 2); or pretreated with rat anti-CD44 IgG plus HA (lane 3) or pretreated with normal rat IgG plus HA (lane 4)) were then used for immunoblotting using anti-involucrin antibody (**a**) or antiprofilaggrin antibody (**b**) or anti-K-10 antibody (**c**) or antiactin antibody (as a loading control) (**d**), respectively. (**h** and **i**) Expression of differentiation markers (involucrin, profilaggrin, and K-10) in CHK treated with CD44siRNA or scrambled sequences: (**h**): Keratinocytes (transfected with CD44siRNA-scrambled sequences (lane 1) or CD44siRNA (lane 2) were solubilized by 1% Nonidet P-40 buffer and immunoblotted with anti-CD44 antibody (a) or antiactin antibody (b); (**i**): Keratinocytes (transfected with CD44siRNA-scrambled sequences (lanes 1 and 2) or CD44siRNA (lanes 3 and 4)) were incubated with no HA (lanes 1 and 3) or with HA (lanes 2 and 4) at 37°C for 36 hours. These cells were then solubilized by 1% Nonidet P-40 buffer and immunoblotted with anti-involucrin (a) or antiprofilaggrin (b) or anti-K-10 (c) or antiactin (as a loading control) (d).

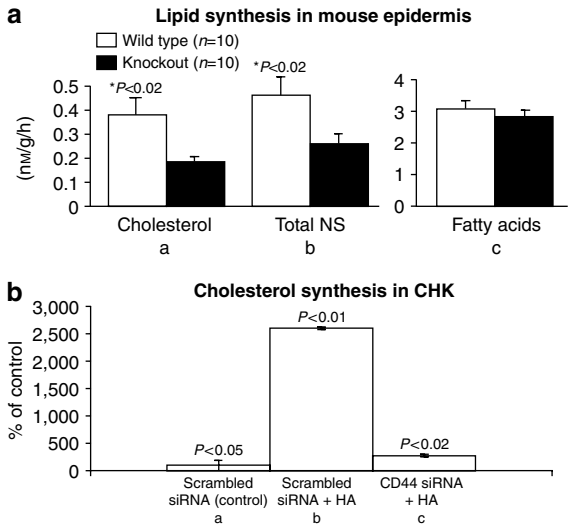

Figure 4. Measurement of lipid and/or cholesterol synthesis. (**a**) Analyses of lipid synthesis in wild-type (CD44 + / +) and CD44 k/o (CD44 − / −) mouse epidermis. Detection of the level of cholesterol synthesis (a); Non-saponifiable lipid synthesis (b); and fatty acid synthesis (c) in the epidermis of wild-type (CD44 + / +) *versus* CD44 k/o (CD44 − / −). (**b**) Analyses of cholesterol synthesis in CHK-transfected with either CD44siRNA-scrambled sequences (a and b) (followed by no HA treatment (a) or HA treatment (b)) or CD44siRNA (followed by HA treatment (c)).

data). Moreover, the SG–SC junction of CD44 k/o mice showed reduced secretion of lamellar material in comparison to the SG–SC junction in wild-type mice (Figure 5c *vs* d; see Table 2 for quantitation data).

Since experimentally induced abnormalities in barrier homeostasis can be due not only to defects in LB formation and secretion, but also to abnormality in either exocytosis or polarization of LB secretion (Elias, 1996), we next examined whether the CD44 k/o mice display abnormalities in the exocytosis, polarity, and/or post-secretory dispersion of LB contents. Two distinctive secretory abnormalities were evident in CD44 k/o mouse epidermis. At 1 hour after acute barrier disruption, not only was LB secretion decreased, as noted above (Figure 5d), but also post-secretory dispersion of secreted lamellar material was delayed within the SG–SC interface of CD44 k/o mouse epidermis (i.e. newly secreted lamellar materials demonstrated a failure to transform into elongated membrane structures at the SG–SC junction) (Figure 5c *vs* d). Secondly, the normal apical polarity of LB secretion towards the SG–SC interface, assessed by ultra-structural cytochemistry utilizing the secretion marker, acid lipase (a LB lipid hydrolase), became aberrant in CD44 k/o epidermis. In wild-type mouse epidermis, secretion of LB content (indicated by extracellular deposition of acid lipase activity) occurs preferentially from the apical region of the outermost SG cell layer, where the great bulk of LB contents are normally secreted (Figure 5e). In contrast, targeting of secretion became redistributed from apical to basolateral membranes in CD44 k/o mice (Figure 5f). Specifically, secreted lipase activity appeared in the intercellular spaces between baso-lateral regions of the mid-to-outer SG cell layers, in addition to the apex of SG cells in CD44 k/o epidermis (Figure 5f). Together, these findings suggest that the transient delay in barrier recovery in CD44 k/o mouse

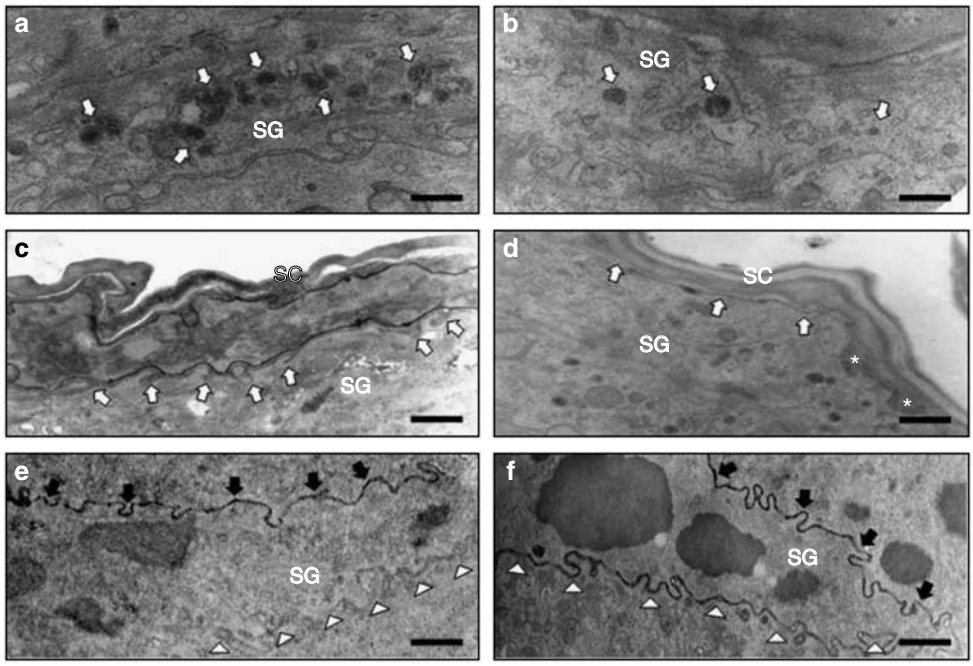

Figure 5. Electron microscopic and ultrastructural cytochemical analyses of LB formation and secretion in wild-type (CD44+/+) *versus* CD44 k/o (CD44−/−) mouse epidermis. Note high density of LB (white arrows) in the cytosol of the SG of untreated wild-type (CD44+/+) mouse epidermis (**a**) *versus* a reduced number of LB (white arrows) in untreated CD44 k/o (CD44−/−) mouse epidermis (**b**); Note increased LB secretion and dispersion of secreted lamellar material (white arrows) in the wild-type (CD44+/+) mouse epidermis (1 hour after acute barrier disruption by tape stripping) (**c**); and note both reduced LB secretion (white arrows) at SG–SC interface and failure of newly secreted lamellar material to disperse (asterisks) in CD44 k/o (CD44−/−) mouse epidermis (1 hour after acute barrier disruption by tape stripping) (**d**); (**e** and **f**) Detection of the LB content marker (acid hydrolase) in the wild-type and CD44 k/o mice. Note secretion of acid lipase activity restricted to the apex (black arrows) (very little in the baso-lateral (white arrow heads)) of wild-type (CD44+/+) mouse epidermis (1 hour after acute barrier disruption by tape stripping) (**e**). In contrast, note aberrant polarity of secreted, acid lipase activity toward the baso-lateral (white arrow heads), plus apical surface (black arrows) of the mid- to outer cell layers of the SG in the CD44 k/o (CD44−/−) mouse epidermis (1 hour after acute barrier disruption by tape stripping) (**f**). (SC: stratum corneum; SG: stratum granulosum; (**a**–**f**) OsO4 postfixation. Bars = (**a**, **c**, and **d**) 0.50 μm upper 0.20 μm lower and (**b**) 0.50 μm.

Table 2. Measurement of LB density and secretion in wild-type (CD44+/+) *versus* CD44 k/o (CD44−/−) mouse skin

Animals	LB density (LB/unit area ±SEM)[1]	LB secretion (mg/cm±SEM)[3]
Wild type (CD44+/+)	8±1 (100%)	0.62±0.03 (100%)
CD44 k/o (CD44−/−)	4±1 (50%)[2]	0.30±0.01 (48%)[4]

[1]Data represent mean±SEM in 20 random electron micrographs from each animal, evaluated by two blinded observers.
[2]Significant different ($P<0.001$) as compared with CD44 wild-type (CD44+/+) samples.
[3]LB secretion was determined by using the ratio between paperweights of traced secretion area at the SG/SC junction over the total length of the first SC cell layer. Data represent the mean±SD (n=10 randomly selected, coded micrographs from five wild-type mice and five CD44 k/o mice).
[4]Significantly different ($P<0.001$) as compared with CD44 wild-type (CD44+/+) samples.

epidermis can be linked to abnormalities in cholesterol synthesis, leading to decreased LB production, aberrant dispersion of secreted LB contents, and loss of the normal apical polarity of LB secretion.

HA–CD44 interaction stimulates LB formation and secretion in CHK

Since CD44 k/o mice display distinct abnormalities in LB formation and secretion, we next assessed the role of HA–CD44 interaction on LB formation and secretion in normal CHK. To dissect whether HA–CD44 interaction regulates one or multiple steps in the LB secretory system, we applied exogenous HA to human keratinocytes, grown in organotypic (air lifted) cultures. When human keratinocytes were grown in the absence of HA (in 0.03 mM Ca^{2+}), as well as in an absence of serum and vitamin C, few LB, little or no LB secretion (Figure 6e) could be detected (Figure 6a; Table 3 for quantitation data). Yet, if exogenous HA (50 μg/ml) is added for 72 hours to these organotypic cultures, large numbers of LB began to appear (Figure 6b; Table 3 for quantitation data), followed by their rapid secretion (Figure 6f). When cells were treated with normal rat IgG, HA-mediated LB formation could also be readily detected (Figure 6d, Table 3 for quantitation data). In contrast, when these organotypic cultures were pretreated with a CD44-specific antibody, followed by HA addition, both LB formation (Figure 6c; Table 3 for quantitation data) and secretion (Figure 6e) were reduced. Together, these observations strongly support a role for HA–CD44 interaction

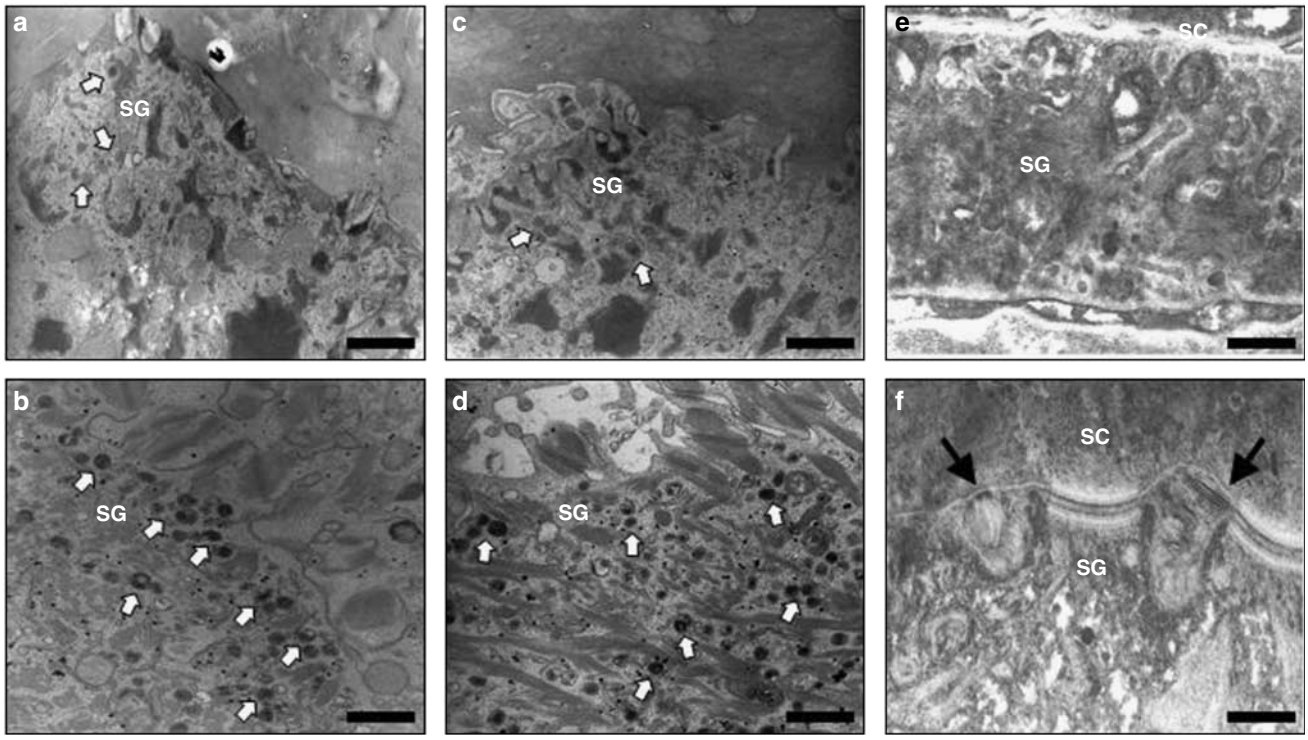

Figure 6. Electron microscopic analyses of LB formation and secretion in human keratinocytes grown in organotypic cultures. Human keratinocytes were grown in a collagen-basement membrane in Transwell dishes according to MatTek organotypic tissue culture conditions as described in the Materials and Methods. (**a** and **b**): (**a**) LB formation (white arrows) in cells treated with no HA; (**b**) LB formation (white arrows) in cells treated with HA; (**c**) LB formation (white arrows) in cells pretreated with rat anti-CD44 plus HA; (**d**) LB formation (white arrows) in cells pretreated with normal rat IgG plus HA; (**e**) Minimal LB secretory processes detected in cells treated with no HA; (**f**) LB secretory processes (dark arrows) detected in cells treated with HA. (**a**–**f**: OsO_4 post-fixation; SC: stratum corneum; SG: stratum granulosum. Bars = (**a**–**d**) 0.50 μm and (**e** and **f**) 0.25 μm.

Table 3. Measurement of LB density in keratinocytes grown in organotypic cultures

Treatments	LB density (LB/unit area ±SEM) (% of control)[1]
No treatment (control)	7±1 (100%)
HA treatment	16±2 (230%)[2]
Rat anti-CD44 IgG + HA treatment	9±1 (129%)[2]
Normal rat IgG + HA treatment	14±2 (200%)[2]

[1]Data represent mean±SEM in 20 random electron micrographs from each sample, evaluated by two blinded observers.
[2]Significant different ($P<0.001$) as compared with no treatment (control) samples.

in the regulation of both LB formation and secretion in epidermal keratinocytes.

DISCUSSION

The major receptor for HA on the surface of epidermal keratinocytes is CD44. Activation of CD44 by HA increases signal transduction and several downstream biological activities (Singleton and Bourguignon, 2002, 2004; Turley *et al.*, 2002; Bourguignon *et al.*, 2004a, b). For example, HA–CD44 interaction induces intracellular signal cascades that regulate cell–cell adhesion, cell migration, and proliferation (Lokeshwar *et al.*, 1996; Singleton and Bourguignon, 2002, 2004; Turley *et al.*, 2002; Bourguignon *et al.*, 2004a), required for morphogenesis (Toole, 1991), wound healing (Alaish *et al.*, 1994), and tumor progression (Bourguignon *et al.*, 1998; Turley *et al.*, 2002). However, neither the extent nor the mechanisms by which keratinocytes respond to HA are well understood at the present time. In both rodents and humans acute disruption of the permeability barrier by mechanical forces, solvents, or detergents increases TEWL, initiating a homeostatic repair response that results in the rapid recovery of permeability barrier function (Feingold, 1991; Elias, 1996). In CD44-expressing wild-type mouse epidermis, we observed a normal rapid repair response following barrier disruption, consisting of secretion of LB contents from cells of the outer SG, accumulating at the SG–SC interface, while leaving the cytosol of these cells temporarily devoid of LB. We report here that CD44 k/o mice exhibit abnormalities in both epidermal structure and permeability barrier homeostasis. Histochemical analyses and immunoperoxidase staining data showed that both CD44 and endogenous HA are expressed in the plasma membranes of epidermal keratinocytes in CD44-wild-type mouse skin, but not in CD44 k/o epidermis. Moreover, very little endogenous HA can be detected in epidermal keratinocyte membranes of CD44 k/o mouse skin. Previous

experiments directed at blocking expression of CD44, using antisense phosphorothioate-based or phosphoramidate-modified oligonucleotides (Chow *et al.*, 1998), or a ribozyme approach (Ge *et al.*, 1995) indicate that reduction of CD44 expression results in an inhibition of a variety of cellular functions (e.g., cell proliferation, migration, and tumor formation) in experimental animal models. Lack of HA-mediated CD44 signaling of proliferation could explain the marked epidermal thinning that we observed in CD44 k/o mice.

In this study, we report a novel effect of HA-mediated CD44 signaling, that is, CD44 activation by HA in keratinocyte culture upregulates the expression of keratinocyte differentiation markers (e.g., involucrin, profilaggrin, and K-10), but not other structural proteins, including actin. Yet, in a previous study we have shown that HA induces actin cytoskeleton reorganization in a CD44-dependent manner during differentiation (Bourguignon *et al.*, 2004a). Specifically, using a CD44-specific siRNAs strategy, we show here that downregulation of CD44 inhibits HA-mediated expression of several differentiation markers. We have also found that several keratinocyte differentiation markers, such as involucrin, profilaggrin, and K-10, are significantly reduced in the skin of CD44 k/o mice as compared to CD44 wild-type mice. These results suggest that CD44 plays an important role in regulating epidermal differentiation and that barrier abnormality can be attributed in part to the formation of effete corneocytes.

Epidermal differentiation leads to the formation of the SC, a heterogeneous tissue composed of lipid-depleted corneocytes embedded in a lipid-enriched matrix with the SC, which subserves the cutaneous permeability barrier (Elias, 1996). In addition to the above-described HA–CD44-mediated effects on corneocytes, we also found that HA–CD44 interactions regulate epidermal lipid (cholesterol) synthesis and/or secretion. Indeed, CD44 k/o mice displayed decreased lipid synthesis, as well as several abnormalities in LBs formation and secretion. LBs are oval-shaped structures located in the SG layers of the epidermis, which secrete the lipid precursors and lipid processing enzymes required to form the barrier. Here, we observed that the density of LB in the cytosol of SG cells is remarkably reduced in CD44 k/o keratinocytes, resulting in decreased secreted lipid within the SC interstices. Moreover, we show that HA stimulates LB formation and secretion in a CD44-depedent manner in human keratinocytes grown in organotypic culture condition. These observations strongly suggest that HA-mediated activation of CD44 is either directly or indirectly regulates LB production and secretion.

As a result of the immediate and subsequent accelerated LB secretory response, the quantity of secreted LB contents continues to increase at the SG–SC interface, so that as early as 1 hour after acute disruption, new lamellar membranes begin to appear within the extracellular spaces of the lower SC. These morphological changes are paralleled by a progressive normalization of permeability barrier function. In contrast, CD44 k/o mice display a disordered polarity of LB secretion and abnormal dispersion of LB content after acute barrier disruption (evidenced by localized retention of non-dispersed, secreted material at the SG–SC junction). Along with the loss of secretion polarity, the apparent abnormality in dispersion appears to account for the delay in barrier recovery in the epidermis of CD44 k/o mice. These observations strongly suggest that HA-mediated activation of CD44 either directly or indirectly regulates LB production and secretion. Thus, we believe that functional CD44 is required not only for keratinocyte differentiation, but also for the LB secretory responses and post-secretory generation of new lamellar membranes, together maintaining and restoring permeability barrier homeostasis.

The rapid formation of nascent LB in SG cells following barrier disruption requires the availability of the major lipid components of LB; that is, cholesterol, glucosylceramides, and phospholipids (Grubauer *et al.*, 1987). The epidermis is a very active site of lipid synthesis and permeability barrier disruption results in a marked increase in the synthesis of cholesterol and an upregulation of several enzymes in the cholesterol biosynthetic pathway (Feingold *et al.*, 1990). These findings suggest that cholesterol synthesis is actively participating in normal barrier homeostasis. Our data indicate that both total cholesterol and total non-saponifiable lipid synthesis are greatly reduced in CD44 k/o mice as compared to wild-type mice. In contrast, the production of other lipid components of the permeability barrier, that is, sphingolipids (e.g., glucosylceramides and ceramides) and saponifiable lipid (fatty acids) remains largely normal in CD44 k/o mouse epidermis. However, reduction of cholesterol synthesis alone can suffice to change LB formation and barrier recovery kinetics (Feingold *et al.*, 1990). Thus, the CD44-linked abnormality in cholesterol synthesis could explain a transient delay in barrier repair in CD44 k/o mouse epidermis. Of course, we cannot preclude the possibility that the LB defects might be a secondary effect due to impaired differentiation of keratinocytes.

Taken together, our findings suggest that HA-mediated CD44 activation regulates two parallel arms of proliferation and differentiation that leads to SC formation: (i) corneocyte production; and (ii) LB formation/secretion (perhaps driven by prior changes in cholesterol synthesis) (Figure 7). Thus, CD44 (via its interaction with HA) should be considered as a potent

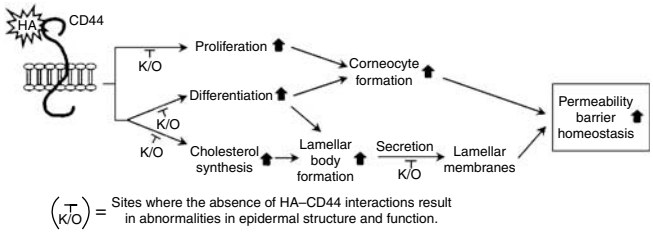

Figure 7. A proposed model for illustrating HA–CD44 interaction in regulating epidermal structure and function. In epidermis, HA interaction with CD44 promotes proliferation, differentiation and cholesterol synthesis. In particular, these parallel arms of proliferation and differentiation promote corneocyte formation; and stimulate both keratinocyte differentiation and LB formation/secretion (perhaps driven by prior changes in cholesterol synthesis), which all are required for normal epidermal permeability barrier function. (CD44 deficiency in k/o mice results in abnormal epidermal structure and function.)

regulator of normal epidermal permeability barrier function. These newly discovered HA/CD44-mediated functions (e.g. keratinocyte differentiation, LB formation/secretion, and epidermal barrier function) could not only provide further insights into the well-known effects of HA–CD44 on wound healing, but also point to direct therapeutic treatments in skin disorders characterized by abnormal differentiation and barrier dysfunction.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal rat anti-human CD44 antibody (clone: 020; isotype: IgG_{2b}; obtained from CMB-TECH Inc., San Francisco, CA.) used in this study recognizes a common determinant of the CD44 class of glycoproteins. Polyclonal mouse anti-involucrin and polyclonal mouse antiprofilaggrin were purchased from Covance Inc. (Princeton, NJ), and Zymed Laboratories Inc. (South San Francisco, CA), respectively. Mouse anti-PCNA antibody and ABC peroxidase reagents were obtained from Caltag Labs (Burlingame, CA) and Vector Labs (Burlingame, CA), respectively. Mouse anti-K-10 was obtained from Abcam Inc. (Cambridge, MA). Healon HA was purified by gel filtration column chromatography using a Sephacryl S1000 column. The purity of HA was further verified by anion exchange HPLC. HA preparations used in this study are free from proinflammatory or other contaminants.

CHK

Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as described previously (Bourguignon *et al.*, 2004a).

Animal model systems

Six week-old male CD44 k/o and wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Preparations of CD44siRNA

The siRNA sequence targeting human CD44 (from mRNA sequence, GeneBank, Acc No. AJ251595) corresponds to the coding region relative to the first nucleotide of the start codon. Target sequences were selected using the software developed by Ambion Inc., UK. As recommended by Ambion, CD44-specific targeted regions were selected beginning 50–100 nucleotides downstream from the start codon. Sequences close to 50% G/C content were chosen. Specifically, CD44 target sequence (5′-AACTCCATCTGTGCAG CAAAC-3′) and scrambled sequences (5′-AAAAACGGTAGATG CATCAGC-3′) were used.

Immunoblotting techniques

Keratinocytes (untreated cells or cells treated with 50 pmole CD44siRNA or 50 pmole siRNA-containing scramble sequences) grown in 0.03 mM Ca^{2+} were treated with no HA or with HA (50 μg/ml) or pretreated with rat anti-CD44 antibody (clone:020, isotype:IgG2b) followed by adding HA (50 μg/ml) at 37°C for 36 and/or 48 hours. These cells were then solubilized in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1.0% Nonidet P-40 (NP-40), 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin and immunoblotted with various immuno-reagents (e.g., rat anti-CD44 IgG (5 μg/ml), or mouse anti-involucrin (5 μg/ml), or mouse antiprofilaggrin (5 μg/ml), or mouse anti-K-10 or mouse antiactin (5 μg/ml)) followed by incubating with horseradish peroxidase-labeled goat anti-rat IgG, or horseradish peroxidase-labeled goat anti-rabbit IgG or horseradish peroxidase-labeled goat anti-mouse IgG. The blots were then developed by the ECL™ system (Amersham Co., Piscataway, NJ). Routinely, we apply 20 μg of proteins per well on the SDS-PAGE for immunblotting analyses.

Organotypic cultures

Human keratinocytes were grown in a collagen-basement membrane at a density of 200,000–250,000 cells per 2.5 cm (3-μm pore) culture inserts in a transwell according to MatTek *in vitro* organotypic tissue culture conditions. The seeded human keratinocytes (in the upper chamber) together with the lower chamber were then immersed in DMEM (0.03 M Ca^{2+}) containing no HA, HA (50 μg/ml) and anti-CD44 (50 μg/ml) plus HA (50 μg/ml) for 72 hours. When the cells reached stratification, the medium was removed from the upper chamber to expose the keratinocytes to air (at day 5). The medium in the lower chamber containing DMEM (0.03 M Ca^{2+}) plus HA (50 μg/ml) or no HA for 5–7 days at 37°C in the 5% CO_2 incubator, with transwells harvested for electron microscopic analyses.

Immunohistochemistry staining techniques

Paraffin-embedded skin tissues (isolated from CD44 k/o or wild-type mice) were cut in 5 μm sections. After deparaffinization and rehydration, the sections were stained hematoxylin and eosin or incubated with various reagents (e.g., rat anti-CD44, biotin-conjugated HA binding protein, anti-PCNA, anti-involucrin, and antiprofilaggrin) followed by adding ABC peroxidase reagent. Subsequently, peroxidase activity was localized with diaminobenzidine substrate (Vector Labs, Burlingame, CA). As controls, skin sections were incubated with preimmune serum followed by incubating with ABC peroxidase reagents. Epidermal thickness was measured on hematoxylin- and eosin-stained sections using $\times 20$ objectives and an ocular micrometer (final magnification $\times 100$). Experimental thickness was defined as the distance between the basement lamina and the apical surface of the uppermost nucleated keratinocytes as described previously (Komuves *et al.*, 2000). To quantitate changes in epidermal proliferation, the numbers of PCNA-positive cells/unit length of epidermis was assessed in randomized, coded digital images as described previously (Komuves *et al.*, 2000).

Electron microscopy and acid lipase assay

Samples (CD44 k/o and wild-type mouse skin; and human keratinocytes (treated with HA, no HA, or pretreated with anti-CD44 followed by HA addition) grown under organotypic cultures) were fixed first in 2% glutaraldehyde and 2% formaldehyde. After fixation, the samples were processed and embedded. To assess the extent and direction of acid lipase (a lipid hydrolase) secretion, glutaraldehyde/formaldehyde-fixed samples were microwave-incubated with a triacylglycerol (triolein) substrate, containing 0.2% Tween 85, by a lead-capture, cytochemical method (Rassner *et al.*, 1997).

Assessment of LB density and secretion

The procedures used for quantifying LB density were the same as described previously (Rassner *et al.*, 1999). In order to quantify LB

secretion in electron micrographs, at lease 10 pictures were taken randomly from each sample at 16,000 × magnification by an unbiased observer, and the area filled by secreted LB content within the SG–SC interface was then traced, cut out and weighted. LB secretion was defined as the ratio of secreted contents (as total weight), over the length (in μm) of the bottom surface of the first SC cell layer, determined with a planimeter.

Measurement of lipid synthesis

Skin samples (isolated for CD44 k/o and wild-type mice) or CHK (untreated or treated with 50 pmole CD44siRNA or 50 pmole siRNA containing scramble sequences in the presence or absence of 50 μg/ml HA for 36 hours) were incubated with in a solution containing 10 mM EDTA in Dulbecco's phosphate-buffered saline, calcium, and magnesium free, containing 40 μCi ^{14}C acetate for 2 hours. The incorporation of ^{14}C acetate into cholesterol, fatty acids, and ceramides was determined in the epidermis after saponification, extraction, and thin layer chromatography, as described previously (Grubauer *et al.*, 1987).

Assessment of permeability barrier function and barrier recovery after acute perturbations

The integrity of the permeability barrier of CD44 k/o and wild-type skin was assessed by TEWL using an electrolytic water analyzer (Meeco Co., Warrington, PA) as described previously (Yang *et al.*, 1995). The barrier was disrupted by either sequential cellophane tape stripping or acetone treatment until TEWL levels exceeded 5 mg/cm^2 per hour, and measurements of TEWL were then repeated at 1, 3, and 6 hours following tape stripping. Samples of skin were also obtained for electron microscopy prior to and 1, 3, and 6 hours following barrier disruption later to evaluate cellular basis for recovery. All studies were approved by the Animal Use Committee at the Veteran Affairs Medical Center, San Francisco.

Statistical analysis

Statistical comparisons were made using Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We gratefully acknowledge Ms Sandra Chang's assistance in the immunohistochemical staining experiments described in this paper. We are also grateful for Ms Christine Camacho for her help in preparing graphs and illustrations. This work was supported by United States Public Health Grants (P01 AR39448, R01 CA66163, R01 CA78633, R01 AR050629, R01 AR649932, and R01 AR19908), and VA Merit Review grants.

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