Expression of Epidermal CAMP Changes in Parallel with Permeability Barrier Status

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Two critical defensive functions of the outer epidermis, the permeability barrier and antimicrobial defense, share certain structural and biochemical features. Moreover, three antimicrobial peptides (AMPs), i.e., mouse β-defensin 3 (mBD3), mouse cathelicidin antimicrobial peptide (mCAMP), and the neuroendocrine peptide, catestatin (Cst), all localize to the outer epidermis, and both mBD3 and mCAMP are secreted from the epidermal lamellar bodies with other organelle contents that subserve the permeability barrier. These three AMPs are upregulated in response to acute permeability barrier disruption, whereas conversely, mCAMP−/− mice (unable to combat Gram-positive pathogens) also display abnormal barrier homeostasis. To determine further whether these two functions are co-regulated, we investigated changes in immunostaining for these three AMPs in skin samples in which the permeability barrier function in mice had been either compromised or enhanced. Compromised or enhanced barrier function correlated with reduced or enhanced immunohistochemical expression of mCAMP, respectively, but conversely with Cst expression, likely due to the role of this AMP as an endogenous inhibitor of cathelicidin expression. mBD3 expression correlated with experimental barrier perturbations, but poorly with developmental changes in barrier function. These studies show that changes in cathelicidin and Cst expression parallel changes in permeability barrier status, with a less clear relationship with mBD3 expression.

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INTRODUCTION

The stratum corneum (SC) of mammalian epidermis mediates several critical protective functions (Elias, 2005), two of which, maintenance of permeability barrier homeostasis and cutaneous antimicrobial defense (distal innate immunity), exhibit certain physical–chemical and biochemical features that contribute simultaneously to both functions (Supplementary Table S1 online) (reviewed in Elias (2007), Elias and Steinhoff (2008), and Elias and Schmuth (2009)). For example, the low pH of the SC creates an ecological milieu that is hostile to microbial pathogens, while simultaneously favoring growth of the normal flora (Aly *et al.*, 1978; Korting *et al.*, 1990). Moreover, the highly cohesive and anhydrous characteristics of the normal SC comprise a formidable physical barrier to invading microorganisms, whereas, conversely, pathogens invade between dyshesive corneocytes when the permeability barrier is compromised (Miller *et al.*, 1988; Elias, 2007). Furthermore, certain lipids that are required for the permeability barrier, such as free fatty acids of both epidermal (Miller *et al.*, 1988; Drake *et al.*, 2008) and sebaceous (Bibel *et al.*, 1989; Georgel *et al.*, 2005) origin, as well as the sphingoid bases of ceramides, also exhibit potent antimicrobial activity. Thus, the increased residence of *Staphylococcus aureus* and other pathogens on lesions of atopic dermatitis (AD) could be explicable not only by alterations in barrier function and innate immunity (Radek and Gallo, 2007), but also by the (i) high pH (cited in Hatano *et al.*, 2009); (ii) lipid-depleted extracellular matrix (Chamlin *et al.*, 2002); (iii) reduced free fatty acid/sphingosine content (Arikawa *et al.*, 2002; Proksch *et al.*, 2003); and (iv) poor cohesion (Cork *et al.*, 2006) of the SC in lesional AD. Notably, the cathelicidin protein, hCAP18, and its carboxy-terminal peptide, LL-37, also are downregulated in lesional AD, which is explicable by the increased T helper type 2 signaling (Howell, 2007) and/or excess serine protease activity (Morizane *et al.*, 2010).

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Abbreviations: AD, atopic dermatitis; AMP, antimicrobial peptide; Cst, catestatin; IMQ, imiquimod; mCAMP, mouse cathelicidin antimicrobial peptide; PS, psychological stress; SC, stratum corneum

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The link between permeability barrier status and antimicrobial defense is shown not only by their shared physical and biochemical characteristics, but also by the fact that acute perturbations in permeability barrier function stimulate metabolic responses that rapidly restore permeability barrier homeostasis in parallel with enhanced antimicrobial peptide (AMP) expression, e.g., mouse cathelicidin AMP (mCAMP), mouse β-defensin 3 (mBD3), catestatin (Cst), RNase 7, and psoriasin production all increase rapidly after acute barrier disruption (Elias and Choi, 2005; Aberg *et al.*, 2008; Radek *et al.*, 2008; Glaser *et al.*, 2009a). Conversely, mCAMP knockout mice display abnormal permeability barrier homeostasis, demonstrating that cathelicidins are required for normal permeability barrier function (Aberg *et al.*, 2008). Notably, both the lipids that mediate permeability barrier function (Grayson *et al.*, 1985), and at least three AMPs, i.e., mCAMP (LL-37), mBD3(hBD2), and Cst, were expressed in the outer epidermis. Moreover, both mCAMP (LL-37) and mBD3 (hBD2) are cargoes within the epidermal lamellar bodies (Oren *et al.*, 2003; Braff *et al.*, 2005; Aberg *et al.*, 2007). Hence, their colocalization and presumed co-secretion insures that constituents of both the permeability and antimicrobial barriers are delivered in parallel to SC extracellular domains.

Our results suggest close, bidirectional changes in mCAMP expression under a variety of conditions where permeability barrier function is either compromised or enhanced, but an apparent, converse relationship with Cst expression, which could reflect its function as a β-muscarinic inhibitor of cathelicidin expression (Radek *et al.* (2010) and cited therein).

RESULTS

Permeability barrier status in various models

In normal mice, acute abrogations of the epidermal permeability barrier function, induced by either organic solvent applications or repeated tape strippings, provoke a transient decline in AMP levels, followed by rapid upregulation of the expression of several AMPs, i.e., mCAMP, mBD3, Cst, and psoriasin, over 2–6 hours in parallel with barrier restoration (Schroder and Harder, 2006; Aberg *et al.*, 2008; Radek *et al.*, 2008; Glaser *et al.*, 2009a). In these studies, we assessed changes in AMP expression in four situations in which permeability barrier homeostasis is subnormal, i.e., after sustained *psychological stress* (PS) (Denda *et al.*, 1998; Choi *et al.*, 2006), in young adult male mice (testosterone replete) (Kao *et al.*, 2001), after *erythemogenic* UVB exposure (Haratake *et al.*, 1997), and in *chronologically* (*intrinsically*) *aged epidermis* (Choi *et al.*, 2007). As our previous studies showed that PS downregulates both mCAMP and mBD3 expression (Aberg *et al.*, 2008), samples from PS mice served as positive controls for the other models. AMP status also was assessed in a library of paraffin-embedded materials from our previously published studies where permeability barrier homeostasis had been altered either experimentally or developmentally (Kao *et al.*, 2001) (Table 1) in testosterone-replete and chronologically aged mice. In these studies, erythemogenic UVB induced a dose- and time-dependent abnormality in permeability barrier function (see below), as reported previously (Haratake *et al.*, 1997).

Table 1. Changes in barrier function in various mouse models

	Basal barrier function	Barrier recovery kinetics
Barrier perturbant		
Psychological stress	Declines[1,2]	Delayed[1,2]
Testosterone replete (male)	Declines[3]	Delayed[3]
Erythemogenic UVB (5–10 MED)	See Figure 5b[4]	Delayed[4]
Intrinsic aging	Declines[5,6]	Delayed[6]
Improved barrier		
Suberythemogenic UVB	Improves[7]	Accelerates[7]
Calcipotriol	Improves[8]	N/D
Endogenous GC blockade	Improves[9]	Accelerates[1,2]
Imiquimod	Improves[10]	Accelerates[10]
Triple lipids	Improves[11]	Accelerates[11]
Petrolatum	N/D	Accelerates[11]
PPARα	No changes	Accelerates[12]
LXR	No changes	Partially normalizes[13]
Chinese herbal mixture	No changes	Accelerates[14]
Urea	Improves[15]	N/D

Abbreviations: GC, glucocorticoid; LXR, liver X receptor; MED, minimal erythema doses; N/D, not demonstrated; PPAR, PP activated receptor.
[1]Denda *et al.* (2000).
[2]Choi *et al.* (2006).
[3]Kao *et al.* (2001).
[4]Haratake *et al.* (1997).
[5]Ghadially *et al.* (1995).
[6]Choi *et al.* (2007).
[7]Hong *et al.* (2008).
[8]Bikle *et al.* (2010).
[9]Aberg *et al.* (2007).
[10]Barland *et al.* (2004).
[11]Man *et al.* (1995). *Arch Dermatol* 131:809–16.
[12]Man *et al.* (2004).
[13]Komuves *et al.* (2002). *J Invest Dermatol* 118:25–34.
[14]Man *et al.* (2011).
[15]Grether-Beck *et al.* (2011).

Compromised permeability barrier function correlates closely with decreased mCAMP expression

Psychological stress. As reported previously, immunostaining for both mCAMP and mBD3 declined following PS (Figure 1a; see also Aberg *et al.*, 2007). Moreover, we now show further that Cst immunostaining also declines after short-term PS (i.e., 24–36 hours), but Cst instead appears to normalize, or even supernormalize, following exposure to more prolonged periods of PS (4 days of restraint) (Figure 1b).

Androgen status (gender). Previous studies have shown that testosterone-replete (adult) mice and humans display normal

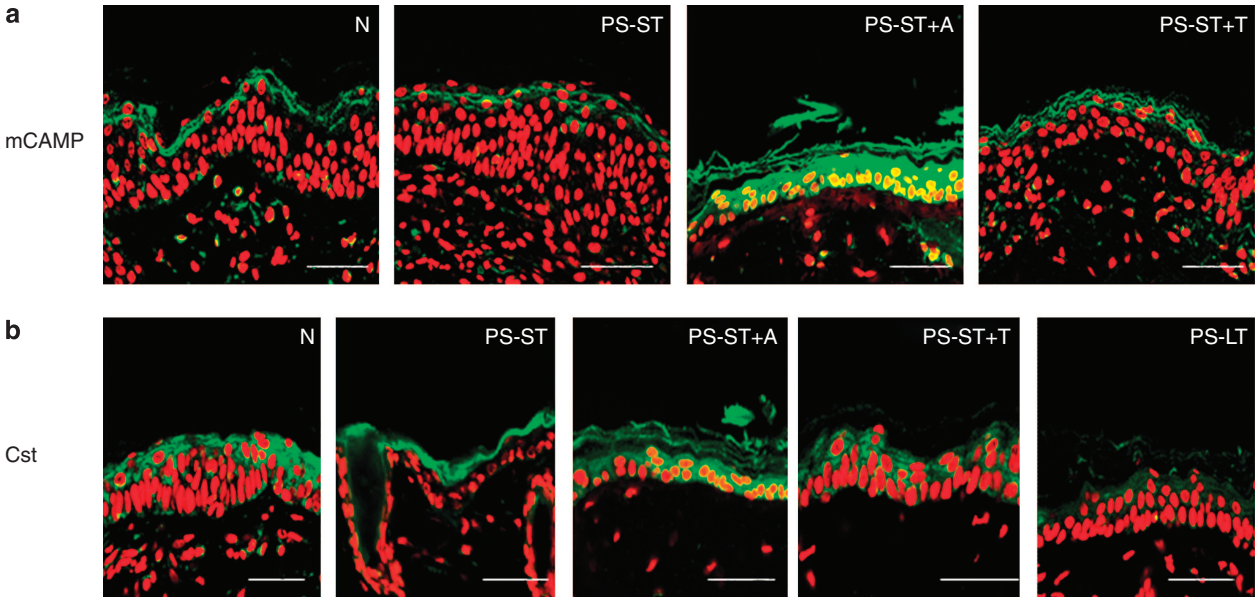

Figure 1. Psychological stress (PS) decreases immunostaining for mouse cathelicidin antimicrobial peptide (mCAMP), β-defensin 3 (mBD3), and catestatin (Cst) in both a glucocorticoid (GC)- and a β-adrenergic-dependent manner. Hairless mice ($n = 4$ or 5 each) were exposed to either insomnia-induced PS for 36–48 hours (short-term PS, PS-ST) or restraint-induced stress for 96 hours (long-term PS, PS-LT), while parallel groups of PS mice ($n = 4$ or 5 each) were co-treated with intraperitoneal antalarmin or Ru486 (not shown; see Aberg *et al.*, 2008), or topical timolol (T) (0.38% in saline) (see Materials and Methods for further details). In all, 5 μM frozen sections were labeled with primary antibodies against mCAMP, mBD3, or Cst. Propidium iodide was used to counterstain the nuclei. Green immunostaining represents AMP labeling. Bar = 40 μm. A, antalarmin; N, normal; red staining, propidium iodide.

basal barrier function, but delayed permeability barrier recovery (Kao *et al.*, 2001) (Table 1). Therefore, we next compared epidermal mCAMP, mBD3, and Cst immunostaining in library skin samples from young adult male versus female mice. Although male mice displayed a marked decline in immunostaining for mCAMP, they appeared to display a modest enhancement of immunostaining for mBD3, and a marked increase in Cst expression (Supplementary Figure S1 online). These results suggest that the decline in permeability barrier with testosterone repletion is paralleled by a concomitant reduction in mCAMP, whereas mBD3 and Cst expression instead appear to increase in androgen-replete males.

Erythemogenic UVB. Although *suberythemogenic* doses of UVB have been shown previously to enhance permeability barrier function (Hong *et al.*, 2008), *erythemogenic* UVB instead provokes a transient, delayed (by 48–96 hours), and dose-dependent barrier abnormality, as we reported previously (Haratake *et al.*, 1997) (Figure 2). Therefore, we next examined whether erythemogenic UVB produces parallel alterations in AMP expression in mice. In these studies, the intensity of AMP immunostaining was quantitated by a blinded observer on multiple, pooled, coded images at each time point. Erythemogenic UVB (5 minimal erythema doses (MED)) resulted in a progressive decline in mCAMP levels, which returned to normal at day 5 (Figure 2; Supplementary Figure S2A online). In contrast, erythemogenic UVB did not alter mBD3 immunostaining (Supplementary Figure S2B online), whereas it simultaneously stimulated a sustained increase in Cst expression immediately after exposure, with immunostaining remaining elevated until day 5, when it began to decline (Figure 2; Supplementary Figure S2C online). Taken together, these results suggest that the transient defect in permeability barrier function, which resulted from erythemogenic UVB irradiation, is paralleled by a marked decline in mCAMP, a minimal decline in mBD3, but a marked enhancement of Cst expression.

Chronologically aged mouse skin. Permeability barrier homeostasis progressively declines during chronologic aging (Ghadially *et al.*, 1995; Choi *et al.*, 2007) (Table 1). Therefore, we next examined age-related abnormalities in AMP expression in library tissue samples from young versus moderately aged mouse epidermis (15–18 months), analogous to human age 50–65 years (Choi *et al.*, 2007). Under basal conditions, the epidermis of young mice displayed low constituent levels of immunostaining for both mCAMP and mBD3, with a prominent decline in mCAMP immunostaining in chronologically aged mouse epidermis. In contrast, both mBD3 and Cst levels appeared to markedly increase in aged mouse epidermis (Supplementary Figure S3 online).

Improved permeability barrier function correlates with enhanced mCAMP expression

Imiquimod and calcipotriol treatment. Both the immune enhancer, imiquimod (IMQ), and the 1,25$(OH)_2$ vitamin D3 analog, calcipotriol, improve barrier function under a variety of experimental and clinical conditions (Barland *et al.*, 2004). Therefore, we next delineated the effects of repeated applications of topical IMQ or calcipotriol on mCAMP expression in normal mouse epidermis. Untreated murine

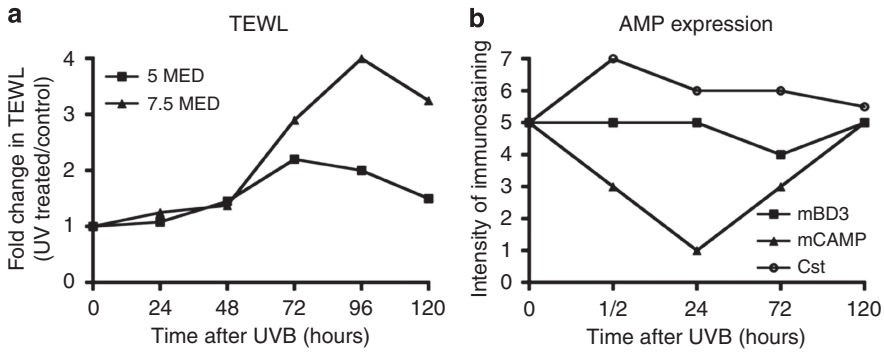

Figure 2. Quantitation of decline in mouse cathelicidin antimicrobial peptide (mCAMP) immunostaining parallels development of a permeability barrier abnormality. (**a**) UVB-induced changes in permeability barrier function are modified from Haratake *et al.* (1997). (**b**) Micrographs (⩾10 each) from mice treated with erythemogenic UVB ($n=4$, as in Supplementary Figure S2 online) were coded, randomized, and graded according to the intensity of staining for mCAMP, β-defensin 3 (mBD3), and catestatin (Cst) by a blinded observer. AMP, antimicrobial peptide; MED, minimal erythema doses; TEWL, transepidermal water loss.

epidermis again clearly demonstrated low, but readily detectable immunostaining for both mCAMP and mBD3, localized to the outer epidermis (Aberg *et al.*, 2007, 2008). Although both IMQ and calcipotriol treatments appeared to increase immunostaining for mBD-3 and mCAMP in comparison to vehicle alone, the increase in mCAMP appeared to be greater than that achieved in parallel, calcipotriol-treated mice (Supplementary Figure S4A vs. S4B online). The increase in mCAMP and mBD3 in calcipotriol- and IMQ-treated mice displays a linear pattern in the SC, corresponding to membrane domains, and it also further localized to vesicles in the cytosol of stratum granulosum cells (Supplementary Figure S4B online, inset, arrows), consistent with its known localization in epidermal lamellar bodies (Oren *et al.*, 2003; Aberg *et al.*, 2007). Finally, we examined AMP expression after several other unrelated maneuvers previously shown to enhance barrier function. In each of these examples, mCAMP expression inevitably increased, but mBD3 and Cst did not always change in parallel (Table 2). Taken together, these results demonstrate first that IMQ and calcipotriol treatment appear to increase the expression of both mCAMP and mBD3 in the outer epidermis. Second, several other, unrelated approaches that improve barrier function also enhance mCAMP expression, with more variable results for mBD3 and Cst (not shown).

Table 2. Changes in antimicrobial peptide expression in relation to altered barrier function

	AMP expression		
Permeability barrier status	**mCAMP**	**mBD3**	**Cst**
Decreased			
PS	↓	↓	↑
Exogenous GC	↓	↓	N/D
Testosterone replete	↓	No change	No change
Erythemogenic UVB	↓	(↓)	↑↑
Aging	↓	↑	↑
Increased			
PS+Ru486/antalarmin	↑[1]	↑	↑
Suberythemogenic UVB	↑[2]	↑[2]	N/D
Imiquimod	↑	↑	N/D
Chinese herbal medicine	↑	↑	N/D
Calcipotriol	↑	↑	N/D
Urea	↑	↑	N/D

Abbreviations: AMP, antimicrobial peptide; Cst, catestatin; GC, glucocorticoid; mBD3, mouse β-defensin 3; mCAMP, mouse cathelicidin antimicrobial peptide; N/D, not demonstrated; PS, psychological stress.
[1]Aberg *et al.* (2007).
[2]Hong *et al.* (2008).

DISCUSSION

We addressed here the hypothesis that permeability barrier function and antimicrobial defense are integrated and co-regulated functions (Elias, 2005), examining whether experimental perturbations or developmental changes that either reduce or enhance permeability barrier status are accompanied by parallel changes in epidermal AMP expression. The impetus for these studies came first from a previous work that showed that these two functions are co-regulated and interdependent in the normal epidermis (Aberg *et al.*, 2008; Hong *et al.*, 2008; Proksch *et al.*, 2008) and that at least one perturbant of the permeability barrier (PS) downregulates mBD3 and mCAMP expression (Aberg *et al.*, 2007). Several studies have already shown that the converse is true; e.g., epidermal AMP expression, including Cst expression (Radek *et al.*, 2008), increased after acute barrier insults in parallel with barrier recovery (Elias and Choi, 2005; Aberg *et al.*, 2008) and after blockade of both glucocorticoid production and action in PS mice (Aberg *et al.*, 2007). We extend these previous observations here by showing not only that short-term PS reduces mCAMP (LL-37) and mBD3 (hBD2) expression, but also that as PS is prolonged, Cst expression also begins to decline.

Our results provide several additional examples to support a putative relationship between permeability barrier and

antimicrobial status, at least for mCAMP. *Testosterone depletion* by either surgical or medical means improves permeability barrier function, whereas conversely, testosterone-replete mice and humans display diminished permeability barrier function (Kao *et al.*, 2001). Although we showed here an apparent, parallel decline in mCAMP expression in male versus female mice, immunostaining for both mBD3 and Cst instead appeared to increase in the epidermis of adult male mice. Thus, it is possible that changes in androgen status could impose potentially important variations in cutaneous antimicrobial defense.

Suberythemogenic doses of UVB have been shown to upregulate permeability barrier function and mBD3/mCAMP expression simultaneously (Aberg *et al.*, 2007), further supporting a putative relationship between these two functions. Conversely, we now show that *erythemogenic* doses of UVB that compromise permeability barrier function (Haratake *et al.*, 1997) also markedly appear to downregulate mCAMP, but produce only minimal, transient alteration in mBD3 expression. The progressive decline (and recovery) of mCAMP expression parallels the time course over which the permeability barrier defect evolves and then recovers. Yet, Cst expression instead appeared to increase at all time points after erythemogenic UVB irradiation. Our previous studies showed that the UVB-induced permeability barrier abnormality correlates with passage of a band of secretion-incompetent, apoptotic cells through the stratum granulosum–SC interface (Holleran *et al.*, 1997). Although such a toxic mechanism could contribute to the observed decline in production of mCAMP, it certainly did not impede Cst expression. Thus, toxicity alone likely cannot account for the selective decline in mCAMP expression after UVB irradiation. The anti-parallel changes in Cst can be explained instead by its role as a muscarinic inhibitor of cathelicidin expression (Radek *et al.*, 2008). Thus, these studies further support a close link between UVB-induced changes in permeability barrier function and cathelicidin expression. Furthermore, together with the work of Hong *et al.* (2008) on suberythemogenic UVB, these studies provide potential clinical implications on how UVB irradiation should be deployed for the treatment of inflammatory dermatoses. Although current recommendations propose ''pushing'' UVB phototherapy doses upwards into the erythemogenic range, this approach clearly could pose adverse consequences not only for permeability barrier function but also for cutaneous antimicrobial defense.

Permeability barrier function begins to decline in adult humans above the age of 50 years (Choi *et al.*, 2007), becoming further compromised above age 75 years (Ghadially *et al.*, 1995), and/or with superimposed photoaging (Reed *et al.*, 1997). We showed here that 15–18-month-old mice, analogous to humans over age 50 years, displayed reduced mCAMP levels, whereas both mBD3 and Cst immunostaining instead appeared to increase in this age group (Table 2). In attempting to explain these and other divergent results for mCAMP versus mBD3, it should be noted that these families of AMPs are regulated by entirely different mechanisms (Oren *et al.*, 2003; Braff *et al.*, 2005; Choi *et al.*, 2005; Aberg *et al.*, 2007; Peric *et al.*, 2008; Eyerich *et al.*, 2009). Although endogenous $1,25(OH)_2$ vitamin D3 and other VDR ligands regulate cathelicidin expression (Zasloff, 2005; Elias, 2007; Aberg *et al.*, 2008; Drake *et al.*, 2008; Hong *et al.*, 2008; Schauber and Gallo, 2008), a variety of cytokines instead stimulate β-defensin production (Nomura *et al.*, 2003; Elias and Choi, 2005; de Jongh *et al.*, 2005; Yano *et al.*, 2008; Kobayashi *et al.*, 2009). Accordingly, endogenous vitamin D levels typically decline with age (Holick, 1987), perhaps accounting for the decrease in mCAMP levels that were observed here in aged murine epidermis. In contrast, cytokine levels vary widely during aging (Ye *et al.*, 1999; Corsini *et al.*, 2009), but IL-1α levels in particular decline with chronologic aging, and are associated with decreased epidermal lipid production (Ye *et al.*, 1999; Barland *et al.*, 2005). In contrast, other epidermal cytokines (e.g., TNFα) increase in the aged epidermis (Corsini *et al.*, 2009), consistent with our observation that mBD3 immunostaining persists or even increases in moderately aged mouse skin. The basis for the apparent, age-related increase in Cst expression is unclear at present, but it could again be related to the role of this neuropeptide as an endogenous inhibitor of cathelicidin production. Whether further abnormalities in antimicrobial defense occur in the moderately aged epidermis and/or with still more-advanced aging and/or photoaging is not yet known. Nevertheless, these age-related differences in AMP expression, which do not strictly parallel changes in permeability barrier status, could also have important clinical implications, as they suggest that cutaneous antimicrobial defense becomes compromised relatively early during the aging process.

We also examined here the opposite situation, asking whether maneuvers that are known to enhance barrier function also upregulate AMP expression. The immune response modifier, IMQ, acts through two members of the Toll-like receptor family, Toll-like receptor 7 and/or 8, which recognize microbial pathogens or their metabolic products and function as primary sensors of the innate immune system (Ambach *et al.*, 2004; Sauder, 2004; Lai and Gallo, 2008). These Toll-like receptors are cell surface receptors that, when activated, stimulate production of epidermis-derived, IFN-α, TNF, and IL-1α (Sauder, 1990; Barland *et al.*, 2004; McInturff *et al.*, 2005; Takeuchi and Akira, 2009). We have shown that topical IMQ enhances barrier function in normal and aged epidermis, by stimulating IL-1α production, which in turn stimulates epidermal lipid synthesis (Ye *et al.*, 1999; Barland *et al.*, 2004). As human β-defensins are upregulated by multiple cytokines, it is highly likely that hBD2 (mBD3) upregulation by topical IMQ is signalled by epidermal production of cytokines. Yet, although the apparent increase in mBD3 immunostaining after calcipotriol treatment was unexpected, it could be linked to the well-known effects of VDR ligands on epidermal differentiation (Bikle *et al.*, 2010). Finally, we examined changes in AMP expression in two other unrelated situations where barrier function is enhanced, i.e., after treatment with topical 5–20% urea (Grether-Beck *et al.*, 2011) and after topical applications of Chinese herbal medications to normal mouse skin (Man *et al.*, 2011). In both

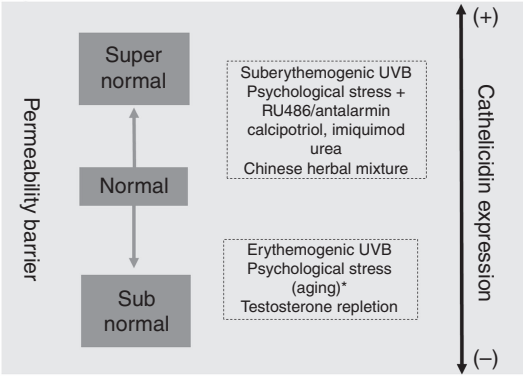

Figure 3. Summary of results—maneuvers that alter barrier functions are paralleled by bidirectional changes in cathelicidin expression. *Parentheses indicate changes in CAMP, but not other AMPs. AMP, antimicrobial peptide; CAMP, cathelicidin antimicrobial peptide.

of these situations, mCAMP expression increased in parallel with enhanced permeability barrier function (Figure 3 and Table 2).

MATERIALS AND METHODS

Models with compromised permeability function

Psychological stress. Our previous studies have shown that both sustained PS and exogenous glucocorticoids downregulate barrier function (Denda *et al.*, 2000; Choi *et al.*, 2005) in parallel with reduced expression of mCAMP and mBD3 (Aberg *et al.*, 2007). Hence, library biopsy samples from PS (short-term) served as positive controls for the additional conditions studied here, where barrier function also is compromised.

Male hairless mice (Skh1/hr) were purchased from Charles River Laboratories (Wilmington, MA). To assess the effects of more long-term PS, animals were placed in motion-restricted environments for 12 hours once daily during nighttime for 72–96 hours. Food and water were restricted in parallel in a control non-motion-restricted group. A plastic container (4.0 (W) × 3.0 (H) × 11.5 (L) cm^3) with mesh walls on the top was used for PS environments, of which the inner space was minimized to allow animals to rotate their bodies. All animals were studied between 8 and 10 weeks of age. The animal experiments described in this study were conducted in accordance with accepted standards of humane animal care, under the protocols approved by the local institutional animal care and use committee at the San Francisco VA Medical Center.

Testosterone-replete (adult male vs. adult female) mice. To assess the impact of physiological levels of testosterone, previously shown to compromise permeability barrier function (Kao *et al.*, 2001), we compared AMP expression in library samples of adult male versus female mice (aged 8–10 weeks; $n=4$ each), processed for immunofluorescence, as described below. Serum testosterone levels were $>500\,pg\,ml^{-1}$ in the male animals and $<200\,pg\,ml^{-1}$ in the female animals (Kao *et al.*, 2001).

Erythemogenic UVB exposure. Hairless, 8–10-week-old female hairless mice were purchased from Charles River Laboratories (Wilmington, MA), and fed Purina mouse diet (Ralston Purina, St Louis, MO) and water *ad libitum*. Natural sunlight was excluded and animals were exposed only to low levels of incandescent light before UVB irradiation. UVB irradiation was delivered with Phillips TL20W/12 fluorescent lamps (Eindhoven, The Netherlands), emitting 280–320 nm. The dorsal skin of each mouse was either sham irradiated or irradiated with single-dose equivalents of either 5 or 10 minimal erythemal doses ($n=5$ each). One minimal erythema dose, determined previously on the same strain of mice, equals approximately $20\,mJ\,cm^{-2}$ ($60–100\,mJ\,cm^{-2}\,hour^{-1}$ equals 1 minimal erythema dose in human beings with type II/III pigmentation). In all, 20 animals were treated in each group, and samples were taken before, immediately after, and then 1, 3, and 5 days following UVB exposure, followed by processing for immunofluorescence studies (see below).

Chronologically aged mouse skin. Epidermal AMP expression was compared in library samples from aged (15–18 months, equivalent to an age range of 50–60 years in humans) versus young adult (3–4 months) hairless mice (Skh1; Jackson Labs, Bar Harbor, ME; $n=4$ each) (Choi *et al.*, 2007). The analogous age of mice and humans was determined from optimal life spans (≈120 years in humans and 24 months in mice). Hairless mice began to display a progressive permeability barrier abnormality after 15 months (Ghadially *et al.*, 1995; Choi *et al.*, 2007).

Models with enhanced permeability barrier function

Not only blockade of glucocorticoid production/action (Aberg *et al.*, 2007), but also suberythemogenic UVB irradiation has already been shown to stimulate mCAMP and mBD3 production (Hong *et al.*, 2008; Glaser *et al.*, 2009b) (Table 1). Here, we assessed changes in mCAMP expression after several additional approaches that enhance barrier function. We focused on changes in mCAMP in this subset of studies, because it most closely paralleled changes in barrier status in the previously assessed models with reduced function.

IMQ and calcipotriol. Previous studies have shown that both 1,25 $(OH)_2$ vitamin D3 and its analogs (Bikle, 2010), as well as IMQ (Barland *et al.*, 2004), enhance barrier function in a variety of settings. The dorsal skin of each mouse was treated with topical IMQ (Aldara, Bristol, TN) 5% cream, calcipotriol (Dovonex, Rockaway, NJ) cream $50\,\mu g\,g^{-1}$, or vehicle two times daily for 7 days ($n=4$ mice in each group). Parallel control groups of hairless mice were treated with the vehicles for the equivalent drug alone at the same time points.

Library biopsy samples from comparable cohorts of 4–5 normal hairless mice each also were assessed after following approaches that are known to enhance barrier function.

Chinese herbal mixture and urea. We recently showed that various Chinese herbal mixtures improve barrier function in normal hairless mice (Man *et al.*, 2011). Recent studies also have shown that topical urea at concentrations ⩾5% improves barrier function in normal human and mouse skin (Grether-Beck *et al.*, 2011).

Tissue processing and immunofluorescence

Biopsy specimens for immunostaining were obtained at time points when maximal changes in barrier function occurred (see figure and table legends, as well as cited references for further details). Full-thickness skin biopsy specimens, which had either been snap-frozen

in liquid nitrogen or library samples embedded in paraffin, were utilized for immunofluorescence studies. Frozen sections (5 μm) were soaked in acetone for 10 minutes, washed in phosphate-buffered saline (PBS), and blocked with 4% BSA and 0.5% cold-water fish gelatin in PBS for 30 minutes. In all, 10 μm paraffin-embedded tissue sections were de-paraffinized, rehydrated, and then rinsed with de-ionized water, followed by three washes in PBS. Sections were incubated for 30 minutes in blocking buffer (4% BSA, 0.5% cold water fish gelatin in PBS), and then incubated overnight at 4 °C with the primary antibodies in blocking buffer. The next morning, sections were washed three times in PBS and incubated for 40 minutes at room temperature with the Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody, diluted 1:2,000 in blocking buffer. Slides were then incubated overnight at 4 °C with primary antibodies (1:500 or 1:1,000) against Cst (from Phoenix Labs, Phoenix, AZ, and Richard Gallo, University of California, San Diego, San Diego, CA), mBD-3 (Alpha Diagnostics, Owings Mills, MD), or mCAMP (from Dr Richard Gallo, UCSD), followed by incubation with FITC-conjugated, goat anti-rabbit secondary antibody (Alpha Diagnostics) for 45 minutes at room temperature, as described (Aberg *et al.*, 2007; Radek *et al.*, 2008). Sections were counterstained with propidium iodide and visualized on a Leica TCS-SP laser confocal microscope at excitation and emission wavelengths of 488 and 532 nm, respectively, photographed at original magnification ×40, and the intensity of AMP immunostaining was scored blindly in randomly mixed micrographs ($n = 20$ in each group) as 0 (subnormal), 1 (normal = basal), or 2–5 (increased, with 5 = most intense, antigen-positive immunostaining). Sections labeled with only the secondary antibody, and/or sections from mCAMP knockout mice (Nizet *et al.*, 2001; Aberg *et al.*, 2008), served as controls.

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

REFERENCES

Aberg KM, Man MQ, Gallo RL *et al.* (2008) Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J Invest Dermatol* 128:917–25

Aberg KM, Radek KA, Choi EH *et al.* (2007) Psychological stress down-regulates epidermal antimicrobial peptide expression and increases severity of cutaneous infections in mice. *J Clin Invest* 117:3339–49

Aly R, Shirley C, Cunico B *et al.* (1978) Effect of prolonged occlusion on the microbial flora, pH, carbon dioxide and transepidermal water loss on human skin. *J Invest Dermatol* 71:378–81

Ambach A, Bonnekoh B, Nguyen M *et al.* (2004) Imiquimod, a Toll-like receptor-7 agonist, induces perforin in cytotoxic T lymphocytes *in vitro*. *Mol Immunol* 40:1307–14

Arikawa J, Ishibashi M, Kawashima M *et al.* (2002) Decreased levels of sphingosine, a natural antimicrobial agent, may be associated with vulnerability of the stratum corneum from patients with atopic dermatitis to colonization by *Staphylococcus aureus*. *J Invest Dermatol* 119:433–9

Barland CO, Elias PM, Ghadially R (2005) The aged epidermal barrier: basis for functional abnormalities. In: *Skin Barrier*. (Elias PM, Feingold KR, eds) Vol New York: Marcel Dekker, 535–52

Barland CO, Zettersten E, Brown BS *et al.* (2004) Imiquimod-induced interleukin-1 alpha stimulation improves barrier homeostasis in aged murine epidermis. *J Invest Dermatol* 122:330–6

Bibel DJ, Miller SJ, Brown BE *et al.* (1989) Antimicrobial activity of stratum corneum lipids from normal and essential fatty acid-deficient mice. *J Invest Dermatol* 92:632–8

Bikle DD (2010) Vitamin D and the skin. *J Bone Miner Metab* 28:117–30

Bikle DD, Teichert A, Arnold LA *et al.* (2010) Differential regulation of epidermal function by VDR coactivators. *J Steroid Biochem Mol Biol* 121:308–13

Braff MH, Di Nardo A, Gallo RL (2005) Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. *J Invest Dermatol* 124:394–400

Chamlin SL, Kao J, Frieden IJ *et al.* (2002) Ceramide-dominant barrier repair lipids alleviate childhood atopic dermatitis: changes in barrier function provide a sensitive indicator of disease activity. *J Am Acad Dermatol* 47:198–208

Choi EH, Brown BE, Crumrine D *et al.* (2005) Mechanisms by which psychologic stress alters cutaneous permeability barrier homeostasis and stratum corneum integrity. *J Invest Dermatol* 124:587–95

Choi EH, Demerjian M, Crumrine D *et al.* (2006) Glucocorticoid blockade reverses psychological stress-induced abnormalities in epidermal structure and function. *Am J Physiol Regul Integr Comp Physiol* 291:R1657–62

Choi EH, Man MQ, Xu P *et al.* (2007) Stratum corneum acidification is impaired in moderately aged human and murine skin. *J Invest Dermatol* 127:2847–56

Cork MJ, Robinson DA, Vasilopoulos Y *et al.* (2006) New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene–environment interactions. *J Allergy Clin Immunol* 118:3–21; quiz 22–3

Corsini E, Racchi M, Lucchi L *et al.* (2009) Skin immunosenescence: decreased receptor for activated C kinase-1 expression correlates with defective tumour necrosis factor-alpha production in epidermal cells. *Br J Dermatol* 160:16–25

de Jongh GJ, Zeeuwen PL, Kucharekova M *et al.* (2005) High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis. *J Invest Dermatol* 125:1163–73

Denda M, Tsuchiya T, Elias PM *et al.* (2000) Stress alters cutaneous permeability barrier homeostasis. *Am J Physiol Regul Integr Comp Physiol* 278:R367–72

Denda M, Tsuchiya T, Hosoi J *et al.* (1998) Immobilization-induced and crowded environment-induced stress delay barrier recovery in murine skin. *Br J Dermatol* 138:780–5

Drake DR, Brogden KA, Dawson DV *et al.* (2008) Thematic review series: skin lipids. Antimicrobial lipids at the skin surface. *J Lipid Res* 49:4–11

Elias PM (2005) Stratum corneum defensive functions: an integrated view. *J Invest Dermatol* 125:183–200

Elias PM (2007) The skin barrier as an innate immune element. *Sem Immunopathol* 29:3–14

Elias PM, Choi EH (2005) Interactions among stratum corneum defensive functions. *Exp Dermatol* 14:719–26

Elias PM, Schmuth M (2009) Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 9:437–46

Elias PM, Steinhoff M (2008) ''Outside-to-inside'' (and now back to ''outside'') pathogenic mechanisms in atopic dermatitis. *J Invest Dermatol* 128:1067–70

Eyerich K, Pennino D, Scarponi C *et al.* (2009) IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J Allergy Clin Immunol* 123:59–66 e4

Georgel P, Crozat K, Lauth X *et al.* (2005) A toll-like receptor 2-responsive lipid effector pathway protects mammals against skin infections with Gram-positive bacteria. *Infect Immun* 73:4512–21

Ghadially R, Brown BE, Sequeira-Martin SM *et al.* (1995) The aged epidermal permeability barrier. Structural, functional, and lipid biochemical abnormalities in humans and a senescent murine model. *J Clin Invest* 95:2281–90

Glaser R, Meyer-Hoffert U, Harder J *et al.* (2009a) The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. *J Invest Dermatol* 129:641–9

Glaser R, Navid F, Schuller W *et al.* (2009b) UV-B radiation induces the expression of antimicrobial peptides in human keratinocytes *in vitro* and *in vivo*. *J Allergy Clin Immunol* 123:1117–23

Grayson S, Johnson-Winegar AG, Wintroub BU *et al.* (1985) Lamellar body-enriched fractions from neonatal mice: preparative techniques and partial characterization. *J Invest Dermatol* 85:289–94

Grether-Beck S, Felsner I, Brenden H *et al.* (2011) Urea uptake enhances barrier function and antimicrobial defense in humans by regulating epidermal gene expression. *J Invest Dermatol* (in press)

Haratake A, Uchida Y, Schmuth M *et al.* (1997) UVB-induced alterations in permeability barrier function: roles for epidermal hyperproliferation and thymocyte-mediated response. *J Invest Dermatol* 108:769–75

Hatano Y, Man MQ, Uchida Y *et al.* (2009) Maintenance of an acidic stratum corneum prevents emergence of murine atopic dermatitis. *J Invest Dermatol* 129:1824–35

Holick MF (1987) Photosynthesis of vitamin D in the skin: effect of environmental and life-style variables. *Fed Proc* 46:1876–82

Holleran WM, Uchida Y, Halkier-Sorensen L *et al.* (1997) Structural and biochemical basis for the UVB-induced alterations in epidermal barrier function. *Photodermatol Photoimmunol Photomed* 13:117–28

Hong SP, Kim MJ, Jung MY *et al.* (2008) Biopositive effects of low-dose UVB on epidermis: coordinate upregulation of antimicrobial peptides and permeability barrier reinforcement. *J Invest Dermatol* 128:2880–7

Howell MD (2007) The role of human beta defensins and cathelicidins in atopic dermatitis. *Curr Opin Allergy Clin Immunol* 7:413–7

Kao JS, Garg A, Mao-Qiang M *et al.* (2001) Testosterone perturbs epidermal permeability barrier homeostasis. *J Invest Dermatol* 116:443–51

Kobayashi M, Yoshiki R, Sakabe J *et al.* (2009) Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br J Dermatol* 160:297–304

Komuves LG, Schmuth M, Fowler AJ *et al.* (2002) Oxysterol stimulation of epidermal differentiation is mediated by liver X receptor-beta in murine epidermis. *J Invest Dermatol* 118:25–34

Korting HC, Hubner K, Greiner K *et al.* (1990) Differences in the skin surface pH and bacterial microflora due to the long-term application of synthetic detergent preparations of pH 5.5 and pH 7.0. Results of a crossover trial in healthy volunteers. *Acta Derm Venereol* 70:429–31

Lai Y, Gallo RL (2008) Toll-like receptors in skin infections and inflammatory diseases. *Infect Disord Drug Targets* 8:144–55

Man M, Hupe M, Mackenzie D *et al.* (2011) A topical Chinese herbal mixture improves epidermal permeability barrier function in normal murine skin. *Exp Dermatol* 20:285–8

Man MQ, Fowler AJ, Schmuth M *et al.* (2004) Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *J Invest Dermatol* 123:305–12

McInturff JE, Modlin RL, Kim J (2005) The role of toll-like receptors in the pathogenesis and treatment of dermatological disease. *J Invest Dermatol* 125:1–8

Miller SJ, Aly R, Shinefeld HR *et al.* (1988) *In vitro* and *in vivo* antistaphylococcal activity of human stratum corneum lipids. *Arch Dermatol* 124:209–15

Morizane S, Yamasaki K, Kabigting FD *et al.* (2010) Kallikrein expression and cathelicidin processing are independently controlled in keratinocytes by calcium, vitamin D(3), and retinoic acid. *J Invest Dermatol* 130:1297–306

Nizet V, Ohtake T, Lauth X *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454–7

Nomura I, Goleva E, Howell MD *et al.* (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 171:3262–9

Oren A, Ganz T, Liu L *et al.* (2003) In human epidermis, beta-defensin 2 is packaged in lamellar bodies. *Exp Mol Pathol* 74:180–2

Peric M, Koglin S, Kim SM *et al.* (2008) IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J Immunol* 181:8504–12

Proksch E, Brandner JM, Jensen JM (2008) The skin: an indispensable barrier. *Exp Dermatol* 17:1063–72

Proksch E, Jensen JM, Elias PM (2003) Skin lipids and epidermal differentiation in atopic dermatitis. *Clin Dermatol* 21:134–44

Radek K, Gallo R (2007) Antimicrobial peptides: natural effectors of the innate immune system. *Semin Immunopathol* 29:27–43

Radek KA, Elias PM, Taupenot L *et al.* (2010) Neuroendocrine nicotinic receptor activation increases susceptibility to bacterial infections by suppressing antimicrobial peptide production. *Cell Host Microbe* 7:277–89

Radek KA, Lopez-Garcia B, Hupe M *et al.* (2008) The neuroendocrine peptide catestatin is a cutaneous antimicrobial and induced in the skin after injury. *J Invest Dermatol* 128:1525–34

Reed JT, Elias PM, Ghadially R (1997) Integrity and permeability barrier function of photoaged human epidermis. *Arch Dermatol* 133:395–6

Sauder DN (1990) The role of epidermal cytokines in inflammatory skin diseases. *J Invest Dermatol* 95:27S–8S

Sauder DN (2004) Mechanism of action and emerging role of immune response modifier therapy in dermatologic conditions. *J Cutan Med Surg* 8(Suppl 3):3–12

Schauber J, Gallo RL (2008) The vitamin D pathway: a new target for control of the skin's immune response? *Exp Dermatol* 17:633–9

Schroder JM, Harder J (2006) Antimicrobial skin peptides and proteins. *Cell Mol Life Sci* 63:469–86

Takeuchi O, Akira S (2009) Innate immunity to virus infection. *Immunol Rev* 227:75–86

Yano S, Banno T, Walsh R *et al.* (2008) Transcriptional responses of human epidermal keratinocytes to cytokine interleukin-1. *J Cell Physiol* 214:1–13

Ye J, Calhoun C, Feingold K *et al.* (1999) Age-related changes in the IL-1 gene family and their receptors before and after barrier abrogation. *J Invest Dermatol* 112:543

Zasloff M (2005) Sunlight, vitamin D, and the innate immune defenses of the human skin. *J Invest Dermatol* 125:xvi–i