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ORIGINAL ARTICLE

Serine Protease Activity and Residual LEKTI Expression Determine Phenotype in Netherton Syndrome

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Mutations in the *SPINK5* gene encoding the serine protease (SP) inhibitor, lymphoepithelial-Kazal-type 5 inhibitor (LEKTI), cause Netherton syndrome (NS), a life-threatening disease, owing to proteolysis of the stratum corneum (SC). We assessed here the basis for phenotypic variations in nine patients with "mild", "moderate", and "severe" NS. The magnitude of SP activation correlated with both the barrier defect and clinical severity, and inversely with residual LEKTI expression. LEKTI co-localizes within the SC with kallikreins 5 and 7 and inhibits both SP. The permeability barrier abnormality in NS was further linked to SC thinning and proteolysis of two lipid hydrolases (β-glucocerebrosidase and acidic sphingomyelinase), with resultant disorganization of extracellular lamellar membranes. SC attenuation correlated with phenotype-dependent, SP activation, and loss of corneodesmosomes, owing to desmoglein (DSG)1 and desmocollin (DSC)1 degradation. Although excess SP activity extended into the nucleated layers in NS, degrading desmosomal mid-line structures with loss of DSG1/DSC1, the integrity of the nucleated epidermis appears to be maintained by compensatory upregulation of DSG3/DSC3. Maintenance of sufficient permeability barrier function for survival correlated with a compensatory acceleration of lamellar body secretion, providing a partial permeability barrier in NS. These studies provide a mechanistic basis for phenotypic variations in NS, and describe compensatory mechanisms that permit survival of NS patients in the face of unrelenting SP attack.

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INTRODUCTION

Netherton syndrome (NS) is an autosomal recessive disorder, characterized by scaling ichthyosis, atopic dermatitis, and a diagnostic hair shaft abnormality (''bamboo hairs''), a phenotype that varies dramatically in severity (Griffiths *et al.*, 1989). The genetic defects in NS comprise a mutations in the *SPINK5* gene on chromosome 5q32 resulting in a reduction or loss of expression of a serine protease (SP) inhibitor (SPI), the lymphoepithelial Kazal-type-5 SPI (LEKTI) (Magert *et al.*, 1999; Chavanas *et al.*, 2000; Sprecher *et al.*, 2001), expressed in keratinizing and non-keratinizing epithelia, thymus (Hassall's bodies), tonsils, parathyroid glands, and trachea (Magert *et al.*, 1999; Walden *et al.*, 2002; Bitoun *et al.*, 2003). Decreased LEKTI activity in NS results in unopposed SP activation that compromises stratum corneum (SC) cohesion, resulting in SC thinning (Komatsu *et al.*, 2002), delayed growth, and hypernatremic dehydration, with sometimes fatal outcomes in affected infants (Stoll *et al.*, 2001; Moskowitz *et al.*, 2004). Through elaboration of the consequences of SP/SPI imbalance for disease expression, NS therefore illustrates the critical importance of SP/SPI balance for the regulation of normal epidermal function.

Both variations in disease severity and clinical outcomes in NS relate to the severity of permeability barrier defect (Moskowitz *et al.*, 2004), which could correlate with the extent of unopposed SP activation (Komatsu *et al.*, 2002). Excess SP activity is associated with SC thinning, abnormalities in SC lamellar body (LB) generation, lamellar membrane ultrastructure, and impaired barrier function (Fartasch *et al.*, 1999; Moskowitz *et al.*, 2004). Yet, neither of these observations *per se* provide a mechanistic link between excess SP activity and the putative barrier abnormality. Moreover, whether the variable phenotypes in NS reflect differences in either SP/LEKTI balance or localization, and

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Abbreviations: aSMase, acidic sphingomyelinase; β-GlcCer'ase, β-glucocerebrosidase; DSC, desmocollin; DSG, desmoglein; klk, kallikrein; LB, lamellar body; LEKTI, lymphoepithelial-Kazal-type 5 inhibitor; NS, Netherton syndrome; SC, stratum corneum; SCCE, stratum corneum chymotryptic enzyme; SG, stratum granulosum; SP, serine protease; SPI, serine protease inhibitor

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how SP/LEKTI imbalance affects permeability barrier function and SC integrity remains unknown. Although an attempt to address these questions was made by studies in *SPINK5* knockout mice, where complete loss of LEKTI produces a severe, NS-type phenotype that leads to death shortly after birth (Descargues *et al*., 2005), these studies provided neither insights into the basis for the variable clinical phenotypes in NS nor into compensatory mechanisms that allow survival of most NS patients.

SC contains several types of SP activities, including two SC-specific SP, kallikrein (klk) 5 and klk7, known to regulate desquamation (Caubet *et al*., 2004; Komatsu *et al*., 2005). Whether LEKTI is an inhibitor of both klks, thereby regulating one or both SP activities in SC, remains unknown. Moreover, the described localization of LEKTI to the stratum granulosum (SG), and its absence from the SC (Walden *et al*., 2002; Bitoun *et al*., 2003), diverges from the SC localization of klk5 and klk7 (Hansson *et al*., 1994; Brattsand and Egelrud, 1999; Bitoun *et al*., 2003; Ishida-Yamamoto *et al*., 2005). Thus, the current view of klk *versus* LEKTI localization must be reconciled to reflect the putative enzyme–inhibitor relationships of these proteins.

SP-mediated attenuation of the SC (Stoll *et al*., 2001; Moskowitz *et al*., 2004) and abnormalities in SC lamellar membrane organization (Fartasch *et al*., 1999) could contribute to the barrier abnormality in NS. Yet, the mechanisms whereby unrestricted SP activity produces such abnormalities remains unknown. We have shown previously that prolonged, experimental elevations in the pH of the SC activate SP, resulting in progressive degradation of two key, extracellular, lipid-processing enzymes, β-glucocerebrosidase (β-GlcCer'-ase) and acidic sphingomyelinase (aSMase) (Hachem *et al*., 2005b). As both of these enzymes are required to generate the extracellular lamellar membrane system that mediates the permeability barrier (Holleran *et al*., 1993; Mao-Qiang *et al*., 1996), we hypothesized that an elevated pH would further increase SP activity, providing a second mechanism that could lead to membrane structural and barrier abnormalities in NS.

Therefore, we addressed here several unresolved issues about how SP/LEKTI imbalance and localization determine phenotype severity, as well as the pathogenic basis for the abnormalities in SC integrity/cohesion and permeability barrier function in NS. To gain further insights into the consequences of excess SP activity, we also assessed a transgenic murine model of increased SP activity (klk7 overexpression) that develops an NS-like dermatosis. Finally, we identified two compensatory responses that explain the survival of NS patients, despite the devastating consequences of unopposed SP activity for epidermal homeostasis. These studies provide new insights into the pathogenesis of NS and the importance of maintenance of SP/SPI balance for survival in a terrestrial environment.

RESULTS

SP activation and LEKTI expression are inversely related to phenotype severity in NS

Nine NS subjects displayed ''mild'', ''moderate'', and ''severe'' disease phenotypes (see Table 1 for severity grading based on the criteria described in Ganemo *et al*. (1999), and Table 2 for genotyping). ''Moderate'' and ''severe'' NS subjects displayed a severe permeability barrier abnormality, assessed as rates of transepidermal water loss. Transepidermal water loss levels in the ''mild'' NS subject were comparable to levels of subjects with other inflammatory dermatoses, such as atopic dermatitis and psoriasis. Pertinently, the ''mild'' NS subject displayed the lowest levels of SP activity among all of the NS subjects by *in situ* zymography, an observation that correlated with immunohistochemical evidence of residual LEKTI expression in the outer epidermis in this subject (Figure 1b and f).

Conversely, ''moderate'' and ''severe'' NS cases demonstrated a progressive increase in SP activity with low-to-absent LEKTI expression (Figure 1c, d, g and h). Together, these results demonstrate an inverse correlation between SP activity and residual LEKTI expression that further predicts clinical severity.

Co-localization of SP with LEKTI in normal epidermis, and selective inhibition of both klk5 and klk7 by rLEKTI

As reported previously (Hansson *et al*., 1994; Brattsand and Egelrud, 1999), SP activity predominated in the anucleated SC layers of normal human epidermis (Figure 1e). Whereas prior immunohistochemical studies claimed that LEKTI is expressed primarily in SG (Bitoun *et al*., 2003; Ishida-Yamamoto *et al*., 2005), our immunofluorescence studies of normal human skin clearly show that LEKTI protein is present not only in SG but also in SC (Figure 1a). Moreover, *en face* images of SC, with merged images, show that LEKTI protein co-localizes with klk7 to SC membrane domains (Figure 1j and k). Further, definitive evidence for LEKTI expression in SC is shown by Western immunoblotting of SC protein extracts, where both full-length and processed LEKTI are present in normal human subjects, but absent in the SC of NS subjects (Figure 1i1+2 vs. i3). The specificity of the anti-klk7 antibody was tested against 5- to 10-fold excess recombinant klk5, klk8, and klk14, without detection of any cross-reactivity. Because of its robust immunoreactivity in immunoblotting (Raghunath *et al*., 2004), the monoclonal 1C11G6 anti-LEKTI antibody was subsequently used as the detecting antibody for both immunohistochemistry and Western immunoblotting.

To ascertain which SPs are the preferred substrates of LEKTI, we next incubated three endogenous epidermal SP (klk5, klk7, and cathepsin G), and one SP that is not present in SC (chymotrypsin), with a range of concentrations of recombinant human LEKTI (rLEKTI). rLEKTI inhibited klk5 and klk7, with less inhibitory activity against cathepsin G, and no activity against chymotrypsin, as demonstrated previously (Mitsudo *et al*., 2003) (Table 3). Finally, addition of rLEKTI to *in situ* zymography sections demonstrated complete suppression of all endogenous SP activity in the epidermis of klk7-overexpressing mice (Figure 2a–c). Together, these studies demonstrate that SP and LEKTI co-localize within membrane domains of normal SC, and that rLEKTI selectively targets both klk5 and klk7.

Table 1. Clinical characteristics of study group and summary of clinical/laboratory studies

							Microscopy				
No.	Race	Age	Skin phenotype	Bamboo hair	Nikolsky or fragility	Genotyped	EM	IHC	Zymography	SC ph	TEWL forearm
1	Caucasian	16	Mild	P	–	+	+	+	+	5.0	10.3
2	Caucasian	13	Moderate	P	–	+	+	+	+	6.8	35.7
3	Caucasian	13	Severe	P	–	+(NF)	+	+	+	7.2	68.8
4	Caucasian	7	Moderate	NF	–	+	–	+	+	NA	40.30
5	Caucasian	38	Severe	P	–	NA	+	+	–	NA	58.80
6	Caucasian	39	Severe	P	–	NA	+	–	–	NA	NA
7	Asian	9	Severe	P	–	+[1]	+	–	–	NA	23.7[2]
8	Asian	12	Severe	P	–	+[1]	+	–	–	NA	63.2[2]
9	Caucasian	15	Severe	P	–	+[1]	+	–	–	NA	57.4[2]
10	Caucasian	8	Normal subject	NA	NA	NA	–	+	+	NA	6.5
11	Caucasian	30	Normal subject	NA	NA	NA	–	+	+	4.8	5.3
12	Caucasian	16	Normal subject	NA	NA	NA	+	–	–	NA	4.8
13	Caucasian	24	Atopic dermatitis	NA	NA	NA	+	–	–	NA	–
14	Caucasian	27	Psoriasis	NA	NA	NA	+	–	–	NA	–
15	Caucasian	28	RXLI	NA	NA	+	+	–	–	NA	–

Patient ages >7 years came from the investigators' practices as follows: nos. 1–3, 10+11+14 from Brussels; nos. 4–6+15 from Innsbruck; and nos. 7–9, 12+13 from San Francisco. Inclusion criteria included scaling dermatoses; bamboo hair shaft abnormalities; and severe atopic dermatitis-like rash. Skin biopsy specimens were obtained from recently (7+ days) untreated sites (i.e., avoidance of corticosteroid application for at least 2 weeks) and processed for immunohistochemical staining, *in situ* zymography (protease- and lipid-processing enzymes), light and electron microscopy, including ruthenium tetroxide post-fixation, aSMase ultrastructural cytochemistry, and lanthanum staining. TEWL was measured on the ventral forearms using a Tewameter 210 (Courage and Khazaka) and expressed as the mean of three measurements in mg/cm^2/h (normal range: 2–5 mg/cm^2/h). Scoring for disease severity was based on the investigators' assessment of erythema, scaling, pruritus, atopic features, growth failure, episodes of dehydration, and social consequences as follows: 0 (absent) to 4 (extremely severe). Severity score for ''mild'' NS was defined as <10, moderate $\geqslant 10$ and $\leqslant 20$, and severe as >20.NF=not found (see Table 2); NA=not assessed; RXLI=recessive X-linked ichthyosis; P=present; +=available; −=not available; EM=electron microscopy; and IHC=immunohistochemistry.
[1]Courtesy of Dr Gabriela Richard.
[2]From Moskowitz *et al.* (2004).

Basis for loss of SC cohesion, with retention of epidermal integrity, in NS

To assess the consequences of excess SP in NS, we demonstrated by Western immunoblotting a phenotype- and SP-dependent decline of the desmosomal cadherins, desmoglein-1 (DSG1) and desmocollin-1 (DSC1) in SC extracts from NS patients (Figure 3a–q, cf. Figure 1). Both DSG1 and DSC1 were significantly reduced in the SC of ''severe'' NS (Figure 3a–q).

As excess SP activity extends downward into the nucleated layers in ''moderate'' and ''severe'' NS, we next evaluated desmosomal cadherins expression within the nucleated layers of NS epidermis. Although neither DSG1 nor DSC1 levels appeared reduced in ''mild'' NS, DSG1 immunolabeling largely disappeared from all epidermal layers in ''moderate'' and ''severe'' NS (Figure 3a–p), whereas immunofluorescence showed additional patchy downregulation of DSC1 in the outer nucleated layers (Figure 3a–p). Together, these results demonstrate phenotype-dependent degradation of both corneodesmosome and desmosomal cadherins in NS.

An analogous loss of DSG1 occurs in human intraepidermal blistering disorders, such as pemphigus and staphylococcal scalded skin syndrome (Amagai *et al.*, 2000; Wu *et al.*, 2000). Yet, even though SC cohesion is perturbed in NS, our patients did not display excess skin fragility such as blisters formation (i.e., negative Nikolsky sign; Table 1). Hence, we assessed whether other desmosomal cadherin(s) are upregulated in NS, thereby providing compensatory structural integrity. Although DSG3/DSC3 expression is restricted to the basal and lower spinous layers in normal human epidermis (Figure 3a–p), immunostaining for both of these cadherins extended apically to all of the suprabasal, nucleated layers in ''moderate'' and ''severe'' NS (Figure 3a–p). Moreover, an increase in the content of both of these proteins was also shown by Western immunoblotting (Figure 3a–q). Together, these results show that compensatory upregulation of other desmosomal cadherins could protect against intra-epidermal vesiculation from excess SP activity in NS.

We next asked whether altered expression profiles of desmosomal cadherins could have structural consequences for desmosomes in NS. Within the epidermal nucleated layers, desmosomes display typical, mid-line structures in both mild NS and in normal controls (Figure 3r–y), as well as in other acquired and inherited dermatoses; that is, atopic dermatitis, psoriasis, and recessive X-linked ichthyosis (not shown). But these central, mid-line core structures are

Table 2. *SPINK5* genotyping

No.	Type of mutation	Genotyping	Level
1	c.209+1G>T	Intron 3	Altered splicing
	c.1302+4A>T	Intron 14	Altered splicing
2	c.1732C>T	Exon 19	p.Arg578X
	c.1732C>T	Exon 19	p.Arg578X
3	c.-19C>T (?)	Exon 1	?
4	c.316_317delAG	Exon 5	p.Asp106ArgfsX6
	c.2240+1G>A	Intron 23	Altered splicing
5	c.462insGT	Exon 6	Frame-shift
	c.462insGT	Exon 6	Mutation
6	c.882+1_882+3del	Intron 10	Splice site mutation
	Not found	Not found	
7	Available at the National Registry of Ichthyosis and Related Disorders, confidential		
8	Complete gene deletion		
9	Complete gene deletion		

SPINK5 mutations were found in all NS subjects except patient no. 4. This patient is a carrier of a C to T substitution in front of the ATG translation initiation codon; second potential mutation was not found. It is not yet known whether this substitution represents a polymorphism or a mutation. Patient nos. 7–9 were genotyped ''positive'' by Dr G. Richards and patient nos. 8 and 9 are siblings.
Underscore indicates three deleted bases.

reduced or absent from most desmosomes in ''moderate'', and ''severe'' NS (Figure 3v–y). Thus, while thinning of SC in NS can be attributed to loss of corneodesmosome, excess SP activity also modifies desmosomes within the epidermal nucleated layers in NS.

Basis for permeability barrier abnormality in NS

Patients with NS can develop severe electrolyte abnormalities (Stoll *et al.*, 2001) and sufficient caloric loss to impair growth (Moskowitz *et al.*, 2004). As shown in Table 1, NS patients displayed a permeability barrier abnormality, which largely, but incompletely, correlates with clinical phenotype. In addition to SC thinning, as one cause for the permeability barrier abnormality (see above and Komatsu *et al.*, 2002), extracellular lamellar membranes that subserve normal barrier function were disorganized in all NS subjects (Figure 4a–e). Whereas ''mild'' NS displayed few changes in membrane structure, the extracellullar lamellar membranes were progressively disrupted in ''moderate'' and ''severe'' NS (Figure 4a–e). As a similar spectrum of lamellar membrane disorganization was also seen in the klk7-overexpressing mice (Figure 5), excess SP activity clearly accounts for the membrane structural abnormalities in NS.

We next explored the relationship between elevated SP activity and membrane disorganization in NS. Our previous studies showed that either experimentally induced elevations of pH that activate SP, or incubations of normal SC extracts with recombinant klk7, decrease the content of β-GlcCer'ase and aSMase (Hachem *et al.*, 2005b). As a consequence of either or both increased pH (Table 1) and/or elevated SP activity in NS, the content of both of these lipid-processing enzymes decreased in the outer epidermis in NS, again in a phenotype-dependent manner (shown by Western blotting in Figure 6h, and by immunofluorescence in Figure 6). Yet, aSMase protein, although lost from SC, increased in the nucleated layers in NS (Figure 6f and g). Furthermore, a complete absence of extracellular aSMase activity in the outer SG and SC of ''moderate'' to ''severe'' NS, with a potentially compensatory increase in the nucleated layers, was demonstrated by ultrastructural cytochemistry (Figure 4f–j). These studies show that proteolytic degradation of lipid-processing enzymes likely accounts for the membrane structural abnormalities responsible for the barrier defects in NS, with evidence for compensatory upregulation of lipid-processing enzymes in the underlying nucleated layers.

Could upregulated LB secretion partially compensate for the defective barrier in NS?

Abnormal or decreased lamellar membranes could result from degradation of lipid-processing enzymes and impaired LB secretion. Yet, we show instead that LB production accelerates in NS (Figure 4a–j), as described previously (Fartasch *et al.*, 1999). Furthermore, accelerated LB secretion is found in klk7-overexpressing mice (Figure 5), suggesting a link between increased SP activity and premature (accelerated) organelle secretion. Finally, to ascertain whether accelerated LB secretion comprises a compensatory mechanism that allow survival of NS patients, we assessed whether accelerated LB secretion in NS provides a partial permeability barrier. Specifically, we assessed the pathways of colloidal lanthanum tracer egress and blockade across NS epidermis. Externally applied water-soluble tracer moved freely through the SC interstices in ''severe'' NS (Figure 4k and l). However, inward-to-outward movement of tracer was impeded within the nucleated layers at sites of premature LB secretion (Figure 4k and l), well beneath the normal site of tracer blockade, at or just under the SG–SC interface. These results demonstrate that accelerated LB secretion occurs in NS, and that these prematurely deposited, extracellular contents provide an initial (partial) barrier to transcutaneous water loss.

DISCUSSION

In this study, we attempted first to address important, unresolved issues related to the variable phenotype and pathogenesis of NS. Second, we utilized NS as a model to ascertain how SP/SPI interactions are modulated to allow normal life in a terrestrial environment. Prior studies have shown that LEKTI mutations can result in relatively unopposed SP activity in the SC of affected individuals (Komatsu *et al.*, 2002). Increased SP activity, in turn, correlates with attenuation of SC, which has been invoked as one explanation for the permeability barrier abnormality (Komatsu *et al.*, 2002). Although the structure of LEKTI predicts activity primarily against tryptic SP (Mitsudo *et al.*, 2003), rLEKTI exhibited relatively selective, inhibitory activity not only

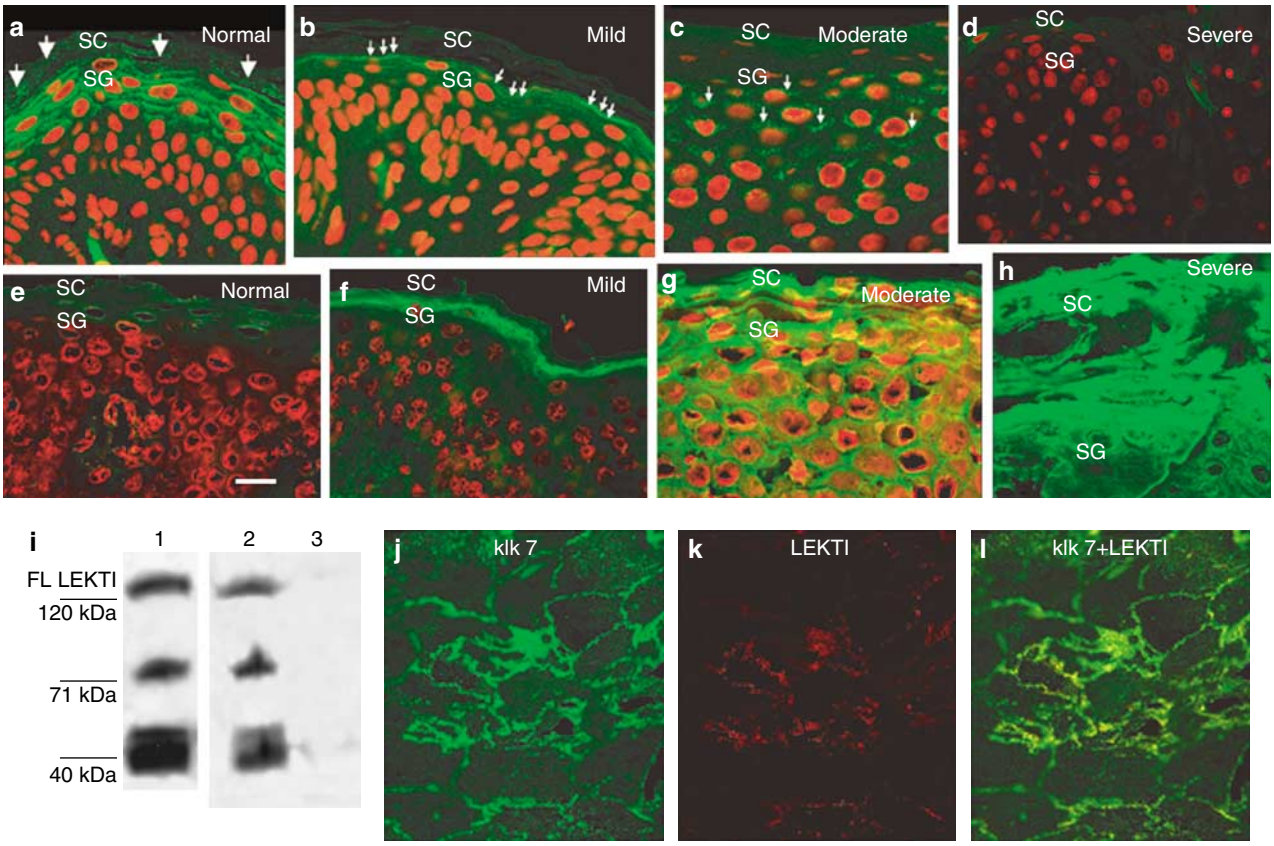

Figure 1. LEKTI localizes to both the SG and SC in normal skin; residual expression correlates with serine protease activation and phenotypic severity in NS. (**a–h**) LEKTI immunofluorescence staining was performed on paraffin sections of skin biopsies using anti-LEKTI monoclonal mouse anti-human in both (**a**) normal control, and (**b**) ''mild'', (**c**) ''moderate'', (**d**) ''severe'' NS. NS patients show a significant decrease with patchy LEKTI immunostaining in (**b**) mild and (**c**) moderate to complete absence in (**d**) severe forms of NS. *In situ* zymography for SP activity was performed on frozen sections (5 μm) from punch biopsies taken from the forearms of three patients with NS (mild: **f**; moderate: **g**; and severe: **h**) and (**e**) a normal control. (**e**) NS patients show a phenotype-dependent increase in proteolytic activity, extending to the lower levels of epidermis in comparison to normal controls. Sections were counterstained with propidium iodide and visualized under a confocal microscope at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Arrows show residual LEKTI in mild and moderate NS. SC: stratum corneum; SG: stratum granulosum. Bar = 10 μm. (**i**) Western immunoblotting for LEKTI protein performed on extracts from SC of normal subjects (1 and 2) and NS control (3) show the presence of pro- and processed forms of LEKTI in isolated SC. (**j–l**) SC paraffin-embedded extracts from normal human skin were used to assess the co-expression of klk7 (green) and LEKTI (red). Both enzyme and inhibitor colocalize to the membrane domains of the SC.

against the epidermal tryptic SP, klk5, but also equipotent *in vitro* activity against the SC chymotryptic enzyme, klk7. Together, the highly selective activity of LEKTI against klk5 and klk7, the variable levels of resident LEKTI expression in NS, and the co-localization of LEKTI with klk7 explain the phenotypic variability in NS. In contrast to prior predictions (Komatsu *et al.*, 2002), the type of mutation and disease severity did not correlate well, but information about specific mutations was not available for some of our patients. Moreover, the relatively small sample size of our study limits assertions about genotype–phenotype relationships. Yet, complete gene deletion in sibling patients 8 and 9, leading to a complete loss of residual LEKTI protein, correlated with disease severity, and the level of deletion in patient no. 1, categorized as ''mild'' and patient no. 4 categorized as ''moderate'', correlated with the presence of five and 10 residual, inhibitory domains, respectively. The variations between the ''moderate'' and ''severe'' in these cases could be explained by either different potencies in the LEKTI inhibitory domains or the added environmental stress that could result in the atopic manifestations in these and other NS patients.

Whereas Bitoun *et al.* (2003) described LEKTI localization to the SG, and absence from SC, their observations can be ascribed either to artifactual extraction of secreted extracellular proteins, along with lipids during tissue processing, or to the inaccessibility of their anti-LEKTI antibodies to protein epitopes in the SC. Here, we localized LEKTI in normal SC both by immunohistochemistry, using antigen enhancement techniques, and, further, by its prominence in Western immunoblots of protein extracts from normal SC. LEKTI has also been described to localize to the hair follicle (Bitoun *et al.*, 2003) and presumably its absence causes the diagnostic bamboo hair phenotype. But how the absence of LEKTI impacts hair morphology is still unknown and remains to be determined.

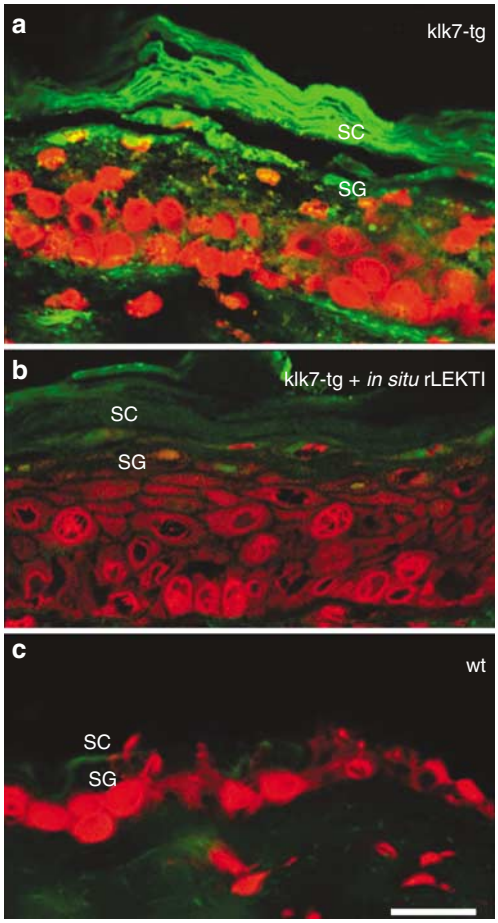

Figure 2. LEKTI specifically inhibits SP activity in klk7-overexpressing mice. (**a**) *In situ* zymography of SP activity in untreated klk7-overexpressing mice. (**b**) Abolition of SP activity in sections of klk7-overexpressing mice, treated with rLEKTI. (**c**) Low SP activity in wild-type (wt), control mice. Bar = 10 μm.

Table 3. Inhibitory activity of recombinant LEKTI against various serine proteases

Protease	LEKTI[1] (nM)	*I/E*[2]	Inhibition (%)	Substate (mM)
Cathepsin G	100	>3	81	0.75
klk7	40	~0.6–0.5	82	1
klk5	50	~0.6–0.4	84	1
Chymotrypsin	150	22	0	1

LEKTI inhibits klk7 and klk5, but has lesser activity and no activity against cathepsin G and chymotrypsin, respectively. All proteases were human recombinant, except chymotrypsin, which was bovine.
[1]*I/E* is the rLEKTI concentration/proteinase concentration ratio.
[2]Percent inhibition=100[1−velocity in the presence of LEKTI/velocity of uninhibited control)].

Human SC contains other SP inhibitors (Alkemade *et al.*, 1994; Pfundt *et al.*, 1996), including secretory leukocyte protease inhibitor; elafin/SKALP, and plasminogen activator inhibitor (type 2), which could in theory compensate for reduced LEKTI in NS, but in reality do not. The inability of these SPI to restrict klk5 and klk7 activity may be attributed to their localization within the cornified envelope (these SPI all possess substrates for transglutaminase 1-induced cross-linking; (Alkemade *et al.*, 1994; Pfundt *et al.*, 1996). Knowing that SLPI is a potent inhibitor of klk7 (Franzke *et al.*, 1996), its restriction to the cornified envelope would make it unavailable to interact with extracellular SP. Alternatively, elafin would not suffice to restrict increased SP activity in NS, even if upregulated, because it displays no inhibitory activity against klk7 (Franzke *et al.*, 1996). Thus, LEKTI could represent the principal, extracellular SPI available to interdict klk5 and klk7 within the SC interstices.

Prior work has established a link between increased SP and SC thinning in NS (Komatsu *et al.*, 2002; Descargues *et al.*, 2005). We show further that the SP-induced thinning of SC is owing to degradation of corneodesmosome, also shown in *SPINK5* knockout mice (Yang *et al.*, 2004; Descargues *et al.*, 2005). DSG1 is a key constituent of corneodesmosome, and a known target of endogenous SP activity (Caubet *et al.*, 2004). In other experimental models, where proteolysis is activated within SC by an increase in pH, we have shown that the murine homolog, dsg1, is also a target of unrestricted SP activity (Hachem *et al.*, 2003; Fluhr *et al.*, 2004; Hachem *et al.*, 2005b).

In NS, the extraordinary increase in SP activity extends beneath the SC into the nucleated cell layers, where DSG1 and DSC1 are degraded in an SP- and phenotype-dependent manner. In ''moderate'' and ''severe'' NS, where SP activity extends deeper into the epidermis, DSG1, and to a lesser extent, DSC1, are progressively degraded. In contrast, in ''mild'' NS, less SP activity appears within the epidermis, and both DSG1 and DSC1 immunolabeling persist. In two, unrelated human bullous disorders, pemphigus foliaceus (Wu *et al.*, 2000) and staphylococcal scalded skin syndrome (Amagai *et al.*, 2000), DSG1 is targeted specifically by an antibody and a bacterial exotoxin, respectively, which disrupt epidermal integrity, resulting in vesiculation and bulla formation. Yet, in contrast to these bullous disorders, the nucleated epidermis retains its structural integrity in NS, apparently owing to a compensatory upregulation of DSG3 and DSC3 in suprabasal epidermal layers, where these proteins normally are expressed minimally (Arnemann *et al.*, 1993; Legan *et al.*, 1994). Although DSG3/DSC3 upregulation appears to be protective, the normal mid-line structure of intra-epidermal desmosomes is largely lost in ''severe'' and ''moderate'' NS. Pertinently, forced, suprabasal expression of DSG3 in transgenic mice both alters phenotype and produces abnormal desmosomal structure (Elias *et al.*, 2001), whereas conversely, DSG1 overexpression can compensate for loss of DSG3 in another transgenic mouse model (Merritt *et al.*, 2002). Thus, suprabasal overexpression of DSG3/DSC3 appears to represent one compensatory response that contributes to the survival of NS patients in the face of a concerted proteolytic attack.

''Moderate'' and ''severe'' NS display a thin SC (Bitoun *et al.*, 2002), which we propose is due not only to reduced LEKTI but also attributable to the increased pH of SC. Klk5 and klk7 both display neutral-to-alkaline pH optima

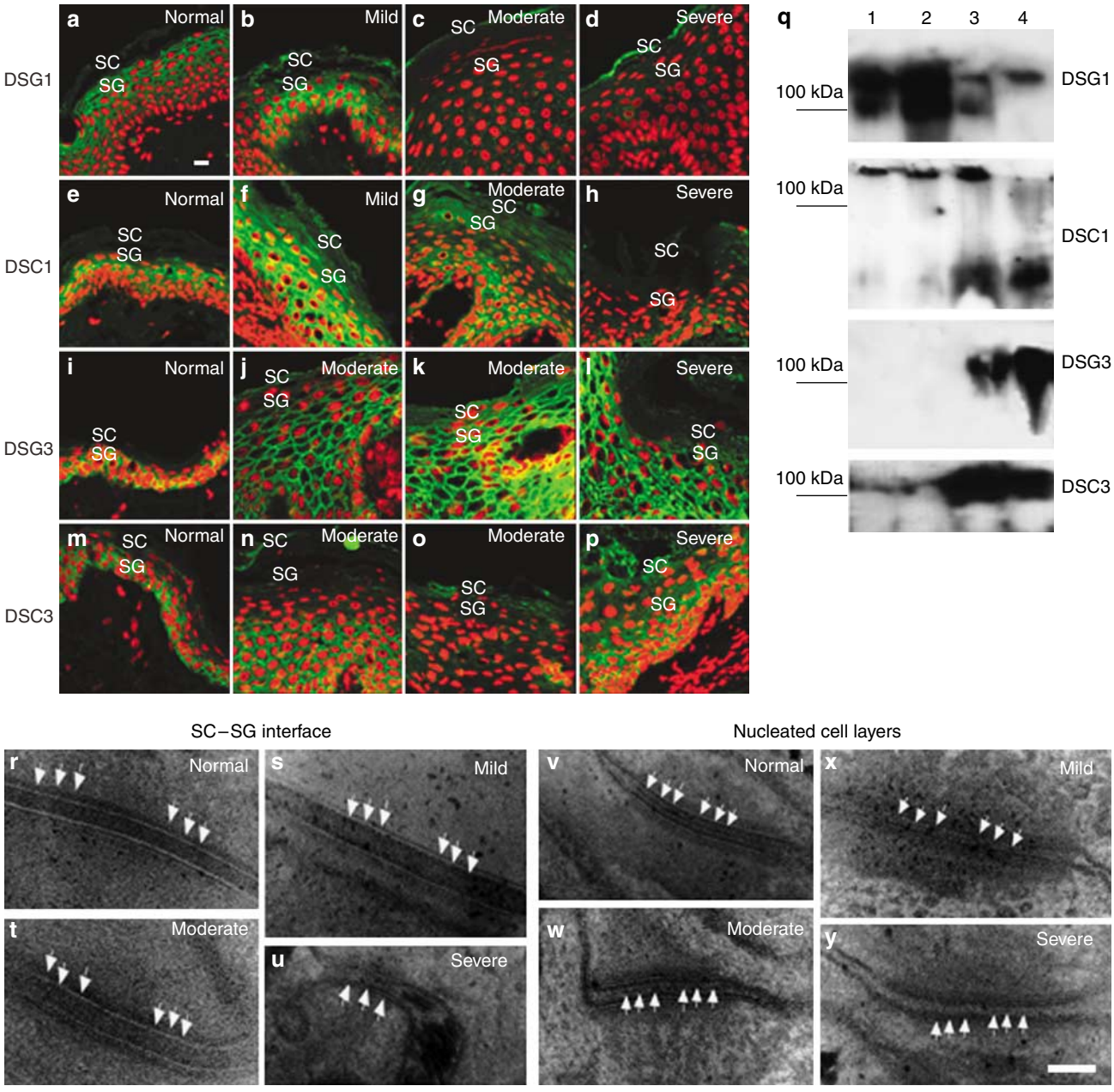

Figure 3. Decreased DSG1, DSC1 in the SC is paralleled by overexpression of DSG3, DSC3, accounting for corneodesmosome/desmosome structural abnormalities in NS. (**a–p**) Immunofluorescence (IF) staining for DSG1, DSC1, DSG3, and DSC3 were performed on paraffin and frozen sections, respectively, in both normal control subjects and three patients with NS. Extent of disorganization and decrease of both DSG1 and DSC1 correlates strongly with the extent of SP activity and clinical severity. Unlike normal control skin, DSG3 and DSC3 are observed in the upper nucleated layers of the epidermis in parallel with the decrease in DSG1 and DSC1. All sections were counterstained with propidium iodide and visualized under a confocal microscope (Leica TCS SP, Heidelberg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Bar = 10 μm. (**q**) Western immunoblot analysis was performed on SC protein extracts from (1) normal control and (2) ''mild'', (3) ''moderate'', and (4) ''severe'' NS. Immunoblotting for either DSG1, DSC1, β-GlcCer'ase, and aSMase show a phenotype-dependent decrease with the presence of degradation bands for DSC1. In contrast, DSG3 and DSC3, normally basal cell-localized epidermal cadherins, are highly expressed in the SC. (**r–y**) Compared to (**r**) normal, corneodesmosomes from the (**s–u**) SG–SC interface (arrows) were shortened in all NS patients. (**u**) The degradation of desmosomal midline structures is more pronounced in NS. In the deeper nucleated layers, (**w**) ''mild'', (**x**) some ''moderate'' NS, and (**v**) control subjects show typical midline structure, with alterations of electron-lucent and electron-dense midline structures. In most ''moderate'' and ''severe'' NS, the (**y**: arrows) electron dense midline is absent. Bar = 100 nm.

(Brattsand *et al.*, 2005), and the surface pH in ''moderate'' to ''severe'' NS, as in other inflammatory dermatoses (Eberlein-Konig *et al.*, 2000; Rippke *et al.*, 2002), approaches neutrality (Table 1), which would further activate SP (Hachem *et al.*, 2003). In other studies, experimental barrier disruption has been shown not only to increase surface pH (Hachem *et al.*, 2005a) but also to enhance SP activity (Hachem *et al.*, 2005c). This pH increase is detrimental to the

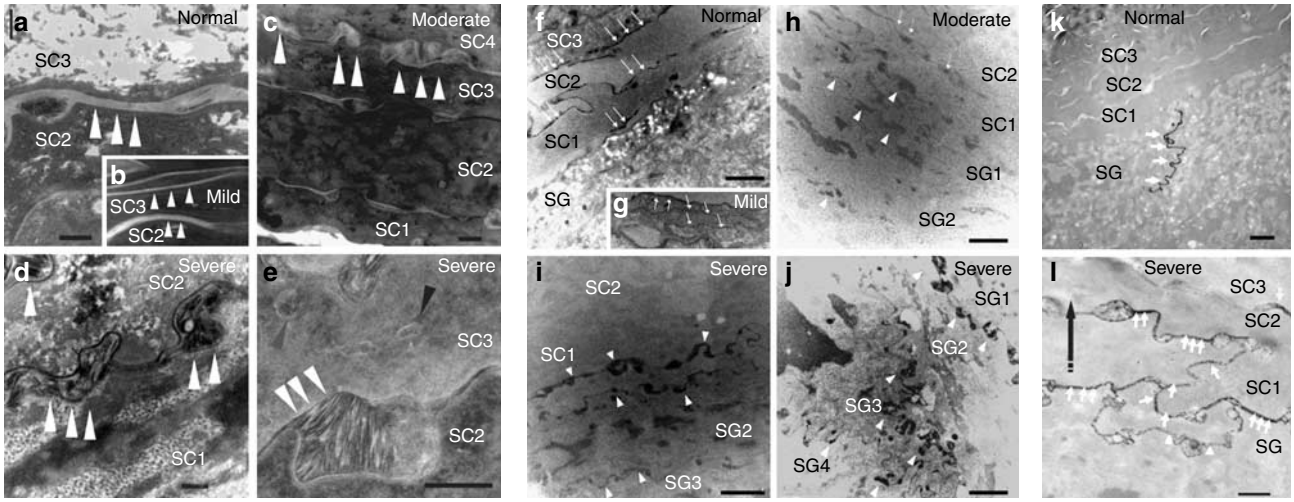

Figure 4. Lamellar membrane abnormalities correlate with permeability barrier dysfunction and phenotype in NS. (**a–e**) *Disorganization of extracellular lamellar membranes*. Ultrastructural images reveal areas of lamellar/non-lamellar phase separation in (**b**) ''mild'' NS, and (**d** and **e** vs. **a**) extensive membrane disorganization in moderate-to-severe NS. (**e**) Some LBs content are entombed bodies within corneocyte cytosols in ''severe'' NS. (**f–j**) *aSMase activity*. Ultrastructural cytochemical staining for aSMase was performed on aldehyde-fixed sections from skin biopsies from normal control and patients with NS. Decreased aSMase cytochemical staining (arrows) is observed within the SC in NS patients that correlates with disease severity. (**i** and **j**) Severe NS patients, however, show compensatory increase in aSMase activity (arrowheads) in the nucleated layers that correlates with premature secretion of LBs within the epidermal nucleated layers. (**k** and **l**) *Water-soluble tracer traverses the SC interstices, butTracer egress is blocked by prematurely secreted LB*. Lanthanum tracer freely moves between corneocytes within SC in (**l**, white arrows) NS, whereas tracer is excluded from (**k**) normal SC. Outward egress of tracer is blocked by secreted LB contents within (**l**, arrowheads) lower SG. Black arrow showing the direction of Lanthanum movement. Bar = 0.5 μm.

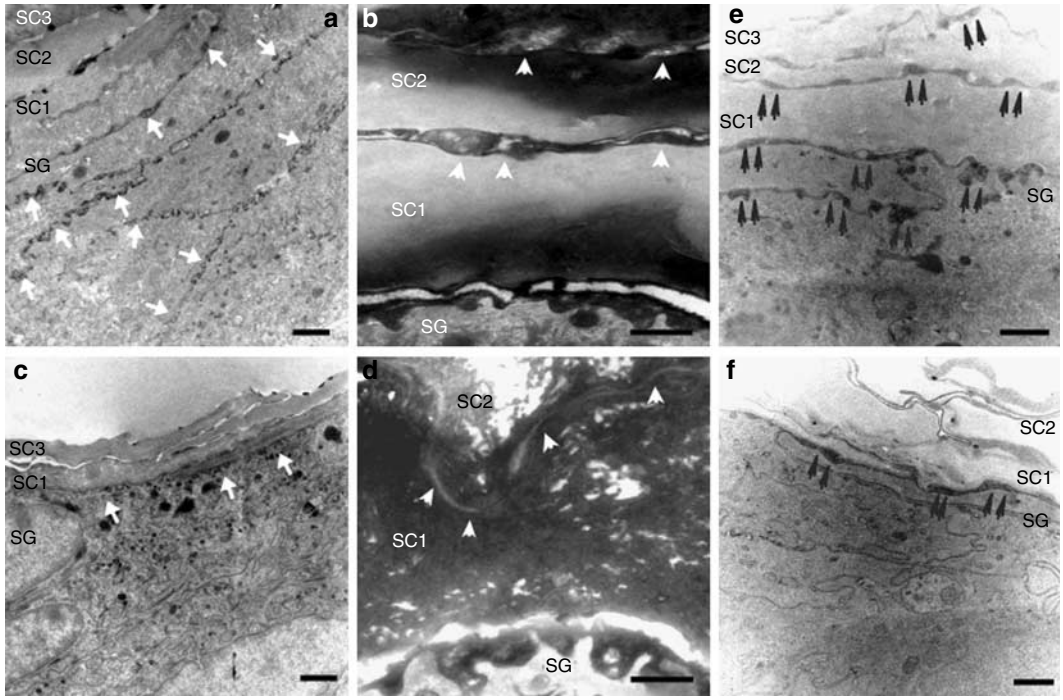

Figure 5. Klk7-Overexpresssing mice display enhanced LB secretion and disorganized extracellular lamellar membranes. Enhanced LBs secretion (white arrows) is observed deep in the (**a**) epidermis of klk7 transgenic mice (klk7-tg), but not in (**c**) wild-type (wt) littermates. Increased neutral lipase cytochemical staining in (**e**) klk7-tg mice *versus* (**f**) wt further confirms enhanced LB secretion, as observed on (cf., **a** and **b**) conventional EM. Finally, compared to (**d**) wt littermates, (**b**) klk7-tg mice display incompletely processed lamellar bilayers as low as the interface between the (SC1) first and the (SC2) second SC layer. Bars = 1 μm.

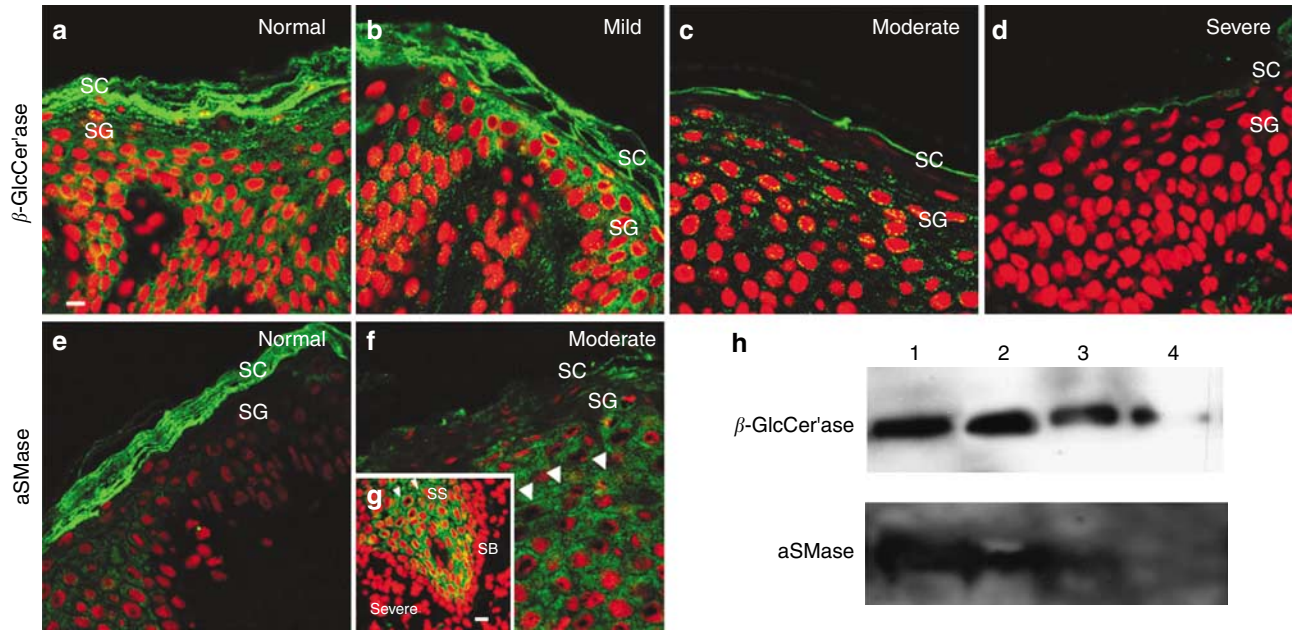

Figure 6. Extent of proteolytic activation correlates with degradation of lipid-processing enzymes in NS. (**a**–**g**) Immunofluorescence staining for β-GlcCer'ase and aSMase was performed on paraffin and frozen skin sections, respectively. Decrease expression of both lipid-processing enzymes within the SC correlates with the extent of the barrier abnormality in NS. (**f**–**g** vs. **e**). Increased aSMase expression, but not β-GlcCer'ase, is found in the deeper nucleated layers in ''moderate'' and ''severe'' NS SC: stratum corneum; SG: stratum granulosum, SS: stratum spinosum. Bar = 10 μm. (**h**) Immunoblotting for both β-GlcCer'ase and aSMase show a phenotype-dependent decrease in moderate and severe NS patients.

permeability barrier, even in normal skin, as shown by the ability of topical SPI to accelerate barrier recovery in normal rodent skin after experimental perturbations (Denda *et al.*, 1997; Hachem *et al.*, 2005c). Hence, an extraordinary vicious cycle could amplify both the extent of SP activation and its functional consequences in NS.

$$\begin{bmatrix} \downarrow \text{LEKT1} \rightarrow & \uparrow \text{SP} \rightarrow & \text{Thin SC} \\ & \uparrow & \\ & \uparrow \text{pH} \leftarrow & \downarrow \text{Barrier} \end{bmatrix}$$

Yet, SP-mediated loss of SC integrity/cohesion alone is unlikely to form the basis for the permeability barrier abnormality in NS. Extensive mechanical removal of normal SC has minimal consequences for the permeability barrier (Bashir *et al.*, 2001), because this critical function becomes largely established at, and just above, the SG–SC interface (Menon *et al.*, 1992; Elias *et al.*, 1998). Instead, we show here that the sustained proteolytic attack in NS prevents the remodeling and organization of secreted lipid precursors into the hydrophobic species that form the extracellular lamellar membranes that mediate normal permeability barrier function (Elias and Friend, 1975). Normally, LB secrete a mixture of polar lipid precursors, including large quantities of glucosylceramides and sphingomyelin (Vielhaber *et al.*, 2001), which then are ''processed'' by co-secreted lipid hydrolases, including β-GlcCer'ase, aSMase, secretory phospholipase A2, and steroid sulfatase, into their more non-polar products (i.e., ceramides, free fatty acids, and cholesterol) that form these unique, extracellular lamellar membranes (Holleran *et al.*, 1991, 1993; Mao-Qiang *et al.*, 1996). We show here that two key, extracellular hydrolases, β-GlcCer'ase and aSMase, are degraded in NS, indubitably from an unrestricted proteolytic attack (Hachem *et al.*, 2005b). Similarly, SP activation, if prolonged by sustained increases in the pH of the SC, would degrade these enzymes (Hachem *et al.*, 2005b). The net result is a phenotype-dependent loss of lamellar membrane architecture in NS, which correlates with both the severity of the clinical phenotype and barrier abnormality. The barrier abnormality, in turn, likely underlies most of the clinical consequences of severe NS; that is, growth failure, electrolyte disturbances, and dehydration (Griffiths *et al.*, 1989).

Despite the potentially devastating epidermal abnormalities, most NS patients survive into adulthood. Our studies demonstrate a second, compensatory mechanism that could facilitate survival in NS. Although accelerated LB secretion is useful as a diagnostic morphological marker in NS (Fartasch *et al.*, 1999), the secreted contents, although not effectively re-organized into lamellar membranes, appear to provide bulk hydrophobic constituents that partially restrict transcutaneous water loss in the extracellular spaces, as shown by the lanthanum perfusion studies (Figure 4k and l).

In summary, in this limited cohort of patients, we show here how SP and LEKTI activity/expression and co-localization determine phenotype in NS. We show further that LEKTI inhibits two epidermis-localized SP (klk5 and klk7). The extent of SP activation also correlates with the degree of barrier dysfunction, attributable both to phenotype-dependent loss of desmosomal cadherins and secreted

lamellar hydrolases. But compensatory upregulation of both DSG3–DSC3 and LB secretion appear to protect against fragility and provide a partial barrier to water loss. Elucidation of the pathogenesis of NS has further illuminated how SP activity is restricted by LEKTI in normal epidermis to allow formation of a functional permeability barrier.

MATERIALS AND METHODS

Patient inclusion

Patients were recruited from the investigators' practices from Brussels, Innsbruck, and San Francisco, as summarized in Table 1. Inclusion criteria included a diagnosis of NS based on: (1) scaling dermatoses, with atopic dermatitis-like features; (2) diagnostic bamboo hair shaft abnormalities, atopic dermatitis, and (3) genotyping and phenotyping (LEKTI immunolocalization). Control subjects included age-matched normals, and subjects with atopic dermatitis, psoriasis, and recessive X-linked ichthyosis. The ethical committees from all three institutions approved the study protocol. Patients or parents (if under-aged) provided informed consent. The study was conducted according to the Declaration of Helsinki Principles. Clinical scoring was performed using previously published criteria (Ganemo *et al.*, 1999), with the addition of two supplementary criteria; that is, prior documented episodes of dehydration and social consequences.

Skin biopsies from NS patients and control individuals were obtained from recently untreated sites (i.e., avoidance of corticosteroids for at least 2 weeks before study; however, some subjects continued to use emollients), and processed as summarized in Table 1. Transepidermal water loss was measured on the ventral forearms of all subjects using a Tewameter (Courage and Khazaka, Köln, Germany). *SPINK5* genotyping was performed according to the method described by Sprecher *et al.* (2001). The University of California, San Francisco patients were genotyped by Dr Gabriela Richard (Thomas Jefferson University, Philadelphia, PA).

Materials

Human neutrophil cathepsin G was purchased from Calbiochem (Nottingham, UK) and pancreatic bovine chymotrypsin was from Roche (Bromma, Sweden). Chromogenic substrates for proteases were as follows: klk7 and chymotrypsin substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA-HCl, 10 mM in deionized water) and klk5 substrate S-2288 (7.1 mM in deionized water, H-D-Ile-Pro-Arg-pNA-2HCl) were from Chromogenix (Milan, Italy), and cathepsin G substrate (20 mM in DMSO, Suc-Ala-Ala-Pro-Phe-pNA) was from Calbiochem (Nottingham, UK). Tris-buffered saline buffer (NaCl, 137 mM; Tris-HCl, 20 mM; pH 7.6) was used as assay buffer in inhibition studies. Human recombinant pro-klk7, klk5, and full-length rLEKTI were purified as described previously (Hansson *et al.*, 1994; Brattsand and Egelrud, 1999; Mitsudo *et al.*, 2003). The primary and secondary antibodies utilized in this study are summarized in Table 4. EnzChek® Protease Assay Kit containing BODIPY-FL-Casein (green fluorescence) was obtained from Invitrogen (Merelbeke, Belgium).

Transgenic klk7-overexpressing mice were generated as previously described by Hansson *et al.* (2002). Tissue specimens were collected from adult mice (older than 5 month) showing obvious signs of clinical dermatitis and excess scale.

Immunofluorescence

Immunofluorescence was performed on either paraffin or cryopreserved frozen sections from NS patients and normal controls,

Table 4. Sources and specifications of antibodies

	Reactivity	Origin	Usage	Source
Primary antibodies				
LEKTI (Clone 1C11G6)	h	m	IF, WB	Raghunath *et al.* (2004)
β-GlcCer'ase	h, m	rb	IF, WB	Gift from Dr Ellen Sidransky, NIH, Bethesda, MD
aSMase	h, m, r	rb	IF, WB	Santa Cruz Biotechenology, Santa Cruz, CA
DSG 1 (Clone P124)	h	m	IF, WB	Biodesign International, Brussels, Belgium
DSG 3 (Clone 5G11)	h	m	IF, WB	Cell Science, Canton, MA
DSC 1	h	m	IF, WB	Progen, Heidelberg, Germany
DSC 3	h	m	IF, WB	Progen, Heidelberg, Germany
Klk7	h,	rb	IF	Arexis AB, Göteborg, Sweden
Secondary antibodies				
Alexa® Fluor 488	m	g	IF	Invitrogen, Merelbeke, Belgium
Alexa® Fluor 488	m	d	IF	Invitrogen, Merelbeke, Belgium
Alexa® Fluor 488	rb	d	IF	Invitrogen, Merelbeke, Belgium
Alexa® Fluor 657	m	c	IF	Invitrogen, Merelbeke, Belgium
HRP conjugated	rb	g	WB	Biochain, Brussels, Belgium
HRP conjugated	m	g	WB	Biochain, Brussels, Belgium

Sources and specifications of antibodies: h, human; m, mouse; r, rat; rb, rabbit; g, rabbit; d, donkey, c, chicken; IF, immunofluorescence; WB, Western blot.

using the antibodies indicated in Table 4. After deparaffinization, paraffin sections were re-hydrated to distilled water, and boiled for 1 minute in a Presto®-containing, antigen-unmasking buffer solution (Vectorlabs, Brussels, Belgium). For cryo-preserved biopsies, sections were first fixed in 95% ethanol chilled at −20°C for 5 minutes. Both paraffin and cryo-sections were washed in phosphate-buffered saline (pH = 7.5), incubated first for 30 minutes in blocking buffer (1% bovine serum albumin, 0.1% cold-water fish gelatin in phosphate-buffered saline), and then for 24 hours at 4°C with the primary antibody in blocking buffer. Tissue sections then were washed with phosphate-buffered saline, and incubated for 1 hour at room temperature with Alexa® Fluor 488 secondary antibody, in blocking buffer, counterstained with propidium iodide (Sigma-Aldrich, Bornem, Belgium), and visualized under a confocal microscope (Leica TCS SP, Heidelberg, Germany) at an excitation and emission wavelength of 485 and 530 nm.

Zymographic assays

In situ SP activity. Frozen sections (5 μm) from the forearms of NS patients, normal control, klk7-overexpressing mice, and wild-type control incubated at 37°C for 2 hours with BODIPY-Fl-casein (1 μg/μl) in deionized water (2 μl/ml). For klk7-overexpressing mice protease assay, rLEKTI (0.01 μg/μl) was added to the reaction milieu. All sections then were rinsed with phosphate-buffered saline, mounted, and visualized under a confocal microscope as described above.

Inhibition studies with rLEKTI. The inhibitory effects of rLEKTI on the SPs, klk5, klk7, cathepsin G, and chymotrypsin were tested in a spectrophotometric assay with the corresponding chromogenic substrates as described above. Different concentrations of rLEKTI were pre-incubated with each protease for 2 minutes in 50 μl assay buffer. Proteolysis reaction was initiated by the addition of 150 μl of the appropriate chromogenic substrate in Tris-buffered saline buffer, and the reaction was followed in a spectrophotometer (Spectramax 250, Molecular Devices, Sunnyvale, CA) by measuring absorbance at 405 nm at 25°C for 20 minutes.

Western immunoblotting

SC was isolated from NS patients and normal control subjects using sequential D-squame tape strippings (20 D-squames per individual; Cuderm, Dallas, TX). Tapes then were incubated overnight at 4°C in 1% Triton X-100 and a protease inhibitor cocktail (Complete Mini, Roche, Brussels, Belgium) in phosphate-buffered saline, and then sonicated for 5 minutes at room temperature to extract proteins from the tapes, followed by measurement of protein content (Bio-Rad Protein Assay kit). Equal amounts of protein from NS and control subjects were loaded onto 10% Tris-glycine polyacrylamide gels (Invitrogen, Merelbeke, Belgium). After electrophoresis, proteins were transferred onto nitrocellulose membranes and immunoblotted with the primary antibodies (Table 4) using the Attoglow Western Blot System (Biochain, Brussels, Belgium).

Electron microscopy

Ultrastructural analysis of LBs and lamellar membranes. Biopsy samples were minced to $<0.5\,mm^3$, fixed in modified Karnovsky's fixative overnight, and post-fixed in either 0.2% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. Whereas osmium tetroxide post-fixation method optically depicts LBs, ruthenium tetroxide post-fixation permits visualization of extracellular lamellar membranes in the SC. After post-fixed fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon–epoxy mixture. Ultrathin sections were examined in an electron microscope (Zeiss 10A; Carl Zeiss, Thornwood, NY).

Ultrastructural cytochemistry for aSMase and lipase. aSMase was detected in samples from NS patients and control subjects as described previously (Rassner *et al.*, 1997, 1999). As ultrastructural cytochemical markers for LB in klk7-overexpressing mice, we utilized neutral lipase known to be concentrated in this organelle as described previously (Rassner *et al.*, 1999).

Lanthanum perfusion. To depict pathways of water movement through epidermis, biopsy samples from two patients with ''severe'' NS were immersed in 4% colloidal lanthanum, the smallest electron-dense tracer, in 0.05 M Tris buffer, pH 7.4, containing 2% glutaraldehyde and 2% formaldehyde for 2 hours, and processed as described above.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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