See related commentary on page 1609

Corneodesmosomal Cadherins Are Preferential Targets of Stratum Corneum Trypsin- and Chymotrypsin-like Hyperactivity in Netherton Syndrome

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SPINK5 (serine protease inhibitor Kazal-type 5), encoding the protease inhibitor LEKTI (lympho-epithelial Kazal-type related inhibitor), is the defective gene in Netherton syndrome (NS), a severe inherited keratinizing disorder. We have recently demonstrated epidermal protease hyperactivity in *Spink5*$^{-/-}$ mice resulting in desmosomal protein degradation. Herein, we investigated the molecular mechanism underlying the epidermal defect in 15 patients with NS. We demonstrated that, in a majority of patients, desmoglein 1 (Dsg1) and desmocollin 1 (Dsc1) were dramatically reduced in the upper most living layers of the epidermis. These defects were associated with premature degradation of corneodesmosomes. Stratum corneum tryptic enzyme (SCTE)-like and stratum corneum chymotryptic enzyme (SCCE)-like activities were increased, suggesting that these proteases participate in the premature degradation of corneodesmosomal cadherins. SCTE and SCCE expression was extended to the cell layers where Dsg1 and Dsc1 immunostaining was reduced. In contrast, a subset of six patients with normal epidermal protease activity or residual LEKTI expression displayed apparently normal cadherin expression and less severe disease manifestations. This suggests a degree of correlation between cadherin degradation and clinical severity. This work further supports the implication of premature corneodesmosomal cadherin degradation in the pathogenesis of NS and provides evidence for additional factors playing a role in disease expression.

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INTRODUCTION

Netherton syndrome (NS, OMIM 256500) is a rare autosomal recessive genodermatosis that combines congenital ichthyosiform erythroderma, hair shaft anomalies (trichorrhexis invaginata), and atopic diathesis (Comel, 1949; Netherton, 1958). The syndrome presents at birth or soon thereafter with generalized exfoliative erythroderma, which is similar clinically to other types of infantile erythroderma including erythrodermic psoriasis, atopic dermatitis, or non-bullous congenital ichthyosiform erythroderma (OMIM 242100). Continuous peeling of the skin can persist in NS similarly to peeling skin syndrome (OMIM 270300) (Schneider *et al.*, 2000; Sardy *et al.*, 2002). The congenital ichthyosiform erythroderma-like features can persist throughout life in the most severe cases or evolve into a unique milder condition known as ichthyosis linearis circumflexa, consisting of migratory, serpiginous, and erythematous plaques, which are bordered by a peculiar double-edged scale (Traupe, 1989). Like ichthyosis linearis circumflexa, trichorrhexis invaginata ('bamboo hair') is pathognomonic for NS and corresponds to the invagination of the distal part of the hair shaft into its proximal part (Netherton, 1958; Wilkinson *et al.*, 1964; Ito *et al.*, 1984). Other hair shaft abnormalities including pili torti (twisted hair) and trichorrhexis nodosa (hair of varying diameter) have been reported (Stevanovic, 1969). Affected individuals have a broad range of atopic

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Abbreviations: CDSN, corneodesmosin; DP, desmoplakin; Dsc1, desmocollin 1; Dsg1, desmoglein 1; klk, kallikrein; LEKTI, lympho-epithelial Kazal-type related inhibitor; NS, Netherton syndrome; PAR2, proteinase-activated receptor 2; SCCE, stratum corneum chymotryptic enzyme; SCTE, stratum corneum tryptic enzyme; SPINK5, serine protease inhibitor Kazal-type 5

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manifestations, including eczema-like rashes, hay fever, and angio-edema associated with high levels of IgE in the serum (Judge *et al.*, 1994; Smith *et al.*, 1995). NS patients with ichthyosis linearis circumflexa have a normal general development, whereas those presenting generalized exfoliative erythroderma display life-threatening complications such as hypernatremic dehydration, electrolyte imbalances, hypothermia, failure to thrive, and recurrent infections, resulting in a high postnatal mortality (Hausser and Anton-Lamprecht, 1996). These complications might be attributed to the severe alteration of the epidermal barrier function (Fartasch *et al.*, 1999).

Histopathologic examination of NS skin reveals a 'psoriasiform' epidermis, which associates hyperplasia of the subcorneal epithelium (acanthosis) with varying degrees of epidermal invaginations into the dermis (papillomatosis) (Hausser and Anton-Lamprecht, 1996). The stratum corneum often displays persistent nuclei in the cytoplasm of corneocytes (parakeratosis) with a focal absence of the granuler layer, and can be found detached from the underlying epidermis or entirely missing (De Wolf *et al.*, 1996; Hausser and Anton-Lamprecht, 1996; El Shabrawi-Caelen *et al.*, 2004). Using transmission electron microscopy, lamellar bodies, which are normally secreted at the transition between the granular layer and the stratum corneum (Fartasch, 2004), were seen to be fewer in number and poorly structured. They are prematurely secreted into the intercellular spaces and their content remains unprocessed in the basal stratum corneum (Fartasch *et al.*, 1999). A moderate to severe perivascular inflammatory infiltrate with regions of exocytosis has been reported in the dermis (Hausser and Anton-Lamprecht, 1996; Sardy *et al.*, 2002).

We previously identified *SPINK5* (serine protease inhibitor Kazal-type 5) as the defective gene in NS (Chavanas *et al.*, 2000). *SPINK5* encodes the multidomain serine protease inhibitor LEKTI (lympho-epithelial Kazal-type related inhibitor) (Magert *et al.*, 1999), which is expressed in the granular layer of the epidermis and in the most differentiated stratified epithelial tissue layers (Bitoun *et al.*, 2003). All *SPINK5* mutations identified so far cause premature termination codons and are associated with complete loss of LEKTI expression in NS epidermis (Bitoun *et al.*, 2002; Ong *et al.*, 2004; Raghunath *et al.*, 2004; Sprecher *et al.*, 2004). LEKTI consists of 15 Kazal-type inhibitory domains, which are capable of blocking *in vitro* the activity of a battery of serine proteases, including trypsin, plasmin, subtilisin A, cathepsin G, and elastase (Magert *et al.*, 2002; Mitsudo *et al.*, 2003; Jayakumar *et al.*, 2004).

In order to investigate the biological(s) function of LEKTI, we have generated and characterized a mouse model for NS. We demonstrated with this model that unregulated epidermal protease activities, such as SCTE (*stratum corneum* tryptic enzyme) and SCCE-like (*stratum corneum* chymotryptic enzyme), result in the degradation of the desmosomal proteins desmoglein 1 (Dsg1) and desmoplakin (Dpk), as well as enhanced profilaggrin proteolytic processing (Descargues *et al.*, 2005). Mutant mice with a similar clinical phenotype have also been developed by others groups (Yang *et al.*, 2004; Hewett *et al.*, 2005). Our findings are consistent with the co-localization of LEKTI with SCTE and SCCE in lamellar bodies in the granular layer (Ishida-Yamamoto *et al.*, 2005) and the demonstration of elevated levels of trypsin-like activity in the stratum corneum of patients with NS (Komatsu *et al.*, 2002). Herein, we have analyzed the molecular mechanism underlying the epidermal defect in NS. In a majority of severely affected patients, Dsg1 and desmocollin 1 (Dsc1) expression was decreased in the upper spinous and granular layers with corneodesmosomes appearing reduced in size and number in the basal stratum corneum. In these patients, the activity of stratum corneum tryptic- and chymotryptic-like enzymes was enhanced, suggesting that these proteases cause early degradation of corneodesmosomal cadherins in the epidermis of NS patients. In contrast, a subset of less severely affected patients, two of whom expressed residual LEKTI levels, showed apparently normal corneodesmosomal cadherins expression and stratum corneum protease activity. Taken together, these results suggest that reduced Dsg1 and Dsc1 staining correlates with clinical severity in NS.

RESULTS

Clinical and molecular features of NS patients

We have examined retrospectively a total of 15 NS patients from diverse ethnic backgrounds (for details see Materials and Methods), aged from less than 1 year to 41 years old at the time of the skin biopsy (Table 1). All patients presented with the characteristic clinical triad consisting of scaly erythroderma associated with trichorrhexis invaginata and atopic manifestations, except for patient 3 who has no scaly erythroderma. To compare the severity between patients, we developed a severity score modified from Sprecher *et al.* (2001). Patients from nine families (2, 6–11, 13, and 14) have severe clinical manifestations including generalized skin involvement with severe hair defect, associated with a history of failure to thrive and/or growth retardation, hypernatremic dehydration, and recurrent infections. In the six other families (1, 3–5, 12, and 15), patients had less severe disease manifestation with moderate skin involvement and rare complications. Patient 3 presented with mild skin phenotype at the time of the skin biopsy, but died during the neonate period as a result of pulmonary complications. We have identified at least one mutation in all patients by direct sequencing of the entire coding sequence of *SPINK5* (Table 1). Mutations with their international nomenclature are shown in Table S1. The mutations were reported previously for a majority of patients (1–3, 7, 9, 10, 13, and 14) (Chavanas *et al.*, 2000; Bitoun *et al.*, 2002). All mutations lead to premature termination codons or splice site defects. Loss of LEKTI expression in the epidermis was found in all patients (Figure 1d), except for patients 12 and 15. Instead, these two patients showed a faint and diffuse immunostaining of LEKTI in the upper spinous and granular layers of the epidermis (Figure 1e). Only one *SPINK5* mutation has been identified in patient 12 (c.238_239insG), whereas patient 15 is homozygous for c.2579delA. These mutations were previously reported in other patients in Bitoun *et al.* (2002)

Table 1. Clinical and molecular features of NS patients

				Skin involvement			Hair Shaft abnormalities (TI, PT, TN)		Serum IgE levels (IU/mL)			Associated Features			
				1	2	3	1	2	1	2>	3>				
Patient	Age at biopsy	*SPINK5* mutations	LEKTI	ILC	Mild scaly erythroderma	Generalized scaly erythroderma	Mild	Severe	10^2–10^3	10^3–10^4	10^4	Hypernatremic dehydration (0/1)	Failure to thrive (0/1)	Recurrent infections (0/1)	Severity score
1	1	c.1888-1G>A (homoz)	–	1			1			2		0	0	1	5
2	17	c.2313G>A (homoz)	–	1		3		2		2		1	1	1	11
3	<1[1]	c.153delT (homoz)	–	1			1			ND		1	1	1	5
4	2	c.2468_2469insA/nd	–		2		1		1			1	0	1	6
5	17	p.Arg217X/ nd	–	1				2	1			0	0	1	6
6	<1	c.268_269insT (homoz)	–			3		2	1			1	1	1	9
7	27	c.283-2A >T/ c.2468_2469insA	–	1		3		2		2		ND	1	1	10
8	15	c.238_239insG/ nd	–			3		2		2		1	1	1	10
9	5	c.1888-1G>A (homoz)	–			3		2			3	0	1	1	10
10	5	c.81+2T>G/ c.153delT	–	1		3	1				3	1	1	1	11
11	41	c.1888-1G>A (homoz)	–			3		2			3	1	ND	1	10
12	21	c.238_239insG/nd	+		2		1			2		0	0	1	6
13	27	c.238_239insG/ p.Arg217X	–			3		2			3	1	1	1	11
14	12	c2041_2042del/ p.Arg371X	–	1		3		2		2		1	1	0	10
15	3	c.2579delA (homoz)	+		2		1			2		0	0	0	5

homoz, homozygous; IgE, immunoglobulin E; ILC, ichthyosis linearis circumflexa; ND, not determined; PT, pili torti; SE, scaly erythroderma at birth; TI, trichorrhexis invaginata; TN, trichorrhexis nodosa.
[1]The patient died during the neonate period as a result of pulmonary complications, with minimal skin involvement.

and Sprecher *et al.* (2004), respectively. The c.238_239insG mutation resulted in a frame shift with a predicted premature termination codon 18 residues downstream of the mutation (p.Ala80GlyfsX18). This suggests that the weak immunostaining of LEKTI in the epidermis of patient 12 could be the consequence of the still unidentified mutation on the second *SPINK5* allele. The c.2579delA mutation localized in exon 27 of *SPINK5* leads to a premature codon stop 63 residues downstream of the mutation (p.Lys860SerfsX63). This indicates that patient 15 with the homozygous c.2579delA mutation could express a truncated from of LEKTI in the epidermis. Interestingly, patients 12 and 15 have a less severe clinical phenotype, with no failure to thrive and no hypernatremic dehydration (Table 1), suggesting that residual LEKTI expression in the epidermis could retain some functional activity, but is not sufficient to prevent the disease.

Alteration of desmosomes ultrastructure in NS epidermis

Transmission electron microscopy examination of the epidermis from seven NS patients (1–3, 9–11, 13, and 15) revealed pronounced defects. In all patients studied, lamellar bodies, which are normally secreted at the interface between the granular layer and the stratum corneum, were prematurely fused with the plasma membrane (Figure 2a). They were secreted into the intercellular spaces of the upper spinous and granular layers, as reported previously (Fartasch *et al.*, 1999) (Figure 2a). In these cell layers, lamellar bodies presented with abnormal morphology as described (data not shown) (Fartasch *et al.*, 1999). In these patients, the cornified envelope was prematurely mature in the granular layer as shown by the increase in the plasma membrane thickness (Figure 2b). Unprocessed lamellar body-derived sheets and also intermingled electron-dense material dilated the extracellular spaces of the granular layer (Figure 2b). In this layer, desmosomes were prematurely changed into corneodesmosomes, which are characterized by a well-defined electron-dense intercellular plug (desmoglea) (Figure 2b). Individual corneocytes showed numerous lipid droplets (Figure 2c), undelivered lamellar body discs in the horny cell matrix (data not shown), nuclear remnants (Figure 2d), and other

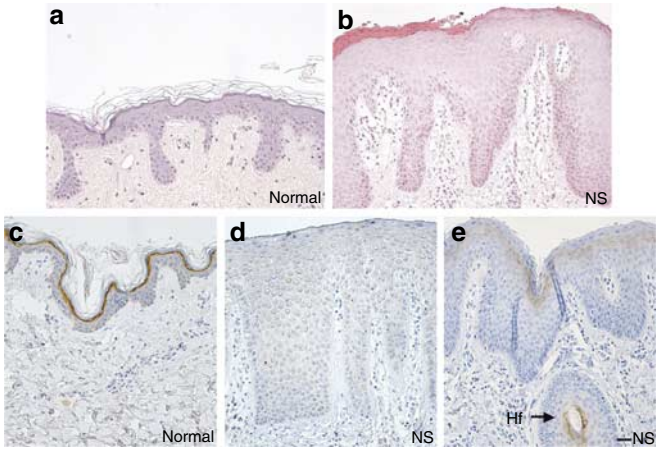

Figure 1. Histopathology of NS epidermis. (**a**) Hematoxylin–eosin staining shows a normal epidermis with approximately a thickness of five cell layers in the stratum Malpighi (living cell layers) and the characteristic basket wave conformation of the stratum corneum. (**b**) NS patient epidermis shows an increased epidermal thickness (acanthosis) and marked invagination of the epidermis into the dermis (papillomatosis). The stratum corneum is lacking on the right side of the picture and is replaced by parakeratotic cells (corneocytes with persistent nuclei) on the left side. The granular layer is focally present in the epidermis of the NS patient on the right side of the picture and is lacking in the region with parakeratosis. Immunohistochemical staining of paraffin-embedded sections with anti-LEKTI D1–D6 monoclonal antibody showing that LEKTI is mainly expressed in the (**c**) granular layer in normal epidermis and is absent in the (**d**) epidermis of NS patients, as shown previously (Bitoun *et al.*, 2003). (**e**) In contrast, patient 15 displays a faint and diffuse immunostaining of LEKTI in the upper spinous and granular layers as well as in the inner root sheath of hair follicles. Hf: hair follicle. Scale bar: 50 μm.

inclusions. In addition to these anomalies, intercellular splits could be found in some patients, at the transition between the granular layer and the stratum corneum (Figure 2c) or in the lower stratum corneum (Figure 2d). Interestingly, corneodesmosomes appeared considerably altered, with degradation of the desmoglea (Figure 2e), in the lower stratum corneum for all the patients analyzed, except for patient 15 (Figure 2c and d). Monitoring of corneodesmosomes properties at the granular layer and stratum corneum interface revealed a significant reduction (28%) in their number in the epidermis of NS patients in comparison with controls (Table S2). The average corneodesmosome size was also diminished by 37% in the epidermis of patients with NS, compared to controls (Table S2). A minority of patients showed altered desmosomes in the upper spinous and granular layers with dilation of intercellular spaces (Figure 2f). Microsplits were also occasionally observed for these patients in the lower granular layer (Figure 2g) with desmosomes associated entirely with one of the two cells and not split in half (Figure 2h). Anomalies of (corneo)desmosome ultrastructure and number were not observed in the epidermis of patients with other keratinizing disorders, including lamellar ichthyosis (two cases), ichthyosis vulgaris (one case), and psoriasis (one case) (data not shown). For these patients, no split could be seen at the transition between the stratum corneum and the granular layer or in the lower stratum corneum (data not shown). All together, these results indicate that premature degradation of

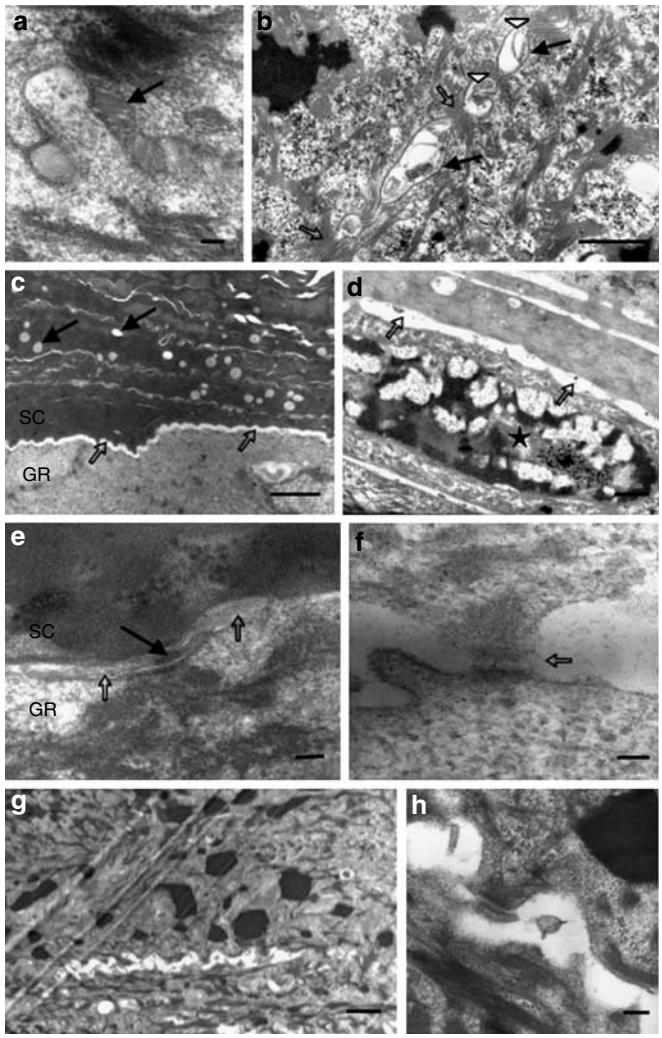

Figure 2. Ultrastructure analysis of NS epidermis. In normal epidermis, exocytosis of lamellar bodies was completed at the interface between the granular layer and the stratum corneum (data not shown). (**a**, arrow) In the epidermis of NS patients, lamellar bodies are prematurely fused with the plasma membrane of keratinocytes in the granular layer and are secreted into the intercellular spaces. (**b**) In the granular layer of the epidermis of patient with NS, the plasma membrane is thicker (arrows), suggesting accelerated maturation of the cornified envelope. (**b**, open arrowheads) Unprocessed lamellar body sheets dilate the extracellular spaces of the overlying granular layer. (**b**, open arrows) In this same layer, desmosomes are prematurely transformed into corneodesmosomes with well-defined electron-dense plugs corresponding to the reinforcement of the desmoglea. In the stratum corneum, the cytoplasm of the corneocytes contains numerous (**c**, arrows) lipid droplets and (**d**, star) nuclear remnants. (**c** and **d**, open arrows) At the interface between the granular layer and the stratum corneum, or in the lower stratum corneum, splits can be seen with partial or complete loss of corneodesmosomes, (**e**, arrow) as a consequence of a premature degradation of the extracellular plug (desmoglea). (**e**, open arrow) Note the presence of lamellar sheets surrounding the corneodesmosome remnant. (**f**) In the upper spinous and granular layers, some desmosomes show abnormal ultrastructure associated with the dilation of the intercellular space. (**g**) Micro-splits can be occasionally found with (**h**) desmosomes associated entirely with one of the two cells . SC: stratum corneum; GR: granular layer. Scale bars: (**a**) 0.1 μm; (**b**) 1 μm; (**c**) 3.3 μm; (**d**) 1.4 μm; (**e**) 0.2 μm; (**f**) 0.1 μm; (**g**) 1.4 μm; and (**h**) 0.25 μm.

corneodesmosomes and splits formation could be a unique feature of the NS epidermis.

Abnormal expression of epidermal terminal differentiation markers reveals premature cornification

We investigated the expression of epidermal terminal differentiation markers including involucrin, loricrin, and filaggrin in the epidermis of patients with NS. Involucrin, which is an early marker of cornification, was expressed in the upper spinous layer and in the granular layer of normal epidermis (Figure 3a). In NS epidermis, involucrin was slightly overexpressed in the same layers (Figure 3b). Loricrin, a much later marker of cornification, was restricted to the upper granular layer in normal epidermis (Figure 3c). Its staining was markedly increased and diffused in the cytoplasm of keratinocytes in the superficial spinous and granular layers of NS epidermis (Figure 3d). Abnormal expression of involucrin and loricrin was observed in a majority of patients analyzed. Filaggrin, which is mainly present in the stratum corneum of normal epidermis, showed a discontinuous pattern in the stratum corneum of NS patients (Figure 3f).

Reduction of desmosomal proteins staining in the granular layer

We have analyzed the expression of the major desmosomal components including Dsg1 and -3, Dsc1 and -3, Dpk, plakoglobin, and corneodesmosin (CDSN). As shown previously, Dsg1 and Dsc1 expression were restricted to the upper spinous and granular layers in normal epidermis (Figure 4a and c). In the epidermis of NS patients, Dsg1 was present in the suprabasal cell layer, but was markedly reduced or absent in the upper spinous and granular layers of the epidermis in nine patients (1,2, 7–11, 13, and 14) (Figure 4b). Anti-Dsc1 staining was also diminished in these layers in the epidermis of six patients (2, 8–11, and 13) (Figure 4d). Dsg3 and Dsc3 staining predominated in the basal layer of normal epidermis and extended to almost all epidermal cell layers (Figure 4e and g). In the epidermis of NS patients, these two desmosomal proteins were redistributed to suprabasal layers, leaving the uppermost part of the acanthotic epidermis unstained (Figure 4f and h). DP, which is a constitutive desmosomal component linking desmosomes to the cytoskeleton, was expressed in all the layers of normal and NS epidermis (Figure 4i), but was reduced in the upper spinous and granular layers in three NS patients (8, 10, and 11) (Figure 4j). Interestingly, desmosomal protein expression (Dsg1, Dsc1, and DP) was normal in patients 12 and 15 who showed moderate LEKTI expression (data not shown). Plakoglobin, which is a component of both desmosomes and adherents junctions, showed no obvious anomalies in NS epidermis when compared with control samples (data not shown). CDSN, which is incorporated into the extracellular core domain (desmoglea) of desmosomes at the transition between the granular layer and the stratum corneum, was expressed in the upper granular layer in normal epidermis (Figure 4k). This protein was overexpressed in the upper spinous and granular layers up to the stratum corneum in the epidermis of eight NS patients for which Dsg1 staining was

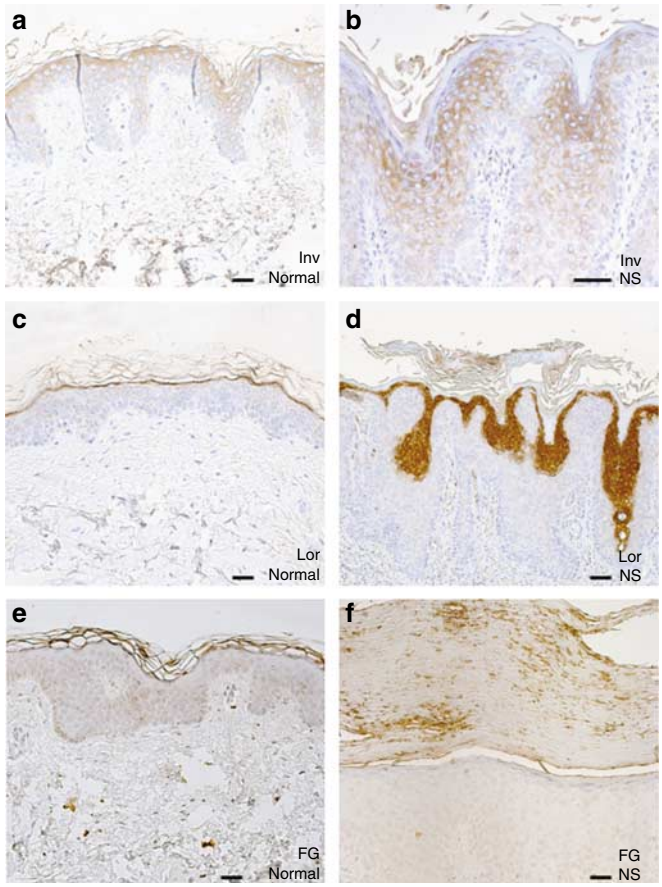

Figure 3. Immunohistochemistry of epidermal terminal differentiation markers. (**a**, **b**) Distribution of involucrin, a major precursor of the cornified envelope, in the epidermis of normal control and NS patient showing expression in the upper spinous and granular layers in both cases, but with a moderate increase in NS patient skin. (**c**, **d**) Loricrin staining is more intense in the granular and the upper spinous layers of the epidermis of NS patient epidermis compared with that of normal skin. (**e**, **f**) Filaggrin shows a discontinuous expression in the stratum corneum of NS patients, the intensity of which is comparable with those seen in normal stratum corneum. Scale bar: 50 μm.

decreased (Figure 4l). CDSN immunostaining was mainly polarized at the upper periphery of the keratinocytes in the epidermis of patient with NS (Figure 4l). The anomalies of Dsg1 and Dsc1 expression described above were not observed in the pathological epidermis of patients with psoriasis, atopic dermatitis, or lamellar ichthyosis (9, 4, and 5 cases analyzed, respectively) (data not shown).

Redistribution of the epidermal proteases SCTE and SCCE and proteinase-activated receptor-2

We have analyzed the expression of SCTE and SCCE, two major serine proteases involved in the desquamation process through the degradation of desmosomal proteins in the lower layers of the stratum corneum in normal epidermis. In controls, both SCTE and SCCE were expressed in the stratum corneum and also at the transition between the granular layer and the stratum corneum (Figure 5a and e). Of the nine NS patients with reduced Dsg1 expression, four patients (9, 11,

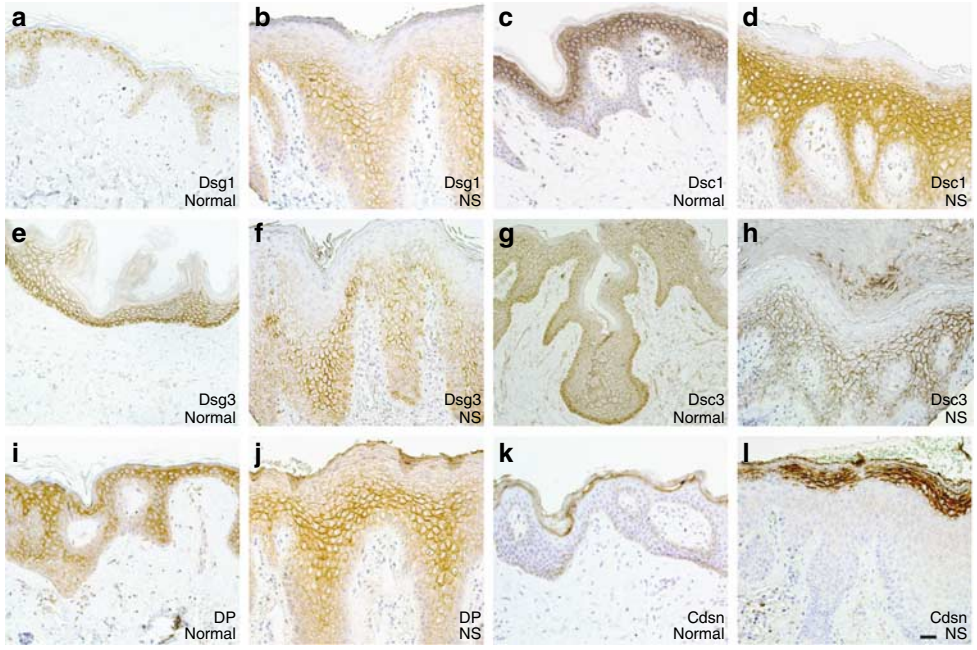

Figure 4. Immunodetection of epidermal desmosomal components. (**a**, **c**) Dsg1 and Dsc1 are expressed in the upper spinous and granular layers of normal epidermis. (**b**) The cell surface staining of Dsg1 is detectable in the suprabasal epidermis, but is remarkably reduced or absent in the upper spinous and granular layers of NS patient epidermis. (**d**) Similarly, Dsc1 immunostaining is also decreased in these cell layers. (**e** and **g**) Dsg3 and Dsc3 staining is mainly seen in the basal layer of normal epidermis and extended to almost all epidermal cell layers. (**f** and **h**) In the epidermis of NS patients, these two desmosomal proteins were redistributed to suprabasal layers, leaving the uppermost part of the epidermis unstained. DP detection, a component of the desmosomal plaque expressed in (**i**) all cell layers of normal epidermis, is also decreased in the (**j**) granular layer of NS epidermis . CDSN expression is increased and extended to the (**k**) upper spinous and granular layers of NS patient epidermis, compared to its expression in the (**l**) stratum corneum and the granular layer of normal epidermis. Scale bar: 50 μm.

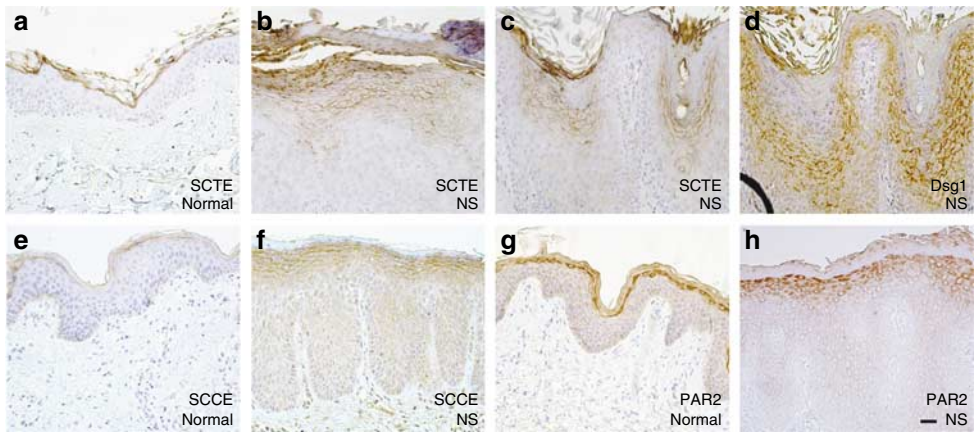

Figure 5. Immunohistochemical analysis of stratum corneum proteases and proteinase-activated receptor-2. (**a**) Anti-SCTE staining shows a restricted distribution of this protease in the stratum corneum and at the interface with the underlying granular layer in normal epidermis. SCTE is slightly increased in the (**b**, **c**) stratum corneum of NS patients and is focally expressed at the (**b**, **c**) cell surface of several cell layers just beneath the stratum corneum including the upper spinous and granular layers. Remarkably, the extension of SCTE labeling correlates with the cell layers in which Dsg1 staining is reduced (compare **c** with **d**). SCCE is mainly present in the (**e**) lower stratum corneum in normal epidermis, whereas its expression is also detected in the (**f**) upper spinous and granular layers in NS patient epidermis. PAR2 cytoplasmic staining is present in an increased number of cell layers in the (**h**) upper spinous and granular layers in the epidermis of patients with NS, in comparison with its distribution restricted to the (**g**) upper granular layer in normal epidermis. Scale bar: 50 μm.

13, and 14) showed redistribution of SCTE immunostaining at the periphery of keratinocytes in the upper spinous and granular layers (Figure 5b). Interestingly, SCTE staining matched the cell layers where Dsg1 expression was reduced (Figure 5c and d). Redistribution of SCCE was also seen in the upper spinous and granular layers in five of the nine patients mentioned above (8–10, 13, and 14) (Figure 5f). In contrast, none of the patients with apparently normal Dsg1 expression showed abnormal expression of SCTE or SCCE (data not shown). We next studied the expression of the protease-activated receptor 2 (PAR2), a receptor that can be activated by trypsin-like proteases and which is involved in skin

inflammation. In normal epidermis, PAR2 immunostaining was cytoplasmic and restricted to the granular layer (Figure 5g). In all NS patient epidermis studied (7–14), PAR2 staining was seen in an increased number of cell layers, spanning from the upper spinous layer to the granular layer (Figure 5h).

Enhanced stratum corneum trypsin- and chymotrypsin-like activity in NS patients

We next assessed whether the diminished corneodesmosome density and desmosomal protein expression in the upper spinous and granular layers resulted from enhanced epidermal protease activity in seven NS patients (2–4, 6, 11, 13, and 15). *In situ* zymography using casein conjugated to FITC as a substrate showed that protease activity was markedly increased in the epidermis of three NS patients (2, 11, and 13) in comparison with normal epidermis (Figure 6a and b). This enzymatic activity mainly localized to the stratum corneum (Figure 6b). We used a preferential substrate of SCTE to evaluate the *in situ* trypsin-like activity of the epidermis from seven NS patients. In normal epidermis, trypsin-like hydrolytic activity mainly localized to the stratum corneum and could be attributed to SCTE-like activity (Figure 6d). SCTE-like activity was increased in the stratum corneum of the epidermis in patients 2, 11, and 13 (Figure 6e). *In situ* zymography was also performed with a preferential substrate of SCCE showing that SCCE-like activity was present, although weak, in the stratum corneum of normal epidermis (Figure 6g). SCCE-like activity was markedly increased in the stratum corneum of patients 2, 11, and 13 (Figure 6h). Interestingly, increased SCTE- and SCCE-like activity in the stratum corneum of NS patients 2, 11, and 13 coincided with the diminution of Dsg1 and Dsc1 expression in the upper spinous and granular layers. In the four other NS epidermis analyzed (3, 4, 6, and 15), protease activity was not enhanced, and expression of these desmosomal cadherins was normal.

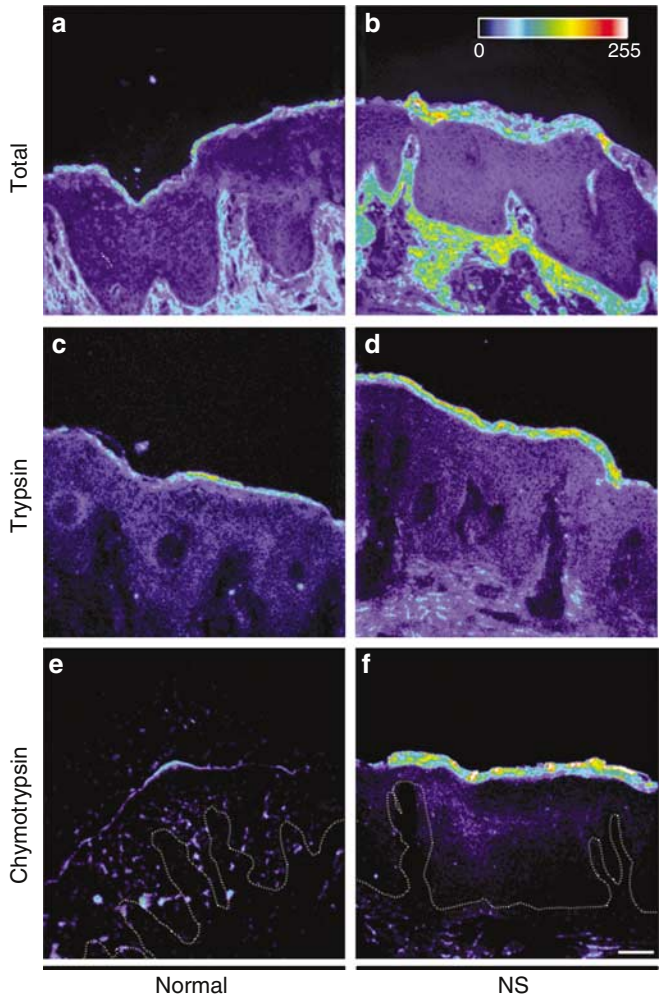

Figure 6. *In situ* zymography analysis. (**a**) In normal epidermis, total protease activity detected by the degradation of the BODIPY FL casein substrate is mainly found in the stratum corneum. (**b**) In the epidermis of patient 13 with NS, the caseinolytic activity is increased in the stratum corneum. (**c**, **d**) Trypsin-like activity shown by cleavage of the synthetic peptide Boc-Val-Pro-Arg-AMC is increased in the stratum corneum of patient 13 in comparison with normal epidermis. (**e**, **f**) Incubation of frozen skin sections with Suc-Leu-Leu-Val-Tyr-AMC peptide solution reveals that chymotrypsin-like activity is also markedly enhanced in the stratum corneum of patient 13, compared with a normal control. The color gradient represents the intensity values of the fluorescence signals ranging from 0 (dark) to 255 (white). Scale bar: 50 μm.

DISCUSSION

In this study, we provide evidence that the absence of LEKTI expression in NS results in enhanced SCTE- and SCCE-like activity with premature degradation of corneodesmosomes in the lower stratum corneum. In the epidermis of patients with NS, corneodesmosomes displayed abnormal ultrastructure with alteration of the well-defined electron-dense plugs, as seen in the upper stratum compactum of normal epidermis, where loss of cohesion between corneocytes leads to desquamation (Fartasch *et al.*, 1993). These defects are associated with a reduction of Dsg1 and Dsc1 immunostaining in the uppermost living layers of the most severely affected NS patients. This reduction is likely to result from an abnormal degradation of these desmosomal cadherins by the unregulated SCTE- and SCCE-like activity. The redistribution of SCTE and SCCE immunostaining in the cell layers in which Dsg1 and Dsc1 staining was reduced supports this hypothesis. However, increased proteolytic activity was not detected in these cell layers, suggesting a possible role of other proteases in desmosomal proteins premature degradation. Taken together, these results are consistent with the recent demonstration that *Spink5*$^{-/-}$ mice reproduce the NS phenotype through the degradation of Dsg1 by epidermal proteases (Descargues *et al.*, 2005).

The SCTE and SCCE serine proteases, two members of the kallikrein family (klk5 and klk7, respectively), are major regulators of the desquamation process (Hansson *et al.*, 1994; Ekholm *et al.*, 2000), and are expressed in the granular layer of normal epidermis (Ishida-Yamamoto *et al.*, 2005). *In vitro* studies have demonstrated that SCTE degrades both Dsg1 and Dsc1, whereas SCCE cleaves only Dsc1 (Caubet *et al.*, 2004). These results are strongly concordant with the reduction of desmosomal cadherins staining in the granular layer and the

increased activity of SCTE- and SCCE-like enzymes in the stratum corneum of NS patients that we report. As LEKTI can inhibit both SCTE and SCCE *in vitro* (Egelrud *et al.*, 2005; Schechter *et al.*, 2005), loss of LEKTI expression in NS epidermis could result in an unregulated activity of these two proteases, with a consequent degradation of Dsg1 and Dsc1. Other trypsin-like proteases that are members of the kallikrein family (klk1, klk4, klk6, klk9–11, klk13, and klk14) are also expressed in the granular layer of normal epidermis (Yousef and Diamandis, 2001; Komatsu *et al.*, 2003, 2005). Therefore, the enhanced trypsin-like activity in the stratum corneum of NS patients could also be attributed to other unregulated proteases.

Whereas epidermal desmosomes contain four isoforms of Dsgs and three isoforms of Dscs, corneodesmosomes contain only Dsg1 and Dsc1 isoforms (Yin and Green, 2004). Therefore, premature degradation of these two cadherins in the epidermis of NS patients by SCTE- and SCCE-like proteases could well result in the loss of stratum corneum integrity and split formation. These anomalies could account for the severe skin barrier defect observed in NS patients and their propensity to develop hypernatremic dehydration (Moskowitz *et al.*, 2004).

Out of the 15 patients studied, six patients (3–6, 12, and 15) presented with normal Dsg1 and Dsc1 immunostaining in the epidermis. SCTE or SCCE immunostaining were also distributed normally in the stratum corneum of these patients. In patients 12 and 15, residual LEKTI expression could in part inhibit epidermal serine proteases activity and prevent premature proteolysis of Dsg1 and Dsc1. This could account for limited corneodesmosome alterations as seen in patient 15, and for a less severe clinical phenotype. The other four patients (3–6) with normal Dsg1 and Dsc1 expression displayed SCTE- and SCCE-like activity in the stratum corneum, equivalent to that detected in normal skin. Interestingly, three of these patients (3–5) showed a mild to moderate cutaneous phenotype at the time of the biopsy. These results suggest that in the absence of LEKTI, the activity of the stratum corneum proteases could be attenuated by other skin-derived protease inhibitors, such as SKALP (skin-derived antileukoprotease) (Franzke *et al.*, 1996), which has been shown to be overexpressed in the epidermis of NS patients (Raghunath *et al.*, 2004; Shimomura *et al.*, 2005) and SLPI (secretory leukocyte protease inhibitor), which is also present in the epidermis (Franzke *et al.*, 1996; Magert *et al.*, 2005).

Several defects seen in NS patients are concordant with the alteration of the keratinization process, which is a characteristic feature of NS (Hausser and Anton-Lamprecht, 1996). The defective skin barrier function, resulting from the loss of stratum corneum integrity, could result in typical compensatory mechanisms of adult epidermis. These include abnormal differentiation as well as hyperproliferation, accounting for acanthosis, and hyperkeratosis (Elias, 2005). The degradation of the desmosomal cadherins in the granular layer of the epidermis in NS patients coincides with CDSN overexpression. This glycoprotein, which is normally incorporated into the extracellular part of corneodesmosomes, has been hypothesized to stabilize them and to shield Dsg1 and Dsc1 from premature proteolysis (Serre *et al.*, 1991; Lundstrom *et al.*, 1994; Simon *et al.*, 1997). CDSN could also be a target for both SCTE and SCCE (Caubet *et al.*, 2004), but its overexpression in NS epidermis has precluded precise analysis of its proteolysis.

Serine proteases can mediate specific regulatory effects via proteinase-activated receptors (PARs). Among the four PARs currently known (PAR1–4), PAR2 is of particular interest as it has been localized to the granular layer of the epidermis (Steinhoff *et al.*, 1999) and is overexpressed in atopic dermatitis (Buddenkotte *et al.*, 2005). Interestingly, PAR2 is also overexpressed in the upper spinous and granular layers of the epidermis in NS patients. In the absence of LEKTI expression, uncontrolled activity of SCTE and/or SCCE may lead to an overactivation of PAR2, as this receptor can be activated by various trypsin-like proteases (Coughlin and Camerer, 2003). As PAR2 is involved in keratinocyte differentiation and proliferation (Santulli *et al.*, 1995; Derian et al., 1997), as well as skin inflammation (Coughlin and Camerer, 2003), its overactivation may contribute to the skin defect observed in NS.

In conclusion, our findings indicate that unregulated desquamation plays a major role in the disease mechanism of NS. In a majority of patients, staining of Dsg1 and Dsc1 is reduced and stratum corneum tryptic- and chymotrytic-like enzyme activity are enhanced. These patients are severely affected with a pronounced barrier abnormality and a marked propensity for hypernatremic dehydration (Moskowitz *et al.*, 2004). Others NS patients do not display these abnormalities and are less severely affected, indicating that the level of LEKTI expression and others factors could play a role in disease expression. These results designate SCTE and SCCE as key players in the diseases process. They also indicate that specific inhibitors of these enzymes have the potential to compensate for LEKTI deficiency and thus could constitute a powerful therapeutic approach to restore epidermal integrity and barrier permeability in NS.

MATERIALS AND METHODS

Patients and clinical material

We ascertained 15 NS families including 15 patients with NS, with their informed consent. Parental consanguinity was known for six patients (1–3, 9, 10, and 15). Patients originated from Europe (France, Italy, and Kosovo) and North Africa (Morocco and Mali). Patient ages ranged from newborn to 41 years old at the time of biopsy and clinical examination. The diagnostic of NS was based on family history and clinical examination according to the following parameters: (i) scaly erythroderma in the first year of life, (ii) ichthyosis linearis circumflexa; (iii) hair shaft defect including trichorrhexis invaginata, and (iii) atopic manifestations (IgE serum levels) (Table 1). Extracutaneous involvement was also recorded. To compare the severity between individuals, we developed a severity score (Table 1) based on the following criteria: skin involvement (ichthyosis linearis circumflexa = 1, mild scaly erythroderma = 2, generalized scaly erythroderma = 3); hair shaft anomalies (mild = 1, severe = 2); IgE levels (10^2–10^3 IU/ml = 1, $>10^3$–10^4 IU/ml = 2, $>10{,}000$ IU/ml = 3), and associated features (absent = 0,

present = 1). Mutations were identified for these patients by direct sequencing of the coding region of *SPINK5* as reported previously (Bitoun *et al.*, 2002). The medical ethical committee CCPPRB (Comité consultatif de protection des personnes se prêtant à des recherches biomédicales) of Toulouse hospitals approved all described studies (Research Project No. 0102908). The study was conducted according to the Declaration of Helsinki Principles and patients gave their written informed consent.

Histopathology

Light microscopy analysis of the epidermis from the 15 NS patients showed numerous abnormalities in comparison with normal epidermis (Figure 1a and b). A marked acanthosis associated with papillomatosis was a constant feature. The stratum corneum was often parakeratotic in NS patients (11 patients out of 15) and hyperkeratosis (increased thickness of the stratum corneum) could be detected in seven patients (6 and 9–14). The granular layer was often mostly diminished in NS epidermis or completely lacking in regions with parakeratosis. A marked hypergranulosis was seen in the epidermis of patients 9 and 14 in association with hyperkeratosis of the stratum corneum without parakeratosis. Only a minimal to moderate inflammatory infiltrate, mostly consisting of mononuclear cells, was present in the papillary dermis of a majority of patients.

Transmission electron microscopy

Small pieces of skin samples were fixed in 4% glutaraldehyde with 0.1 phosphate (Sorensen's) buffer, pH 7.4, for 1 hour at 4°C, and rinsed twice in Sorensen's buffer, pH 7.4, for 12 hours at 4°C. They were subsequently post-fixed for 1 hour at room temperature in 0.25 M saccharose with 0.5 M Sorensen's buffer and 2% osmium. The samples were then dehydrated in a graded series of ethanol solutions and embedded with the Embed 812 kit (Electron Microscopy Sciences, Hatfield, PA). 'Near-surface' sections (80–90 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope. Corneodesmosome properties were analyzed as follows: total individual corneodesmosomes were quantified at electron microscopy magnification (× 15,000) at the granular layer and stratum corneum interface, and factorized by total continuous membrane length previously normalized against scale bar within each image. Individual corneodesmosome length along the plasma membrane at the granular layer and stratum corneum interface was also measured. The statistical significance of differences between groups (normal and NS) of continuous variables was analyzed using the Mann–Whitney *U*-test.

Antibodies

Primary antibodies were directed against: involucrin, SCCE, and Dsg1 (Santa Cruz Biotechnology, Santa Cruz, CA); loricrin and filaggrin (Covance Research Products, Denver, PA); Dsg3 and plakoglobin (Zymed Laboratories, South San Francisco, CA); Dsc1 and -3 (Progen, Deutschland); DP (Serotec, UK); and SCTE (Abcam, UK). These antibodies were used following the manufacturer's recommendations. The anti-CDSN polyclonal antibody was generated by Dr A. Ishida-Yamamoto (Department of Dermatologie, Midorigaoka Medical College, Asahikawa, Japan) and was raised against the central part (AAGPPISEGKYFSS) of the CDSN protein. This antibody, as well as the anti-LEKTI D1-D6 monoclonal antibody, was used as previously reported in Descargues *et al.* (2005) and Bitoun *et al.* (2003), respectively. The anti-PAR2 A5 polyclonal antibody was a generous gift from Dr N. Vergnolle and Dr M.D. Hollenberg (Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Canada). This antibody was raised against a peptide sequence derived from the N-terminus of the mouse PAR2 protein (GPNSKGRSLIGRLDTPYGGC) and was used at the dilution of 1:1,000 onto paraffin-embedded sections.

Histology and immunohistochemistry

Skin biopsies were fixed for 24 hours in 10% neutral-buffered formalin, dehydrated for 24 hours in 70% ethanol, and embedded in paraffin. Four-micrometer sagittal sections were cut and stained with hematoxylin and eosin. For immunohistochemical analysis, the reactivity to various antibodies was investigated with the 4 μm sections. A specific signal was detected using the appropriate Dako EnVision System, HPR (DAB) kit (Dako, Denmark).

In situ zymography

Frozen sections (5 μm thickness) were rinsed with a washing solution (2% Tween 20 in deionized water) and incubated at 37°C overnight with 100 μl of BODIPY FL casein using the EnzChek Ultra Protease Assay kit (Invitrogen, Carlsbad, CA) in deionized water (10 μg/ml) in order to visualize global protease activity. Cryostat sections were incubated in the same conditions with 100 μl of Boc-Val-Pro-Arg-AMC or Suc-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich, St Louis, MO) at 100 μM in Tris 50 mM, $CaCl_2$ 10 mM for the detection of trypsin- and chymotrypsin-like activity, respectively. All sections were rinsed with the washing solution, mounted, and visualized with the inverted high-end microscope Axiovert 200 (Zeiss, UK) at an excitation wavelength of 485 and 400 nm and an emission wavelength of 530 and 460 nm for BODIPY FL and AMC fluorescent dyes, respectively. Frozen sections from normal and NS skin were photographed at equal time points and exposure time. The specificity of the fluorescence signal was controlled by incubating frozen sections with 1 mM of serine protease inhibitor AEBSF (Sigma-Aldrich, St Louis, MO). Images were captured and analyzed with Metamorph Imaging system software, version 3.6 (Universal Imaging Corporation, Downingtown, PA). The intensity of the fluorescence signals was coded as color gradient, ranging from 0 (dark) to 255 (white).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. *SPINK5* mutations with international nomenclature notation.

Table S2. Quantification of corneodesmosomes at the GR/SC interface.

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