DNase1L2 Degrades Nuclear DNA during Corneocyte Formation

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The removal of keratinocyte (KC) nuclear DNA by deoxyribonucleases (DNases) is an important step in the formation of normal stratum corneum (SC). However, the molecular identity of the DNA-degrading enzymes has so far remained elusive. Here we show that the endonuclease DNase1-like 2 (DNase1L2) is preferentially expressed in the epidermis and that its expression correlates with terminal differentiation of KC *in vitro* and *in vivo*. In biopsies of normal skin, DNase1L2 mRNA was regularly found in suprabasal KC and DNase1L2 protein was highly abundant in the stratum granulosum. In contrast to normal skin, DNase1L2 expression was downregulated in parakeratotic epidermis such as in psoriatic lesions. When DNase1L2 gene expression was knocked down by small interfering RNA in a human skin equivalent model, nuclei were maintained through all layers of the SC. Taken together, our data demonstrate that DNase1L2 plays an essential role in DNA degradation during terminal differentiation of epidermal KC.

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INTRODUCTION

Normal terminal differentiation of epidermal keratinocytes (KC) leads to the loss of all cellular organelles, including the nucleus during the conversion of living cells to corneocytes (Haake and Holbrook, 1999). At the transition of the granular layer KC into stratum corneum (SC), nuclear DNA is degraded in a manner that resembles DNA breakdown during apoptosis. Both processes occur within less than 6 hours and involve the formation of double-strand DNA breaks that are detectable by labeling of the free 3′-OH termini using the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling assay (Gavrieli *et al.*, 1992; Holbrook, 1994). Defective breakdown of nuclear DNA during terminal KC differentiation is a characteristic feature of diseases such as psoriasis and chronic dermatitis and manifests histologically as parakeratosis. Yet, the regulation of this process and the molecular identity of the deoxyribonucleases (DNases) involved have remained unknown.

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Abbreviations: ALAS1, aminolevulinate synthase 1; CAD, caspase-activated DNase; DLAD, DNase2-like acid DNase; DNase, deoxyribonuclease; DNase1L2, DNase1-like 2; endoG, endonuclease G; KC, keratinocyte PBG-D, porphobilinogen deaminase; RNAi, RNA interference; SC, stratum corneum; SE, skin equivalent; siRNA, short interfering RNA

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Distinct DNases are active in various physiological and pathological settings (Thompson, 1995; Nagata, 2005; Samejima and Earnshaw, 2005). Extracellular DNA in the alimentary tract and in blood is degraded primarily by DNase1, also known as pancreatic DNase. Lack of DNase1 causes antichromatin autoimmunity and glomerulonephritis (Napirei *et al.*, 2000). Intracellular DNA degradation is a critical part of various forms of programmed cell death. In the context of apoptosis, DNA breakdown is initiated by cell-autonomous DNases within the nucleus of the dying cell (Samejima and Earnshaw, 2005). After the apoptotic cell is phagocytosed by a macrophage or a neighboring cell, lysosomal DNases finish the degradation of DNA (Samejima and Earnshaw, 2005). The most prominent member of cell-autonomous apoptotic DNases is caspase-activated DNase (CAD) (Nagata 2005; Samejima and Earnshaw, 2005). In addition, endonuclease G (EndoG), Nm23-H1, and DNase1-like 3 (DNase1L3) have been shown to degrade nuclear DNA in apoptotic cells (Li *et al.*, 2001; Shiokawa and Tanuma, 2001; Fan *et al.*, 2003). DNA breakdown in phagocytes is mediated by DNase2 (Krieser *et al.*, 2002). Cell type-specific forms of programmed cell death such as terminal differentiation of erythroid precursor cells and enucleation of cells in the eye lens deviate from the two-step process active during apoptosis. The nucleus of erythroid precursors is expelled and phagocytosed by macrophages in which DNase2 digests the DNA (Kawane *et al.*, 2001). In fiber cells of the eye lens, nuclear breakdown occurs completely in a cell-autonomous manner and is mediated by DNase2-like acid DNase (DLAD), an enzyme almost exclusively expressed in eye lens fiber cells (Nishimoto *et al.*, 2003). This process is critical for organ function as evidenced by the development of cataracts of the

nucleus lentis in mice deficient for DLAD (Nishimoto *et al.*, 2003).

We hypothesized that (I) DNA degradation in KC of the upper stratum granulosum was, similar to the enucleation process in lens fiber cells, mediated by a specific DNase and (II) expression of this DNase was associated with terminally differentiated KC. A screening strategy devised on the basis of these assumptions allowed us to identify DNase1-like 2 (DNase1L2) as a likely candidate DNase. Comparative analysis of orthokeratotic and parakeratotic skin supported an involvement of DNase1L2 in terminal differentiation of KC and short interfering RNA (siRNA)-mediated knockdown of DNase1L2 expression in an *in vitro* human skin equivalent (SE) model demonstrated its essential role for DNA degradation in the course of orthokeratotic SC formation.

RESULTS

DNase1L2 mRNA is preferentially expressed in the skin and is upregulated during KC differentiation *in vitro*

To screen for DNases that are upregulated during differentiation of epidermal KC, we compared the mRNA expression levels of nine DNases in human KC undergoing spontaneous differentiation *in vitro*. Differentiation was induced by maintaining cells in progressively confluent culture for up to 5 days. These culture conditions had previously been shown to induce expression of filaggrin, loricrin, involucrin, and caspase-14 (Lee *et al.*, 1998; Eckhart *et al.*, 2000). Real-time PCR quantification revealed that the mRNA levels of most DNases remained either unchanged (DNase1, DNase1L1, DNase2, EndoG) or were even reduced (CAD, Nm23-H1) in day 3 and day 5 postconfluent KC as compared to preconfluent KC (Figure 1a). The expression of DLAD was low in preconfluent KC and fell below the detection limit in differentiated KC (not shown). DNase1L3 mRNA was increased on the third day after KC had reached confluence, but decreased in abundance later. DNase1L2 was the only DNase that was upregulated at both time points analyzed. Expression of DNase1l2 increased stronger than the expression of any other DNase during confluent KC culture and finally reached a level approximately 100-fold higher than that found in preconfluent KC (Figure 1a). These screening results prompted us to focus our further investigations on DNase1L2.

Reverse transcription-PCR amplification of the open-reading frame of DNase1L2 revealed that only the large splice variant of DNase1L2 (Shiokawa *et al.*, 2004) was expressed in human KC (not shown). Western blot analysis confirmed that DNase1L2 was also strongly upregulated at the protein level, relative to the product of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase in differentiated KC (Figure 1b). Next, we determined the expression level of the DNase1L2 gene in skin and in various human tissues. Using real-time PCR, abundance of DNase1L2 mRNA was found to be at least 16 times higher in the skin than in any other organ tested (Figure 1c). Together, these data suggested that DNase1L2 represented a likely candidate for a specific function during terminal differentiation of KC.

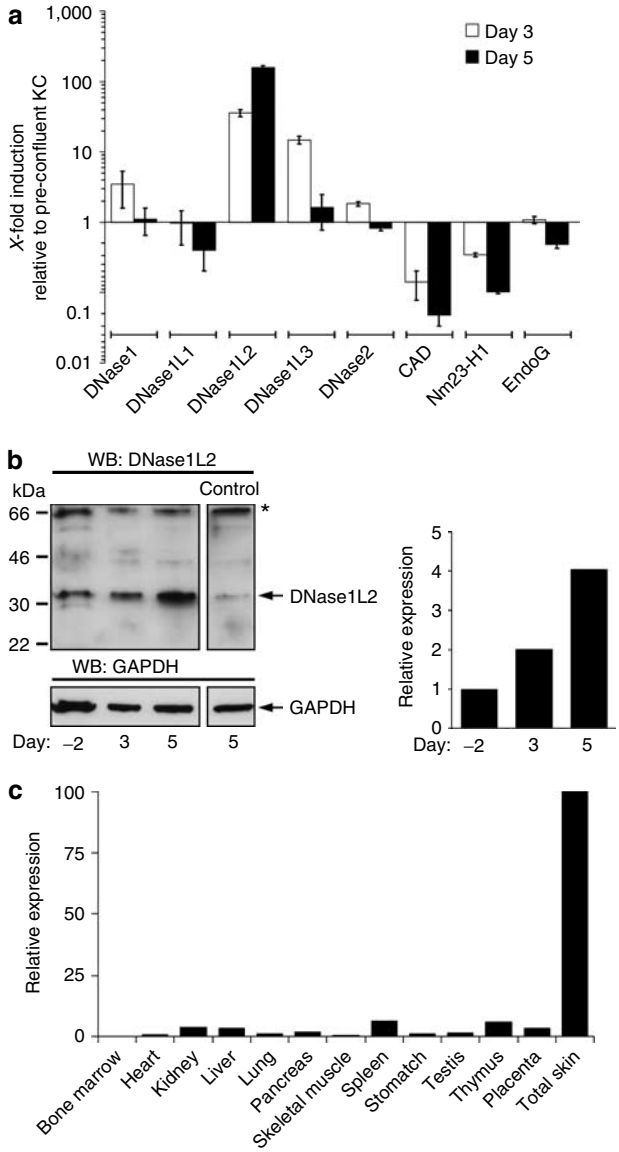

Figure 1. DNase1L2 expression is stronger in skin than in other tissues *in vivo* and is associated with terminal KC differentiation *in vitro*. (**a**) The expression levels of different DNases were determined by real-time PCR in KC on days 3 and 5 after reaching confluence. Experiments were performed in quadruplicates. The results were normalized to the expression of ALAS1 and the mean values were displayed in relation to preconfluent KC on a logarithmic scale. (**b**, upper left panel) DNase1L2 protein was detected by Western blot with a rabbit anti-DNase1L2 antiserum. The specificity of the antiserum was evaluated by preincubating the antiserum with recombinant DNase1L2 (control), which largely reduced the intensity of the band at the size predicted for DNase1L2 but did not alter the signal intensity of an unspecific band (asterisk). (**b**, right panel) Quantification of DNase1L2 protein relative to glyceraldehyde-3-phosphate dehydrogenase was performed by densitometric analysis after (**b**, lower left panel) re-probing the blot with anti-glyceraldehyde-3-phosphate dehydrogenase antibody. Expression of DNase1L2 mRNA was analyzed in a panel of human tissues by quantitative real-time PCR. (**c**) The results were normalized to PBG-D expression in the respective tissues and displayed in relation to skin.

DNase1L2 is expressed in suprabasal KC in normal human epidermis

To localize DNase1L2 expression in human skin, we analyzed normal human skin by *in situ* hybridization and immunohistochemistry. DNase1L2 mRNA expression in the epidermis was weak in basal layer cells, but strongly increased toward the granular layer (Figure 2a). Immunostaining with anti-DNase1L2 antiserum showed that DNase1L2 protein was highly abundant in the stratum granulosum but neither in less-differentiated KC nor in other cell types of the skin (Figure 2c). Immunoreactivity in the SC was weak to negative. DNase1L2 was also detected in the cortex of hair follicles (Figure 2e) and in sebaceous glands (Figure 2g). The detection of DNase1L2 was completely blocked by preincubation of the anti-DNase1L2 antiserum with recombinant DNase1L2 (Figure 2d, f, h), but not with purified DNase1 (not shown).

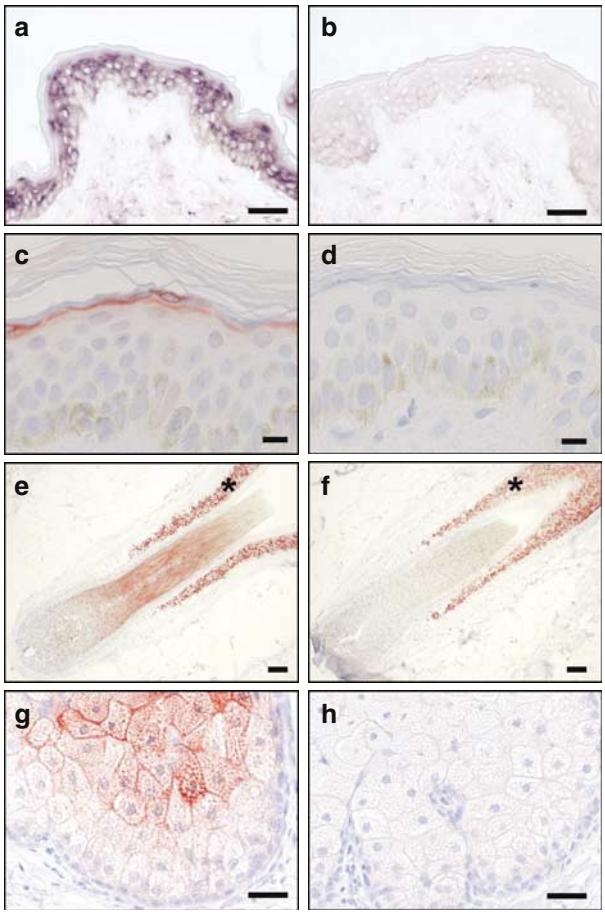

Figure 2. DNase1L2 expression by human KC *in vivo* correlates with their differentiation status. Human skin sections were subjected to (**a**, **b**) DNase1L2-specific *in situ* hybridization or (**c–h**) immunostaining using mouse anti-DNase1L2 antiserum. (**a**) *In situ* hybridization with DNase1L2 antisense probe detected DNase1L2 mRNA in the epidermis with increasing abundance towards the granular layer, (**b**) whereas the control reaction with the sense probe was negative. (**c**) Immunostaining detected DNase1L2 protein in the stratum granulosum. DNase1L2 was also detected within the (**e**) cortex of the hair follicle and in (**g**) terminally differentiated sebocytes in the center of sebaceous glands. (**d**, **f**, **h**) The specificity of the staining was demonstrated by preabsorption of the first step antibody with recombinant DNase1L2, which blocked the staining. (**e**, **f**) Unspecific staining within the hair follicle is marked by an asterisk. Bars = (**c**, **d**) 10 μm, (**a**, **b**, **g**, **h**) 40 μm, and (**e**, **f**) 80 μm.

Expression of DNase1L2 is strongly reduced in parakeratotic epidermis of psoriasis lesions and in Bowen's disease lesions

As compared to normal skin (Figure 3a) and non-involved skin adjacent to psoriasis lesions (Figure 3b), DNase1L2 immunoreactivity was strongly downregulated in lesional psoriatic epidermis with parakeratosis (Figure 3b). Essentially the same pattern was observed in and around parakeratotic lesions in Bowen's disease (Figure 3c). In other skin diseases associated with aberrant KC differentiation such as common warts and eczema, expression of DNase1L2 was in most but not all cases reduced (not shown). In normal skin and in all diseased skin samples, DNase1L2 localized primarily to the cytoplasm of KC and rare, nuclear staining was strictly confined to terminally differentiated KC in areas of orthokeratotic SC formation.

Knockdown of DNase1L2 induces parakeratosis in a human SE model

To characterize the function of DNase1L2 in terminal differentiation of KC, we knocked down the expression of this gene by RNA interference (RNAi). Proliferating KC were transfected with DNase1L2-specific siRNAs and appropriate control small interfering RNAs. Subsequently, the transfected cells were cultured as the epidermal component of an *in vitro* SE model in which KC undergo the full program of terminal differentiation (Rendl *et al.*, 2002). After 7 days of culture, the SEs were analyzed for the expression of DNase1L2 by Western blot and immunohistochemistry. Nuclear DNA was visualized on thin sections by hematoxylin staining and labeling with Hoechst dye, respectively. RNAi suppressed

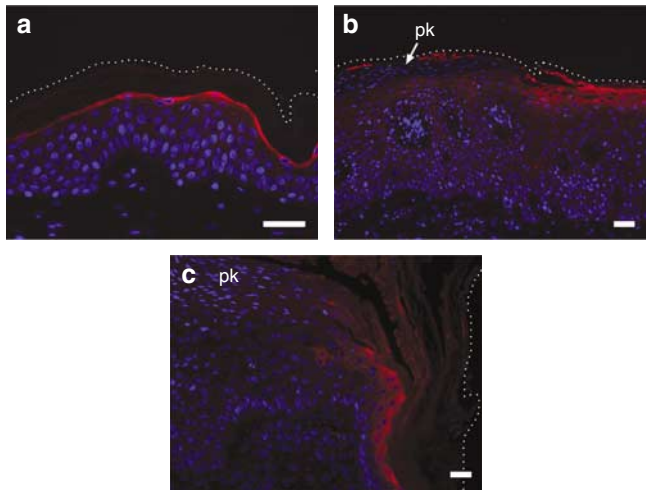

Figure 3. Epidermal DNase1L2 expression is downregulated in parakeratotic epidermis of psoriasis lesions and Bowen's disease. Expression of DNase1L2 was detected by immunofluorescence labeling in (**a**) normal skin, (**b**) psoriasis, and (**c**) Bowen's disease. Note the coincidence of DNase1L2 expression and orthokeratosis in normal and non-lesional skin and the lack of DNase1L2 in parakeratotic (pk) lesions. Dotted lines indicate the border of the SC. Bars = 40 μm.

DNase1L2 expression to less than 5% of the normal level as estimated by semiquantitative Western blot analysis (Figure 4a). At least 90% of the corneocytes were free of nuclear DNA in SEs consisting of KC treated with control siRNA, reflecting efficient DNA degradation (Figure 4b).

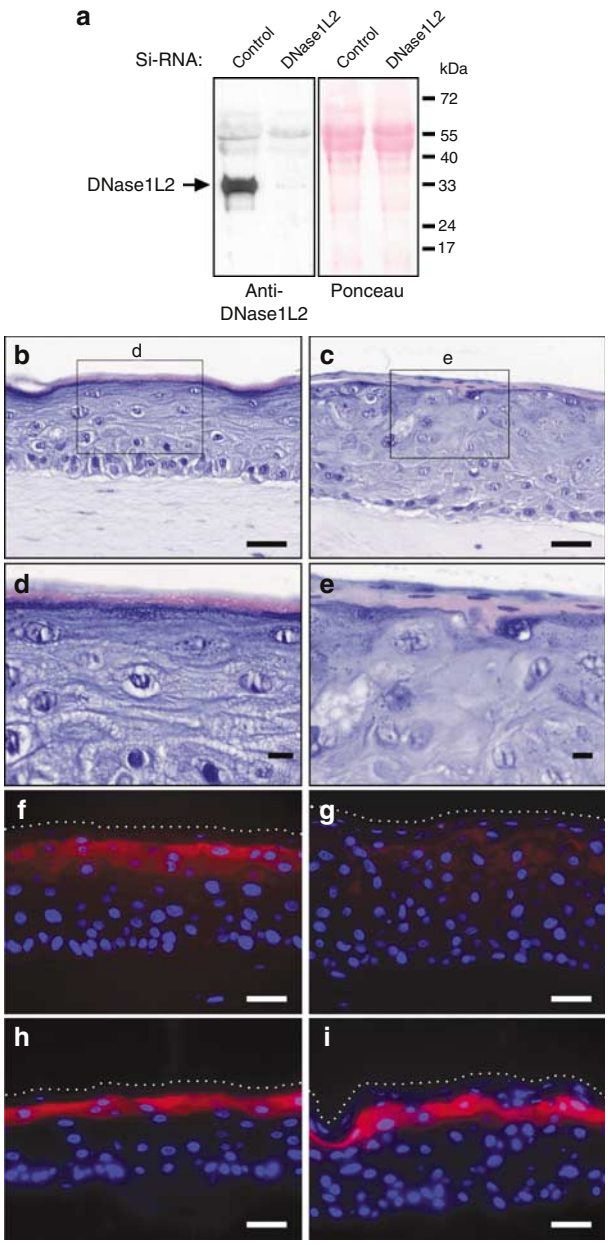

Figure 4. siRNA-mediated knockdown of DNase1L2 in KC results in parakeratosis. KC were transfected with siRNA specific for DNase1L2 and control siRNA and used for the establishment of SE models. After 7 days of differentiation in the SE culture, protein lysates were analyzed by Western blotting using mouse anti-DNase1L2 antiserum (**a**). Equal loading of lanes was confirmed by Ponceau staining of the membrane. SEs were analyzed by (**b–e**) hematoxylin and eosin staining as well as immunofluorescence labeling for (**f**, **g**) DNase1L2 and (**h**, **i**) loricrin. (**d**) and (**e**) represent a higher magnification of (**b**) and (**c**), respectively. (**f–i**) Nuclei were counterstained with Hoechst dye. (**f–i**) Dotted lines indicate the border of the SC. The experiment was performed three times with identical results. Bars = (**b**, **c**, **f**, **g**, **h**, **i**) 40 μm and (**d**, **e**) 10 μm.

By contrast, the SC of siRNA-treated SEs contained nuclear DNA in virtually the same density as present in the living layers of the epidermal compartment, suggesting that DNA breakdown was inhibited by knockdown of DNase1L2 (Figure 4c). Essentially the same results were obtained with two additional DNase1L2-specific siRNAs (not shown), whereas neither of the control siRNAs induced parakeratosis (Figure 4b and results not shown). The expression pattern of the late differentiation marker loricrin was unaffected by the treatment of KC with the DNase1L2 siRNA and by control siRNAs (Figure 4i, h), which confirmed the specific effect of RNAi in this system. Taken together, these data demonstrate that DNase1L2 is required for the degradation of nuclear DNA in terminally differentiated KC.

DISCUSSION

Although the molecular machinery that build up the cornified envelope has been characterized well in recent years (Candi *et al.*, 2005), astonishingly little is known about the simultaneous processes that remove KC components, which are characteristic for living cells, for example, DNA, RNA, ribosomes, mitochondria, and a plethora of metabolic enzymes. Our study identifies the first DNase, and actually the first degradative enzyme at all, which is essential for this special form of KC programmed cell death.

The present study shows that terminal differentiation of epidermal KC involves a change in the expression of DNases. The apoptosis-associated enzyme CAD is downregulated in KC differentiating *in vitro*, whereas an essentially epidermis-specific DNase, that is, DNase1L2, is strongly upregulated. This expression pattern gives support to the postulates of previous reports (Gandarillas *et al.*, 1999; Lippens *et al.*, 2000; Rendl *et al.*, 2002) that the differentiation-associated death of KC does not depend on the molecular machinery of apoptosis but rather on a specific set of enzymes. The physiological role of suppression of CAD and NM23-H1 expression as well as of the upregulation of DNase1L3 in the early phase of differentiation will be subject to further investigations in our laboratory.

Our data demonstrate that DNase1L2 is tightly controlled at the transcriptional level. We found that DNase1L2 mRNA is expressed in the epidermis at much higher levels than in any other organ analyzed and that it is strongly upregulated in terminally differentiated KC. The increase in mRNA expression was consistently associated with an increase in the abundance of the DNase1L2 protein. These findings extend information provided by two previous studies, which reported low levels of DNase1L2 mRNA in several human tissues (Rodriguez *et al.*, 1997; Shiokawa and Tanuma, 2001; Shiokawa and Tanuma, 2004), as these studies provided no data on DNase1L2 expression in the skin. DNase1L2 mRNA was reported to be upregulated by proinflammatory cytokines in the HaCaT cell line (Shiokawa *et al.*, 2004). Based on this finding, the authors suggested that DNase1L2 may have a role in inflammation. Our finding that DNase1L2 is downregulated during skin inflammation *in vivo*, that is, in psoriatic epidermis, argues against such a role for this enzyme. In addition, our data clearly demonstrate that KC

differentiation, even in the absence of exogenous inflammatory cytokines, suffices to strongly upregulate DNase1L2 expression.

Knockdown of DNase1L2 in SE results in parakeratosis, which demonstrates that DNase1L2 is an essential mediator of nuclear DNA degradation in terminally differentiated KC. Although it remains to be investigated whether other DNases are also involved in this process, our data show that the role of DNase1L2 is non-redundant. The results of our immunohistochemical investigation of skin diseases strongly suggest that *in vivo* DNase1L2 is also for orthokeratotic KC differentiation, as its reduction is consistently associated with parakeratosis. The detection of strong DNase1L2 expression not only in subcorneal KC of interfollicular epidermis but also in epidermal appendages, where terminally differentiated KC undergo cell death, namely in hair follicles, sebaceous glands (this study), and nail units (Jaeger K *et al.*, submitted), indicates that DNase1L2 is active in programmed cell death of several or all differentiation lineages of KC.

An important aim of future investigations will be to clarify the regulation of DNase1L2 activity. We observed that DNase1L2 is localized predominantly to the cytoplasm and, only in few cases, to the nucleus of differentiating KC. This is in accordance with the previous finding that transient expression of recombinant DNase1L2 in 293 cells does not result in nuclear translocation of the enzyme and has no immediate impact on the integrity of chromosomal DNA (Shiokawa and Tanuma, 2001). DNase1L2 lacks a nuclear targeting signal (Shiokawa and Tanuma, 2001) and, therefore, can gain access to nuclear DNA only after disruption of nuclear membranes, which in KC presumably occurs in the course of their transition to the SC. In preliminary investigations of the mechanism of DNase1L2-mediated DNA degradation in KC, we could confirm that DNase1L2 is able to degrade chromatin-associated DNA after permeabilization of the nuclear membrane (H. Fischer, L. Eckhart and E. Tschachler, unpublished). The fact that the optimal pH for DNase1L2 activity, that is, pH 5.6 (Shiokawa and Tanuma, 2001), corresponds to the acidic milieu within the SC (Ohman and Vahlquist, 1994) may indicate that full enzyme activity occurs only during or after conversion of KC into corneocytes.

Although parakeratosis is a well-recognized diagnostic feature of many diseases, it is presently not known to what extent degradation of nuclear DNA during corneocytes formation is crucial for SC structure and functions. The identification of DNase1L2 as a critical DNA breakdown enzyme during this process provides the basis for a better molecular definition of differentiation-associated KC cell death and its physiological roles.

MATERIALS AND METHODS

Cell culture

Normal human epidermal KC (Cambrex, San Diego, CA) were cultured as described previously (Rendl *et al.*, 2002). Cells seeded into six-well plates (Corning Incorporated, Corning, NY) were harvested either on the following day at 50–60% confluence or 3–5 days after reaching confluence.

Tissue samples

Normal human skin from mammary reduction surgery was kindly provided by the Department of Plastic Surgery, Medical University of Vienna, Austria. Paraffin-embedded specimens of human skin diseases including psoriasis ($n=6$), common warts ($n=3$), Bowen's disease ($n=3$), and eczema ($n=7$) were obtained from the biopsy archive of the Department of Dermatology, Medical University of Vienna, Austria. All experimental procedures were approved by the Regional Committee for Medical Research Ethics and were conducted in compliance with the Declaration of Helsinki Principles.

siRNA-mediated gene silencing in human SEs

A detailed description of the methodology of RNAi in SE and its validation with established KC target genes will be presented in a separate report (Mildner *et al.*, 2006). In brief, KC were transfected with the following siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA): DNase1L2-siRNA1, 5′-GAGAUCGACGCGCUCUAC GACGUGU-3′; DNase1L2-siRNA2, 5′-GACAUGCUGUUCCUGGG CGACUUCA-3′; DNase1L2-siRNA3, 5′-CGGCCUGGACCAGACUC AGGCUCUU-3′; control siRNA1 (unrelated sequence), 5′-GGCAU UACAGUGUGUCUCACCCAAA-3′; control siRNA2 (scrambled sequence of DNase1L2-siRNA1), 5′-GAGAGCCGCUCGAUCAGCG CAUUGU-3′; and control siRNA3 (scrambled sequence of DNase1L2-siRNA3), 5′-CGGAGGUGACCCUCACGGAUCCCUU-3′. siRNAs with chemical modifications according to the Stealth™ RNAi technology (Invitrogen, Carlsbad, CA) were used. Twenty-four hours after transfection, KC were seeded onto a fibroblast collagen gel suspension and maintained in culture inserts as described by Rendl *et al.* (2002) for 7 days to facilitate differentiation into SEs.

Molecular cloning of DNase1L2

The open-reading frame of DNase1L2 was amplified from KC cDNA using the primers DNase1L2-s1 (5′-TAGGATCTCTGAGCCTCGG-3′) and DNase1L2-a1 (5′-CAGCCGACTCTGCCTTG-3′) and cloned into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). For bacterial expression, full-length DNase1L2 was cloned into pET-23a(+) vector (Novagen, Madison, WI) which allowed the expression of a fusion protein containing an N-terminal T7-tag and a C-terminal 6xHis-tag. The primers used were DNAse1L2-s2 (5′-CGC**GGATCC**A TGGGCGGGCCC-3′) and DNAse1L2-a2 (5′-CCG**CTCGAG**TCGGT GGAACTTGAGGGT-3′). *Bam*HI and *Xho*I sites flanking the coding sequences are indicated in bold. For expression in *Pichia pastoris*, DNase1L2 was re-amplified from the vector pET-23a(+)-DNase1L2, omitting the putative N-terminal signal sequence of DNase1L2 (amino acids 1–18; Shiokawa and Tanuma, 2001), but including the C-terminal 6xHis-tag. The sense primer was DNase1L2-s3 (5′-CC**ATCGAT**AACCGCCGCGCTTCGCATC-3′) and the antisense primer was DNAse1L2-a3 (5′-GC**TCTAGA**TCAGTGGTGGTGGTGG TGGTG-3′). *Cla*I and *Xba*I sites flanking the coding sequences are shown in bold. The fragment was cloned in-frame with the α-factor signal sequence of the *P. pastoris* expression vector pPICZC (Invitrogen).

Reverse transcription-PCR and quantitative real-time PCR

RNA from KC and total human skin as well as polyA+ RNA from multiple human tissues (Clontech, Palo Alto, CA) was reverse-transcribed using the Gene Amp RNA PCR kit (Applied Biosystems,

Foster City, CA). Quantitative real-time PCR was performed with the LightCycler technology (Roche Applied Science, Mannheim, Germany) as described previously (Mrass *et al.*, 2004). The following primers were used: DNase1-s, 5′-CAGGATGCACCAGACACCTA-3′; DNase1-a, 5′-ACAATGGCAAACTCCCTGAC-3′; DNase1L1-s, GCT GCAGGAGGTGGTAGACT-3′; DNase1L1-a, 5′-GGCAAAGACGTC ATCCTCAT-3′; DNAse1L2-s4, 5′-ACTCAGACTGCGCCTACGAC-3′; DNAse1L2-a4, 5′-CTCGAGTCATCGGTGGAACT-3′; DNase1L3-s, 5′-ATCAGGATGGAGACGCAGAT-3′; DNase1L3-a, 5′-CACGTCCG TGTAGACCTCAA-3′; DNase2-s, 5′-TCGCCTTCCTGCTCTACAAT-3′; DNase2-a, 5′-CCCATCTTCGAGAACTGAGC-3′; CAD-s, 5′-CAA TGGCAGCTACTTCGACA-3′; CAD-a, 5′-GGAATGATGGTGCGTTT CTT-3′; Nm23-H1-s, 5′-ACCATCCGTGGAGACTTCTG-3′; Nm23-H1-a, 5′-GAAGGAGGGGAAATGGATGT-3′; EndoG-s, 5′-TGGACG ACACGTTCTACCTG-3′; EndoG-a, 5′- CTTGCCGATGACCTGGTA CT-3′; DLAD-s, 5′-CATCCCTCAGTTTCCTCCAA-3′; and DLAD-a, 5′-GCCAGGAATCTCTGATGAGC-3′. Relative expression of the target molecule was normalized to the expression of the housekeeping genes aminolevulinate synthase 1 (ALAS1), and porphobilinogen deaminase (PBG-D), which were amplified using the primer pairs ALAS1-s 5′-CCACTGGAAGAGCTGTGTGA-3′, ALAS1-a 5′-AC CCTCCAACACAACCAAAG-3′ and PBG-D-s 5′-TCGAGTTCAGTG CCATCATC-3′, PBG-D-a 5′-CAGGTACAGTTGCCCATCCT-3′, respectively. The specificity of the reactions was confirmed by sequencing of the PCR products.

In situ hybridization

A 595 bp fragment of DNase1L2 was re-amplified from the vector pET-23a(+)-DNase1L2 and cloned into the vector pCRII-TOPO (Invitrogen) in sense and antisense direction downstream of the SP6 promoter. The primers were DNase1L2-s5 (5′-GTACAGGAAA GACGCGGTGT-3′) and DNase1L2-a4 (5′-CTCGAGTCATCGGTG GAACT-3′). Both sense and antisense probes were generated by *in vitro* transcription with SP6 polymerase using the DIG RNA labeling kit (Roche, Basel, Switzerland). *In situ* hybridization of paraffin sections was performed according to a protocol published previously (Eckhart *et al.*, 2000).

Production of recombinant DNase1L2

For the expression of recombinant protein in bacteria, DNase1L2 cDNA was cloned into pET-23a(+) (Novagen). Expression of recombinant DNase1L2 in *E. coli* BL-21 (DE3) was induced with isopropyl-β-D-thiogalactopyranoside (1 mM) at 37°C for 3 hours. Inclusion bodies were prepared by lysozyme treatment of cells and harvested by low-speed centrifugation. DNase1L2 was solubilized with 10% SDS, dialyzed against phosphate-buffered saline, and purified by Ni^{2+} affinity chromatography using the Probond purification kit (Invitrogen). Recombinant DNase1L2 was also produced in *P. pastoris* using the Pichia expression kit (Invitrogen) according to the manufacturer's instructions. During expression DNase1L2 protein was processed at the N-terminus by proteolytic removal of the α-factor secretion signal. The active enzyme was purified from the culture supernatant by Ni^{2+} affinity chromatography and dialyzed against 0.1 × phosphate-buffered saline.

Production of antisera against DNase1L2

Polyclonal antisera against DNase1L2 were produced by immunizing rabbits and mice with purified recombinant DNase1L2 preparations derived from *E. coli* and *P. pastoris*. The specificity of the antisera was evaluated by preabsorption with recombinant DNase1L2, which prevented the appearance of Western blot bands at the size predicted for DNase1L2. Both sera were used in Western blot analysis with identical results and the mouse anti-DNase1L2 serum was used for immunohistochemistry and immunofluorescence as described below.

Immunohistochemistry and immunofluorescence

Diseased and normal human skin as well as SEs were fixed with phosphate-buffered 4.5% formaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. Immunohistochemical analysis of paraffin sections was performed according to a protocol published previously (Weninger *et al.*, 1996). Briefly, paraffin sections were prepared for staining by heat antigen retrieval consisting of 2 × 5 minutes microwave cycles at 500 W in Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) cooled to room temperature and incubated with phosphate-buffered saline, pH 7.2, plus 2% BSA and plus 10% goat serum (DAKO) for 20 minutes to block nonspecific binding of the secondary antibody. Endogenous peroxidase was blocked with 0.3% H_2O_2/methanol. The sections were incubated with polyclonal mouse anti-DNase1L2 antibody diluted 1:5000 in phosphate-buffered saline, pH 7.2, plus 2% BSA overnight at 4°C. After washing, slides were incubated with biotinylated goat anti-mouse IgG (1:200; (Amersham Biosciences, Chalfont, UK) and exposed to streptavidin–biotin complex (DakoCytomation). 3-Amino-9-ethylcarbazole was used as chromogen (DakoCytomation). The slides were counterstained with hematoxylin (Merck KGAa). The specificity of the staining was confirmed by preabsorption of the first step antibody with recombinant DNase1L2, which blocked the staining and preabsorption with purified bovine DNase1 (Sigma-Aldrich, St Louis, MO), which did not decrease immunoreactivity even when used at an eight-fold higher concentration than DNase1L2. Immunofluorescence labeling was performed as described previously (Rendl *et al.*, 2002). The results were controlled by antigen preabsorption tests as described for immunohistochemistry. Loricrin was detected with polyclonal rabbit anti-human antiserum ($1:10^3$; Covance, Berkeley, CA).

Western blot analysis

KC were lysed in phosphate-buffered saline containing 1% NP-40 (Sigma-Aldrich) and complete protease inhibitor cocktail (Roche, Mannheim, Germany). SEs were lysed in lysis buffer containing 50 mM Tris (pH 7.4), and 2% SDS by sonication. Insoluble cell debris of lysed KC and SEs were removed by centrifugation and protein concentration was measured by the BCA (bicinchoninic acid) method (Pierce, Rockford, IL). Western blot analysis was performed as described previously (Rendl *et al.*, 2002). The following first step antibodies were used for the detection of specific antigens: rabbit polyclonal anti-DNase1L2 ($1:10^3$), mouse polyclonal anti-DNase1L2 ($1:10^3$), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Biogenesis, Poole, UK, 1:200). For semiquantitative analysis of protein expression, the intensities of Western blot bands were determined by densitometry.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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