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Mechanisms and emerging functions of DNA degradation in the epidermis

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1. ABSTRACT

Degradation of DNA is involved in key processes maintaining the integrity of the epidermis such as the cornification of keratinocytes, the removal of damaged cells and the defense against potentially harmful microorganisms. Recent studies have characterized some of the molecular mechanisms and physiological functions of DNA degradation in the epidermis. Deoxyribonuclease (DNase)1L2 and TREX2 were identified as epidermis-specific DNases and DNase 2 was shown to be the predominant DNA-degrading enzyme on the surface of the skin. Here we review the latest insights into the DNA catabolism in the skin and discuss open questions pertaining to the molecular biology of epidermal DNA breakdown.

2. INTRODUCTION

2.1. Skin barrier function and the catabolism of biological macromolecules

The skin is the outermost organ of the body and forms a barrier to the environment. The protection against environmental insults is achieved by different mechanisms in the various layers of the skin, i.e. the layer of cornified cells (stratum corneum) on the surface, the living epidermis, the dermis and the subcutis. Epidermal keratinocytes play a central role for the maintenance of the outer barrier by constantly renewing the stratum corneum, by synthesizing specialized barrier proteins and lipids and by modulating the function of other skin cells including immune cells. The delicate homeostasis of epidermal differentiation involves processes in which major biological

macromolecules such as DNA, RNA and proteins are degraded. Within the stratum corneum several proteins are cleaved at distinct amino acid sequence motifs (1) whereas nucleic acids are efficiently degraded in a sequence-unspecific manner. It is currently unclear to which extent the processes of degradation of proteins and nucleic acids are interconnected in the skin. Some degree of interdependency is suggested by evidence for the activation of RNA degradation by proteases of the stratum corneum (2). Moreover, the breakdown of RNA and DNA might be partially linked by nucleases that target both nucleic acids (3). In general, the regulation of the catabolism of nucleic acids in the skin has become more clear in recent years. Here we summarize the evidence for multiple roles of DNA breakdown in the skin and the implications on skin homeostasis.

2.2. Degradation of DNA in the skin

2.2.1. Degradation of endogenous DNA during cornification of keratinocytes

The degradation of DNA is part of the regular terminal differentiation program of keratinocytes (4, 5). Cornification, i.e. the last step of this differentiation program, converts living keratinocytes into anucleate and essentially DNA-free remnants, i.e. corneocytes, that are interconnected by corneodesmosomes. Lipids secreted by keratinocytes seal the intercellular spaces between corneocytes. Besides the formation of corneocytes, keratinocyte differentiation along alternative pathways and with different modes of cornification lead to distinct end products, i.e. nails, hair and filiform papillae on the upper surface of the tongue. In mice, keratinocytes also cornify in the outermost layers of the esophagus and forestomach (6). Still another differentiation pathway leads to sebocytes which build up the sebaceous glands and ultimately produce sebum by holocrine secretion. All above-mentioned cell differentiation processes culminate in non-classical modes of programmed cell death during which DNA is degraded. Strikingly, DNA degradation has been detected even in a simple form of cornification in the epidermis of the frog (7) which suggests a conserved role of this process in keratinocyte cornification.

The degradation of nuclear DNA during cornification has been characterized only partially. The breakdown of the nucleus of differentiated keratinocytes has been estimated to take less than 6 hours (8). DNA fragments with free 3´-OH ends are formed in this process and can be detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of thin sections (5). Because of the rapid completion of DNA degradation during cornification, only few TUNEL-positive nuclei – all situated within the stratum granulosum-stratum corneum transition layer – can be found within the epidermis at any given point in time (9). An acceleration of cornification and DNA degradation can be induced by the disturbance of the skin barrier by tape stripping (10). The enzymatic machinery for DNA degradation during cornification is discussed in section 4.

2.2.2. Degradation of DNA during removal of damaged or superfluous cells in the skin

While DNA is protected and maintained intact by different repair mechanisms in living cells, it is degraded when cells die. Several forms of cell death are active in most or all types of tissues, including the epidermis. Initially, the morphological classification of cell death distinguished between apoptosis and necrosis. Later autophagic cell death (cell demise accompanied but not mediated by autophagic vacuolization), pyroptosis and other types of cell death have been defined (for review, see reference 11). The mode of DNA degradation has been best characterized for apoptosis.

Apoptosis, also known as programmed cell death, is a ubiquitous mode of cell demise that is characterized by rounding-up of the cell, condensation of chromatin, fragmentation of the nucleus and maintenance of an intact plasma membrane. The main mechanism of DNA degradation in apoptosis is the hydrolysis of DNA by DNA fragmentation factor B (DFFB), also known as caspase-activated DNase (CAD) (12). DFFB is an evolutionarily ancient endonuclease that is regulated by DNA fragmentation factor A (DFFA), also known as inhibitor of CAD (ICAD) (12, 13). Cleavage of DFFA/ICAD by the proapoptotic protease, caspase-3, releases DFFB to cleave nuclear DNA. In addition, acinus, apoptosis-initiating factor (AIF), cyclophilin A, L-DNase II, DNase1L3, and endonuclease G are implicated in DNA degradation within apoptotic cells, however, their respective contributions are unclear (11). Apoptotic cell fragments are phagocytosed by neighboring cells or macrophages where the breakdown of nuclear DNA is completed. Within the lysosomes of the phagocyte, the DNA fragments of the dying cell are degraded by DNase 2 (14).

Necrosis was originally described as uncontrolled destruction of cells. However, recent studies have established the concept that necrosis can also occur in a programmed manner for which the term necroptosis has been coined (11). DNA released from necrotic cells is taken up by phagocytosing cells such as macrophages but probably also by resident cells of the skin. DNA released into the extracellular space may be degraded by DNase 1, i.e. the predominant neutral DNase of the serum and most other body fluids (15). *In vitro* DNase 1 is also able to contribute to intracellular degradation of nuclear DNA in cells undergoing necrosis (16). The fate of DNA from necrotic cells remains to be investigated *in vivo*.

Pyroptosis is a caspase-1-dependent pro-inflammatory mode of cell death that is triggered by various pathological stimuli (17). It is associated with the secretion of interleukin (IL)-1-beta and IL-18 as well as with exocytosis of lysosomes (18). Different from apoptosis, DFFA/ICAD is not cleaved suggesting that DFFB/CAD is not activated in this form of cell death. However, an as-yet unidentified caspase 1-activated nuclease cleaves nuclear DNA during pyroptosis (17).

In the epidermis, keratinocytes can undergo apoptotic, necrotic and probably also pyroptotic death. When keratinocytes of the interfollicular epidermis are irreversibly damaged by UVB radiation, they form so-called sunburn cells, i.e. cell corpses with eosinophilic cytoplasm and condensed nuclei containing TUNEL-

positive DNA fragments. The process of sunburn cell formation is thought to resemble apoptosis. However, sunburn cells are probably not taken up by phagocytes but are moved to the skin surface where they integrate into the stratum corneum and ultimately are shed during desquamation (19). An example of classical apoptotic death is the demise of keratinocytes of the hair follicle during the catagen phase of the hair cycle in which TUNEL-positive DNA fragments are detectable in dying cells (20, 21). Apoptosis is also activated during various skin diseases, e.g. toxic epidermal necrolysis and graft-versus-host disease (5).

Physical and chemical insults can result in necrosis of skin cells. The molecular processes that are induced by the rupture of the cell membrane are not fully understood. Interestingly, recent studies have implicated RIP kinases in a regulated form of necrosis termed necroptosis (11) which also occurs in the epidermis (22). Importantly, the uncontrolled release of nucleic acids may contribute to the induction of inflammation after skin injury (23-25).

2.2.3. Degradation of DNA on the skin surface

DNA-degrading enzymes are present on the skin surface, as it has been initially reported fifty years ago (26). Several reports have partially characterized DNase activities of the skin. Tabachnick and Freed reported on an extracellular DNase activity that could be eluted from the surface of the skin and hair of rodents (26). Probably, for technical reasons, only DNase activity at neutral pH was detected in this study (26), whereas only acid DNase activity was detected in human and rat epidermis in another study (4). Later extracellular neutral and acid DNase activities were detected in the epidermis of the guinea pig (27). Likewise, DNase activities at acid and neutral pH were reported to be present in preparations of human epidermis and isolated stratum corneum as well as in cow snout epidermis (28, 29). The DNase activity at pH 5 was found to be higher in normal human stratum corneum than in psoriatic scales (30). DNase activity at pH 7.4 was detected in normal stratum corneum whereas it was virtually absent in psoriatic scales. According to electrophoretic analyses, the acid DNase activity may be derived from a single enzyme whereas two different DNases appeared to be active at neutral pH (30). By affinity chromatography on DNA-cellulose, 3 distinct neutral DNase activities and one acid DNase were enriched from the epidermis and from the stratum corneum (28). Only the acid DNase could be detected in extracts from psoriatic scales (28). In another study, the total amount of neutral DNase activity on the skin surface of guinea pigs was found to increase after skin wounding by shaving and to increase even more strongly by the combination of shaving and beta-irradiation (31). The epidermal DNases were classified as DNase I (activity optimum around pH 7 and dependent of magnesium ions) and DNase II (activity optimum around pH 5 and independent of magnesium ions) but the molecular identities of these enzymes have not been determined in studies using material purified from the skin.

3. PHYSIOLOGICAL ROLES OF DNA AND DNA DEGRADATION

The breakdown of DNA has different effects depending on the physiological context. This is because DNA has several roles in the organism and in the interaction with pathogens as well as commensal microbes. The following sections provide a brief overview of the roles of DNA and the impact of DNA degradation on physiological processes, focusing on the relevance for the barrier functions of the skin.

3.1. Primary function of DNA as carrier of genetic information

Obviously, the most important biological function of DNA in general and also in the skin is to serve as the carrier of genetic information. DNA can fulfil this function because it is a relatively stable molecule. However, spontaneous hydrolysis of the base-sugar bond, the attack by active oxygen species and UV irradiation damage DNA (32). These types of damage are restricted to a small portion of the DNA molecule and, by themselves, do not lead to degradation of DNA under physiological conditions.

In living cells DNA damage is effectively repaired by several mechanisms adapted to different forms of injury (33, 34). It is important to note that limited enzymatic degradation of DNA at the site of damage is a key element of both DNA repair and DNA recombination (33, 34). Some of the DNA-degrading enzymes implicated in DNA repair may have additional roles in the removal of DNA during cell death and, *vice versa*, some DNases with primary functions in cell death-associated DNA degradation have been proposed to mediate local DNA cleavage in living cells (35, 36).

The degradation of DNA is associated with an irreversible halt to the proliferation of cells. It also shuts down the production of cellular mRNAs and encoded proteins. Thus, the breakdown of DNA is a highly efficient means to control dysfunctional, pre-malignant and malignant cells. This process is particularly relevant for the epidermis which is exposed to various insults from the environment. Accordingly, DNA degradation in irreversibly damaged cells contributes to the homeostasis of the epidermis and to the maintenance of the skin barrier.

3.2. DNA as a structural component of the nucleus

DNA occupies a significant portion of the volume of the nucleus and serves as an element of the nucleoskeleton (37). In agreement with this role, the nucleus including its DNA is degraded during differentiation of some cells, i.e., mammalian erythroblasts to allow for a reduction in cell size (38). Moreover, the presence of a nucleus affects the optical properties of a cell, and degradation of the nucleus contributes to the establishment of an optically uniform cell interior in the lens cells of the eye (39). In the context of the epidermis, the role of DNA as a structural component of the cell may also be relevant in cornifying keratinocytes because the DNA-rich nucleus may sterically limit the formation of a keratin cytoskeleton as will be discussed in section 4.1 (40).

Besides its interactions with other cell components, DNA also interferes with ultraviolet radiation. Nuclear DNA represents a major UVB-absorbing substance of skin cells. The absorption of UVB radiation generates DNA photoproducts such as cyclobutane pyrimidine dimers (CPDs) and (6–4)-photoproducts. The CPDs in UV-damaged DNA have a crucial role in UV-induced immunosuppression, which can be inhibited by topical application of liposomes containing the T4 endonuclease V (41). Accordingly, T4 endonuclease V has been proposed as a therapeutic agent for the repair of UV-induced DNA damage (42).

The aberrant retention of nuclear chromatin in cornified keratinocytes, also known as parakeratosis, is an important diagnostic marker of multiple skin diseases such as psoriasis, granular parakeratosis, pityriasis rubra pilaris, and seborrheic dermatitis (43). The main site affected by parakeratosis is the stratum corneum, however, also the nail can be parakeratotic (44). Parakeratotic lesions are associated with altered mechanical properties of corneocytes in the stratum corneum and pitting of nails (44). However, the specific contribution of DNA retention to these functional changes is difficult to estimate as parakeratosis is typically just one of several alterations in the cornification process associated with these diseases (45).

3.3. The role of DNA in the pathogenesis of infectious diseases

DNA is the genetic material of pathogens relevant to the skin barrier, i.e., bacteria and DNA viruses such as papilloma viruses, Herpes viruses, and poxviruses (Molluscum contagiosum, vaccinia, cowpox). In general, the degradation of DNA is a means of efficiently killing DNA-dependent pathogens. Bacteria that have evolved the restriction-modification system and the clustered regularly interspaced short palindromic repeat (CRISPR) interference system to destroy infectious foreign DNA in a sequence-dependent manner (46). Restriction endonucleases cleave foreign DNA at specific sites that are protected by methylation in the DNA of the host bacteria (46). CRISPR has similarities with eukaryotic RNA interference but has also been shown to target foreign DNA directly (47).

In mammals the DNA of pathogens may be attacked by endogenous, sequence-unspecific DNases. At present this hypothesis is supported only by a limited amount of experimental evidence. For example, DNA intermediates of the replication cycle of retroviruses were found to be targets of a cellular exonuclease (48), and the study of infection of human keratinocytes by human papillomavirus 31 (HPV31) suggested that structural changes of the viral capsid in the endosome increase the susceptibility of the viral genome to DNases, at least *in vitro* (49). Studies in genetically defined models with and without the activity of distinct DNases may help to determine the relevance of the anti-infectious roles of DNases.

In this context it is noteworthy that DNA degradation can also be mediated by DNases of pathogens and commensal microbes. Many bacteria produce and, to some extent, secrete DNases (see also section 3.5.) whereas only few viruses encode DNases (50). The DNases of herpes viruses are thought play a role in the viral life cycle. In addition, the alkaline DNase of Epstein–Barr virus (EBV), also known as human herpes virus 4, has recently been shown to induce genomic instability in human epithelial host cells (50).

3.4. DNA as a ligand for molecular sensors of innate immunity

In the recent years DNA has been identified as an important danger signal that triggers innate immune responses (51, 52). Most likely, the sensitivity to DNA has evolved to detect infectious DNA and to limit the spread of DNA-dependent pathogens. However, the DNA sensors are not specific for foreign DNA and endogenous DNA has been shown to induce similar response as pathogen-derived DNA. DNA is detected by an endosomal membrane-bound receptor, i.e. toll like receptor 9 (TLR9), and by cytoplasmic receptors, i.e. AIM2, DAI, IFI16, and DDX41 (53-56). As has been reviewed elsewhere (51, 52), signaling from these receptors induces the production of type I interferons or the caspase-1-dependent maturation and secretion of interleukin-1 (IL-1) and IL-18. TLR9 and AIM2 have been implicated in the initiation of pro-inflammatory signaling in the initiation phase of psoriasis (23, 25). In these studies, endogenous DNA released from damaged or necrotic keratinocytes has been suggested to be the trigger of TLR9 and AIM2 signaling. At present it is not clear whether the pro-inflammatory action of DNA is counteracted by enzymatic degradation of DNA.

3.5. DNA as a component of extracellular traps

The antimicrobial activity of neutrophils, eosinophils and mast cells involves the formation of so-called extracellular traps (ETs), i.e. structures consisting of DNA, histones and other proteins that trap and kill bacteria (57-59). The DNA is released from cells in a process named ETosis (60). Both nuclear and mitochondrial DNA can contribute to the formation of ETs (61). ETs have been implicated in human skin diseases such as psoriasis, hypereosinophilic syndrome, allergic contact dermatitis, dermatitis herpetiformis and others (59, 62).

The degradation of the DNA component of ETs is an escape strategy of bacterial pathogens such as group A *Streptococcus* and *Staphylococcus aureus* (63, 64). For tissue homeostasis, ETs are disassembled by endogenous DNases such as DNase 1 present in the serum. An impairment of neutrophil extracellular trap degradation is associated with lupus nephritis in a subset of patients with systemic lupus erythematosus (SLE) (65).

3.6. Extracellular DNA as a component of bacterial biofilms

The skin surface is colonized by a wide variety of bacterial species some of which are able to form biofilms (66). Biofilms are surface-attached bacterial communities within a matrix of substances secreted from the bacteria. DNA has been demonstrated to be a main component of these biofilms (67). *In vitro*, the formation of biofilms of

some pathogenic bacteria can be suppressed by extracellular DNases (67). The anti-biofilm activity of cutaneous DNases has been demonstrated for bacteria frequently causing skin infections (68), as discussed in section 4.1.2.

3.7. DNA as a target of autoimmune antibodies

DNA is the antigen of anti-DNA antibodies which are characteristic of SLE, a disease that involves cutaneous lesions in the majority of patients (43). Although the role of DNA as an immunogen and the mechanism of the induction of anti-DNA antibodies are not fully understood at present, exposure of chromatin fragments upon cell death has been shown to trigger autoimmunity (69, 70). Importantly, DNase 1 activity was proposed to prevent the exposure of the immune system to extracellular DNA (70). Besides SLE, anti-DNA antibodies can appear in some other diseases and after treatment with certain drugs (71, 72).

3.8. Potential roles of DNA degradation products

DNA catabolism leads to the formation of nucleotides and nucleosides from which the nucleobases are cleaved off. In living cells purines are further degraded to uric acid whereas pyrimidines are degraded to beta-alanine and beta-aminoisobutyrate (73). Studies using radioactively labeled thymidine have suggested that DNA degradation in the granular layer releases thymidine some of which diffuses through the stratum corneum to the skin surface while another fraction of thymidine may be reused by living cells (74). The presence of uric acid in the stratum corneum indicates that purines are, at least to some extent, degraded in the upper layers of epidermis (75). Comprehensive investigations of the nucleotide catabolism in terminally differentiated keratinocytes are needed to unravel the physiological roles of DNA breakdown products in the skin.

4. MOLECULAR MECHANISMS OF DNA DEGRADATION IN THE EPIDERMIS

4.1. DNase1L2

4.1.1. Structure and biochemical characteristics of DNase1L2

DNase1L2 is a member of the DNase1 family of endonucleases. Initially, DNase1L2 was reported to be expressed at low levels in multiple tissues (76). Later investigations revealed that DNase1L2 is, by far, most strongly expressed in epidermal keratinocytes and undergoes transcriptional upregulation during terminal differentiation of keratinocytes (40, 77, 78). These features have made DNase1L2 a prime candidate for a specific role in DNA degradation during keratinocyte differentiation.

DNase1L2 is encoded by the *DNASE1L2* gene on human chromosome 16p13.3 and by the *Dnase1l2* gene on murine chromosome 17. *DNASE1L2* consists of 7 coding exons (79). Retention or removal of the intronic sequence between exons 4 and 5 during pre-mRNA splicing leads to two alternative human DNase1L2 mRNA variants, DNase1L2-L and DNase1L2-S, respectively (79, 80). The short variant has been detected in peripheral blood leukocytes and brain but the long variant predominates in all human tissues including the epidermis (77). The DNase1L2-L-specific sequence insert codes for a proline-rich domain of unknown functional significance. Expression of the two DNase1L2 protein variants in 293 cells and comparative analysis of enzymatic activity *in vitro* showed no difference between the two isoforms (79). The alternative splicing of DNase1L2 is not conserved and only one mRNA, corresponding in structure to DNase1L2-S, is expressed in the mouse (our unpublished data).

The expression of DNase1L2 mRNA is tightly regulated. Proliferating keratinocytes express minute amounts of DNase1L2. However, upon differentiation of epidermal keratinocytes *in vitro* and *in vivo*, as shown by *in situ* mRNA hybridization (77), the production of DNase1L2 mRNA is upregulated by more than hundred-fold. Since this transcriptional upregulation is accompanied by an upregulation of the abundance of DNase1L2 protein, the main level of control of DNase1L2 expression appears to be transcription. In immortalized keratinocytes of the HaCaT cell line the expression of DNase1L2 is induced by pro-inflammatory cytokines TNF-α and IL-1β whereas interferons, IL-4 and IL-13 have no effect on DNase1L2 expression (79). A binding site for the transcription factor NF-κB was localized to nucleotides -221 to -212 upstream of the start codon of human *DNase1L2* and confirmed to be active in HaCaT cells (79). The physiological significance of these data remains unclear because the expression of DNase1L2 was not found to be upregulated but rather downregulated in human skin samples affected by inflammatory diseases (77). The mechanism of transcriptional control of DNase1L2 during keratinocyte differentiation remains to be elucidated.

DNase1L2 proteins contain an N-terminal hydrophobic signal peptide of a length of 20 amino acids. This protein segment is homologous to propeptides of other members of the DNase1 family which have been shown to be cleaved off during biosynthesis and to allow for protein targeting to the endoplasmic reticulum, nuclear membrane Golgi, or for extracellular secretion (76). Overexpression experiments suggested that DNase1L2 is largely localized to the endoplasmic reticulum (our unpublished data), at least when the integrity of cellular organelles is preserved. Partial secretion of DNase1L2 was observed when DNase1L2 was overexpressed in 293 cells (76).

The structure of the mature DNase1L2 protein belongs to the endonuclease/exonuclease/phosphatase protein family (pfam 03372) with the catalytic residues of the DNase1 family members being present at conserved amino acid sequence positions (80). *In vitro* studies using purified recombinant DNase1L2 showed that DNase1L2 efficiently degrades supercoiled plasmid DNA (76). The reaction results in DNA fragments with 5´-phosphate and 3´-OH ends which are substrates of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). In contrast to the maximum activity of other members of the DNase1 family at neutral pH, recombinant DNase1L2 was most active at pH 5.6 but virtually inactive at pH 7 (76). A different version of recombinant

DNase1L2, that was not fused to a C-terminal Myc tag (76) and lacked the prodomain, showed high activity over a broad pH range from pH 5.0 to 7.6 (our unpublished data). The reason for this discrepancy in the pH optimum remains to be determined. The catalytic activity of DNase1L2 depends on divalent cations. Calcium and magnesium ions synergize in the activation of DNase1L2, and cobalt and manganese ions activate the enzyme even when present alone. By contrast, the activity of DNase1L2 is suppressed by zinc ions (76).

Ectopic expression of DNase1L2 in HeLa S3 cells does not induce cell death by itself nor does it enhance DNA fragmentation upon proapoptotic stimulation of these cells (76). Likewise, transient overexpression of DNase1L2 in keratinocytes is not associated with cell death (our unpublished data). However, permeabilization of cultured keratinocytes and application of recombinant DNase1L2 leads to efficient degradation of nuclear DNA (our unpublished data).

4.1.2. DNA degradation by DNase1L2 in the epidermis and in skin appendages

Based on the hypotheses that a DNase involved in the breakdown of DNA in cornifying keratinocytes might be upregulated during terminal differentiation of keratinocytes, the expression of a series of candidate DNases was determined in proliferating keratinocytes and keratinocytes in postconfluent culture, which represents a strong stimulus of differentiation *in vitro* (81). DNase1L2 was the only DNase of the panel under investigation that was consistently upregulated (77). Further investigations showed induction of DNase1L2 at the protein level in differentiated keratinocytes both *in vitro* and *in vivo* (77). High abundance of DNase1L2 was detected in human stratum granulosum, the hair matrix, the inner part of the sebaceous gland and the nail matrix (77, 82). By contrast, DNase1L2 was not expressed at significant levels in other tissues investigated. The confinement of DNase1L2 gene expression to differentiated keratinocytes strongly suggested a role of DNase1L2 in this phase of the keratinocyte life cycle or in the structures formed by dead remnants of keratinocytes, i.e. the stratum corneum, sebum, hair, and nails.

The role of DNase1L2 in cornification was tested in a human skin equivalent model in which keratinocytes form a stratified epidermis *in vitro* and ultimately are converted to enucleate corneocytes (77). Treatment of keratinocytes with DNase1L2-specific short interfering RNAs (siRNAs) efficiently suppressed the expression of DNase1L2 and led to the retention of nuclear DNA in the stratum corneum, a phenomenon also observed in inflammatory, hyperkeratotic skin diseases, most notably psoriasis, and known as parakeratosis. Although the skin equivalent model used in this study more closely resembles the *de novo* formation of the epidermis during development or the regeneration of the epidermis after wounding than the steady state conditions of homeostatic epidermis, this study demonstrated for the first time that a distinct DNase, i.e., DNase1L2, is essential for the breakdown of nuclear DNA during cornification of human keratinocytes, thus providing a molecular underpinning for the classification of cornification as a distinct mode of cell death different from apoptosis (11).

The contribution of DNase1L2 to DNA degradation in epidermal keratinocytes was also investigated using a mouse model in which the *Dnase1l2* gene was constitutively inactivated by targeted deletion (40). In the mouse, DNase1L2 is mainly expressed in the matrices of hair follicles and nails, in the epithelia of the tongue and the esophagus, the Hassall´s bodies of the thymus, and at variable levels in the tail, the interfollicular and plantar epidermis (40). Deletion of *Dnase1l2* did not compromise viability and health of mice, at least under non-stressed conditions, but led to distinct changes at the microscopic level. Nuclear remnants were aberrantly retained in hard corneocytes of hair and nails, in the scales on the tail as well as on the surface of the tongue and the esophagus. Quantification of DNA by real-time PCR revealed the presence of high amounts of nuclear DNA in hair and nails of DNase1L2 knockout mice, suggesting that DNase1L2 is essential for DNA degradation in these skin appendages. Mitochondrial DNA was increased in the nails but not in hair of DNase1L2-deficient mice, indicating that DNase1L2 also contributes to the breakdown of mitochondria during maturation of the nails. In contrast to the abovementioned keratinocyte-derived structures, the stratum corneum of the interfollicular epidermis was orthokeratotic in DNase1L2 knockout mice (40). Hence, murine epidermis and human skin equivalents differ in the dependence on DNase1L2 in the breakdown of nuclear DNA during stratum corneum formation. It remains to be determined whether DNase1L2 plays a role under conditions of *de novo* formation of the stratum corneum, as in the human skin equivalents.

The retention of DNA in hair of DNase1L2-deficient mice was associated with aberrant retention of histone proteins, ultrastructural disturbances in hair corneocytes and increased sensitivity of hair to mechanical stress (40). Thus, DNase1L2-mediated degradation of nuclear DNA is essential for establishing the full mechanical resilience of hair. A similar role of DNA degradation in nails is likely but has not been investigated. Moreover, the first demonstration of a negative effect of DNA on the stability of cornified cells indicates that the removal of DNA, irrespectively of an involvement of DNase1L2 in this process, may also enhance the stability of corneocytes of the stratum corneum.

The studies on the DNase1L2 knockout mice included the development of a novel method to visualize DNA in hair by permeation of the hair cuticle and incubation with a fluorescent dye specific for DNA. Modifications of this method allowed to study DNA retention in human hair (83). This study showed that nuclear DNA was virtually completely degraded in the great majority hair corneocytes of all human individuals of the study cohort. However, some individuals contained DNA-rich nuclear remnants in a small portion of hair corneocytes suggestive of incomplete DNA breakdown as in parakeratosis of the stratum corneum. The presence of

fluorescence-labeled DNA in hair was associated with high yields of DNA extraction, and the method of *in situ* DNA labeling was proposed for predicting the probability of success of forensic genotyping of hair found at crime scenes (83).

In addition to the putative role of DNase1L2 in the degradation of endogenous DNA, DNase1L2 may also degrade exogenous DNA to prevent the formation of bacterial biofilms. DNase1L2 is present as a catalytically active protein in human stratum corneum (68). At this site it may encounter biofilm-forming bacteria. Since extracellular DNA is an essential component of the biofilm matrix of many bacterial species (67) including the skin pathogen *Staphylococcus aureus* (84), the activity of DNase1L2 on the skin surface may restrict the formation or maintenance of cutaneous bacterial biofilms. Indeed, recombinant DNase1L2 suppressed biofilm formation by *Pseudomonas aeruginosa* and *S. aureus in vitro* (68). In addition to DNase1L2, other DNases (see below, section 4.2) may contribute to the anti-biofilm defense of the stratum corneum.

An association of DNase1L2 with a disease has not been reported yet. The human *DNASE1L2* gene shows little or no variation with regard to single nucleotide polymorphisms (SNPs) in various human populations (85, 86). However, reduced levels of DNase1L2 protein were detected in the lesions of various inflammatory skin diseases (77). The evaluation of the potential role of DNase1L2 in the pathophysiology and diagnosis of human diseases requires further studies.

4.2. DNase 2

4.2.1. Structure and biochemical characteristics of DNase 2

DNase 2 is an acid DNase present in the lysosomes of essentially all cell types. A recent investigation of DNase activities of the stratum corneum has shown that DNase 2 is the predominant DNase of the human and murine skin surface (78). Therefore, some key properties of DNase 2 and the evidence for its activity in the epidermis are described here. For more details, the reader is referred to excellent reviews of DNase 2 that have been published previously (14, 87).

DNase 2, also known as DNase 2-alpha, is encoded by the *DNASE2* gene on human chromosome 19p13.2 and by the *Dnase2a* gene on murine chromosome 8. *DNASE2* consists of 6 coding exons (87). DNase 2 mRNA is expressed ubiquitously and it expression level is not altered during differentiation of keratinocytes (77). The DNase 2 protein contains an N-terminal hydrophobic signal peptide of a length of 16 amino acids, and four N-glycosylation sites to which mannose-6-phosphate is attached (87), thus facilitating translocation to the lysosome. Upon overexpression of DNase 2 in 293 cells, up to 30% of the recombinant enzyme is secreted (88). Proteolytic processing of DNase 2 into so-called alpha and beta-chains has been reported but may represent an artifact of preparation (87, 89).

DNase 2 is an endonuclease that generates DNA fragments with 5´-OH and 3´-phosphate ends that are not labeled by the TUNEL reaction. Compatible with an activity in lysosomes, the pH optimum of DNase 2 is in the range of pH 4.5 to pH 5.0. Divalent cations are not required for catalytic activity of DNase 2.

The main physiological role of DNase 2 is the degradation of phagocytosed DNA (14). Macrophages and other phagocytes take up apoptotic bodies including DNA and target them for breakdown in lysosomes. A special function of DNase 2 is the degradation of DNA in nuclei expelled from erythroblasts in the course of differentiation to erythrocytes (90). An additional role of DNase 2 in the suppression of horizontal gene transfer from apoptotic to phagocytosing cells has been proposed (91), yet the physiological significance of this role is unclear.

Deficiency of DNase 2 is associated with embryonic or perinatal lethality in mice (92). Undigested DNA induces a massive interferon response and autoimmunity (93). This phenotype can be rescued by deletion of the *Ifnar* gene which encodes the type I interferon receptor. However, even in the absence of IFNAR the loss of DNase 2 is associated with autoimmune reactions leading to the development of rheumatoid arthritis (94, 95).

A mutation in the promoter of human *DNASE2* gene is associated with a reduced production of DNase 2 protein and decreased acid DNase activity in human tissues (89). This variant was reported to increase the risk for developing rheumatoid arthritis, thus supporting the physiological significance of the mouse model described above (96).

4.2.2. DNA degradation by DNase 2 in the epidermis

Being a tissue-ubiquitous DNase with acidic pH optimum, DNase 2 was considered a candidate for the acid DNase activity on the skin surface (78). Other candidates, such as DNase1L2 and L-DNase II, which are present in the stratum corneum, were excluded as main contributors to this catalytic activity (40, 78, our unpublished data). DNA degradation assays and zymographic analyses showed that the predominant DNase of human stratum corneum has an activity optimum below pH 5.0. siRNA-mediated specific knockdown of DNase 2 expression in human *in vitro* skin models and the genetic ablation of DNase 2 led to strong suppression of acid DNase activity in the epidermis and in the stratum corneum (78), demonstrating that DNase 2 is indeed the main acid DNase of the outermost layers of the epidermis.

Interestingly, the DNase 2-dependent activity of the stratum corneum was associated with a zymography band corresponding to an apparent molecular weight of approximately 130 kD which is by far larger than the molecular weight predicted for DNase 2 (78). This finding may indicate the existence of a DNase 2 protein complex in the stratum corneum (78). However, this hypothesis has not been tested yet and the physiological relevance of putative interactions of DNase 2 with other proteins is unclear.

The absence of DNase 2 in human skin models and in gene-targeted mice did not compromise the breakdown of endogenous DNA during cornification of keratinocytes, suggesting that DNase 2 is not essential for this process (78). Accordingly, we have proposed that the main substrate of DNase 2 in the stratum corneum may be exogenous DNA derived from infectious agents (78). Alternatively, DNase 2 might degrade endogenous DNA that is released in the course of skin wounding.

It is important to note that the DNase 2-deficient mice that have been investigated for skin DNase activity so far, lack the type I interferon receptor I (IFNAR1) (78). Deletion of IFNAR1 is necessary to avoid the embryonic lethality associated with constitutive ablation of DNase 2 (94). Therefore, the potential effects of undegraded DNA on epidermal interferon signaling needs to be studied in a different model system.

4.3. TREX2

4.3.1. Structure and biochemical characteristics of TREX2

Three prime repair exonuclease 2 (TREX2) is an exonuclease with a tissue expression pattern of striking similarity to that of DNase1L2 (97). Although its biological functions are incompletely understood at present, TREX2 is likely to play a specific role in the epidermis.

TREX2 is closely related to TREX1, which is also known as DNase III (98, 99) and will be described in section 4.4. The *TREX2* gene is located on human chromosome Xq28, and murine *Trex2* is located on chromosome X A6. *TREX2* consists of a single coding exon that is preceded by a non-coding exon (100). Alternative pre-mRNA splicing of human TREX2 mRNA is predicted to generate 3 alternative variants that code for proteins of 236, 278 and 279 amino acids (100). The short variant of human TREX2 is equivalent in structure to murine TREX2, appears to be functional and is present at higher abundance than the longer variants in human tissues (97). In addition, TREX2 mRNA transcripts containing exons of the *HAUS7* gene, which is located on the 5´-side of *TREX2*, have been identified in human cells (100). The functional significance of these fused gene transcripts is not clear.

The TREX2 protein, like TREX1, contains three conserved exonuclease sequence motifs known as ExoI, ExoII, and ExoIII but lacks a proline-rich region and a carboxy-terminal domain of about 75 amino acids that is present in TREX1 (101). The exonuclease motifs contain four conserved acidic residues that participate in the binding of divalent metal ions required for catalysis. The pH optimum of TREX2 activity is at pH 8.0 (102). TREX2 removes nucleotides from single-stranded and double-stranded DNA (97). Shevelev and colleagues have proposed that TREX2 interacts with polymerase-delta to support proofreading during DNA replication and repair (103). TREX2 is localized both in the cytoplasm and the nucleus where it has been suggested to play a role in maintaining chromosomal stability. However, no chromosomal instability was observed in a TREX2-deficient mouse model (97).

4.3.2. DNA degradation by TREX2 in the epidermis

The expression of TREX2 has been reported for many tissues and cell lines (100, 104). However, a systematic comparison of expression levels in murine tissues and validation of the results in a TREX2 knockout mouse model revealed that TREX2 expression in non-epithelial tissues is low relative to that in the skin, the tongue, the esophagus and the forestomach (97). TREX2 protein was detected by Western blot and immunofluorescence analysis exclusively in these epithelial tissues (97).

The comparison of the TREX2 knockout mice with wildtype mice showed that TREX2 does not contribute to the 3´-exonuclease activity of the dermis but accounts for approximately half the total 3´-exonuclease activity in the epidermis and in keratinocytes (97). The deletion of *Trex2* did not compromise the proliferation characteristics of cells nor was it associated with an autonomous tumorigenic phenotype or reduced survival (97). These findings argue against a role of TREX2 in DNA repair. TREX2 knockout mice show normal immunological development and, in contrast to TREX1 knockout mice (105), do not develop autoimmunity. When TREX2-deficient mice were treated with 7,12-dimethylbenz(a)anthracene (DMBA) alone or followed by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) they developed significantly more papillomas than wildtype control mice. Both reduced rates of apoptosis and increased clonogenic survival of TREX2-deficient keratinocytes as compared to wildtype keratinocytes were identified as mechanisms possibly underlying the increased susceptibility to skin carcinogenesis (97).

The characterization of the TREX2 knockout mouse by Soler and colleagues has changed the concept of the physiological function of TREX2 (97). The preferential expression in the epidermis and related tissues points to a distinct role of TREX2 in epithelia. As the TREX2 knockout mouse does not show an obvious phenotype under non-stressed conditions, a role of TREX2 in some kind of stress response seems likely. The results of the carcinogenesis study in mice may point in this direction (97). However, the molecular processes altered in TREX2-deficient mice remain to be determined precisely to understand the difference to normal mice. Equally important, the potential role of TREX2 in types of stress specific for the epidermis should be tested in future studies.

4.4. Other DNases active in the epidermis

Besides DNase1L2, DNase 2 and TREX2, several other known DNases are expressed in the epidermis. These include, but are not restricted to, DNase 1, DNase1L3, CAD, endonuclease G, L-DNase II, TREX1, APE1, and others (77, 97, 106). In some cases, the roles of these DNases in the epidermis have been investigated with regard to distinct hypothetical functions. The results of these studies are summarized below. However, additional functions in the DNA catabolism of the epidermis may be uncovered by more comprehensive studies of various existing DNase gene knockout mouse models. More importantly, there is the possibility that currently

uncharacterized DNases may be present and active in the epidermis. Unbiased purification of DNA-degrading activities from the epidermis and subsequent sequencing of active proteins might be a way forward to identify such hypothetical DNases.

DNase 1 is the prototypical neutral DNase. It is most strongly expressed in the parotid gland and at lower levels in other tissues (107). DNase 1 is secreted from cultured cells and represents the predominant DNase of the serum, where it partly originates from the liver (15, 108). Epidermal keratinocytes were shown to express DNase 1 mRNA whereas the enzymatic activity of DNase 1 in keratinocytes has not been investigated yet (77). Targeted deletion of DNase 1 in the mouse led to an SLE-like phenotype, however, gross alterations of the skin of DNase 1 knockout mice were not reported (109).

DNase1L3, also known as DNase-gamma, is expressed preferentially in the liver and the spleen (110). Its mRNA is also detected in human epidermal keratinocytes in which its abundance is upregulated during during the first phase of differentiation *in vitro* (77). DNase1L3 shows perinuclear localization in living cells and translocates into the nucleus where it contributes to DNA degradation during apoptosis (76). Two mouse lines carrying the same spontaneous point mutation in the *Dnase1l3* gene develop SLE (111), and a loss-of-function variant of human DNASE1L3 causes a recessive form of SLE (112). The role of DNase1L3 in the epidermis is presently unknown.

Caspase-activated DNase (CAD), also named DNA fragmentation factor (DFF) is an evolutionarily ancient endonuclease that cleaves nuclear DNA during apoptotic cell death (12, 13). No epidermal phenotype has been reported for the CAD knockout mouse (113). In a two-stage model of skin carcinogenesis consisting of subsequent treatment of mouse skin with DMBA and TPA, the number of papillomas per mouse was increased 4-fold in CAD knockout mice relative to wild-type control mice. In addition, the growth rate and the maximum size of papillomas of CAD-deficient mice exceeded those of controls (114), indicating that CAD suppresses tumorigenesis induced by exogenous chemical carcinogens.

Endonuclease G degrades both DNA and RNA (3) and has been implicated in caspase-independent apoptosis (11). However, inactivation of the endonuclease G gene does neither compromise the susceptibility of cells to apoptotic stimuli nor viability of mice (115). A skin phenotype of endonuclease G-deficient mice has not been reported.

L-DNase II is an acid DNase that is generated by proteolytic cleavage of serpin B1 (116). *In vitro* L-DNase II contributes to apoptotic cell death in response to several stimuli (117). However, its role in programmed cell death *in vivo* is elusive. Serpin B1 was detected in protein extracts from human stratum corneum but knockdown studies in human *in vitro* skin models and investigations of the skin of serpinB1 knockout mice argued against a role of L-DNase II in epidermal DNA degradation (78).

TREX1 resembles TREX2 with regard to structure and catalytic activity, however, it is expressed at higher levels in non-epidermal tissues. Both DNA repair and apoptotic DNA degradation have been proposed to involve the activity of TREX1 (98, 118, 119). TREX1 knockout mice develop inflammatory myocarditis (105), which is possibly induced by autoimmune reactions. TREX1 was implicated in antiviral defence because it acts as an effector of the type I interferon response (120). Moreover, TREX1 attacks DNA that is formed by reverse transcription of genomic RNA of HIV-1 and possibly other retroviruses (48). Interestingly, TREX1 also benefits HIV-1 by digesting non-productive reverse transcripts that otherwise would trigger an interferon response (48). The primary role of TREX1 may be the degradation of single-stranded DNA, that is generated by endogenous retroelements (121). Thus, TREX1 limits the induction of type I interferon by endogenous retroviral elements and prevents autoimmunity. Deleterious mutations in the *TREX1* gene are indeed associated with the autoimmune diseases such as Aicardi-Goutieres syndrome and familial chilblain lupus which is a form of cutaneous lupus erythematosus (122).

Besides TREX1 and TREX2, further proteins with 3´-5´ exonuclease activity, e.g. APE1, MRE11, RAD1, RAD9, and VDJP exist in humans (for review, see reference 123). APE1 was reported to have both endonuclease and exonuclease activity (123, 124). It has been implicated both in DNA repair and in DNA fragmentation during apoptosis (124). In human epidermis and in cultured human keratinocytes, APE1 is induced to translocate to the nucleus upon irradiation with UV (106). Recently, APE1 was shown to be involved in TLR2-dependent proinflammatory signaling in the skin with a possible role in psoriasis (125). Further studies are needed to understand the function of APE1 in the skin.

5. OUTLOOK

Despite significant progress in unraveling the mechanisms and functions of epidermal DNA catabolism in recent years, several open issues need to be addressed in future studies. For example, the regulation of DNA degradation during cornification of keratinocytes is still not fully understood. DNase1L2 is involved in the hard cornification of hair and nails in mice (40) and in stratum corneum formation in a human *in vitro* skin model. Nevertheless, other DNases which participate in the cornification-associated programmed cell death remain to be identified, particularly in the interfollicular epidermis. Genetic or pharmacological blockade of these DNases will allow to determine physiological functions of DNA degradation in terminal differentiation of keratinocytes in addition to the maturation of mechanical resilience of cornification products (40). A comprehensive characterization of the effects of incomplete DNA degradation may open a new view of the role of parakeratosis in diseased skin. Currently, parakeratosis is

considered a consequence of the inflammatory and hyperproliferative process. However, it is conceivable that the incomplete removal of the nuclear components could be a driving force of inflammation or, in a kind of vicious circle, contribute to the chronicity of the process.

The physiological role of DNase activity on the skin surface is still unknown. DNases of the stratum corneum may be required to degrade residual DNA of cornifying keratinocytes, DNA of damaged cells in superficial wounds or DNA of bacterial biofilms. Studies in DNase 2-deficient murine skin and complementary investigations in models of human skin will help to determine the physiological significance of DNases on the skin surface. Importantly, the epidermal DNases are likely to degrade DNA in the course of topical gene transfer. Both cutaneous gene therapy and vaccination with DNAs encoding immunogenic proteins have to overcome the DNase barrier of the stratum corneum. As DNase 2 is the predominant DNase of the stratum corneum (78) and also contributes to the suppression of transfection of foreign DNA into living cells (126), the inhibition of DNase 2 may be a promising strategy to enhance the efficiency of DNA transfer in the epidermis.

Another open question relates to the emerging role of nucleic acids in skin inflammation. Do endogenous DNases act as negative regulators of inflammation? It is conceivable that DNase 2, TREX2, and other epidermal DNases degrade DNA that would otherwise stimulate the production and release of cytokines and interferons in the skin. This role may be restricted to distinct situations such as the response to tissue damage. Accordingly, future studies will have to involve stress experiments in appropriate models. In the long run it may be possible to modulate DNA degradation in the skin, either by altering the expression or activity of endogenous DNases or by applying exogenous DNases, for therapeutic purposes.

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Abbreviations: AIF: apoptosis-initiating factor, AIM2: absent in melanoma 2, APE1: apurinic or apyrimidinic site endonuclease class 1, CAD: caspase-activated DNase, CPD: cyclobutane pyrimidine dimer, CRISPR: clustered regularly interspaced short palindromic repeat, DAI: DNA-dependent activator of IRFs, DDX41: DEAD (Asp-Glu-Ala-Asp) box polypeptide 41, DFF: DNA fragmentation factor, DMBA: 7-12-dimethylbenz(a)anthracene,DNase: Deoxyribonuclease, EBV: Epstein-Barr virus, ET: extracellular trap, Exo: exonuclease, IFI16: interferon gamma-inducible protein 16, HIV: human immunodeficiency virus, HPV: human papillomavirus, ICAD: inhibitor of CAD, IFNAR: type I interferon receptor, IL: interleukin, MRE: meiotic recombination 11, NF-kappa B: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, PCR: polymerase chain reaction, RAD: radiation, siRNA: short interfering RNA, SLE: systemic lupus erythematosus, SNP: single nucleotide polymorphism, TLR: toll like receptor, TNF: tumor necrosis factor, TPA: 12-O-tetradecanoylphorbol-13-acetate, TREX: three prime repair exonuclease, TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, UV: ultraviolet, VDJP: V(D)J RSS-dependent DNA joining protein

Key Words: Epidermis, Endonuclease, Exonuclease, Stratum Corneum, Apoptosis, Psoriasis, Antimicrobial Defense, Biofilm, Inflammation, Review

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