Biochimica et Biophysica Acta 1833 (2013) 3471–3480

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Cell death by cornification ☆

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 7 June 2013
Accepted 8 June 2013
Available online 20 June 2013

Keywords:
Keratinocyte
Cornification
Apoptosis
Necrosis
NF-κB

ABSTRACT

Epidermal keratinocytes undergo a unique form of terminal differentiation and programmed cell death known as cornification. Cornification leads to the formation of the outermost skin barrier, i.e. the cornified layer, as well as to the formation of hair and nails. Different genes are expressed in coordinated waves to provide the structural and regulatory components of cornification. Differentiation-associated keratin intermediate filaments form a complex scaffold accumulating in the cytoplasm and, upon removal of cell organelles, fill the entire cell interior mainly to provide mechanical strength. In addition, a defined set of proteins is cross-linked by transglutamination in the cell periphery to form the so-called cornified envelope. Extracellular modifications include degradation of the tight linkages between corneocytes by excreted proteases, which allows corneocyte shedding by desquamation, and stacking and modification of the excreted lipids that fill the intercellular spaces between corneocytes to provide a water-repellant barrier. In hard skin appendages such as hair and nails these tight intercorneocyte connections remain permanent. Various lines of evidence exist for a role of organelle disintegration, proteases, nucleases, and transglutaminases contributing to the actual cell death event. However, many mechanistic aspects of kearatinocyte death during cornification remain elusive. Importantly, it has recently become clear that keratinocytes activate anti-apoptotic and anti-necroptotic pathways to prevent premature cell death during terminal differentiation. This review gives an overview of the current concept of cornification as a mode of programmed cell death and the anti-cell death mechanisms in the epidermis that secure epidermal homeostasis. This article is part of a Special Section entitled: Cell Death Pathways. Guest Editors: Frank Madeo and Slaven Stekovic.

1. Introduction

The body surface of terrestrial animals is exposed to air and to mechanical stress, both incompatible with the persistence of living cells at the direct interface between an organism and its environment. While terrestrial arthropods have evolved an exoskeleton that protects underlying living epidermal cells against desiccation and mechanical damage, terrestrial tetrapods utilize a layer of dead cell corpses and extracellular deposited material as a barrier to the environment. In addition to the role of the skin in protecting against mechanical stress and in limiting the diffusion of chemical substances, the skin is also an eminent barrier to microbial infections. A plethora of antimicrobial proteins are produced by differentiating keratinocytes in a constitutive or inducible manner that protect against bacteria, viruses, fungi and parasites [1,2]. During homeostasis the most superficial cornified layers of the outer skin barrier are continuously shed by a process called desquamation and replaced by differentiating keratinocytes derived from a pool of epidermal stem cells. Corneocytes, the building blocks of the epidermal barrier, are formed by a unique form of programmed cell death referred to as cornification.

Cornification comprises three key elements, (1) the replacement of intracellular organelles and intracellular content by a compact proteinaceous cytoskeleton, (2) the cross-linking of proteins at the cell periphery to form a cornified cell envelope, and (3) the linkage of corneocytes into a multicellular, functional but biologically dead structure. The latter is in contrast with the classical apoptotic and necrotic cell death modes that are mainly aimed to delete excessive or damaged cells. There are numerous variants of cornification that lead to different cornified structures, such as the stratum corneum of the interfollicular epidermis, the stratum corneum of palmoplantar skin, the nails, the hair shaft, the inner root sheath of the hair and the papillae of the tongue. The main focus of this review is on our current understanding of the mechanism and special features of cell death by cornification for the maintenance of the skin barrier (stratum corneum). We will also address the distinctive features of hard cornification in hair, nail and tongue.

Abbreviations: FLG, filaggrin; IKK, IκB kinase; K, keratin; KLK, kallikrein; NMFs, natural moisturizing factors; RIPK, receptor interacting protein kinase; SC, stratum corneum; SG, stratum granulosum; TAK1, TGFβ-activated kinase; TGase, transglutaminase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UCA, urocanic acid

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http://dx.doi.org/10.1016/j.bbamcr.2013.06.010

2. Differentiation of epidermal keratinocytes

2.1. A brief guide to keratinocyte differentiation

The structures of the epidermis and skin appendages are maintained by differentiation of keratinocytes from a pool of stem cells. The epidermal stem cells are located in the basal layer of the epidermis and in special niches of the hair follicle [3]. They give rise to transiently amplifying cells that are still located in the basal layer. By asymmetric division, proliferating keratinocytes generate cells that stop to divide and start terminal differentiation (Fig. 1). These daughter cells move into the suprabasal layers of the epidermis or into suprabasal positions in the bulge of the hair follicle. Once keratinocytes are detached from the basement membrane of the epithelium, they change their gene expression profile under the control of p63 and other transcription factors [4,5]. Instead of keratins K5 and K14, expressed by all proliferating keratinocytes, the differentiating keratinocytes of the interfollicular epidermis express K1 and K10. In the hair follicle and in the nail unit differentiating keratinocytes express cysteine-rich keratins able to form multiple disulfide bridges that confer additional mechanical strength (so-called "hair keratins"). Later during differentiation, expression of a gene cluster named the "epidermal differentiation complex" (EDC) generates proteins such as involucrin and loricrin [5,6]. Both are cross-linked by enzymes of the transglutaminase (TGase) family [7]. As the main

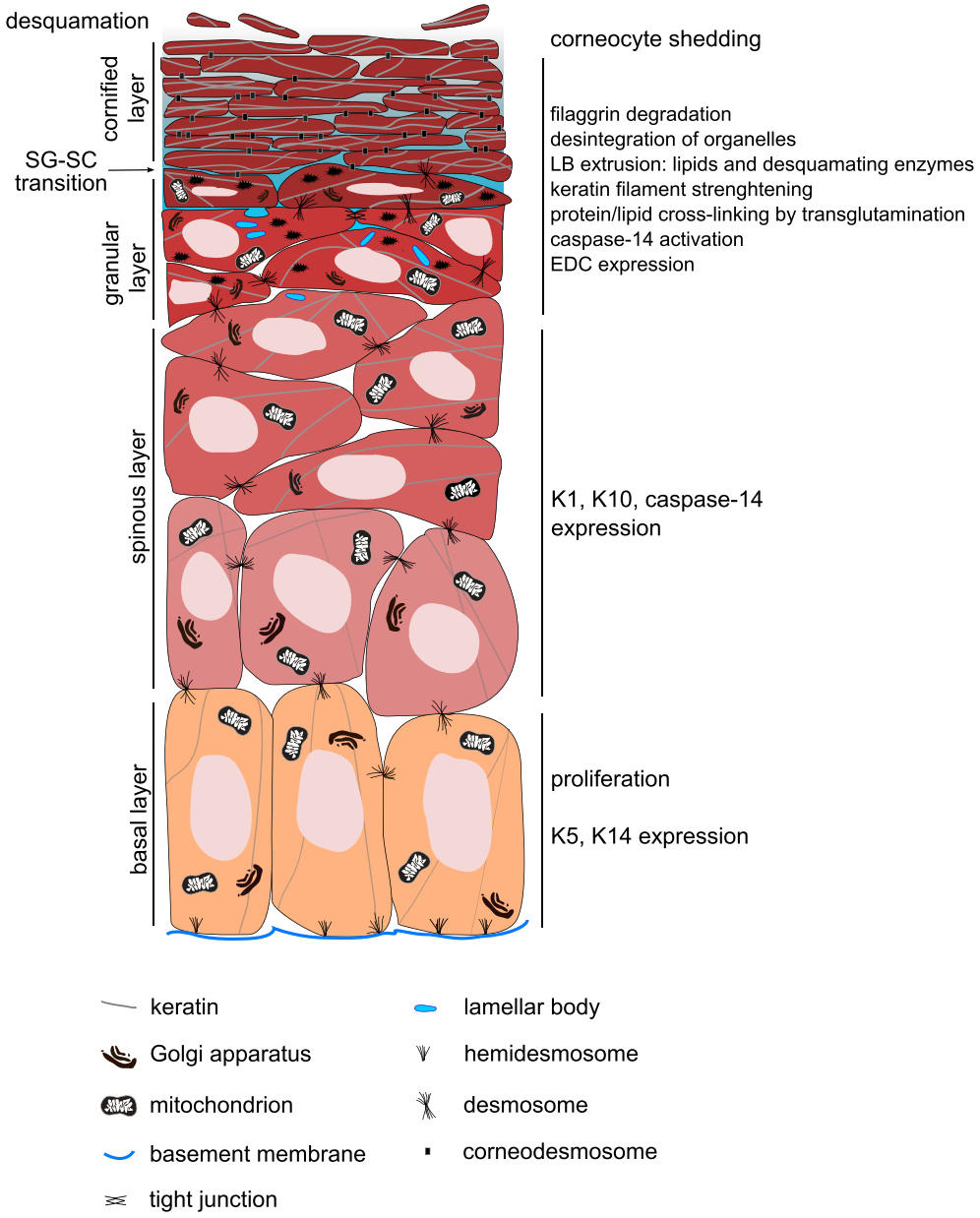

Fig. 1. The process of terminal differentiation. The epidermis consists of layers (indicated on the left) of different stages of keratinocyte differentiation distinguished by morphological hallmarks and expression markers. The cells of the basal layer are attached to the basement membrane by hemidesmosomes, have the capacity to proliferate and provide new cells that will differentiate towards the surface of the skin. Cells of the spinous layer no longer divide and express typical markers of differentiation such as keratins K1 and K10 and caspase-14. In the granular layer, keratohyalin granules are present and proteins of the epidermal differentiation complex (EDC) are expressed. At the transition from the granular to the cornified layer, a cascade of events occurs: caspase-14 becomes active and contributes to filaggrin degradation, and keratins and other proteins are cross-linked by transglutaminases. At this point the content of the lamellar bodies (LBs) is extruded into the intercellular space because the LBs fuse with the plasma membrane. At the cytoplasmic side of the plasma membrane cross-linking of proteins forms the cornified envelope (CE) that is tightly connected to neighboring CEs via corneodesmosomes. The actual physical skin barrier is formed by the tight junctions, which form strong intercellular interactions, and the lipids in the intercorneocyte spaces. Eventually the corneodesmosomes are proteolytically degraded by extracellular enzymes and the corneocytes are shed during desquamation.

transglutaminase, TGase 1, is localized at the cell membrane, the proteins form an insoluble structure named "cornified envelope" close to the cell surface [7]. Filaggrin (FLG), also encoded in the EDC, is the main component of keratohyalin granules to which the granular layer (stratum granulosum, SG) owes its name. Upon dephosphorylation and proteolysis of the profilaggrin precursor, filaggrin is dispersed and causes the aggregation of the keratin intermediate filaments by a yet incompletely understood mechanism [8]. Simultaneously the nucleus is degraded and cell organelles disappear by an unknown mechanism. Ultimately, keratins remain as the prevailing proteins inside the cornified envelopes [9], strongly contributing to the mechanical resistance of the cornified layer (stratum corneum, SC). In addition, keratins can also regulate pathways involved in growth, proliferation, migration and apoptosis of epithelial cells. This topic has been recently reviewed by Pan et al. [10].

In the interfollicular and palmoplantar epidermis, the conversion of living keratinocytes to corneocytes is associated with a reduction of cell volume [11], perhaps due to a reduction of intracellular water content. Moreover, lipid lamellae preformed in intracellular lamellar bodies are extruded from the apical side of granular layer cells. Lamellar bodies also contain enzymes that modify the secreted lipids outside of the cells. Glucosylceramides and sphingomyelin are converted to ceramides and phospholipids are converted to free fatty acids and glycerol [12]. Subsequently, a portion of omega-hydroxyceramides and free fatty acids is linked by the action of TGase1 and by other mechanisms to proteins at the outer side of the cornified envelope [7,13,14]. When the dead corneocyte is integrated into the SC, it is thus completely surrounded by lipids that seal the intercellular space and protect against water loss. For further details on the involvement of lipids in forming the skin barrier we refer to a number of excellent reviews on this topic [15–17]. Furthermore, corneocytes remain tightly connected to each other via corneodesmosomes, which are cross-linked to the cornified envelope [18–20]. Therefore, the dead keratinocytes are part of a supracellular structure that is resistant to mechanical stress. Proteolysis of corneodesmosomes releases the bonds between corneocytes of the outer cornified layer, leading to desquamation. The entire differentiation process is tightly controlled at the levels of gene expression (in living keratinocytes) and enzyme activities (in living and dead keratinocytes).

In many aspects differentiation of keratinocytes in hair and nails is similar to that of keratinocytes in the epidermis. However, hair and nail keratins are more extensively cross-linked to each other as well as to a special matrix of keratin-associated proteins (KRTAPs) [21]. This is achieved by the formation of disulfide bonds between these highly cysteine-rich proteins. The resulting structures are much harder than the stratum corneum. Transglutamination does occur in the hair [22], but may be less critical than in the epidermis. An intercellular lipid matrix like that of the stratum corneum is not formed [23], possibly due to a lack of lamellar body excretion; however, this remains to be determined. Therefore, proteases for the disconnection of corneocytes are perhaps not secreted in the hair fiber and desmosomal junctions are, at least partially, maintained [24]. Together with the particular spatial arrangement of the proliferating compartments within hair follicles and the nail apparatus, these features cause unidirectional growth and the formation of skin projections in the form of hairs and nails [25].

Cornification in hair and nails shares many features including the dependence on DNase1L2 for nuclear DNA degradation [26]. Therefore, it is likely that nails resemble or even represent the evolutionary precursors of hair [27]. Notably, hairs are more complex structures than nails as they consist of multiple onion-like concentric layers of keratinocytes that are all derived from the hair bulb but express different structural proteins [28]. The modes of cornification also differ significantly between 1) the medulla, which comprises the innermost cells that cornify incompletely; 2) the cortex that forms a quasi-crystalline arrangement of keratin intermediate filaments; 3) the cuticle, which forms the external layer of thioester-bound fatty acids of the hair fiber; 4) the inner root sheath, showing faster cornification than in the hair fiber followed by disintegration in the upper hair canal; and 5) the outer root sheath, for which the mode of terminal differentiation is uncertain [21,23]. In addition to cornification during the growth of the hair, keratinocytes of the hair follicle also undergo classical apoptosis during the regression phase of the hair cycle [29]. These apoptotic cells are efficiently removed by phagocytozing neighboring cells [30] to prevent leakage of cellular content and tissue inflammation. Hair follicles are associated with sebaceous glands that consist of specialized keratinocytes that follow still another pathway of differentiation. The terminal differentiation of these "sebocytes" is characterized by accumulation of lipid droplets in the cytoplasm and nuclear degeneration eventually leading to programmed cell death and holocrine secretion of sebum [31]. This form of cell death shares little, if any, features with cornification. The molecular pathways resulting in sebocyte elimination and cell death are still unknown.

2.2. Suppression of premature cell death during terminal keratinocyte differentiation

Although terminal differentiation of keratinocytes includes cell death, it has become clear during the last decade that it also employs pro-survival mechanisms (Fig. 2). This apparent paradox results from the need to ensure a productive way of coordinated cornification leading to the formation of mechanically resilient and interconnected cell corpses rather than the premature demise of cells due to various forms of intracellular or extracellular stress. Moreover, disorganized apoptotic or necrotic cell death would lead to the release of DAMPs (danger associated molecular patterns), inflammation and disturbance of the differentiation process of adjacent keratinocytes. Therefore, keratinocytes adopt efficient anti-apoptotic and anti-necrotic mechanisms to prevent untimely keratinocyte death before cornification.

In vivo, epidermal keratinocytes differentiate upon detaching from the basement membrane. The differentiation signal is transduced by unoccupied β1-integrins [32]. Because unoccupied β1-integrins can initiate apoptotic cell death [33], this would argue for the existence of efficient intrinsic survival mechanisms required to maintain the viability of suprabasal cells in vivo. Interestingly, it has been shown that NF-κB, a transcription factor conferring resistance to apoptosis, is activated and translocated to the nucleus upon differentiation as witnessed by nuclear translocation of the RelA subunit [34]. NF-κB is a complex formed by homo- and hetero-dimerization of the NF-κB family members p50, p52, RelA (p65), RelB, and c-Rel. A multitude of triggers induce signaling pathways that converge on the activation of the IκB kinase (IKK) complex, consisting of the IKK1 and IKK2 catalytic subunits and the NEMO (also known as IKKγ) regulatory subunit. The IKK complex phosphorylates the NF-κB inhibitory IκB proteins, targeting them for proteasomal degradation. This leads to the release of the NF-κB transcription factor and unmasking of the nuclear translocation signal sequence, nuclear translocation and transcriptional activity of NF-κB [35]. Several mouse gene knock-out models in which essential NF-κB mediators were ablated in the keratinocyte compartment, such as TAK1 (TGFβ-activated kinase) or NEMO [36,37], show increased sensitivity to TNF (tumor necrosis factor)-induced apoptosis leading to massive skin inflammation and perinatal death. Depending on the situation, TNF can be derived from infiltrating immune cells or produced locally by keratinocytes. Consequently these mice are phenotypically rescued by crossing them to TNFR1 deficient mice. However, upon RelA or IKK2 ablation [38,39] or overexpression of a dominant-negative p50 subunit in the skin [40], epidermal hypo-activation of NF-κB can also lead to cell death-independent but TNFR1-dependent skin inflammation. This may be explained by the existence of an NF-κB-dependent signaling pathway that suppresses TNFR1-dependent JNK activation leading to epidermal hyperproliferation [40]. However, TAK1 deficient keratinocytes cannot activate JNK upon TNF treatment [37]. Based on these reports, we hypothesize that NF-κB in the skin prevents TNFR1-mediated inflammatory signaling and premature apoptosis in the skin to allow proper cornification and epidermal barrier formation. Whether the

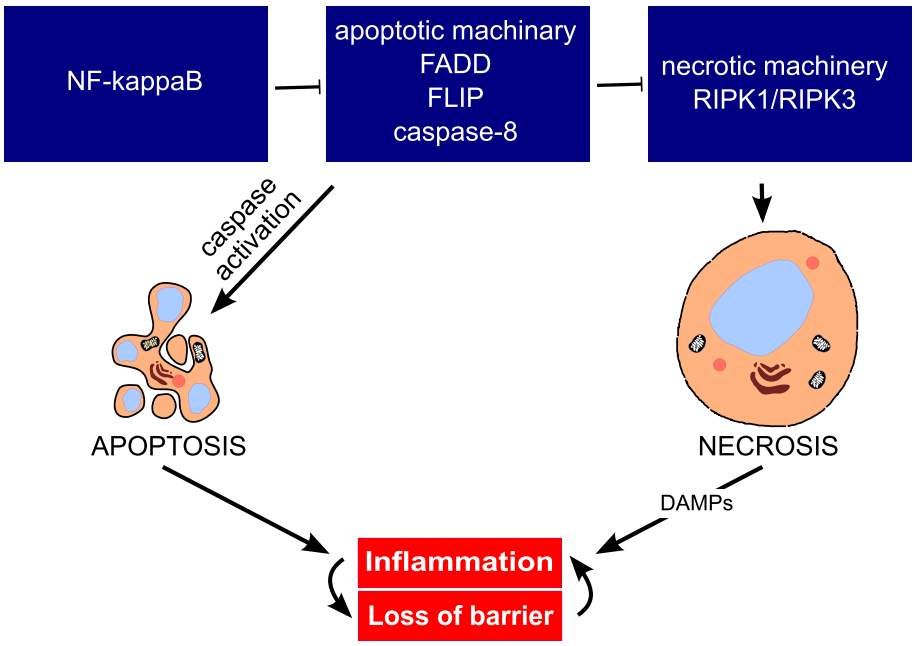

Fig. 2. Control of premature cell death in keratinocytes is required to maintain epidermal homeostasis. Key steps in the signaling pathways leading to apoptotic and necrotic cell death lead to caspase activation and involvement of RIP kinases (RIPK1/RIPK3), respectively. The apoptotic machinery can inactivate the necrotic machinery by cleaving RIP kinases and is kept in check by the NF-κB pro-survival pathway. Apoptosis in the absence of efficient phagocytosis and necrosis of keratinocytes can lead to loss of barrier skin function and inflammation. Danger associated molecular patterns (DAMPs) released from necrotic cells can ignite the inflammatory process. Skin inflammation and barrier loss are intertwined, because both can enforce one another.

protective effect of NF-κB is related to the observation that α-catenin protects suprabasal cells from apoptosis is currently not clear [41].

During recent years it has become clear that necrotic cell death can be as controlled and programmed as apoptosis, therefore called regulated necrosis or necroptosis [42,43]. The latter pathway is initiated by the activation of the kinases RIPK1 and RIPK3, which form the necrosome complex that signals to necroptosis [42,43]. The importance of controlling necroptosis during epidermal development is evident from the neonatal lethality of epidermal keratinocyte-specific (EKO) *Fadd*$^{-/-}$ or *Casp8*$^{-/-}$ mice [44–46], which can be rescued by ablation of RIPK1 or RIPK3 [44,47]. In addition, it was shown that RIPK1 and 3 form a complex with FADD/caspase-8 in which the RIP kinases become inactivated by caspase-8-dependent cleavage [48]. At birth, *Fadd* deficient mice are histologically indistinguishable from control littermates. However, a few days later they show an increase in necrotic cells in the epidermis and develop severe skin inflammation resulting in skin barrier loss and mortality [44]. This indicates that unknown postnatal triggers induce necroptosis and skin inflammation. TNF levels were increased in FADD deficient mice; however the suppression of TNF signaling by epidermal deletion of either TNF or TNFR1 could only delay but not prevent the development of skin lesions. Therefore, additional necroptosis-inducing ligands and receptors could be involved in the development of skin inflammation in Fadd^{EKO} mice. Recently it was shown that caspase-8 deficiency in dendritic cells can lead to RIPK3-mediated activation of the NLRP3 inflammasome [49]. Whether a similar mechanism may act in keratinocytes needs to be investigated. Taken together, all these data indicate that during homeostatic keratinocyte differentiation efficient pathways are activated to protect the keratinocytes against premature apoptosis and necrosis to allow the complex keratinization process to take place.

3. The process of cornification

3.1. Cytoarchitectural aspects of cornification

Cornification involves major changes of the intracellular organization, the formation of a cornified envelope and the integration of the dead cells into a supracellular interconnected structure. In the granular epidermal layer, the continued expression of the suprabasal keratins K1, K2, and K10 adds to the reinforcement of the cytoskeleton, and post-translational modifications as well as the interaction with filaggrin coordinate the formation of keratin bundles [8]. These filaments are linked to desmosomes so that the cytoskeletons of neighboring cells and later corneocytes are interconnected. Remarkably, more than 85% of the corneocyte protein content consists of keratins [50], suggesting that the cytoskeleton is of primary importance for corneocyte function [51]. The high keratin content is not only achieved by the massive production and stabilization of keratins during differentiation but likely also by the removal of other proteins that have originally resided either in the cytoplasm or in organelles. At the microscopic level, cornification is associated with the complete disintegration of subcellular compartments, i.e. organelles such as the nucleus, the mitochondria, endoplasmic reticulum, and lysosomes. To date, the mechanisms of cornification-associated proteolysis and organelle removal (see Section 3.2) is not well understood. Apart from bulk degradation of proteins, also limited proteolysis that yields functional protein products has been characterized during cornification. Remarkably, keratin intermediate filaments outlast this process unaffected.

Though keratinocyte differentiation is a process different from apoptotic cell death [52], the caspase family member caspase-14 is involved in the execution of limited proteolysis during cornification [53,54]. In the epidermis this caspase is increasingly expressed during keratinocyte differentiation and proteolytically maturated at the SG–SC transition [55–57]. The development of caspase-14 deficient mice showed that terminal keratinocyte differentiation does not depend on caspase-14; however the degradation of filaggrin was defective in these mice [53,54]. The precursor profilaggrin, with an N-terminal conserved S100 Ca-binding domain and B-domain followed by multiple filaggrin monomer units, is found in keratohyalin granules in the granular layer. At the SG–SC transition, the highly phosphorylated precursor is dephosphorylated, proteolysed into FLG monomers and the N-terminal S100 domain is released. Several proteases have been postulated to be directly or indirectly involved in this step, such as matriptase, furin, PACE4, SASPase and calpain [8]. During cornification, the FLG monomer

becomes partially proteolysed, and is then further cut by caspase-14 to allow FLG degradation into amino acids, some of which act as natural moisturizing factors (NMFs) [54]. Therefore, caspase-14 deficiency results in accumulation of partially proteolysed FLG fragments and a major reduction in NMF and filaggrin-derived free amino acids. FLG is a major source of histidine, which is further metabolized to the potent UVB scavenger urocanic acid (UCA) in the cornifying layers [58]. In accordance, caspase-14 deficient mice as well as mutant mice unable to convert histidine to UCA show increased UVB sensitivity that can be restored by topical UCA application [59,60]. In addition, filaggrin knock-down in artificial in vitro skin constructs results in a similar phenotype [61]. These findings indicate that, next to melanin, UCA present in the cornifying layers has an important contribution to the protection of the underlying keratinocytes against UVB-induced damage. The overall importance of FLG is underscored by the fact that FLG mutations predispose to atopic dermatitis and asthma, and FLG deficiency leads to ichthyosis vulgaris [62]. However, these diseases may be caused by skin barrier defects primarily due to the lack of the filaggrin-dependent keratin aggregation.

Next to (limited) proteolysis during terminal keratinocyte differentiation several proteins are covalently cross-linked by transglutamination to function primarily as components of the cornified envelope. These proteins are late differentiation markers and include involucrin, loricrin and small proline rich proteins (SPRRs). The transglutamination reaction is catalyzed by transglutaminases 1, 3 and 5, which are specifically expressed in cornifying structures [7]. Transglutaminases form covalent connections between a glutamine and a lysine residue (Nε-(γ-glutamyl) lysine isopeptide cross-links) as well as between glutamine residues and the ω−hydroxy group of ceramides. The first type of reaction is critical for establishing the mechanically resilient protein backbone of the corneocytes and the second type of reaction is crucial for establishing the extracellular lipid envelope, essential for the barrier to water loss [7,63,64]. In contrast to the so-called "dustbin hypothesis", which proposed that all available substrates, i.e. proteins containing accessible glutamine or lysine residues, would be incorporated into the cornified envelope [65], investigations of the molecular composition of the cornified envelope suggested an ordered and specific assembly process [64,66]. Ultimately, a sealed protein envelope of 5–10 nm thickness is formed underneath the cell membrane, which later even replaces the cellular membrane. At this stage the corneocytes are embedded in a lipid matrix extruded by the lamellar bodies, which functions as water repellent, and are strongly interconnected by corneodesomosomes. The latter differ from desmosomes by the extra incorporation of corneodesmosin and ensure the physical strength of the cornifying layers [18].

Once the cornification process is completed, extensive protease activity is still required during desquamation. Kallikreins (KLKs) are serine proteases with tryptic or chymotryptic-like activity that are involved in this shedding process [67]. The preproform of KLK5 and KLK7 (also known as stratum corneum tryptic enzyme or SCTE and stratum corneum chymotryptic enzyme or SCCE, respectively), is stored in lamellar bodies that are extruded at the apical side of the SG [68]. In the intercellular space KLK5 and KLK7 are proteolytically matured and target corneodesmosomes by cleaving the structural proteins corneodesmosin, desmocollin and desmoglein [69]. Although the intercellular space of the SC has a low water content and an acidic pH, this quite extreme condition is well tolerated by KLKs [69,70]. Major contributions to understanding KLK physiology have come from studying endogenous inhibitors [67]. LEKTI (lympho-epithelial Kazal-type related inhibitor I) is encoded by *Spink5* and mutations in this human gene have been associated with Netherton syndrome, a rare autosomal recessive ichthyosiform disease characterized by skin barrier defects and skin inflammation [71]. Therefore, efficient KLK 5 and/or 7 inhibitors would be very useful in the management of diseases caused by increased KLK protease activity resulting in enhanced cleavage of corneodesmosomes, such as Netherton disease.

3.2. Removal of organelles during cornification

A main characteristic of cornification is the breakdown of intracellular compartmentalization by degradation of organelles. This is evident from the comparison of keratinocytes in the granular layer and corneocytes in the cornified layer of the epidermis. However, there is little information on intermediate stages of organelle breakdown. Lavker and Matoltsy observed an increase in lysosome-like bodies concomitant with the disappearance of mitochondria, Golgi apparatus, ribosomes, and ER during cornification in the keratinizing epithelium of the rumen of sheep [72], which contains a relatively large number of cells in the transitional stage between the granular and cornified layers. Morioka and colleagues reported mitochondrial structures within lysosomes of the granular layer in fetal rat skin [73]. A proposed role of autophagy in mitochondrial breakdown was not supported by the characterization of mice deficient in epidermal autophagy [74,75].

Although an increase in lysosomal cysteine protease activity has been observed during terminal keratinocyte differentiation [76], the fate of lysosomes in the granular layer is not known. However, it is clear that lysosomal proteases execute important functions during cornification: 1) Papillon–Lefèvre syndrome patients, carrying a loss of function mutation in the cathepsin C gene, develop palmoplantar keratodermas (i.e. severe thickening of the cornifying layers) [77]. 2) Cathepsin D deficient mice show a defect in transglutaminase 1 activity, less pronounced cornified envelopes and thickening of the SC [78]. 3) Cathepsin L deficient mice develop epidermal hyperplasia with hyperkeratosis [79]. 4) Mice deficient in the cysteine protease inhibitor cystatin M/E, which inhibits legumain, cathepsin L and cathepsin V, have disturbed cornification, impaired barrier function and dehydration [80]. The same authors found also that cathepsin L is needed for the proteolytic activation of transglutaminase 3 [81], which cross-links structural proteins during cornified envelope formation. An important role of lysosomal cathepsins in upstream events of proteolysis during cornification has been suggested by comparing the proteomes of cathepsin L and cathepsin B-deficient epidermis [82]. Histone 2A and caspase-14 were the most strongly accumulating proteins in response to abrogation of cathepsin B or cathepsin L, respectively. These findings suggest roles of cathepsins in the degradation of chromatin and in controlling the processing of filaggrin. Taken together, all these data indicate that cathepsins play a role in the cornification process (strengthening of the cornified envelope) and desquamation. Because cystatin M/E is a cytosolic inhibitor of cathepsins, this implies that during the final steps of cornification functional proteases are released from the lysosomes, possibly by disintegration of the lysosomes. Interestingly, the timely rise in transglutaminase activity during the final steps of cornification could be the result of organelle degradation, since they need proteolytic processing by lysosomal proteases and increased Ca^{2+} levels that could be released from degrading endoplasmatic reticulum or mitochondria, both organelles that act as Ca^{2+} storages.

Although nuclear changes such as condensation of the nucleolus, occurrence of indentions in the nuclear shape and flattening of the nucleus during terminal keratinocyte differentiation have been described more than 40 years ago [83], the mechanisms of nuclear degradation, as for the other organelles, remain poorly characterized. As the removal of the nucleus appears to be a fast process that may occur within less than 6 h [84], few if any transitional cells are seen on thin sections of the epidermis [84,85]. Only during epidermal wound closure after tape stripping, which involves accelerated cornification, an increase in the abundance of transitional cells can be observed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of fragmented nuclear DNA [86]. In contrast, transitional cells with TUNEL-positive nuclei are frequently observed in the nail matrix in homeostatic conditions [87].

It is only during the last years that we start to identify the mechanism of nuclear DNA degradation during cornification (see Section 4.3). In all types of cornification, nuclear DNA is degraded as evidenced by the

absence of labelling with DNA-specific fluorescence dyes in normal stratum corneum, hair and nails [26,88,89]. Interestingly, hair from different human donors contains variable numbers of DNA-positive nuclear remnants [89], suggesting that the completeness of DNA degradation during cornification of the hair is a polymorphic trait. Interestingly, this lack of DNA degradation also suppresses, at least in part, the removal of histones [89], indicating that the degradation of DNA is upstream of degradation of other chromatin components.

What might be the function(s) of intracellular bulk degradation during cornification? As outlined above, a main function of the interior of a corneocytes is mechanical resilience, mediated by the keratin network [11]. Intuitively, it makes sense that the removal of cellular organelles, that do not contribute to the stability of the cytoskeleton but rather disturb a regular arrangement of intermediate filaments, is beneficial to the achievement of a hard structure. Experimental evidence for this concept has been obtained for cornification in the hair, which involves the removal of nuclear DNA by DNase1L2 [26]. Furthermore, the removal of most cell structures might contribute to the enormous decrease in cell volume (approx. 50% decrease) that is visible on electron micrographs of the outer epidermis [11]. Possibly, this cellular shrinking supports mechanical resilience of the stratum corneum or it allows maturation of the extracellular lipid organization. It is also conceivable that cornification-associated alterations in the protein composition of the cells are beneficial for the establishment of physico-chemical parameters of the stratum corneum such as the pH. Importantly, the cellular water content decreases from 70% to 40% during the stratum granulosum to corneum transition [90]. At present, the mechanism of this dehydration and its impact on hydrolytic reactions during cornification are unclear.

In spite of the many morphological studies performed on cornifying skin and the generation of many gene knockouts affecting epidermal differentiation in the mouse, the mechanisms responsible for organelle degradation in cornifying keratinocytes remain undiscovered. This suggests that the degradation of keratinocyte organelles is controlled by several redundant dismantling enzymatic pathways, making it difficult to identify them. The comprehensive screening of gene knockouts in mice [91], and the continuous progress in electron microscopical techniques such as 3D electron microscopy of cornifying tissues could teach us more about the molecular and morphological aspects of cellular remodelling during cornification.

4. Molecular mechanisms of cell death during cornification

4.1. Cell death mechanisms of normal and pathological cornification

Several steps of the cornification process are irreversible and may suffice on their own to kill the cell. However, the formation of the cornified envelope and the embedding of the cells into a lipid matrix have a rather indirect killing effect as they shut off the energy supply and the communication with living neighboring cells. By contrast, the intracellular changes of cornification include three hallmarks of cell death and can be considered as the actual cell death process: 1) the molecular machinery that constitutes the cell's ability to respond to stimuli from the environment is degraded, 2) the production of energy is stopped by the removal of mitochondria, and 3) the nucleus is dismantled and the DNA is destroyed.

During normal terminal differentiation of keratinocytes all cornification subprocesses are tightly coordinated and the intracellular remodeling appears to cause the decisive changes that qualify cornification as a mode of cell death (Fig. 3). However, even if cornification-associated intracellular remodeling is blocked (e.g. by alterations in the genetic information or in the execution of parts of the terminal differentiation program) keratinocytes eventually die. Yet, these types of cell death are pathological forms of cornification known as parakeratosis [92], which is histologically recognized by the retention of discernible nuclear remnants within corneocytes. At the ultrastructural level, mitochondria have also been detected in the parakeratotic stratum corneum of psoriasis lesions [93]. Parakeratotic cornification is often found in inflamed, hyperproliferative skin that shows increased epidermal turnover. This suggests that under these conditions the remodeling machinery needed for correct cornification cannot follow the increased keratinocyte turnover, leading to incomplete formation of the cornified envelope and defective removal of the organelles. Interestingly, the lack of complete cornification in psoriasis was attributed to premature death of the cornifying keratinocyte, thereby hampering the expression of late differentiation markers such as profilaggrin and loricrin needed to execute full cornification [94]. Alternatively, the lack of late differentiation marker expression in parakeratotic skin could also be explained by the suppressive effect of inflammatory mediators known to drive the disease, e.g. TNF, IL-17 or histamine [95–97].

4.2. Protein transglutamination and proteolysis during keratinocyte cell death

Cell death by cornification is associated with and partially caused by enzymatic processing of cellular proteins by transglutamination and proteolysis. The activity of transglutaminases is the most central aspect of cornification as it builds the supramolecular structures of the cross-linked intermediate filament cytoskeleton and the cornified envelope [64]. However, are keratinocytes killed by transglutamination of their proteins? Deletion of transglutaminase-1 severely disturbs cornification [98,99]. The keratin intermediate filaments were not condensed and a typical cornified envelope did not form in the absence of TGase1. In addition, TGase1-negative epidermis showed aberrant retention of the nucleus and of keratohyalin granules in the stratum corneum [99]. Grafting TGase1 deficient skin onto nude mice led to massive hyperkeratosis and the formation of superficial scales consisting of flattened dead cells without a classical cornified envelope [98]. The observed thickening of the cornifying layers is probably an attempt of the tissue to physically compensate for the defective epidermal barrier. Organelle remnants were not observed in the cornifying layers of TGase1-deficient transplants but the presence of large vacuoles suggested that intracellular remodeling in the granular layer was abnormal. This indicates that the retention of organelles or granules is not a cell-autonomous characteristic of TGase1 deficient keratinocytes, but rather a secondary effect due to the massive skin barrier loss in full TGase1 deficient mice.

Proteases are highly active and their activity is tightly controlled during cornification [100,101] but does their action kill the cell, like proteolysis by caspases kills the cells in apoptosis? Importantly, apoptotic caspases do not contribute to cell death by cornification. The only caspase active in terminally differentiated keratinocytes is caspase-14 [55,56]. Although caspase-14 is not required for cornification-associated cell death [53], it was suggested that caspase-14 plays a redundant role in the degradation of the cellular content during cornification [102]. In addition, caspase-14 deficient mice show aberrant cornification upon acute barrier disruption [86].

Lysosomal proteases are implicated in the execution of apoptosis under certain conditions and contribute to membrane permeabilization in necrotic cell death [103,104]. Assuming that lysosomal cathepsins are critical initiators and effectors of cornification-associated proteolysis, could they fulfill a role in keratinocyte cell death during cornification? Cathepsins reside in lysosomes and have an activity optimum at acidic pH. However, cathepsin V localizes also to lamellar bodies and cathepsin L and legumain were detected by immunogold electron microscopy in the cytoplasm of apparently viable keratinocytes in the granular layer [105]. In addition, several other lysosomal proteins were identified in preparations enriched for lamellar bodies [106] leading to the suggestion that lamellar bodies are a new class of secretory lysosomes. Later during cornification, the lysosomal membrane may be permeabilized by an as-yet unknown mechanism. Currently, it is unclear whether and how lysosomal proteases participate in the degradation of organelles

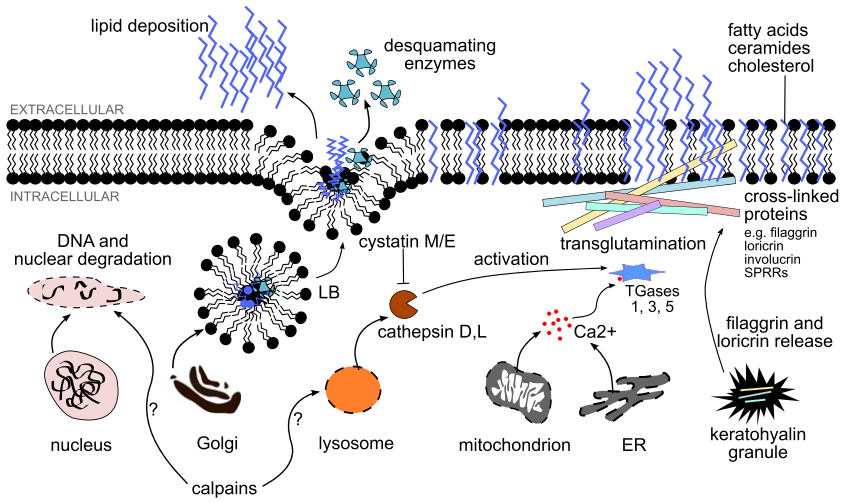

Fig. 3. Cell death by cornification. At the SG–SC transition the different cell organelles are eliminated. The nucleus and its DNA are degraded by unknown mechanisms. Damaged lysosomes release cathepsins, together with Ca^{2+} released from the ER and/or mitochondria needed for transglutaminase activation. A role for calpain in nuclear and lysosome degradation has been suggested. Transglutaminases cross-link intercellular proteins close to the cell membrane. Many of those proteins are stored and released from keratohyalin granules in the granular layer. As an end result the cell membrane is covered with cross-linked proteins at the intercellular side, forming the cornified envelope. Transglutaminases also link lipids to the cornified envelope, forming the lipid envelope. Specialized lipid vesicles known as lamellar bodies, probably arising from the Golgi, fuse with the lipids of the cell membrane and extrude their content. The LBs release lipids that fill the intercorneocyte spaces, resulting in a lipid barrier preventing water loss, and release desquamating enzymes that gradually chew up the corneodesmosomes. The massive accumulation of keratins during cell death by cornification has been omitted from this figure for the sake of clarity. See Section 4 for further details. ER, endoplasmatic reticulum; TGase, transglutaminase.

and cell death during cornification. Results obtained by deletion of individual cathepsins in mice suggest that in case cathepsins would be implicated in keratinocyte cell death this process will be executed by the redundant action of several cathepsins rather than by a specific member of this protease family. To avoid leakage of cellular content into the tissue, causing epidermal inflammation due to cathepsin-mediated damage to the cell membrane, lysosomal leakage will probably only start after the formation of the cornified envelope sealing the cell. Intriguingly, the destabilization of lysosomes by calpain-1 has been suggested to be a key step in epithelial cell death during mammary gland involution [107,108]. Interestingly, calpain activity has been shown to trigger DNA fragmentation in apoptotic neuronal cells [109]. Calpain-1 is expressed and is active in the stratum granulosum [110]. However, no skin phenotype has been reported for the calpain-1 knockout mouse or mice overexpressing the specific calpain inhibitor calpastatin [109,111], making it less likely that calpain would fulfill a non-redundant role in cell death by cornification.

4.3. Nucleic acid degradation during cornification

DNA degradation is a feature of various forms of cornification in mammals and has been detected even in a simple form of cornification in the epidermis of the frog [112], suggesting a conserved role of this process in keratinocyte cornification. The stratum corneum is also devoid of significant amounts of RNA but rather features high levels of RNase activity due to specific upregulation of certain RNases, such as RNase7 and the removal of the endogenous RNase inhibitor protein [113].

DNase1L2 is the only endonuclease gene known so far that is transcriptionally upregulated during terminal differentiation of keratinocytes in mammals [114]. The induction of DNase1L2 leads to an accumulation of DNase1L2 enzyme in keratinocytes of the granular layer of the interfollicular epidermis, the hair and nail matrix and in terminally differentiated sebocytes [87,114]. The expression of DNase1L2 is minimal or negative in non-epidermal tissues suggesting a specific role of DNase1L2 in cornification and sebocyte differentiation. Short interfering RNA (siRNA)-mediated knockdown of DNase1L2 expression in a human organotypic skin model led to parakeratotic stratum corneum [114]. Thus, DNase1L2 is essential for the removal of nuclear DNA during cornification of human keratinocytes in vitro. However, DNase1L2 ablation in the mouse did not abrogate nuclear DNA degradation during stratum corneum formation in the interfollicular epidermis [26]. The apparent difference in the consequences of DNase1L2 abrogation may be caused by differences between the human and mouse cornification machinery and/or by differences between the de novo formation of the stratum corneum in vitro [114] and the homeostatic epidermis in vivo [26]. Investigations addressing this open issue are underway in our laboratories.

However, in contrast to interfollicular soft stratum corneum formation, the modes of terminal differentiation of keratinocytes in hard cornified structures such as the medulla, cortex and cuticle of the hair, in nails, in tail scales of the mouse, and in the epithelia on the surface of the tongue and the esophagus were disturbed by DNase1L2 ablation [26]. Nuclear DNA was detected on thin sections of these skin structures, and elevated levels of nuclear DNA could be extracted from hair and nails of DNase1L2 knockout mice. Interestingly, in the nail the level of mitochondrial DNA was also increased, suggesting that DNase1L2 does not specifically degrade nuclear DNA. Although DNase1L2-deficient mice were macroscopically inconspicuous, the microstructure of hair was disturbed and weakened by the aberrant retention of nuclear remnants containing DNA [26]. It remains an open question whether DNA degradation by DNase1L2 is also required for enhancing the resistance to mechanical stress in other modes of cornification.

Although experimental evidence for distinct DNases other than DNase1L2 in cornification has not been reported yet, at least two candidate enzymes contributing to cornification-associated DNA breakdown have been identified. DNase 2 has been shown to be the main DNase of the stratum corneum in humans and mice [115]. Nevertheless, DNase 2 knockdown in human skin equivalents or DNase 2 knockout in mice does not affect the nucleus or DNA degradation in the epidermis [115]. Of course, if this would be a redundant process, an involvement in the removal of DNA fragments from forming corneocytes is still possible. The other candidate DNase is TREX2, an exonuclease that was not included in the DNase panel for screening DNases upregulated during terminal differentiation of keratinocytes [114]. A later study demonstrated epidermis-specific and apparently keratinocyte-differentiation-associated expression of TREX2 in the mouse [116]. As an exonuclease, TREX2 depends on the initial

endonucleolytic action of other DNases to contribute to DNA breakdown. Future investigations of mice carrying a double deficiency of the DNase genes mentioned above, or others, may reveal the concerted action of multiple DNases in keratinocyte differentiation.

5. Summary and outlook

Many mechanistic aspects of the cornification process have become clear in recent years. These include the definition of distinct sets of genes encoding structural components, enzymes and regulatory proteins of cornifying keratinocytes and the role of subcellular compartmentalization in the control of cornification. Cell death in the context of cornification involves distinct enzyme classes such as transglutaminases, proteases, DNases and others. Although degradation of nucleic acids by DNases and RNases is an intrinsic step of keratinocyte death by cornification, it may not be the decisive event in this process. The fragmentation of DNA and RNA is probably preceded by distinct trigger(s) such as proteolytic events that may, in parallel, trigger other processes, such as the removal of pivotal proteins, that suffice to kill the cell. Yet, the molecular coordination of this mode of programmed cell death is far from understood. Its clarification requires the establishment of model systems that address functional redundancies and backup strategies of cornification. However, depending on the degree of redundancy this may turn out to be less evident. Nevertheless identifying the mechanisms governing cornification may help to develop strategies for improving cornification and skin barrier function in inflammatory skin conditions, such as atopic dermatitis and psoriasis. In addition, such strategies might also help to direct non-differentiating cancerous keratinocytes towards cornification and cell death.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

Research in the lab of W. Declercq was supported by VIB, Ghent University, BELSPO IAP 7/32, Stichting tegen Kanker 2010-162, FWO-Vlaanderen: G.0226.09N, 1.5.169.08 and 1.5.058.12N and a Methusalem grant (BOF09/01M00709) to Peter Vandenabeele. S. Lippens is a postdoctoral fellow with the FWO-Vlaanderen. The labs of W. Declercq, L. Eckhart and E. Tschachler are part of the EU COST SKINBAD BM0903 action.

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