Epidermal Proteases in the Pathogenesis of Rosacea

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A number of different proteases and their inhibitors have a role in skin physiology and in the pathophysiology of inflammatory skin diseases. Proteases are important in the desquamation process and orderly regulation of the skin's barrier function. On the basis of the catalytic domain, proteases are classified into aspartate-, cysteine-, glutamate-, metallo-, serine-, and threonine proteases. Particularly, serine proteases (SPs) contribute to epidermal permeability barrier homeostasis, as acute barrier disruption increases SP activity in skin and inhibition by topical SP inhibitors accelerated recovery of barrier function after acute abrogation. In rosacea, increased levels of the vasoactive and inflammatory host-defense peptide cathelicidin LL-37 and its proteolytic peptide fragments were found, which were explained by an abnormal production of tryptic activity originating from kallikrein-related peptidase (KLK) 5. It is therefore possible that also other proteases, even from microbial or parasite origin, have a role in rosacea by forming alternate angiogenic and proinflammatory cathelicidin peptides. Further, the regulation of protease activity, in particular KLK-5 activity, might have a role in rosacea. This review briefly summarizes our current knowledge about keratinocyte-derived proteases and protease inhibitors, which might have a role in the pathophysiology of rosacea.

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INTRODUCTION

Although rosacea is one of the most frequent inflammatory skin disease, its pathophysiology is not understood yet. Recent findings suggest that proteolytically generated products mediate many of the pathological changes in rosacea. Most interestingly, these enzymatic processes were identified to contribute to epithelial barrier homeostasis and are directly involved in other inflammatory skin diseases. This review will summarize the various proteases and their inhibitors involved in epidermal barrier function and will try to relate their function at the end to the development of rosacea.

PROTEASES

The formation of stratified epithelia requires a specific differentiation program, which includes a timely and spatially well-coordinated proteolytic system to detach the corneocytes from each other without any disturbance of the barrier function. A number of different proteases and their inhibitors have been involved in the desquamation process and demonstrated to contribute to the skin's barrier function. On the basis of the catalytic domain, proteases are classified into aspartate-, cysteine-, glutamate-, metallo-, serine-, and threonine proteases. Particularly serine proteases (SPs) have a prominent role in epidermal permeability barrier homeostasis, as acute barrier disruption increases SP activity in skin and inhibition by topical SP inhibitors accelerated recovery of barrier function after acute abrogation (Hachem *et al.*, 2006).

Cysteine-, aspartate-, and metallo proteases

Cysteine peptidases represent phylogenetically ubiquitous enzymes, which can be classified into clans of independent proteins (based on the structural organization of the active site). Two of the major clans in mammalian genomes are the ''CA'' clan, where members share an evolutionary and structural history with papain, and the ''CB'' clan, which includes the caspases and the legumains.

One of the most skin-relevant caspases is the cysteinyl-aspartate protease caspase-14 (Demerjian *et al.*, 2008). In contrast to other ubiquitously expressed members of the caspase family, caspase-14 is rather specifically expressed within the epidermis, where it is of high importance in the formation of the physical skin barrier. It is expressed in the keratinocytes (KCs) of the uppermost stratum granulosum (SG), where it was found to be associated with the nucleus, the keratohyalin granules, and the desmosomes. Although its localization suggested a role for nuclear degradation during cornification, in caspase-14-deficient mice, nuclear degradation was not affected. The observation that caspase-14 has only been found in terrestrial mammals but not in birds or reptiles, and that profilaggrin is a direct substrate of caspase 14, suggests that it is important for the formation of a soft stratum corneum (SC). This could indicate a coevolution of a soft SC and the *caspase-14* gene (Denecker *et al.*, 2007). Caspase-14 is produced as procaspase within the SG, where it maturates in cornified epithelia. Although it is not clear

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Abbreviations: KLK, kallikrein-related protease; SC, stratum corneum; SG, stratum granulosum; SP, serine protease

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how it maturates, most likely a SP with elastase-like properties could be involved (Denecker *et al.*, 2008). Thus, caspase-14 seems to be involved in the correct processing of profilaggrin, preceding its degradation into hygroscopic amino acids, as well as the formation of ultraviolet B-protective compounds.

Recently, it was reported that chronic loss of epidermal caspase-8 results in the development of an atopic-like disease in mice (Li *et al.*, 2010). Conditional caspase-8 knockout mice exhibited increased metalloproteinase-2 activity and a phenotype similar to atopic dermatitis. Metalloproteinases are thought to contribute mainly to wound-healing processes, but their effects are also important for epithelial barrier function.

Another skin cysteine peptidase is cathepsin C, which represents a lysosomal cysteine peptidase of the papain family CIA, which is important for intracellular degradation, and which has a role in the activation of SPs in immune cells (Rao *et al.*, 1997). Cathepsin C knockout mice indicated that activation and processing of granzymes A and B, which are important for T-cell-mediated cell killing, depends on cathepsin C (Pham and Ley, 1999). Interestingly, cathepsin C deficiency in humans leads to a marked reduction of both levels and activities of the neutrophil SPs elastase, protease-3, and cathepsin G (Pham *et al.*, 2004), which will be described below.

Cathepsin D represents the main aspartic protease of endolysosomes. It is active at the physiological acidic pH of healthy skin and is of relevance in the desquamation process (Horikoshi *et al.*, 1999). Cathepsin D knockout mice showed reduced levels of involucrin and loricrin and lower transglutaminase 1 activity, which indicates that cathepsin D contributes indirectly to the barrier function of human skin (Egberts *et al.*, 2004).

Serine proteases

Serine proteases represent a family of enzymes that use a catalytic triad in the substrate-binding pocket (Ser, His, Asp) to cleave peptide bonds. On the basis of their substrate specificity, these proteases can be subdivided into trypsin-like enzymes (cleaves C-terminally of Arg and Lys), chymotryptic enzymes (cleave behind aromatic or bulky, hydrophobic amino acids), and the elastase-like enzymes (cleave behind small or medium size non-polar amino acids). These enzymes have an important role in the terminal differentiation process and desquamation.

Matriptase and prostasin

One of the most important factors in epidermal barrier function is profilaggrin. It is processed at the SG/SC interphase to filaggrin monomers. These are crosslinked to form macrofibrils and eventually ''natural moisturizing factors'', which are important for maintaining the hydration of the SC (reviewed in Ovaere *et al.* 2009). The importance of profilaggrin proteolysis to maintain epidermal structure and hydration has been underscored by human genetic studies. These have shown that loss-of-function mutations in profilaggrin cause ichthyosis vulgaris and are strongly predisposing to atopic dermatitis and asthma, possibly due to a disturbed epidermal barrier function, which allows entry of allergens and infectious agents (Sandilands *et al.*, 2006).

Major proteases required for initiating profilaggrin processing are the type II transmembrane SP matriptase and prostasin, a glycosyl-phosphatidylinositol-anchored membrane SP. There is now evidence that the autoactivating protease matriptase functions upstream of prostasin in a zymogen activation cascade that regulates terminal epidermal differentiation and is required for prostasin zymogen activation (Netzel-Arnett *et al.*, 2006). A reduced matriptase expression was shown to be associated with incomplete terminal differentiation of the epidermis, epidermal appendages, and oral epithelium (Bugge *et al.*, 2007). Matriptase gene mutations lead to ichthyosis as recently reported (Alef *et al.*, 2008). Matriptase is immediately activated by exposure to an acidic pH, as it occurs in skin, suggesting that matriptase activation may be a direct response to proton exposure (Tseng *et al.*, 2010). Recent evidence showed that during epidermal differentiation, the matriptase–prostasin proteolytic cascade is tightly regulated by two mechanisms, either by prostasin activation temporally coupled to matriptase autoactivation or by the hepatocyte growth factor activator inhibitor-1, which is rapidly inhibiting not only active matriptase but also active prostasin, resulting in an extremely brief window of opportunity for both active matriptase and active prostasin to function on their substrates (Chen *et al.*, 2010). Until now, these two membrane-bound proteases are thought to be mainly involved in skin homeostasis. However, the ability of matriptase to activate kallikrein-related (KLKs) proteases in the skin points to a regulatory role of matriptase in inflammatory skin diseases (Sales *et al.*, 2010).

Kallikrein-related peptidases

Kallikrein-related proteases are the largest family of tryptic and chymotryptic SPs, which are encoded by 15 genes on chromosome 19q13.4. In skin, KLKs are produced by KCs of the SG, where they are released into interstices of the upper SG and lower SC. To date, SP activity of SC is attributed to human tissue KLKs (Borgono *et al.*, 2007). At least eight KLKs have been reported to be expressed in healthy skin, of which KLK5, KLK7, KLK8, and KLK14 seem to be the most important (reviewed in Lundwall and Brattsand, 2008). Their putative function has been extensively studied (reviewed in Eissa and Diamandis, 2008; Lundwall and Brattsand, 2008). A wealth of literature revealed proteolytic function of the two SPs, KLK5 and KLK7, in SC. These proteases, previously termed as ''stratum corneum tryptic enzyme,'' (KLK5), and ''stratum corneum chymotryptic enzyme,'' (KLK7), have an important role in the desquamation process, as it was shown that SP inhibitors were able to inhibit corneocyte shedding from human plantar skin *ex vivo* (Lundstrom and Egelrud, 1988). Both enzymes are maximally expressed in the SG, where they are released from lamellar bodies and located within the SC interstices. Here they are thought to form a proteolytic cascade in which KLK5 activates itself as well as KLK7 (Ovaere *et al.*, 2009). Once active, both enzymes are believed to digest *in vivo* corneodesmosin, DSG1, and

desmocollin-1, as these substrates have been shown to be digested *in vitro*. There is now evidence that also other KLKs participate in desquamation. It was recently shown that KLK14 is responsible for 50% of the total trypsin-like SP activity in the SC. Because KLK14 can activate and be activated by KLK5, it is very likely that it also participates in the cascade pathway.

Apart from these three KLKs, also KLK8 seems to be involved in a proteolytic activation cascade regulating skin desquamation: KLK8 is abundantly expressed and colocalized with other KLKs in human epidermis and sweat glands. It is also transported and exocytosed by lamellar bodies into the SG/SC interface, and thus may have a role in SC barrier functions. Very recent studies showed that recombinant KLK8 is optimally active at pH 8.5, suggesting that it has a role in the upper SG where the pH is rather neutral (Eissa *et al.*, 2011). Active KLK8 has been found in SC extracts and in sweat, where, until recently, only KLK1 and kininase II were identified as active SPs. This raises its potential functional involvement in skin desquamation, although the physiological substrates need to be identified.

Proteases of bacterial, fungal, and parasite origin

Apart from proteases produced by KCs during differentiation and desquamation, the SC might also contain extracellular proteases originating from microbes and/or parasites residing at the skin surface. Bacterial proteases are often accessory proteins, which are not fundamental for cell growth and division, but are considered to be virulence factors, which are often associated with mobile genetic elements such as plasmids, integrated phages, and pathogenic islands. The clustering of bacterial protease genes in operons allows their coordinated expression, which in turn may imply a cooperation of the produced proteins (Wladyka and Pustelny, 2008).

Staphylococci produce a number of extracellular proteases, e.g., epidermolytic toxins, staphylococcal SPs such as a glutamyl endopeptidase referred to as V8, and a cysteine protease in *S. aureus*. Similar and other proteases are produced by skin-relevant bacteria, including commensal *S. epidermidis*, *S. pyogenes*, *Pseudomonas aeruginosa*, and others (Wladyka and Pustelny, 2008). Among the proteases, SPs, cysteine proteases, and metalloproteases represent the most abundant bacterial proteases. These affect the host's innate immune system in a bacterial species-specific manner by targeting phagocytes, cytokines, and cytokine receptors, inflammatory signaling pathways, complement, contact activation, as well as antimicrobial peptides (Potempa and Pike, 2009).

Apart from bacteria, fungi represent an important source of proteases as well. Upon fungal infections, which are mostly seen at mucosal surfaces, *Candida albicans* represents the most common fungal pathogen. *Candida* species are ubiquitous commensal yeasts that reside as part of the normal mucosa microflora without causing infections. Suitable predisposing conditions will let *C. albicans* change to a ''pathogenic'' stage, in which proteases represent major virulence factors. These are exclusively secreted aspartyl proteases (Naglik *et al.*, 2004). It is believed that extracellular proteases of saprophytic microorganisms are primarily secreted to obtain nutrients from decomposition of complex materials. There is, however, strong evidence that secreted aspartyl proteases are also needed for invasion of the host, thereby, interacting with several important host-defense functions eventually causing inflammation (Naglik *et al.*, 2004).

Proteolysis is also a vital element for survival of parasites, which enables them to digest resistant structural proteins. For example, house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) produce cysteine proteases and SPs (Donnelly *et al.*, 2006), which are well known as ''group 1 house dust mite allergens'' to induce allergic reactions. Several reports have shown that these proteases interact with pathways of the innate defense system, suggesting that these might also be directly involved in inflammatory skin reactions.

Neutrophil proteases

Upon skin infection or at conditions causing ''neutrophilic dermatoses'', the primary cell infiltrate consists of neutrophils. A massive infiltrate in the epidermis can lead to pustule formation. Upon infection, neutrophils phagocytose microbes and then kill these microbes within the phagolysosome by oxygen radical-generating systems, the alpha-defensins, as well as proteases, which are released from primary (''azurophilic'') and secondary (''specific'') granules (Faurschou and Borregaard, 2003). Only primary granules contain high amounts of the SPs such as human leukocyte elastase, cathepsin G, and protease 3. These enzymes are not released upon phagocytosis. But upon ''frustrating phagocytosis'' (attempts to phagocytose particles that are bigger than leukocytes) and formation of ''neutrophil extracellular traps'', consisting of neutrophil-derived DNA, where these cationic enzymes are bound (Brinkmann *et al.*, 2004), a release of these enzymes can occur. Indeed, human leukocyte elastase activity is present at the surface of lesional skin of patients with psoriasis, a neutrophilic dermatosis (Wiedow *et al.*, 1992). Neutrophil SPs have been identified as important innate immune regulators (Meyer-Hoffert, 2009a; Meyer-Hoffert and Wiedow, 2011). Thus, neutrophil-derived enzymes may further determine the outcome of an inflammatory skin lesion independent of possible homeostasis of KC-derived proteases and protease inhibitors.

PROTEASE INHIBITORS

Proteolytic activity in the skin, which is often restricted to a few target proteins, its tissue localization, and its enzymatic activity need to be properly controlled in the tissue. Although gene expression and zymogen activation are important regulatory elements to restrict enzymatic activity, the most important one is the expression of more or less specific protease inhibitors within the skin. These inhibitors regulate the activity of diverse proteases, more or less protease-specifically, in a time and concentration-dependent manner.

Kazal-type-related protease inhibitors

The ''lympho-epithelial Kazal-type-related inhibitor'' (LEKTI, at present named LEKTI-1) is an effective inhibitor of multiple SPs (Roelandt *et al.*, 2009). Processing of this multidomain

protease inhibitor into fragments or single domains restricts the inhibitory properties to SPs such as trypsin, plasmin, subtilisin A, cathepsin G, and human neutrophil elastase. LEKTI-1 consists of 15 complete or incomplete Kazal domains. *In vitro,* recombinant LEKTI-1 fragments or single domains inhibit the KC-derived SPs KLK5, -6, -7, -13, and -14. LEKTI-1 is expressed in various stratified epithelia as three splice variants. In the epidermis, LEKTI-1 is expressed in the SG, where LEKTI-1 protein is located in lamellar bodies—separate from KLKs, but secreted into the extracellular space together (Ishida-Yamamoto *et al.*, 2004, 2005). The 145-kDa form comprises all 15 potential inhibitory Kazal domains, but it is cleaved rapidly into multidomain fragments, which might be cleaved further to produce single domains and complexes with KLK5 and KLK7 in the SC. These complexes dissociate at acidic pH, which because of a pH gradient within the SC may lead to a controlled homeostatic desquamation. Mutations in *Spink5* (which encodes LEKTI-1) generates premature termination codons, as seen in Netherton syndrome (Chavanas *et al.*, 2000), result in expression of truncated LEKTI forms lacking several protease-inhibiting domains. This rare ichthyosiform skin disease is characterized by dry skin, increased desquamation, hair abnormalities (''bamboo hair''), and atopy. A decreased level of functional LEKTI correlates inversely with SP activity in SC, a decreased physical barrier function, and severity of the disease.

Another Kazal-type inhibitor is LEKTI-2 (*Spink9*), which has been originally discovered in palmar and plantar SC extracts (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009). LEKTI-2/SPINK9 is mainly expressed in palmar and plantar skin, close to KLK5. Apart from skin, expression was seen in the thymus (thus referred as LEKTI-2). All other tissues showed a very low transcription level of *Spink9*. LEKTI-2/SPINK9 selectively inhibited KLK5, but not other proteases including chymotryptic KLK7 and tryptic KLK14, or several SPs such as trypsin and chymotrypsin. The LEKTI-2/SPINK9 activity differs in this respect from that of LEKTI-1: The K_i of LEKTI-2 was found in the range of 60–250 nM. LEKTI-1 domains have been reported to inhibit KLK5 in the range of 3 nM (domain 8–11) to 120 nM (domain 9–15). Further, the LEKTI-1 domains exhibit a more or less broad activity spectrum. It remains to be determined whether LEKTI-2/SPINK9 has a role in skin diseases. Considering the specific expression of LEKTI-2/SPINK9 at palmar and plantar sites, as well as its specific activity to inhibit KLK5, it is intriguing to speculate that it could be a relevant factor in hand and foot eczema.

By following the hypothesis that likely more Kazal-type inhibitors are present in human skin, we identified SPINK6 as a selective inhibitor of KLKs in the skin (Meyer-Hoffert *et al.*, 2010). Unlike LEKTI-1, but similar to LEKTI-2, SPINK6 possesses only one typical Kazal domain. SPINK6 is strongly expressed, unlike LEKTI-2, in skin from various locations and can be purified from human plantar SC extracts. At low levels, it is expressed in many other tissues and is induced during KC differentiation. Although immunohistochemical analyses revealed SPINK6 expression in the SG of healthy human skin at various anatomical localizations and in the skin appendages, including sebaceous glands and sweat glands, SPINK6 expression was found to be decreased in lesions of atopic dermatitis. Recombinant SPINK6 inhibited KLK4, KLK5, KLK6, KLK7, KLK12, KLK13, and KLK14, but not KLK1, KLK3, and KLK11, suggesting a tissue KLK-selective inhibitory activity, as thrombin, trypsin, plasmin, matriptase, prostasin, mast cell chymase, cathepsin G, neutrophil elastase, and chymotrypsin were not inhibited (Meyer-Hoffert *et al.*, 2010; Kantyka *et al.*, 2011). The finding that SPINK6 inhibited desquamation of human plantar callus in an *ex vivo* model suggests that SPINK6 has a role in modulating the activity of KLKs in human skin. Interestingly, SPINK6 exhibited some proteolytic inhibitory activity against caspase-14 and is so far the only reversible inhibitor of caspase-14 in human skin (Kantyka *et al.*, 2011).

Trappins and SERPINS of human skin

Apart from LEKTIs, KC produce a number of additional protease inhibitors. Members of one group are termed ''trappins'' (acronym for transglutaminase substrate, WAP-domain-containing proteins; Schalkwijk *et al.*, 1999). Human epidermis contains secretory leukocyte protease inhibitor and elafin. Both are efficient inhibitors of neutrophil SPs; secretory leukocyte protease inhibitor inhibits cathepsin G and elastase, and elafin inhibits elastase and protease-3. This suggests that these protease inhibitors are important at inflammatory conditions to protect the tissue from the damage caused by neutrophil SPs. Moreover, their importance in wound repair and host defense is well accepted (Ashcroft *et al.*, 2000; Zhu *et al.*, 2002; Nakamura *et al.*, 2003).

Another group of SP inhibitors are SERPINs, which encompass nearly 40 members, of which many have been implicated in cancer and inflammation (Meyer-Hoffert, 2009b). These protease inhibitors have a unique mechanism to inhibit enzymatic activity: SERPINs cause a conformational change of the protease and then covalently bind to it. A few members of the SERPIN family have been reported to be expressed in human skin, such as SERPINB3 (squamous cell carcinoma antigen-1), SERPINB4 (squamous cell carcinoma antigen-2), and SERPINB13 (headpin/hurpin). SERPINs have possibly a role in protecting tissue from proteolysis by bacterial proteases; SERPINB8 and SERPINB9 inhibits subtilisin A. SERPINA1 inactivates some microbial proteases including protease K. Further, a C-terminal fragment of SERPINA1 inhibits HIV-1 entry by interaction with the gp41 fusion protein.

Cystatins

Apart from SPs, also cysteine protease activity is under the control of inhibitors in skin. These include the members of the cystatine gene family (Zeeuwen *et al.*, 2009). Cystatins represent polypeptides that are members of a superfamily of evolutionarily related proteins that can be divided into three subgroups, and which are widely expressed in several human tissues and secretions. They effectively inhibit various cysteine proteases, such as cathepsins B, L, H, K, and S, at micromolar to picomolar concentrations, in a competitive

and reversible manner. Whereas cystatin A and cystatin C were reported to function as epidermal protease inhibitors with antimicrobial properties—possibly by inhibiting microbial cysteine proteases—cystatin M/E regulates in the epidermis crosslinking of structural proteins by transglutaminase 3 in the cornification process by controlling cathepsin L and legumain activities (Meyer-Hoffert, 2009b). Cathepsin L has been shown to activate transglutaminase 3, an epidermis-specific enzyme that is important in the cornification process where it is responsible for crosslinking of small proline-rich proteins and loricrin. A deregulation of this pathway by uncontrolled cysteine protease activity leads to abnormal SC and disturbance of skin barrier function (Zeeuwen *et al.*, 2009).

Other regulating factors of protease activity

It should not be overlooked that the proteolytic activity of proteinases depends on factors such as pH and ion concentration. All SPs including KLKs decrease their proteolytic activity in acidic environments. The physiological pH of around 5.5 already results in more than 90% less activity compared with optimal *in vitro* conditions. Patients with atopic dermatitis often show an elevated pH at the skin surface, which might likely contribute to observed elevated SP levels in these patients (Voegeli *et al.*, 2009, 2011). Interestingly, the inhibitory activity of LEKTI-1 depends on the pH as well, which might enhance proteolytic deregulation when the epidermal pH is elevated. Moreover, Zn^{2+} inhibits KLK5 (Debela *et al.*, 2007). This might have clinical consequences when Zinc levels are low as in acrodermatitis enteropathica. The exfoliation and inflammation observed in this disease might be a result of decreased KLK5 inhibitions.

PROTEASE-ACTIVATED RECEPTORS

Protease-activated receptor (PARs) represent seven membrane-spanning G-protein-coupled receptors that are activated by SPs, which cleave a ''tethered'' receptor-activating ligand at the N terminus (Steinhoff *et al.*, 2005). To date, four PARs (PAR1–4) have been characterized. PAR-1, PAR-3, and PAR-4 are activated by thrombin, and PAR-2 by trypsin and chymotrypsin. In human skin, PAR-2 is abundantly expressed by KCs (Steinhoff *et al.*, 1999), where it has a role in regulating permeability barrier homeostasis, inflammation, pruritus, pigmentation, and wound healing upon activation by various endogenous and exogenous SPs. Whereas during skin inflammation, PAR-2 is activated by neutrophil elastase and mast cell tryptase; upon infection or skin barrier defects, proteases originating from certain bacteria, house dust mites, cockroaches, or parasites can activate this receptor (Shpacovitch *et al.*, 2007). Activation by *Propionibacterium acnes* protease causes induction of certain proinflammatory proteins, matrix metalloproteinases, and antimicrobial peptides, including LL-37 (Lee *et al.*, 2010).

PROTEASES AND PROTEASE INHIBITORS IN SKIN DEFENSE

Healthy human skin defends itself by a ''physical defense shield'' present in the SC and a layer of various lipids, together with an acidic pH, and a ''chemical'' or ''antimicrobial defense shield'' of the skin, where location-dependent different effector systems are activated. In addition, at the skin surface, defense strategies would be used, which utilize and control the microflora to prevent microbial infection by limiting its growth and/or colonization (Schroder, 2010). It is therefore intriguing to speculate that healthy skin protects itself at the surface by sequestrating essential trace elements and inhibiting bacterial proteases, thus inhibiting, apart from invasion, also degradation of sensitive antimicrobial peptides at the skin surface.

Upon injury or barrier defects, microbes or microbial products will come into contact with living epidermal cells, inducing several innate defense cascades. Among them is the induction of antimicrobial peptides, in particular, two β-defensins, preferentially the Gram-negative bacteria killing hBD-2 and the broad-spectrum antimicrobial peptide hBD-3, as well as the host-defense peptide cathelicidin hCAP18/LL-37 (Schroder, 2011). Whereas hBD-2 is mainly induced via the microbe-associated pattern recognition receptor Toll-like receptor (TLR) 5, the cathelicidin is induced by injury via EGFR ligands released upon wounding and 1,25-dihydroxy-vitamin D3.

Proinflammatory cytokines such as IL-1β, tumor necrosis factor-α, IL-8, and others will be produced upon acute injury and infection by KCs, which have been stimulated via (intracellular) pattern-recognition receptors, mainly the constitutively expressed TLR2 (Miller, 2008). This would induce the recruitment of neutrophils into the skin, where these leukocytes accumulate focally within the epidermis and release proteases such as elastase, cathepsin G, and protease-3. Keratinocytes within inflamed skin lesions produce enhanced amounts of elafin and secretory leukocyte protease inhibitor—which would dampen the activity of neutrophil proteases. Apart from inducing inflammatory cell recruitment, TLR2 ligands, such as cell wall components of Gram-positive bacteria, also mediate induction and a further release of KLK5 in cultured KCs (Yamasaki *et al.*, 2011). In these conditions, a marked increase of KLK5 activity is seen in KC supernatants, which implicates a role *in vivo*—when this activity is not blocked by KC-derived LEKTI-1, LEKTI-2/SPINK9, and SPINK6. As a consequence, increased KLK5 activity would cause a temporarily limited, increased desquamation, which is always seen during healing of banal skin infections. A summary of the putative role of epidermal proteases and epidermal protease inhibitors is given in Figure 1.

PROTEASES AND PROTEASE INHIBITORS IN ROSACEA

A striking picture of rosacea histology is the domination by irregularly dilated capillary vessels in the upper dermis. Unlike many bacterial skin infections, the ''epicentre'' of the inflammation does not appear to be the pilosebaceous follicle. The cellular infiltrate, which mainly consists of lymphocytes, and in papulopustular rosacea also, neutrophils, appears to be distributed perivascularly (Crawford *et al.*, 2004). The observation of dilated capillary vessels in rosacea lesions suggests the presence of more stimulants for

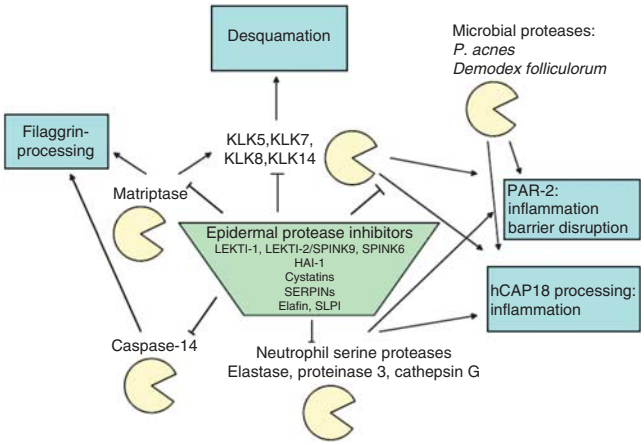

Figure 1. Epidermal proteases and protease inhibitors as regulators of the skin barrier in healthy and inflamed skin. In healthy skin, matriptase and caspase-14 are key enzymes for regular filaggrin processing and kallikreins (KLKs) 5 and 7 (and likely KLK8 and 14) are important for desquamation, thus forming the regular physical barrier. The activity of these enzymes, which is timely and spatially different for each enzyme, is controlled by various more or less specific epidermal protease inhibitors (green box). Apart from these epidermal proteases, also proteases originating from skin microbes and parasites get access to living keratinocytes and infiltrating leukocytes, where they may stimulate proinflammatory cascades (including neutrophil infiltration) via protease-activating receptors (PARs) and/or cleave the cathelicidine (hCAP18/LL-37) precursor, which may originate from either neutrophils and/or keratinocytes, into angiogenic LL-37 fragments, thus inducing rosacea-relevant processes (blue boxes). HAI-1, hepatocyte growth factor activator inhibitor-1; LEKTI, lympho-epithelial Kazal-type-related inhibitor; *P. acnes*, *Propionibacterium acnes*; SERPIN, SERine Proteinase Inhibitor.

vascular endothelial cells, as found for vascular endothelial growth factor, CD 31, and D2-40 (Yamasaki and Gallo, 2009). A recent study investigating the trigger for enhanced stimulant expression and subsequent vascular effects identified the cathelicidin LL37, which induced vasodilatation and neovascularization in animal models. Effects are mediated via the formyl peptide receptor-like 1, a G-protein-coupled receptor expressed on endothelial cells, as well as epidermal growth factor transactivation. However, there is recent evidence that some cleavage products of the human cathelicidin protein hCAP18 have a unique capacity to be both vasoactive and proinflammatory. Thus, LL37 and other cathelicidin fragments induce vascular effects through several signaling pathways and would explain at least parts of the vascular effects seen in rosacea (Yamasaki and Gallo, 2009).

A further study demonstrated that rosacea patients not only revealed abnormally high levels of LL-37 but also a different pattern of LL-37 fragments (Yamasaki *et al.*, 2007). The principal LL-37 peptide in rosacea, FA-29 and LL-37 itself, but not the shorter peptides DI-27 and KR-20, which are abundant in healthy skin, caused erythema and vasodilatation upon injection into mice and have greater proinflammatory properties than the original LL-37 peptide, thus explaining at least parts of the clinical presentation of this disease. The presence of the vasoactive and inflammatory LL-37 peptides in rosacea was explained by abnormal production of the epidermal KLK5, which cleaves LL-37 into vasoactive and proinflammatory peptide fragments (Yamasaki *et al.*, 2007). This, however, does not exclude the possibility that apart from KLK5, other SPs generate LL-37 peptides in rosacea. KLK8, recently identified as abundant SP in healthy skin (Eissa *et al.*, 2011), and KLK14 might have a role in rosacea besides KLK5. KLK5, KLK8, and KLK14 are induced upon terminal differentiation and are produced upon terminal differentiation in the uppermost SG, where LL-37 is also produced in rosacea (Yamasaki *et al.*, 2007). As dilated capillary vessels are seen in the upper dermis, one may speculate that either LL-37 peptides generated in the upper SG are diffusing down to the upper dermis and/or dermal KLK(s) contribute to LL-37 proteolysis. The only KLK produced and released by dermal fibroblasts is KLK14 (Eissa *et al.*, 2011). The mechanism as to why and how KLK5 is induced in rosacea was recently investigated by Yamasaki *et al.*, (2011). They found that ligands of TLR2, a pattern-recognition receptor that is overexpressed in rosacea, caused an increase in KLK5 secretion in KCs, which adds another piece to the puzzle. But what induces LL-37 in rosacea? Although EGFR ligands are likely candidates, proteases of different origin might also be candidate molecules inducing LL-37 and proinflammatory cytokines and matrix metalloproteinases via PAR-2 in rosacea (Lee *et al.*, 2010).

A significant association exists between *Demodex folliculorum* infestation and the development of rosacea. Although not explicitly shown for *Demodex*, it is well documented that house dust mites produce serine and cysteine proteases, which are known to be involved in the pathogenesis of allergies. Recent studies revealed that mite-derived SPs, but not cysteine proteinases, activate KCs via PAR-2 (Kato *et al.*, 2009), making it possible that LL-37 is also induced by *Demodex* SPs in rosacea.

Increased SP activity in rosacea could also be caused by impaired inhibition. The putatively relevant SP inhibitors within the epidermis are LEKTI-1 and SPINK6, but not LEKTI-2/SPINK9 (despite its KLK5-specific inhibition profile), because of its restricted palmar and plantar expression. There is yet neither any information about the lesional expression and activity profile of LEKTI-1 and its cleaved domains, as well as SPINK6, nor any knowledge about the possible role of other protease inhibitors (e.g., SERPINs) that could contribute to increased KLK5 activity in rosacea.

PERSPECTIVE

Today there is strong evidence for a role of proteases, in particular, the SP KLK5, as key factors in rosacea. KLK5 is able to activate signaling pathways, which lead to the production of inflammatory mediators and cytokines and possibly also epithelial production of the key host-defense peptide LL-37 (Figure 1). It activates several matrix metalloproteases, which cause shedding of several EGFR ligands, inducing among other proteins also LL-37. The mechanism as to why KLK5 is enhanced in rosacea is still not fully understood. A reduced expression of KLK5 inhibitors does not seem to be the driving mechanism, as patients with

Netherton syndrome, who lack the KLK5 inhibitor LEKTI-1, do not normally suffer from rosacea. A unique and apparently rosacea-specific finding is the increased TLR2 expression, which is absent in other inflammatory skin diseases such as psoriasis and atopic dermatitis (Yamasaki *et al.*, 2011). TLR2 signaling mediates in KCs induction of KLK5 and, in a 1,25-dihydroxyvitamin D3-dependent manner, LL-37 (Heilborn *et al.*, 2010). Thus, it would explain the two key unique findings in rosacea. But which ligand causes TLR2-dependent signaling? Sera from rosacea individuals, but not from normal controls, contained antibodies against heat shock proteins and lipoproteins from *Bacillus oleronius*, a bacterium living in the *D. folliculorum* gut. As such antigens are known to represent stimuli for TLRs, it is tempting to speculate about the involvement of mites in pilosebaceous unit involvement in rosacea (Yamasaki *et al.*, 2011). Future studies will have to identify the key factors that initiate inflammatory reactions, and all the proteases, as well as its regulators, which are involved in these processes, in rosacea. Doxycycline is an approved drug for rosacea treatment. It became clear that its therapeutic potential does not exclusively depend on its antimicrobial activity but seems to be related more to protease inhibition (Monk *et al.*, 2011). Specific proteolytic regulation might have more therapeutic potential in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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