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Abstract

Antimicrobial peptides are a critical part of the innate immune system of the skin. Epidermal keratinocytes (KCs) express numerous such peptides which establish a protective barrier to invading microorganisms. The antimicrobial peptide S100A7c (psoriasin) is the most efficient killer of *Escherichia coli*, but the regulatory mechanism of S100A7c in KCs was unclear. We investigated the responsiveness of S100A7c expression to microbial components and found that flagellin, a ligand for Toll-like receptor (TLR) 5, strongly induced the expression of S100A7c whereas all other TLR ligands had no significant effect on S100A7c mRNA expression. Supernatant from wild-type *E. coli*, but not from a flagellin-deficient *E. coli* strain (ΔFliC), strongly induced S100A7c mRNA as well as protein expression, identifying flagellin as the principal S100A7c inducing component of *E. coli* supernatant. In line with this finding, small interference RNA-mediated knock-down of TLR5 expression suppressed the ability of KCs to up-regulate S100A7c mRNA and protein expression in response to flagellin or *E. coli* supernatant suggesting that this up-regulation is mediated via TLR5 signaling.

RNase 7, another antimicrobial peptide expressed by KCs, not only contributes to the surface ribonuclease activity of human skin but also participates to the cutaneous antimicrobial defense. RT-PCR screening revealed that in addition to RNase 7 epidermal KCs expressed RNases 1, 4 and 5 as well as RNase inhibitor (RI), an endogenous protein that blocks the ribonucleolytic activity of RNases. *In vitro* assays demonstrated that RI suppresses the ribonuclease activity of stratum corneum (SC) RNases as well as the anti-bacterial activity of RNase 7 and the *Candida*-cidal activity of RNase 5. RI is strongly expressed by the granular layers of the epidermis whereas it was not detectable in the SC. Co-incubation experiments with SC extracts led to degradation of RI protein which could be prevented by the serine protease inhibitor aprotinin. This finding suggests the existence

of a physiological mechanism of RI breakdown in the SC to facilitate skin surface ribonuclease and antimicrobial activity.

Taken together these data bring new insights into the regulatory mechanisms of skin-derived antimicrobial peptides and demonstrates the importance of SC proteolysis activity for establishing appropriate RNase-dependent antimicrobial functions of the skin.

Contents

1. Introduction

1.1. The structure of human skin

Skin is the outer covering of living organisms. It functions as a protective barrier against the external environment; as an important factor in temperature regulation; as a sensory organ; and as an organ of metabolism that has synthesizing, excretory, and absorptive functions. Human skin is composed of the *epidermis*, which is the outermost epithelial layer of the skin and serves as a direct barrier to the environment; the subjacent *dermis*, which harbors skin vessels, skin nerves and skin appendage; and the *subcutis* consisting mainly of fat cells (Bolognia J.L. et al., 2008).

The epidermis is a stratified epithelium which is formed of keratinocytes (KCs) through terminal differentiation, but also harbors other cell types *i.e.* melanocytes, Langerhans cells and Merkels cells (Bolognia J.L. et al., 2008). KCs originate from a stem-cell pool in the basal epidermal layer and undergo progressive terminal differentiation as they move outward, finally forming the stratum corneum (SC). On simple morphological grounds, the epidermis can be divided into four distinct layers: *stratum basale*, *stratum spinosum*, *stratum granulosum* and *SC* (Bolognia J.L. et al., 2008).

Beneath the epidermis, the vascularised dermis provides structural and nutritional support. The dermis is tightly connected to the epidermis by a basement membrane. It also harbors many nerve endings that provide the sense of touch and temperature. It contains the hair follicles, sweat glands, sebaceous glands, apocrine glands and blood vessels. The blood vessels in the dermis provide nutrition and removal of waste to its own cells as well as the stratum basale of the epidermis (Bolognia J.L. et al., 2008).

The subcutis lies below the dermis and serves mainly for fat storage and insulation. It attaches the skin to the underlying bone and muscle (Bolognia J.L. et al., 2008).

1.2. Skin-derived antimicrobial peptides: establishing a defense-line against invading microorganisms

In 1987, Michael Zasloff showed that frog skin protects itself against infections by the release of antimicrobial peptides called magainins (Zasloff, 1987), which exhibit a broad-spectrum of antimicrobial activity. These observations led other investigator to search for the presence of antimicrobial proteins also in human skin (Schröder and Harder, 2006).

Normal human skin is colonized by large numbers of microorganisms. Most of these microorganisms are commensals which live on the skin surface without causing disease. In addition to the normal skin flora, the skin is exposed to pathogenic microorganisms. However, these pathogens rarely survive long enough to produce clinical infection. Whereas the mechanical barrier function of normal skin plays an important role in the clearance of pathogens from the skin surface (Schröder and Harder, 2006), there is evidence from a number of studies that skin-produced antimicrobial proteins also play an important part in the elimination of such pathogens (Gläser et al., 2005; Harder et al., 1997; Harder and Schröder, 2002; Lee et al., 2008; Murakami et al., 2002; Schittek et al., 2001).

In the past 10 years a number of skin-derived antimicrobial peptides have been discovered in human skin as discussed below.

1.2.1. Lysozyme

Lysozyme was the first antimicrobial protein found in skin (Ogawa et al., 1971). It catalyses the hydrolysis of the peptidoglycan layer in the bacterial cell wall. The antimicrobial activity of lysozyme is mainly against Gram-positive bacteria, *e.g. Staphylococcus aureus*, but it is also active against Gram-negative bacteria, *e.g.*

Escherichia coli (Ellison, III and Giehl, 1991) or *Pseudomonas aeruginosa* (Cole et al., 2002), suggesting that it might control the growth of bacteria in healthy skin.

Immunoreactive analysis demonstrates that lysozyme is mainly located in the cytoplasm of cells in the spinosum layers of the epidermis. All parts of the eccrine sweat glands as well as pilo-sebaceous follicle cells, hair bulb and follicle sheat cells were also positive for lysozyme, but not the hair-shaft (Papini et al., 1982). Since immunoreactive lysozyme is absent in the SC and skin washing fluid (Ogawa et al., 1971), its contribution to cutaneous host defense is still unclear.

1.2.2. Dermcidin

Dermcidin appears to be the principal sweat antimicrobial peptide (Schröder and Harder, 2006). It is expressed as a precursor which is then proteolytically cleaved to a 47-amino acid containing peptide resulting in dermcidin-1 (DCD-1). This peptide performs antimicrobial activity against *S. aureus*, *Enterococcus faecalis*, *E. coli* as well as *Candida albicans* at low micromolar concentration (1–10 µg/ml). Therefore suggesting that dermcidin may play a role in the regulation of human skin microflora (Schittek et al., 2001). The antimicrobial activity of dermcidin is maintained at high salt concentrations and over a broad pH range, which are similar to the conditions in human sweat (Schittek et al., 2001).

Dermcidin is specifically and constitutively expressed in sweat glands, secreted into the sweat and transported to the epidermal surface (Schittek et al., 2001). Interestingly, dermcidin protein expression is not induced in human epidermal KCs under inflammatory skin conditions (Rieg et al., 2004). It has been shown that the amount of several DCD-derived peptides in sweat of patients with atopic dermatitis is significantly reduced which could be a reason for pronounced colonization with *S. aureus* and recurrent bacterial skin infections in this patients (Rieg et al., 2005).

1.2.3. Cathelicidin

Human cathelicidin is produced as a preproprotein of 18 kDa (human cationic antimicrobial protein with a molecular size of 18 kDa; hCAP-18) which is proteoliticlly processed by the serine protease proteinase 3, yielding the C-terminal 37-amino acids containing the antimicrobial active LL-37 peptide (Sorensen et al., 2001). Apart from the C-terminal peptide LL-37, the cathelin domain of hCAP-18 is also antimicrobially active as demonstrated with recombinant cathelin (Zaiou et al., 2003). *In vitro*, LL-37 inhibits the growth of a variety of Gram-negative (*E. coli, P. aeruginosa, Salmonella typhimurium*) and Gram-positive bacteria (*Lysteria monocytogenes, S. aureus, Staphylococcus epidermidis* and vancomycin-resistant *Enterococci*) at micromolar concentrations. It is also active against several bacteria at high salt conditions (Turner et al., 1998).

Cathelicidin is expressed in circulating neutrophils and myeloid bone marrow cells, in epithelia of the skin, airways, mouth, tongue, esophagus, intestine, cervix, vagina, epididymis and in testis (Gallo et al., 1997; Gudmundsson et al., 1996; Malm et al., 2000; Zaiou and Gallo, 2002; Zanetti, 2005). Like dermcidin, the human cathelicidin is also expressed in eccrine glands and duct cells. Immunoreactive LL-37 is localized in both the eccrine secretory glands and ducts, where it is found to be diffusely located in the cytoplasm of the secretory gland, and also located in the ductal epithelium (Murakami et al., 2002). This observation and the antimicrobial activity of LL-37 against various bacteria indicate that LL-37 might also contribute to the antimicrobial activity of human sweat (Murakami et al., 2002).

The expression of cathelicidin in skin seems to be tightly regulated because it is only expressed in KCs of inflamed skin but not of healthy skin (Frohm et al., 1997). It was also demonstrated that cathelicidin expression was up-regulated after skin injury (Dorschner et al., 2001). In an *in vitro* model, the highest level of cathelicidin is attained at 48 hours after injury (Heilborn et al., 2003). It has been demonstrated that 1,25-dihydroxyvitamin D(3),

induces the expression of cathelicidin in human KCs (Wang et al., 2004; Weber et al., 2005). This induction was also observed in monocytes and neutrophils (Wang et al., 2004). The mechanism for this up-regulation occurs via a consensus vitamin D response element (VDRE) in the promoter of cathelicidin which is bound by the vitamin D receptor (VDR) (Gombart et al., 2005). Interestingly, the murine homologue to human cathelicidin (cathelicidin-related antimicrobial peptide; CRAMP) is not induced by vitamin D because of the absence of VDRE in murine CRAMP promoter (Gombart et al., 2005).

The *in vivo* relevance of cathelicidin in cutaneous host defense has been demonstrated in a mouse model. Mice deficient in the expression of CRAMP were more susceptible to skin infections caused by group A Streptococcus (GAS) (Nizet et al., 2001). These mice were also susceptible to urinary tract infections caused by uropathogenic *E. coli* (Chromek et al., 2006).

1.2.4. Skin-derived ribonucleases (RNases)

The human RNase A superfamily consists of thirteen members which are encoded by unique genes located on chromosome 14q11.2. Eight genes (RNases 1 to 8) of this family encode for ribonucleolytically active proteins which are relatively small in size (~15 kDa) and to varying degrees catalytically active against standard RNA substrates (Dyer and Rosenberg, 2006). Apart from their ribonuclease activity, some members of this family have antimicrobial properties as well (Domachowske et al., 1998; Lehrer et al., 1989; Rudolph et al., 2006). RNase 2 has antiviral activity, especially against the respiratory syncytial virus (Domachowske et al., 1998). RNase 3 performs antibacterial activity against *S. aureus* and *E. coli* (Lehrer et al., 1989) and has also killing activity toward the helminth *Schistosoma mansoni* (Ackerman et al., 1985). RNase 5 is active against *C. albicans* and *Streptococcus pneumoniae* (Hooper et al., 2003). RNase 7 and RNase 8 exhibit a broad

spectrum of antimicrobial activity against both Gram-negative and Gram-positive bacteria (Harder and Schröder, 2002; Rudolph et al., 2006). To date, among members of these family, only RNase 5 (this dissertation) and RNase 7 were detected in the SC (Harder and Schröder, 2002), which are discussed below.

RNase 7: Analysis of healthy human skin for the presence of endogenous antimicrobial proteins led to the discovery of a 14.5 kDa antimicrobial ribonuclease termed RNase 7 (Harder and Schröder, 2002). The capacity of RNase 7 to digest RNA identifies this molecule as a part of the RNase activities found on human skin, making it necessary to take special precautions when performing experiments with RNA (*i.e.* by wearing gloves) (Harder and Schröder, 2002). RNase 7 is present at 4–8 µg/g of dry weight healthy SC. In contrast, 10–25 µg/g could be recovered from psoriatic-scale material, suggesting that RNase 7 might be inducible (Harder and Schröder, 2002; Harder and Schröder, 2005b). RNase 7 exhibits a broad spectrum of antimicrobial activity against both Gram-negative and Gram-positive bacteria and the yeast *C. albicans*. For unknown reasons RNase 7 is extremely effective at killing a vancomycin-resistant strain of *E. faecium* already at 20 nM concentrations (Harder and Schröder, 2002). It was suggested that RNase 7 antimicrobial activity is due to binding to bacterial membrane which renders the membrane permeability and causes pore formation into the membrane of bacteria. Additional studies have revealed that antimicrobial and ribonuclease activities of RNase 7 are independent of each other. The antimicrobial activity of recombinant RNase 7 is still retained at 4°C (Huang et al., 2007). The pro-inflammatory cytokines interleukin (IL) -1β, interferon (IFN) -γ and to a lesser degree also tumour necrosis factor (TNF) -α induced RNase 7 messenger RNA (mRNA) expression in KCs. Primary KCs treated with heat-killed bacteria such as *P. aeruginosa* and to a lesser degree *S. aureus, E. coli,* and *S. pyogenes* also induced RNase 7 expression in KCs (Harder and Schröder, 2002).

RNase 5: RNase 5, also known as angiogenin, has been implicated in blood vessel formation (Fett et al., 1985). A recent study revealed antimicrobial function for RNase 5 toward *C. albicans* and *S. pneumoniae in vitro* (Hooper et al., 2003). RNase 5 is secreted by reconstructed human epidermis (Rendl et al., 2001), but its contribution to the innate immunity of the skin is unknown.

Ribonuclease inhibitor: Ribonucleas inhibitor (RI) is a leucin-rich repeat protein with the shape of a horseshoe (Kobe and Deisenhofer, 1993). It constitutes about 0.1% of the total protein in the cytosol of mammalian cells (Leland and Raines, 2001). RI binds with high affinity to several members of the RNase A superfamily (Iyer et al., 2005; Johnson et al., 2007; Maeda et al., 2002; Papageorgiou et al., 1997; Shapiro et al., 1986; Shapiro and Vallee, 1987), thus inhibiting their enzymatic activity. Remarkably, RI binds to a series of RNases, *i.e.* RNases 1-6 which share low sequence identity (Papageorgiou et al., 1997). It is of particular importance for cells to protect themselves from exogenous RNases, since extracellular RNases which enter the cytosol and evade the binding of RI, catalyze cleavage of cellular RNA, eventually leading to cell death (Leland and Raines, 2001).

RI is strongly expressed by the granular layer of the epidermis (this dissertation); however, at the time of our investigation it was unclear whether RI has the potential to block the microbicidal activities of RNase 5 and RNase 7, which are both expressed by KCs and are present in the SC.

1.2.5. Human defensins

Defensins are cysteine-rich, cationic peptides with β-sheet structures that are stabilized by three intramolecular disulphide bonds between the cysteine residues (Ganz, 2003). Mammalian defensins are classified into three subfamilies, the α-, β- and θ-defensins, which differ in their distribution of and disulphide links (bonds) between the six

conserved cysteine residues. The disulphide linkages of cysteine residues in α-defensins are between Cys^1–Cys^6, Cys^2–Cys^4 and Cys^3–Cys^5, whereas in β-defensins, the linkages are Cys^1–Cys^5, Cys^2–Cys^4 and Cys^3–Cys^6. By contrast, θ-defensins have a circular structure with the cysteine residues linked as Cys^1–Cys^6, Cys^2–Cys^5 and Cys^3–Cys^4 (Ganz, 2003).

Leukocytes and epithelial cells are the main sources of mammalian defensins. So far, six human α-defensins have been identified (Klotman and Chang, 2006). Although α-defensins are found in granulocytes, at mucosal surfaces and in various tissues (Agerberth et al., 2000; Cunliffe, 2003; Fellermann and Stange, 2001; Hein et al., 2002), but there is no evidence of their expression by KCs (Harder and Schröder, 2005b).

Twenty eight human β-defensins (hBDs) have been identified by gene-based searches, but only six hBDs (hBD-1, -2, -3, -4, -5 and -6) are expressed mainly by epithelial cells (Ganz, 2003; Yang et al., 2004). Whereas hBD-1 is constitutively expressed by epithelial cells, expression of hBD-2 and hBD-3 can be induced by viruses, bacteria, microbial products and pro-inflammatory cytokines (Schröder and Harder, 2006). The mechanism(s) by which microorganisms are killed and/or inactivated by defensins is not understood completely. However, it is generally believed that killing is a consequence of disruption of the microbial membrane. For example hDB-2 can aggregate to form 'channel-like' pores leading to disruption of membrane integrity and function, which ultimately causes the lysis of microorganisms (Hoover et al., 2000; Sahl et al., 2005; Yang et al., 2002). Below are listed the β-defensins expressed by human KCs.

HBD-1 is the first discovered human β-defensin. It was originally isolated from human blood filtrates as a novel peptide with significant sequence homology to bovine β-defensins (Bensch et al., 1995). Mature hBD-1 is a small, cationic peptide of 36 amino acids residues. In blood plasma and urine, several forms of hBD-1 have been isolated ranging in length from 36 to 47 amino acids residues (Valore et al., 1998). Recombinant and natural

hBD-1 forms exhibit salt-sensitive antimicrobial activity against various laboratory and clinical strains of *E. coli* at micromolar concentrations (0.3-10μM). Moreover it was reported that concentrations of 1-10μg/ml of a recombinant baculovirus-derived hBD-1 preparation killed *P. aeruginosa.* LD_{50} (lethal dose that achieves a colony-forming units reduction of 50%) of *P. aeruginosa* was found to be 1μg/ml and 100ng/ml for hBD-1 and hBD-2, respectively (Singh et al., 1998). As yet, no studies have reported activity of hBD-1 against Gram-positive bacteria such as *S. aureus* (Harder and Schröder, 2005a). In contrast to hBD-2 and hBD-3, gene expression of hBD-1 in KCs is not markedly induced by pro-inflammatory cytokines like IL-1β, TNF-α, TNF-γ or by bacteria like *P. aeruginosa* (Harder et al., 2004). Natural hBD-1 protein has not yet been isolated from human skin. However using in situ hybridization, hBD-1 mRNA expression was detected in the suprabasal KCs, sweat ducts of human skin (Fulton et al., 1997) and sebaceous glands (Ali et al., 2001).

HBD-2 is a small, cationic peptide of 43 amino acids which was originally isolated from lesional psoriatic scale extracts using an *E. coli* affinity column (Harder et al., 1997). Natural hBD-2 derived from psoriatic scale shows preferential antimicrobial activity against Gram-negative bacteria such as nonmucoid and mucoid strains of *P. aeruginosa* and *E. coli* (LD_{90}, 10 mg/ml), less activity against *C. albicans* (LD_{90}, 25 mg/ml) and only bacteriostatic activity against *S. aureus* at 100 mg/ml (Harder et al., 1997). Activity of hBD-2 depends on ion composition and is sensitive to the concentration of NaCl. The ability of hBD-2 to inhibit bacterial growth diminishes when salt concentration is increased from 20 to 150 mM (Bals et al., 1998; Tomita et al., 2000), suggesting that hBD-2 is unable to kill bacteria in serum or at skin surface covered with evaporated sweat (Schröder and Harder, 2006). Pro-inflammatory cytokines like IL-1α, IL-1β and TNF-α or stimuli like heat-killed bacteria such as *P. aeruginosa* have proven to be the most effective inducer of hBD-2 in KCs (Harder et al., 1997; Huh et al., 2002; Sorensen et al., 2003).

In normal skin, hBD-2 immunoreactivity is located to the uppermost layers of the epidermis and SC thus providing a first-line defense against invading microbes (Ali et al., 2001). On a subcellular level, hBD-2 is stored in lamellar bodies of stimulated KCs of the spinous and granular layer of the epidermis, suggesting that hBD-2 is released with the lipidlike contents of lamellar bodies (Oren et al., 2003). In contrast to psoriatic skin, healthy skin extracts harbor only low amounts of hBD-2 peptide (Schröder and Harder, 1999). HBD-2 is also seen in the stratified epithelia of the oral cavity as well as in gingival cells (Dale and Krisanaprakornkit, 2001).

HBD-3 is a 5-kDa nonhemolytic and salt-insensitive antimicrobial peptide which has been originally isolated from human psoriatic scales and was cloned from KCs (Garcia et al., 2001a; Harder et al., 2001). In contrast to hBD-2, hBD-3 is a broad-spectrum antimicrobial peptide. It is active against a number of human pathogens, including methicillin-resistant strains of *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE). Furthermore, when in vitro activities of hBD-3 alone or combined with lysozyme, metronidazole, amoxicillin and chlorhexidine were investigated against several oral bacteria, hBD-3 showed bactericidal activity against all of the bacterial species tested (Maisetta et al., 2003). Ultrastructural analyses of hBD-3-treated *S. aureus* have revealed signs of perforation of the peripheral cell wall, with explosion-like liberation of the plasma membrane within 30 min and bacteriolysis (Harder et al., 2001). The morphological effects resemble those seen when *S. aureus* is treated with penicillin (Schröder and Harder, 2006). When comparing the induction of hBD-2 and hBD-3 in KCs, IL-1β was found to be the strongest inducer of hBD-2, whereas IFN-γ, which does not induce hBD-2, is the most powerful hBD-3-inducing cytokine (Harder et al., 2004). HBD-3 is widely expressed in skin, placenta, tonsil, trachea, tongue, and other oral tissues (Dunsche et al., 2001; Garcia et al., 2001a; Harder et al., 2001).

HBD-4 was initially identified by screening the human genome database (Garcia et al., 2001b). The expression of hBD-4 in KCs was slightly up-regulated by contact of *P. aeruginosa* but was strongly induced by phorbol-myristate-acetate (PMA) (Harder et al., 2004). However, so far attempts have failed to isolate hBD-4 peptide from psoriatic scales as well as from healthy human skin-derived SC (Harder and Schröder, 2005a).

1.2.6. S100A7c (psoriasin)

S100 proteins comprise a multigene family of low molecular weight cationic proteins which are characterized by two calcium-binding EF-hand motifs (Donato, 2001). Some of these proteins are expressed within the epidermis and antimicrobial properties have been described for several members of this family (Buchau et al., 2007; Ganz and Lehrer, 1997; Gläser et al., 2005; Gottsch et al., 1999; Wolf et al., 2007).

The human S100A7 was first identified as a protein up-regulated in inflamed psoriatic skin (Madsen et al., 1991). Genomic analysis revealed five copies of *S100A7*-like genes (*S100A7a*-*S100A7e*) within the human genome, but only duplicates a-c were predicted to be coding (Kulski et al., 2003). The S100A7a and S100A7c share the highest amino acid sequence similarity (93%). The hypothetically translated S100A7b sequence has the least similarity, with only 50% of the amino acid sequences common to S100A7c. The S100A7d and S100A7e appear to be fragmented and therefore non-coding (Kulski et al., 2003). Among these five duplications only S100A7c was recovered from skin-washing fluids and extracts of SC (Schröder and Harder, 2006).

Biochemical analyses of *E. coli*-killing activity in extracts of a healthy person's SC identified S100A7c as a principal *E. coli*-cidal antimicrobial protein in healthy skin SC extracts. A detailed biochemical analysis of healthy SC-derived S100A7c, revealed S100A7c to be heterogeneous with the 11.366-Da form as the predominant S100A7c form

in these extracts. In skin, S100A7c is expressed focally and released from KCs. Apart from KCs, sebocytes, the lipid-secreting cells of sebaceous glands, also showed immunoreactive S100A7c indicating that S100A7c is possibly also secreted together with lipids. From skin washings using buffer or acetone (to extract skin lipoids), S100A7c could be recovered from both aqueous and acetone extracts, indicating that the highly hydrophobic S100A7c is also stored in the lipid layer of healthy skin (Gläser et al., 2005).

In vitro, S100A7c shows preferentially antimicrobial activity against *E. coli*, with a LD_{90} near 0.5 µM whereas there is bactericidal activity for *P. aeruginosa* and *S. aureus* at much higher concentrations (LD_{90} >30 µM), S100A7c is far less bactericidal for the commensal *S. epidermidis* (Gläser et al., 2005). Antimicrobial activity of S100A7c has been observed at increased concentrations of salt, neutral and acidic pH which is seen on surfaces of healthy skin and upon conditions, when sweat is evaporating (Gläser et al., 2005). Using a neutralizing monoclonal antibody *in vivo* and *in vitro* confirmed that S100A7c is the principal *E. coli*-bactericidal component of healthy skin. Moreover, pre-treatment of healthy skin with neutralizing S100A7c antibodies also resulted in an increase of commensals, suggesting that a permanent S100A7c 'film' at skin surfaces might also contribute to growth control of the commensal flora (Gläser et al., 2005).

Until now, the exact mechanism by which S100A7c kills *E. coli* is unknown. S100A7c is characterized by two calcium-binding EF-hand motifs and its antimicrobial activity against *E. coli* is sensitive towards treatment with Zn^{2+}but not Ca^{2+}, Mg^{2+}, Fe^{2+} or Mn^{2+}. This suggests that sequestration of $Zn^{2+}$is the mechanism how S100A7c possibly kills *E. coli* (Gläser et al., 2005). Deprivation of Zn^{2+}ultimately affects Zn^{2+}dependent enzymes; Zn^{2+}as well as $Cu^{2+}$are essential transition metal ions for functional *E. coli* superoxide dismutase suggesting that these bacteria have a particular need to defend themselves against oxidative damage by endogenously generated reactive oxygen species (Benov et al., 1995; Benov et al., 1997).

1.3. Chemotactic properties of skin-derived antimicrobial peptides

The earliest clues that defensins might play a role in adaptive immunity were the observations that α-defensin-1 and α-defensin-2 are chemotactic for human monocytes and T cells (Chertov et al., 1996; Territo et al., 1989). In the case of hBD-1 and hBD-2, which attract memory T cells and immature dendritic cells, the chemoattractant activity might be due to defensin binding to the chemokine receptor CCR6, which is also shared by the C-C chemokine ligand 20 (CCL20) (Yang et al., 1999a). Thus, it appears that CCR6 functions as a receptor for both the chemokine CCL20 and β-defensins. Although the physiological importance of this interaction has not yet been shown, the high concentrations of hBD-2 in inflamed skin make it probable that this defensin could compete effectively with the natural CCL20, despite the higher affinity of the latter for CCR6 (Yang et al., 1999a). Recent structural analysis of CCL20 indicated marked similarities to hBD-2 in the putative receptor-binding region of CCL20. This might be of high relevance *in vivo*, because hBD-2 represents one of the major antimicrobial peptide in psoriatic lesions (Harder and Schröder, 2005b).

HBD-3 has been shown to be chemotactic for immature dendritic cells as well as monocytes, but since monocytes do not express CCR6, hBD-3 might use an additional chemotaxin receptor (Garcia et al., 2001a; Yang et al., 1999b). In addition, hBD-4 has been reported to attract monocytes by as yet unknown mechanism (Garcia et al., 2001b).

Despite the functional overlap and sharing of receptors, chemokines and defensins have no apparent homology at the amino acid level, and their evolutionary relationship is unclear at present (Hughes, 1999). On the basis of usage of chemokine receptors and the tertiary structural similarities between defensins and chemokines, the defensins were considered to be ‘microchemokines’ which act on cells of the adaptive immune system (Hoover et al., 2002; Oppenheim et al., 2003).

Sharing a receptor with chemotactic factors is not unique to β-defensins, because another major human antimicrobial peptide LL-37, also uses a chemotactic receptor, formyl peptide receptor-like 1 (FPRL1), to mediate its chemotactic and Ca^{2+}-mobilizing activities (De et al., 2000). In contrast to β-defensins, LL-37 is chemotactic for neutrophils, monocytes and T cells in the micromolar range, but not for dendritic cells (Yang et al., 2001). Besides its direct antimicrobial and chemotactic function, LL-37 also has multiple roles as a mediator of inflammation, influencing diverse processes such as cell proliferation and migration, immune modulation, wound healing, angiogenesis and the release of cytokines and histamine (Bals and Wilson, 2003). By promoting re-epithelialisation of human skin wounds, LL-37 could play a role in repair of damaged tissue (Heilborn et al., 2003).

S100A7c exhibits strong chemotactic activity on $CD4^+$ T lymphocytes and neutrophils (Jinquan et al., 1996). A recent study defined RAGE (receptor for advanced glycation end products) as the S100A7c receptor (Wolf et al., 2008). Taken together the chemotactic capacities of antimicrobial peptides suggest that they play a role in recruiting T cells, immature dendritic cells and monocytes to sites of infection, thereby facilitating (as adjuvants) the initiation of adaptive antimicrobial immune response.

1.4. Antimicrobial peptides and diseases

The observation that antimicrobial peptides are expressed in the uppermost parts of the epidermis in skin suggests the existence of a ‘protective chemical shield’. These antimicrobial peptides function as effector molecules in skin infections and inflammations. It is therefore interesting to speculate that a defect in this protective system may lead to recurrent local infections, which in turn may lead to inflammation (Ong et al., 2002; Schröder and Harder, 2006).

On the other hand, it is possible that overexpression of antimicrobial peptides in skin may lead to increased protection. This hypothesis is supported by a disease concomitance seen in patients with psoriasis, a non-infective inflammatory skin disease, which unexpectedly rarely suffer from infectious skin diseases (Henseler and Christophers, 1995). Biochemical analysis of psoriatic scale extracts revealed the presence of hBD-2, RNase 7 and S100A7c as principal antimicrobial peptides in these extracts (Schröder and Harder, 2006). Atopic dermatitis, is a chronic inflammatory skin disease associated with significant barrier disruption, T-helper type 2 mediated skin inflammation, and an impaired innate immune response. These characteristics increase the susceptibility of atopic dermatitis patients to recurrent skin infections. In contrast to psoriasis, patients with atopic dermatitis often suffer from skin infections with *S. aureus* and a decreased expression of hBD-2, hBD-3 and LL-37 has been observed in acute and chronic lesions of these patients (Ong et al., 2002). However a recent study demonstrated an increase of S100A7c protein in paitents with atopic dermatitis (Gläser et al., 2008).

The involvement of antimicrobial peptides is not only restricted to skin disorders. Cystic fibrosis (CF) is a common autosomal recessive genetic disease caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene. The *CFTR* gene encodes a regulated chloride channel that also functions as a regulator of other ion channels. The main cause of morbidity and mortality in CF is respiratory failure due to progressive destruction of the airways and lungs by recurrent infections and inflammation. These events indicate a local defect in epithelial host defense, as the infection almost always remains localized in the lung and does not spread elsewhere or affect non-respiratory epithelia. It is proposed that the local host defense impairment is, at least in these paitents, due to the inhibition of defensin activity by the abnormal ionic environment of airway fluid. Cl^- concentrations of airway fluid is increased from 80 mM of healthy individuals to 170 mM in patients with CF, respectively (Smith et al., 1996). Indeed, activity of hBD-2

depends on ion composition and is sensitive to the concentration of NaCl. The ability of hBD-2 to inhibit bacterial growth diminishes when salt concentration is increased from 20 to 150 mM (Bals et al., 1998; Harder et al., 2000).

1.5. Recognition of microbial components

The innate immune system is the first line of the defense that protects hosts from invading microbial pathogens. It recognizes microorganisms by specific microbial components designated as pathogen-associated molecular patterns (PAMPs). These PAMPs, ranging from lipids, lipoproteins, proteins and nucleic acids are produced by microorganisms but not by the host. PAMPs are essential for the survival of the microorganism and therefore difficult for the microorganism to alter or to substitute. For instance, lipopolysaccaride (LPS) is a well defined PAMP of Gram-negative bacteria; Gram-positive bacteria, by contrast, produce lipoteichoic acid (LTA) and lipoproteins which are recognized as PAMPs. Peptidoglycan is a PAMP of the cell wall and is produced by Gram-positive as well as Gram-negative bacteria. Flagellin, another important PAMP, is the major protein constituent of bacterial flagella. Single- and double-stranded RNA (ssRNA, dsRNA) make up PAMPs of viruses and β-glucan is an important PAMP of fungi (Ishii et al., 2008).

Host cells express various pattern recognition receptors (PRRs) which are able to sense diverse PAMPs (Akira and Hemmi, 2003). Recognition of PAMPs by PRRs activates intracellular signaling pathways that culminate in the induction of antimicrobial peptides, inflammatory cytokines, chemokines, interferons and up-regulation of co-stimulatory molecules (Ishii et al., 2008). Among the major PRRs are the Toll-like receptors (TLRs) which recognize pathogens at either the cell surface or lysosome/endosome membranes (Ishii et al., 2008) and the nucleotide-binding

oligomerization domain (NOD)-like receptors (NLRs) which recognize pathogens that have invaded the cytosol (Kanneganti et al., 2007).

TLRs: The discovery of the TLR family began with the identification of Toll receptor of drosophila and which was found to be essential for establishing dorsoventral polarity during embryogenesis in drosophila (Hashimoto et al., 1988). Subsequent studies revealed that Toll also has an essential role in the insect innate immune response against fungal infection (Lemaitre et al., 1996). After the discovery of Toll in drosophila, a number of structurally related proteins were identified in mammals and were thus named “Toll-like” receptors. To date, 11 human TLRs and 13 mouse TLRs have been identified, and each TLR appears to recognize distinct PAMPs derived from various microorganisms, including bacteria, viruses, protozoa and fungi (listed in Table 1) (Akira et al., 2006). The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region of the IL-1 receptor, known as the TIR domain, which is required for downstream signaling, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). By contrast, the extracellular region of the TLRs and IL-1Rs differs markedly; the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs which are responsible for recognition of PAMPs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains. The LRR domains are composed of 19–25 tandem LRR motifs, each of which is 24–29 amino acids in length (Akira et al., 2006).

TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs: the subfamily of TLR1, TLR2, and TLR6 recognizes lipids, whereas the highly related TLR7, TLR8, and TLR9 recognize nucleic acids (Akira and Takeda, 2004).

Table 1. TLR Recognition of Microbial Components

Microbial Components	**Species**	**TLR Usage**
Bacteria		
Lipopolysaccharide (LPS)	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
Lipoteichoic acids (LTA)	Group B *Streptococcus*	TLR6/TLR2
Peptidoglycan (PG)	Gram-positive bacteria	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
not determined	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
double stranded RNA	Viruses	TLR3
single stranded RNA	RNA viruses	TLR7 and TLR8

TLRs are expressed on various immune system cells, including macrophages, dendritic cells, B cells, specific types of T cells, but also in cells which are not part of the immune system such as fibroblasts and epithelial cells (Kadowaki et al., 2001; Köllisch et al., 2005; Lebre et al., 2007; Miller et al., 2005; Miller and Modlin, 2007) . TLRs may be expressed extra- or intracellularly. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface, while TLRs 3, 7, 8, and 9 are found almost exclusively in intracellular compartments such as endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible. Some TLR may function as heterodimers. For example, TLR1 and TLR6 serve as co-receptors to TLR2, and TLR2/1

and TLR2/6 heterodimers recognize bacterial tri-acyl lipopeptides and di-acyl lipopeptides, respectively (Ishii et al., 2008).

In human KCs, functional expression of TLRs1, 2, 3, 5 and 9 has been demonstrated (Köllisch et al., 2005; Lebre et al., 2007; Miller et al., 2005), while expression of TLR4 was detected by some but not other authors (Baker et al., 2003; Kawai, 2003; Köllisch et al., 2005). In studies where KCs were derived form plastic surgery skin specimens functional TLR4 activity was detected (Buchau et al., 2007; Lebre et al., 2007; Pivarcsi et al., 2003) whereas in KCs derived from human foreskin specimens none such activity was seen (Köllisch et al., 2005; Mempel et al., 2003). It has been suggested that these controversial reports may be to different culture conditions or origin of KCs (Buchau et al., 2007).

NLRs: NLRs are a large family of cytoplasmic PRRs which are implicated in the recognition of bacterial components. Proteins of this family, like those of the TLR family, possess LRRs that mediates ligand sensing (Kanneganti et al., 2007). For instance, NOD1 and NOD2, members of the NLRs family, detect γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), found on bacterial peptidoglycan, respectively. Lack of either NOD1 or NOD2 in macrophages abolished proinflammatory cytokine production in response to the corresponding ligands (Kobayashi et al., 2005). Expression of NOD2 was detected in primary KCs, and stimulation with MDP induced hBD-2 peptide release (Voss et al., 2006).

1.5.1. TLRs in distinguished skin diseases and infections

Psoriasis: Psoriasis is an inflammatory skin disease that is characterized clinically by cutaneous erythematous plaques covered with a silvery scale. Histologic evaluation of psoriatic plaques reveal a thickened epidermis, with KC hyperproliferation and an inflammatory infiltrate in the dermis comprised of T cells, macrophages, and dendritic cells

(Kupper and Fuhlbrigge, 2004). There are a number of reports that evaluated the expression of TLRs by KCs in psoriasis. One study demonstrated that TLR1 was expressed by KCs in normal skin and in psoriasis. They further demonstrated that there was an enhanced TLR1 expression in the basal layer of KCs in psoriatic plaques compared with normal skin (Curry et al., 2003). Another study demonstrated that KCs of psoriasis lesions had a higher expression of TLR2 and less expression of TLR5 than normal skin (Baker et al., 2003). A study by Miller *et al.* found that TLR5 and TLR9 expression in psoriasis lesion are higher compared to normal skin (Miller et al., 2005). The role and function of TLRs expression by KCs in psoriasis lesions is unknown, but superinfection of lesions of stable plaque psoriasis by bacteria occurs only rarely. This decreased susceptibility to infection may be because of increased levels of antimicrobial peptides such as β-defensins, cathelicidin and S100A7c in psoriatic skin (Harder and Schröder, 2005b).

Acne vulgaris: Acne vulgaris is an inflammatory skin disease that mainly occurs during adolescence and involves inflammation of the pilosebaceous unit. Although the pathogenesis of acne vulgaris is multifactorial, one of the factors that contribute to acne lesions is inflammation caused by the anaerobic bacterium *Propionibacterium acnes* (Miller and Modlin, 2007). It has been demonstrated that the activation of TLR2 by *P. acnes in vitro* induced IL-12 and IL-18 production by primary human monocytes (Kim et al., 2002). Another study demonstrated that all-trans retinoic acid, which is used as a topical treatment for acne, down-regulated the expression and activation of TLR2 on cultured monocytes (Liu et al., 2005). Moreover, it has been demonstrated that *P. acnes* induce hDB-2 and IL-8 in human KCs and this induction is inhibited by anti-TLR2 and anti-TLR4 neutralizing antibodies (Nagy et al., 2005). Taken together these studies indicate the importance of TLRs in the pathogenesis of acne vulgaris.

Atopic dermatitis: Atopic dermatitis is a chronic inflammatory skin disease associated with significant barrier disruption, T-helper type 2 mediated skin inflammation, and an impaired innate immune response. These characteristics increase the susceptibility of atopic dermatitis patients to recurrent skin infections (Ong et al., 2002). A study demonstrated that a polymorphism in TLR2 correlates with a severe phenotype of atopic dermatitis. This study suggests that lack of functional TLR2 may exacerbate atopic dermatitis (Ahmad-Nejad et al., 2004). Although, the activation of TLR2 in KCs induce the expression of hBD-2 (Kawai, 2003), but a direct role of TLR2 in the pathogenesis of atopic dermatitis is still unclear.

***S. aureus* skin infections:** *S. aureus* is a Gram-positive extracellular bacterium that is responsible for the vast majority of skin and soft tissue infections in humans, including impetigo, folliculitis, and cellulitis. Several *in vitro* experiments have demonstrated that lipoteichoic acid of *S. aureus* activate immune cells via TLR2 (Schröder et al., 2003; Takeuchi et al., 1999). In addition, *in vivo* studies of TLR2-deficient mice have demonstrated that these mice are more susceptible to *S. aureus* skin infection and develop larger skin lesions than wild-type mice (Miller et al., 2006).These data provide evidence that TLR2 is important in host defense against *S. aureus* infections in the skin.

Candidiasis: Candidiasis is an infection caused by the yeast *C. albicans* which is a dimorphic fungal pathogen. Clinical infections include mucocutaneous infections as well as invasive and life-threatening infections usually seen in immunocompromised patients (Levitz, 1998). *In vitro* studies have shown that *C. albicans* -derived mannan induces in human monocytes the production of TNF-α in a TLR4-dependent manner (Tada et al., 2002). In an *in vitro* model it has been demonstrated that treatment of KCs with *C. albicans* and mannan resulted in the activation and nuclear translocation of NF-κB. In addition, it was shown that *Candida*-killing activity of KCs was NF-κB-dependent and that this activity could be inhibited by anti-TLR2 and anti-TLR4 neutralizing antibodies (Pivarcsi et al.,

2003). These studies may suggest an important role of TLRs in *C. albicans* skin infections. The clinical relevance of these *in vitro* studies are still unclear because no published report of *in vivo C. albicans* skin infections in context with TLR2 or TLR4 have been found (Miller and Modlin, 2007).

Herpes simplex virus: Herpes simplex virus (HSV) and Varicella-Zoster virus (VZV) are common viral pathogens that infect human skin. It has been demonstrated that VZV activates induced IL-6 cytokine response of human monocytes in a TLR2-dependent manner (Wang et al., 2005). HSV is recognized through both TLR2 and TLR9 in dendritic cells for the induction of IL-6 and IL-12 secretion (Sato et al., 2006). Thus, suggesting for TLR2 and TLR9 an important role in recognition and host defense against HSV and VZV infections in the skin

2. Objectives and format of the dissertation

Human skin is an effective barrier against invading microorganisms. This protective function is partly mediated by the presence of antimicrobial peptides. The overall objective of this dissertation was to identify molecular mechanisms by which antimicrobial peptides of human skin are regulated. The aim of the first part was to determine the mechanism by which *E. coli* induces the up-regulation of the antimicrobial peptide S100A7c, the principal *E. coli*–cidal factor present at the surface of human epidermis (Gläser et al., 2005). The objective of the second part was to investigate the potential role of RNase-RI interaction in human skin and its implication on the RNase-dependent antimicrobial defense system.

The following part of the dissertation is organized in two chapters:

Chapter I has been published in the "Federation of American Societies for Experimental Biology". Abtin, A., Eckhart, L., Mildner, M., Gruber, F., Schröder, J. M., Tschachler, E. (2008). **Flagellin is the principal inducer of the antimicrobial peptide S100A7c (psoriasin) in human epidermal keratinocytes exposed to *Escherichia coli*.** *FASEB J.* 22: 2168-2176

Chapter II has been accepted for publication in the "Journal of Investigative Dermatology". Abtin, A., Eckhart, L., Mildner, M., Ghannadan, M., Harder, J., Schröder, J. M., Tschachler, E. (2009). **Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking antimicrobial activities of RNase 5 and RNase 7.**

Both chapters contain the following sections: Title, Abstract, Introduction, Materials and Methods, Results, Discussion, Figures and References.

References cited in the Introduction, Objectives and Discussion are listed at the end of the dissertation.

3. Chapter I: Flagellin is the principal inducer of the antimicrobial peptide S100A7c (psoriasin) in human epidermal keratinocytes exposed to Escherichia coli.

FASEB J. 22: 2168-2176

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Flagellin is the principal inducer of the antimicrobial peptide S100A7c (psoriasin) in human epidermal keratinocytes exposed to *Escherichia coli*

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ABSTRACT **Epidermal keratinocytes (KCs) express antimicrobial peptides as a part of the innate immune response. It has recently been shown that the culture supernatant of *Escherichia coli* induces the expression of S100A7c (psoriasin) in KCs and that S100A7c efficiently kills *E. coli*. Here we have investigated which of the microbial components triggers the up-regulation of S100A7c expression. Exposure of human primary KCs to ligands of the human Toll-like receptors (TLRs) revealed that only the TLR5 ligand flagellin strongly induced the expression of S100A7c mRNA and protein, whereas all other TLR ligands had no significant effect. In contrast to the supernatant from flagellated wild-type (WT) *E. coli*, the supernatant of a flagellin-deficient *E. coli* strain (ΔFliC) did not induce S100A7c expression. Small interfering RNA-mediated knockdown of TLR5 expression suppressed the ability of KCs to up-regulate S100A7c expression in response to both flagellin and WT *E. coli* supernatant. Taken together, our data demonstrate that bacterial flagellin is essential and sufficient for the induction of S100A7c expression in KCs by *E. coli*.—Abtin, A., Eckhart, L., Mildner, M., Gruber, F., Schröder, J.-M., Tschachler, E. Flagellin is the principal inducer of the antimicrobial peptide S100A7c (psoriasin) in human epidermal keratinocytes exposed to *Escherichia coli*. *FASEB J.* 22, 2168–2176 (2008)**

Key Words: innate immunity · Toll-like receptor · TLR · pathogen-associated molecular pattern · PAMP · antimicrobial defense

HUMAN SKIN IS AN EFFECTIVE BARRIER against invading microorganisms. This protective function is partly mediated by the presence of antimicrobial peptides (AMPs) in the skin (1, 2). Although known for a long time in other species (3, 4) and in other organ systems of humans (5, 6), the presence and importance of AMPs in human skin has only recently been demonstrated (1, 7). AMPs of the skin comprise a heterogeneous group of molecules, such as human β-defensin (hBD) -1, hBD-2, hBD-3, RNase 7, cathelicidin, and S100A7c (2). The antimicrobial activity described for AMPs is due to perforation and disruption of the bacterial membrane (*e.g.*, hBD-2, RNase 7, and cathelicidin) (8–10) by deprivation of essential trace elements (*e.g.*, S100A7c) (1) or by as yet unknown mechanisms (*e.g.*, hBD-1 and hBD-3) (2). Studies using mouse models with deficiencies in AMP genes have emphasized the importance of some of these peptides in the defense against invasive bacterial infections in epithelia of different organs (11–13). For example, mice deficient in the expression of CRAMP (the mouse homolog to the human cathelicidin) were more susceptible to skin infections caused by group A *Streptococcus* (12) and urinary tract infections by *Escherichia coli* (11).

One arm of the antimicrobial defense of the skin is provided by members of the multifunctional S100 protein family. S100 proteins have a molecular mass of 9–13 kDa and are characterized by two calcium-binding EF-hand motifs (14). Eleven proteins of the S100A family, *i.e.*, S100A2, S100A3, S100A4, S100A6, S100A7c, S100A8, S100A9, S100A10, S100A11, S100A12, and S100A15, are expressed in the epidermis (15–17). S100A7c, S100A12, and S100A15 have Gram-negative bacteria-killing activities (1, 18, 19), and a heterodimer complex of S100A8/S100A9 suppresses the growth of the yeast *Candida albicans* (20). S100A7c has been identified as a protein that is up-regulated in lesional psoriatic skin; hence, it was initially named psoriasin (21). However, in the meantime it has been established that S100A7c is also expressed in normal skin (1, 21). S100A7c is encoded by a gene located on chromosome 1q21 within a cluster of five *S100A7*-like genes, *S100A7a* through *S100A7e*. S100A7a–S100A7c transcripts encode functional proteins whereas *S100A7d* and *S100A7e* are pseudogenes (22). S100A7c shows 93 and 50% amino acid sequence similarity compared with S100A7a and S100A7b, respectively (22). A recent report has provided evidence that the human *S100A7* genes have arisen by repeated duplications of an ancient *S100A7* gene whereas the mouse has lost the *S100A7* gene (23).

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Of all the proteins of the S100A7 family only S100A7c is present at high concentrations in human stratum corneum (2). Gläser *et al.* (1) have shown that *E. coli* is effectively killed on human skin and that this killing activity was inhibited *in vivo* by a neutralizing antibody to S100A7c. Furthermore, this report demonstrated that S100A7c is up-regulated by exposing keratinocytes (KCs) to *E. coli* culture supernatants. Together, these data suggest that S100A7c has a critical role in the skin defense against *E. coli* and probably also against related bacteria.

The induction of antimicrobial molecules is a central mechanism of the innate immune defense. Components of different pathogens, *i.e.*, pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors, such as the Toll-like receptors (TLRs) (24), which are expressed on a wide variety of immune and nonimmune cells (25–29). To date, 10 members of the TLR family of genes have been identified in humans (30, 31). Each TLR recognizes distinct PAMPs derived from various microorganisms. For instance, TLR3 recognizes viral double-stranded RNA (32), TLR4 recognizes lipopolysaccharide (LPS) (33), and TLR5 recognizes bacterial flagellin (34). Engagement of TLRs by microbial components initiates signaling cascades that activate mitogen-activated protein kinases and induces nuclear translocation of NF-κB (35), which consequently leads to the production of proinflammatory cytokines (35) and the induction of AMPs (such as hBD-2) in various epithelial cells (28, 36–38). In human KCs, functional expression of TLR1, 2, 3, 5, and 9 has been demonstrated (26–28), whereas expression of TLR4 in KCs was detected by some but not other authors (27, 39, 40). TLR2 has been shown to mediate the activation of NF-κB gene expression in response to *Staphylococcus aureus* (41). The bacterial components by which *E. coli* induces the expression of S100A7c have not yet been identified.

Here we have investigated whether TLR ligands are able to up-regulate S100A7c expression in KCs and whether any of these ligands are involved in the induction of S100A7c by *E. coli* culture supernatants. The regulation of S100A7c was compared with that of the antimicrobial peptide hBD-2. Our results reveal that the induction by *E. coli* of both S100A7c and hBD-2 depends on the presence of bacterial flagellin and TLR5 on KCs.

MATERIALS AND METHODS

Cell culture

Human primary KCs prepared from neonatal foreskin were obtained from Clonetics (San Diego, CA, USA) and cultured in serum-free keratinocyte growth medium (KGM; Clonetics) as described previously (42). For stimulation, third passage KCs were cultured in 12-well tissue culture plates (Corning Incorporated, Corning, NY, USA) and used at a confluence of 60–70%. Stimulation was performed in keratinocyte basal medium (KBM; Clonetics).

Reagents used for treatment of KCs

For *in vitro* assays, recombinant interleukin-1α (IL-1α; R&D Systems, Minneapolis, MN, USA), IL-8 (R&D Systems), and the following TLR ligands (InvivoGen, San Diego, CA, USA) were used: synthetic tripalmitoylated lipopeptide (Pam3CSK4; TLR1/2 ligand), heat-killed *Listeria monocytogenes* (HKLM; TLR2 ligand), poly(I:C), a synthetic analog of double-stranded RNA (TLR3 ligand), ultrapure LPS from *E. coli* K12 (TLR4 ligand), purified flagellin from *Salmonella typhimurium* (TLR5 ligand), synthetic lipoprotein of *Mycoplasma salivarium* (FSL1; TLR6/2 ligand), imiquimod (R837; TLR7 ligand), single-stranded RNA40 (TLR8 ligand), and ODN2006 (CpG oligonucleotide type B; TLR9 ligand). For IL-1α neutralizing experiments, KCs were used at a confluence of 60–70% in 12-well tissue culture plates. Before stimulation with IL-1α and flagellin, cells were preincubated for 90 min with a polyclonal goat IgG IL-1α neutralizing antibody (2 μg/ml; R&D Systems) or a polyclonal goat IgG isotype control (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For blocking IL-1 receptors, cells were incubated for 90 min with 100 ng/ml IL-1 receptor antagonist (IL-1Ra; Strathmann Biotec, Hamburg, Germany) before stimulation with flagellin or IL-1α.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

After stimulation, cells were washed with PBS, and RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For cDNA synthesis RNA was reverse-transcribed with murine leukemia virus-reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and oligo(dT) primers (Roche Diagnostics, Basel, Switzerland). cDNA sequences of the genes under investigation were obtained from GenBank. Primers were designed using PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA, USA). The following forward (F) and reverse (R) intron-spanning primers were used—for β-2-microglobulin: F, 5′-GATGAGTATGCCTGCCGTGTG-3′; R, 5′-CAATCCAAATGCGGCATCT-3′; for S100A7c: F, 5′-GGAGAACTTCCCCAACTTCCTT-3′; R, 5′-GGAGAAGACATTTTATTGTTCCT-3′; for S100A7a: F, 5′-AAATACACCGGACGTGATGG-3′; R, 5′-TCTTGTCCTTTTTCTCAAAGACAGT-3′; for S100A7b: F, 5′-GATGAATATCCCTCTAGGTGAGAAAGT-3′; R, 5′-GGTGCCACTCCATGCATTAT-3′; for hBD-2: F, 5′-ATCAGCCATGAGGGTCTTGT-3′; R, 5′-GAGACCACAGGTGCCATTTT-3′; for hBD-1: F, 5′-CCCAGTTCCTGAAATCCTGA-3′; R, 5′-CTTCTGGTCACTCCCAGCTC-3′; for hBD-3: F, 5′-AGCTGTGGCTGGAACCTTTA-3′; R, 5′-CGATCTGTTCCTCCTTTGGA-3′; for cathelicidin: F, 5′-GCTAACCTCTACCGCCTCCT-3′; R, 5′-GGTCACTGTCCCCATACACC-3′; for TLR5: F, 5′-CTAGAAGTCCCTTCTGCTAGGAC-3′; R, 5′-AAGGGGAAGGATGAAGCAGT-3′; and for IL-8: F, 5′-CTCTTGGCAGCCTTCCTGATT-3′; R, 5′-TATGCACTGACATCTAAGTTCTTTAGCA-3′. Quantitative real-time PCR was performed by LightCycler technology using the Fast Start SYBR Green I Kit for amplification and detection (Roche Diagnostics) as described previously (43). Determination of relative quantification of target gene expression and amplification efficiencies was performed using a mathematical model by Pfaffl (44). The expression of the target gene was normalized to the expression of β-2-microglobulin. All real-time PCRs were performed in triplicate. The specificity of the reactions was confirmed by sequencing of the PCR products.

Bacterial strains and KC stimulation

E. coli NK 9373 and *E. coli* NK 9375 (ΔFliC; a flagellin-deficient strain having an in-frame deletion within the *fliC* gene) (45) were kindly provided by Dr. David Bates (Harvard University, Cambridge, MA, USA). Bacterial culture supernatants were prepared as described previously (1) with minor modifications. Briefly bacteria were grown in tryptic soy broth (TSB) (Fluka, Buchs, Switzerland) with agitation at 37°C until an optical density of 1.0 (OD_{600}) was reached. Then 1 ml of this culture was combined with 9 ml of TSB and incubated overnight in 75-cm^2 flasks (Cellstar, Frickenhausen, Germany) at 37°C without agitation. Optical density (OD_{600}) of overnight cultures of *E. coli* strains was set with TSB to 1.7. Subsequently, bacteria were heat-killed in a water bath at 65°C for 60 min. Heat-killing was verified by a plating culture on Luria-Bertani agar. After heat-killing, cultures were centrifuged at 5000 *g* for 15 min. The resulting supernatants were diluted 1:100 in KBM and used to stimulate KCs from which the previous medium had been removed.

Cytokine measurement

Culture supernatants of stimulated KCs were depleted by centrifugation of detached cells or cell fragments and stored at −20°C until analysis. Concentrations of IL-1α and IL-8 were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Small interfering (si) RNA transfections

Third-passage primary KCs were used at a confluence of 60–70% in 12-well tissue culture plates. KCs were transfected with the following Stealth siRNAs using Lipofectamine 2000 reagent (Invitrogen): TLR5-siRNA1, 5′-CAUCCUUCAUUUGGGAAGUUGAAUU-3′; TLR5-siRNA2, 5′-CCACCAGGAGUAUUUAGCCAUCUGA-3′; control siRNA1 (scrambled sequence of TLR5-siRNA1), 5′-CAUACUUGUUUAAGGGUUGACCAUU-3′; and control siRNA2 (scrambled sequence of TLR5-siRNA2), 5′-CCAGGAUGAUUAGAUACCCUCCUGA-3′. Lipofectamine 2000 (30 μl) was mixed with 60 μl of a 20 μM siRNA solution (1:1 mix of siRNA1 and -2) and 4 ml of OPTI-MEM medium (Gibco BRL, Gaithersburg, MD, USA). After 30 min at room temperature, 4 ml of KBM was added, and the solution was poured onto the KCs in 12-well tissue culture plates (600 μl/well) for 24 h. Afterward the medium was changed to KGM, and cells were left for another 24 h before stimulation.

Western blot analysis

For analysis of protein expression, KCs were lysed in SDS-PAGE loading buffer (50 mM Tris, pH 7.4, 2% SDS). After sonication, insoluble cell debris was removed by centrifugation, and protein concentration was measured by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). Western blot analysis was performed as described previously (46). The following first step antibodies were used: mouse monoclonal IgG_1 anti-S100A7c clone 47C1068 (dilution 1:500; Abcam, Cambridge, UK) and mouse monoclonal IgG2b anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (dilution 1:400; Biogenesis, Poole, UK). The membranes were developed using the Chemiglow reagent (Alphainnotech, San Leandro, CA, USA) according to the manufacturer's instructions.

RESULTS

Flagellin but not other TLR ligands induces S100A7c expression in human primary KCs

To investigate the role of TLRs in the regulation of S100A7c expression in KCs, primary human epidermal KCs were stimulated with ligands for TLR1 through TLR9. The expression of S100A7c and other AMPs was determined by quantitative real-time PCR analysis. S100A7c mRNA was up-regulated by the TLR5 ligand flagellin but not by other TLR ligands (**Fig. 1*A***). The level of up-regulation of S100A7c induced by flagellin equalled that induced by IL-1α (Fig. 1*A*). Among the other AMPs investigated, hBD-1, hBD-3, RNase7, and cathelicidin were not changed by stimulation with TLR ligands (data not shown). hBD-2 was up-regulated by flagellin, poly(I:C) (TLR3 ligand), and ODN2006 (TLR9 ligand) in a manner comparable with the results of previous studies (28, 36) (Fig. 1*B*). Similar to the results obtained with S100A7c, hBD-2 mRNA was almost as strongly induced by flagellin as by IL-1α. Because hBD-2 expression showed responsiveness to flagellin similar to that of S100A7c, it was used for comparison in all further experiments.

Flagellin induces S100A7c expression in an IL-1-independent manner

Next we determined the dose and time dependence of flagellin-induced AMP expression. S100A7c mRNA was significantly induced at a flagellin concentration of 5 ng/ml (**Fig. 2*A***), whereas 1 ng/ml flagellin sufficed to up-regulate hBD-2 mRNA expression (Fig. 2*B*). Increasing concentrations of flagellin up to 10 ng/ml induced higher expression levels of both S100A7c and hBD-2 mRNA, whereas further elevation of the flagellin did not cause significant additional up-regulation (Fig. 2*A, B*) and even reduced the inductive effect of flagellin (Fig. 2*B*). The maximum up-regulation of both S100A7c and hBD-2 mRNA was observed at time points later than 8 h after addition of flagellin to the KC culture medium (Fig. 2*C, D*). Essentially the same temporal expression pattern was induced by stimulation with IL-1α (Fig. 2*C, D*), whereas IL-8 mRNA was up-regulated as early as 2 h after stimulation and decreased later (Fig. 2*E*).

The time course of S100A7c or hBD-2 expression seemed to be compatible with an indirect mechanism of regulation involving flagellin-induced cytokine release and subsequent induction of S100A7c and hBD-2 by cytokines. We therefore investigated the role of two candidate cytokines, *i.e.*, IL-1 and IL-8.

Because IL-1α is stored in KCs and can be released by various stress stimuli (47), we determined by ELISA the IL-1α concentration in KC culture supernatant at 2 h after flagellin stimulation, a time point before the increase of S100A7c and hBD-2 expression (Fig. 2*C, D*). However, IL-1α concentrations did not differ between

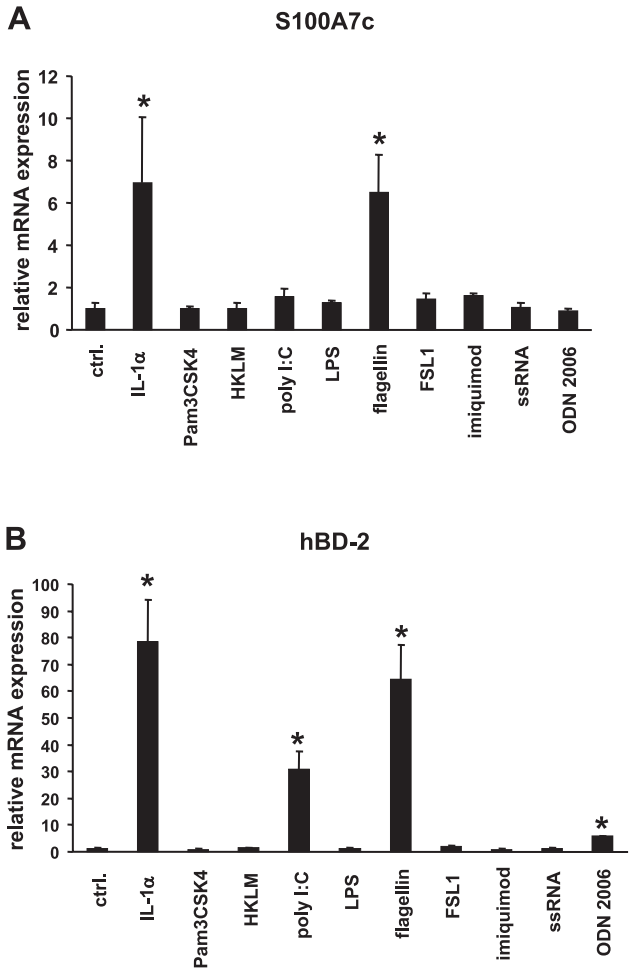

Figure 1. Flagellin induces S100A7c and hBD-2 expression in human primary KCs. Normal human epidermal KCs were stimulated for 16 h with IL-1α (10 ng/ml) and the following TLR ligands: 0.5 μg/ml synthetic tripalmitoylated lipopeptide (Pam3CSK4; TLR1/2 ligand), 10^8 cells/ml heat-killed *Listeria monocytogenes* (HKLM; TLR2 ligand), 15 μg/ml poly(I:C) (a synthetic analog of double-stranded RNA; TLR3 ligand), 100 ng/ml ultrapure LPS from *E. coli K12* (TLR4 ligand), 0.5 μg/ml purified flagellin from *S. typhimurium* (TLR5 ligand), 0.5 μg/ml FSL1 (a synthetic lipoprotein of *M. salivarium*; TLR6/2 ligand), 10 μg/ml imiquimod (R837; TLR7 ligand), 1 μg/ml single-stranded RNA40 (ssRNA; TLR8 ligand), and 2.5 μM ODN2006 (CpG oligonucleotide type B; TLR9 ligand). After stimulation, RNA was harvested and relative S100A7c (*A*) and hBD-2 (*B*) gene expression levels were quantified by real-time PCR and normalized to the expression of the housekeeping gene β-2-microglobulin. The mean values are displayed in relation to untreated controls. Data are means ± SD of one representative experiment of three, each done in triplicate. $*P < 0.05$ *vs.* untreated control; unpaired Student's *t* test.

supernatants of flagellin-stimulated and unstimulated KCs either at 2 h or 24 h after stimulation (**Fig. 3*A***).

To exclude the theoretical possibility that released IL-1α is captured by cellular receptors and exerts its effects without increasing in concentration in the bulk supernatant, KCs were preincubated with an IL-1α neutralizing antibody or its corresponding isotype control, and/or with an IL-1Ra that neutralizes both IL-1α and IL-1β. Neither treatment interfered with S100A7c or hBD-2 induction by flagellin (Fig. 3*B, C*), demonstrating that extracellular IL-1 is not required for the observed effects of flagellin. Consistent with previous reports (48, 49), addition of IL-8 did not alter the mRNA expression levels of S100A7c and hBD-2 (Fig. 2*F*).

Flagellin-deficient *E. coli* does not induce S100A7c expression

To determine the contribution of flagellin in the induction of S100A7c expression by *E. coli* culture supernatant, we compared a wild-type (WT) flagellated *E. coli* strain (NK 9373) and a flagellin-deficient (ΔFliC) *E. coli* strain (NK 9375) with regard to their capacity to induce S100A7c expression. Culture supernatants of *E. coli* strains were diluted 1:100 in KBM and applied to KC cultures for 16 and 48 h. Quantitative real-time PCR revealed that WT *E. coli* induced S100A7c and hBD-2 mRNA expression, whereas ΔFliC *E. coli* did not (**Fig. 4*A*, *B***). Neither WT nor ΔFliC *E. coli* strains were able to induce the expression of hBD-1, hBD-3, RNase7, and cathelicidin (Supplemental Fig. 1). Western blot analysis of cell lysates showed that the increase of S100A7c mRNA in response to flagellin or WT *E. coli* also resulted in a strong increase of S100A7c protein (Fig. 4*C*). In contrast, ΔFliC *E. coli* lacked the ability to induce S100A7c protein production (Fig. 4*C*). Addition of flagellin (10 ng/ml) to the ΔFliC *E. coli* culture supernatant restored the induction of S100A7c protein expression (Fig. 4*C*), demonstrating that the failure of this strain to induce S100A7c is not due to the presence of an inhibitor. Notably, supernatants of ΔFliC *E. coli* were also able to induce secretion of IL-8 by KCs, although less than WT *E. coli* (Fig. 4*D*). These findings demonstrate that flagellin is essential for *E. coli*-mediated induction of S100A7c and hBD-2 but only partially contributes to *E. coli*-mediated cytokine secretion from KCs.

Because it has been reported that S100A7a, misleadingly named human S100A15 (50), is also up-regulated by *E. coli* (18), we compared the potential biological significance of S100A7a and S100A7c in the defense against *E. coli*. Like S100A7c, S100A7a was up-regulated by IL-1α, flagellin, and WT *E. coli* but not by ΔFliC *E. coli* (Supplemental Fig. 2*A*). In contrast with the conclusions of another report (18), we did not observe an increase in S100A7a in response to the TLR4 ligand LPS (data not shown). However, when we compared the absolute expression levels of both S100A7a and S100A7c by quantitative real-time PCR we found that S100A7c was expressed at a 650-fold higher level than S100A7a (12285±836 *vs.* 19±3 mRNA copies per 1 μg of total RNA) (Supplemental Fig. 2*B*). Transcripts of the third potentially functional S100A7 isoform, S100A7b, could not be detected in KCs (Supplemental Fig. 2*B*). These data suggest that S100A7a and S100A7c are regulated by *E. coli* components in a similar manner; however, S100A7c is expressed at a much higher level.

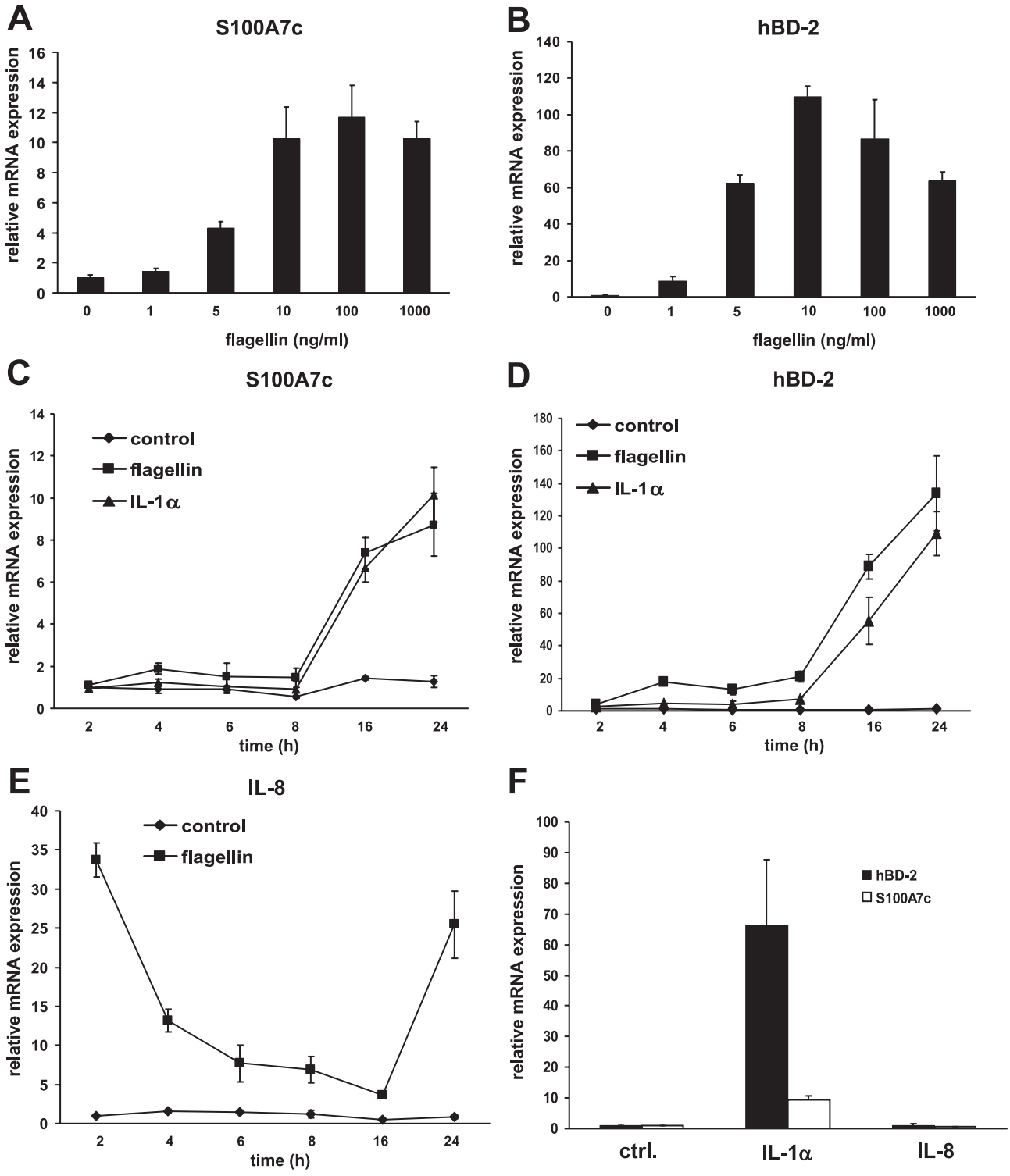

Figure 2. Time- and dose-dependent effect of flagellin on S100A7c and hBD-2 expression. KCs were stimulated with the indicated concentrations of flagellin for 24 h. Thereafter, quantitative real-time PCR analysis of S100A7c (*A*) and hBD-2 (*B*) mRNA expression was performed. KCs were cultured for the indicated times in the presence of 10 ng/ml flagellin, and quantitative real-time PCR analysis of S100A7c (*C*), hBD-2 (*D*), and IL-8 (*E*) mRNA expression was performed. KCs were stimulated with IL-1α or IL-8 (both at 10 ng/ml) for 24 h; after stimulation, relative S100A7c and hBD-2 gene expression levels were determined by quantitative real-time PCR (*F*). All gene expression levels were normalized to the housekeeping gene β-2-microglobulin. The mean values are displayed in relation to untreated controls. Data are means ± SD of one representative experiment of three, each done in triplicate.

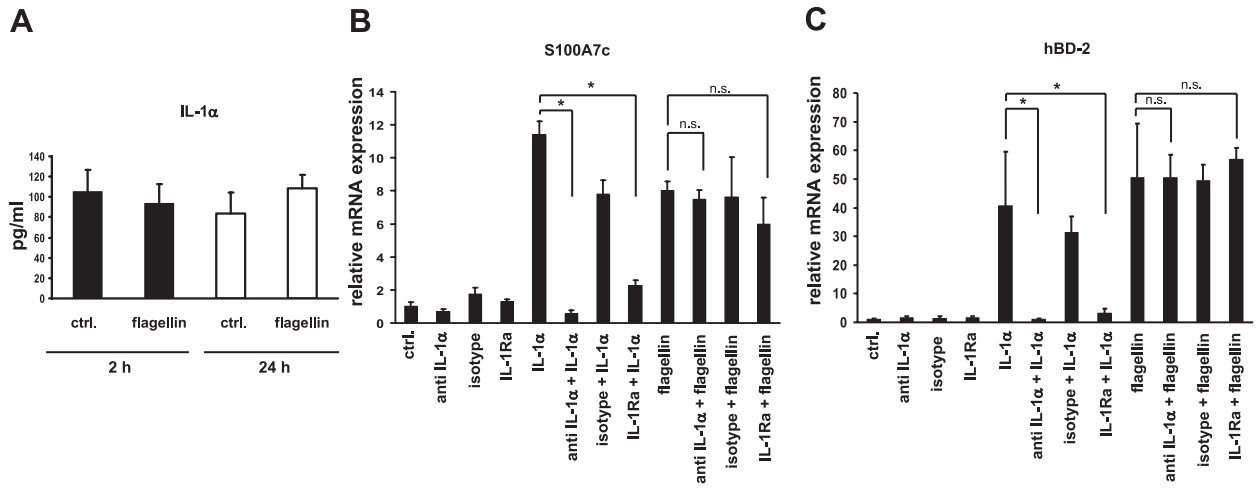

Figure 3. Flagellin induction of S100A7c and hBD-2 is IL-1α-independent. *A*) KCs were stimulated with flagellin (10 ng/ml) for the indicated times, and culture medium was measured for IL-1α secretion by ELISA. Data are means ± SD of one representative experiment of two, each done in triplicate. *B, C*) KCs were preincubated for 90 min with anti-IL-1α (2 μg/ml), with isotype control (2 μg/ml) or with IL-1Ra (100 ng/ml), as indicated. Afterwards, cells were treated with IL-1α or flagellin (both at 10 ng/ml) or left untreated for 24 h in the continued presence of the reagents used for preincubation. Relative gene expressions of S100A7c (*B*) and hBD-2 (*C*) were quantitated by real-time PCR and normalized to the housekeeping gene β-2-microglobulin. The mean values are displayed in relation to untreated controls. Data are means of triplicates ± SD. Similar results were obtained in a second experiment. $*P < 0.05$, determined by unpaired Student's *t* test. n.s., not significant.

Knockdown of TLR5 abolishes S100A7c and hBD-2 induction by *E. coli*

Flagellin is known to induce antimicrobial defense *via* two pathways, namely activation of the intracellular receptor ICE-protease-activating factor (Ipaf) followed by caspase-1-mediated processing of IL-1β (51) and/or binding to TLR5 and MyD88-dependent intracellular signaling (24). Because our results obtained with the IL-1Ra argued against a role of IL-1β and, accordingly, also against a role of the Ipaf pathway (51), we focused our further investigations on the putative role of TLR5 in the response of KCs toward *E. coli*. siRNA technology was used to knock down TLR5 expression in KCs. Transfection of KCs with TLR5 siRNA led to a down-regulation of TLR5 mRNA by 70% compared with siRNA with a scrambled sequence (**Fig. 5*A***). Knock-down of TLR5 strongly inhibited the induction of S100A7c and hBD-2 mRNA by flagellin and WT *E. coli* supernatant (Fig. 5*B, C*). Western blot analysis of cell

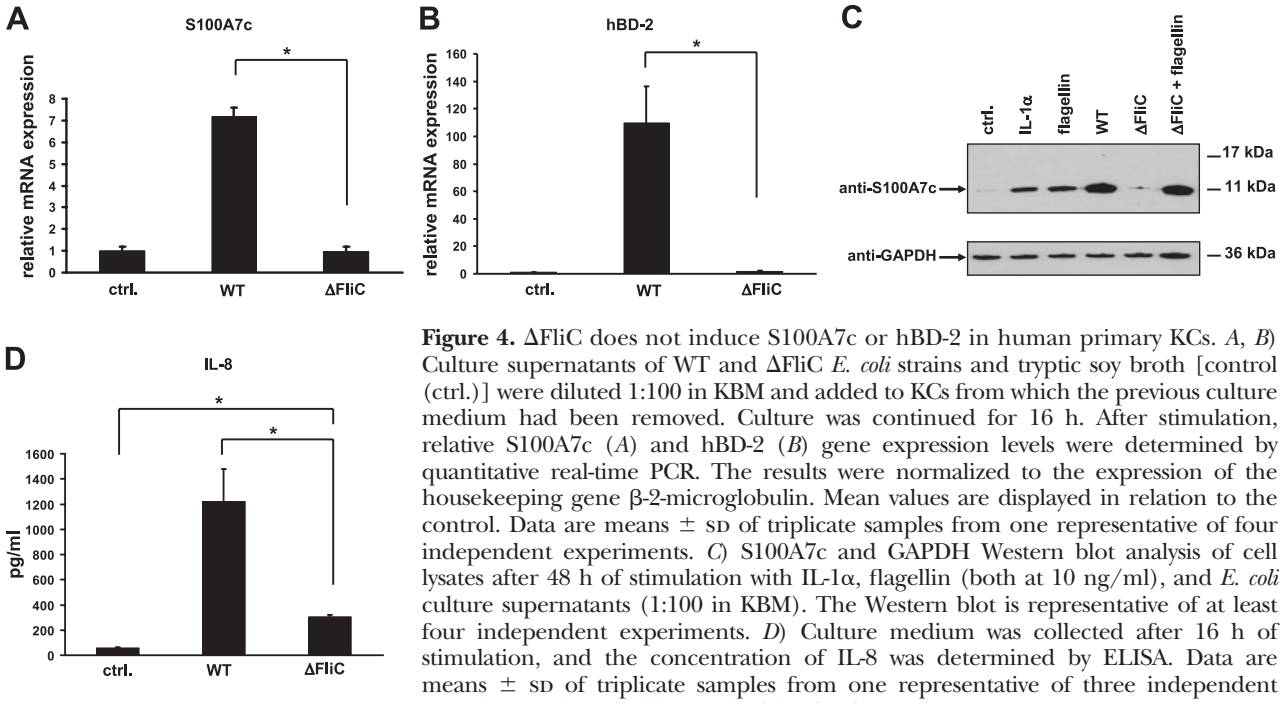

Figure 4. ΔFliC does not induce S100A7c or hBD-2 in human primary KCs. *A, B*) Culture supernatants of WT and ΔFliC *E. coli* strains and tryptic soy broth [control (ctrl.)] were diluted 1:100 in KBM and added to KCs from which the previous culture medium had been removed. Culture was continued for 16 h. After stimulation, relative S100A7c (*A*) and hBD-2 (*B*) gene expression levels were determined by quantitative real-time PCR. The results were normalized to the expression of the housekeeping gene β-2-microglobulin. Mean values are displayed in relation to the control. Data are means ± SD of triplicate samples from one representative of four independent experiments. *C*) S100A7c and GAPDH Western blot analysis of cell lysates after 48 h of stimulation with IL-1α, flagellin (both at 10 ng/ml), and *E. coli* culture supernatants (1:100 in KBM). The Western blot is representative of at least four independent experiments. *D*) Culture medium was collected after 16 h of stimulation, and the concentration of IL-8 was determined by ELISA. Data are means ± SD of triplicate samples from one representative of three independent experiments. $*P < 0.05$, unpaired Student's *t* test.

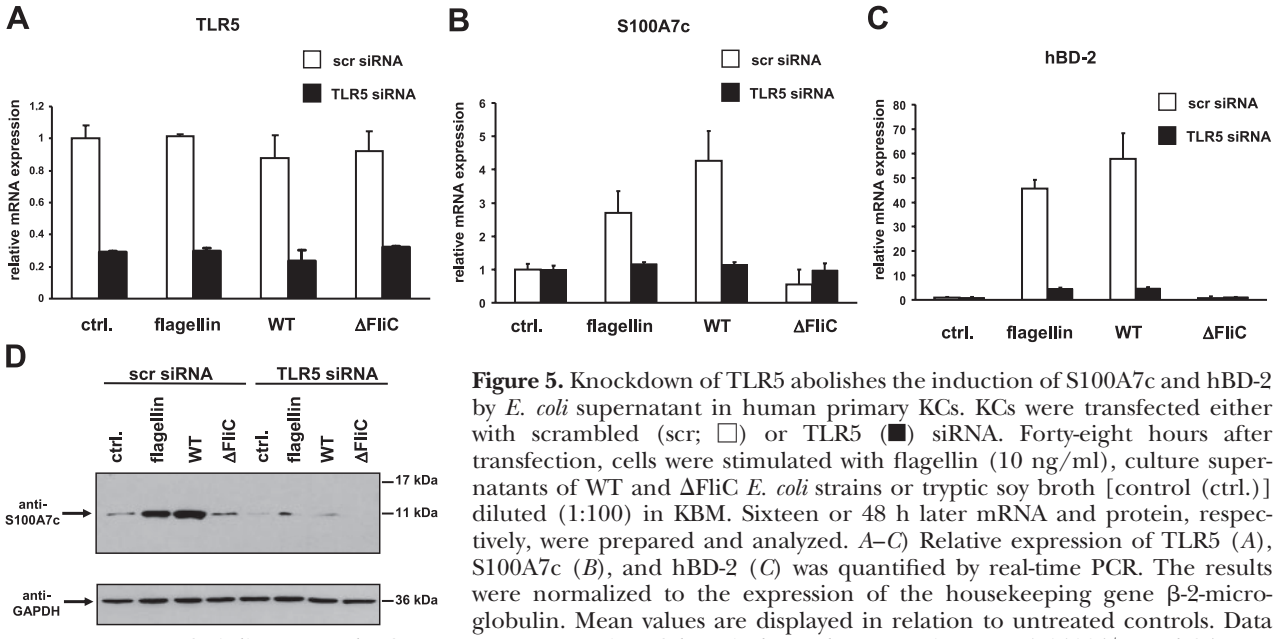

Figure 5. Knockdown of TLR5 abolishes the induction of S100A7c and hBD-2 by *E. coli* supernatant in human primary KCs. KCs were transfected either with scrambled (scr; □) or TLR5 (■) siRNA. Forty-eight hours after transfection, cells were stimulated with flagellin (10 ng/ml), culture supernatants of WT and ΔFliC *E. coli* strains or tryptic soy broth [control (ctrl.)] diluted (1:100) in KBM. Sixteen or 48 h later mRNA and protein, respectively, were prepared and analyzed. *A–C*) Relative expression of TLR5 (*A*), S100A7c (*B*), and hBD-2 (*C*) was quantified by real-time PCR. The results were normalized to the expression of the housekeeping gene β-2-microglobulin. Mean values are displayed in relation to untreated controls. Data are means ± SD of triplicate samples from one representative of three independent experiments. *D*) S100A7c and GAPDH Western blot analysis of TLR5 knockdown KC lysate stimulated for 48 h as indicated. The data are representative of three independent experiments.

lysates showed that knockdown of TLR5 also suppressed the increase of S100A7c protein production by flagellin and WT *E. coli* supernatant (Fig. 5*D*).

DISCUSSION

S100A7c is one of the most potent antimicrobial agents of the skin surface and it appears to be relatively specific for *E. coli* (1). The fact that S100A7c is expressed in a focal pattern in the epidermis and the finding that *E. coli* culture supernatants are able to increase S100A7c abundance on human skin have indicated that S100A7c is up-regulated by bacterial substances entering the epidermis through microwounds (1, 2). However, distinct exogenous molecular triggers of S100A7c have remained elusive. In the present study, we demonstrate that S100A7c is induced by flagellin, a component of flagellated bacteria, including *E. coli*.

A key result of this study was the demonstration that flagellin deficiency of *E. coli* is associated with a lack of induction of S100A7c. Because we compared two *E. coli* strains that differed exclusively at the locus of the *fliC* gene, which was destroyed by targeted deletion in *E. coli* NK9375, mutations affecting other gene products can be excluded as causative factors for the observed difference. Together with our demonstration that purified flagellin is sufficient to induce S100A7c up-regulation, the data obtained with *E. coli* supernatants strongly suggest that flagellin is the only relevant *E. coli* component that stimulates S100A7c expression in KCs. However, there remains the theoretical possibility that an as yet unknown factor attaches to flagellin and contributes to its effects. The ultimate proof may involve the use of ultrapure flagellin or flagellin fragments produced by peptide synthesis *in vitro* instead of purification from flagellated bacteria or bacteria expressing recombinant flagellin.

Complementary to our findings regarding the role of bacterial flagellin in S100A7c regulation, we have identified the flagellin receptor TLR5 as an essential element of the sensory and signaling system for *E. coli* in KCs. Furthermore, our data also demonstrate that TLR5 is critical for the induction of hBD-2. Compared with the results of a previous report (52) that showed ~7-fold up-regulation of hBD-2 promoter activity in response to bacterial muramyl dipeptide (MDP), the 50- to 100-fold up-regulation of hBD-2 mRNA expression by flagellin observed in our study appears to be more relevant in a physiological context. However, it is conceivable that TLR5 on the KC membrane and intracellular NOD2, the receptor of MDP, cooperate in sensing *E. coli* and triggering hBD-2 expression.

S100A7c is up-regulated by flagellin more than 8 h after stimulation of KCs. The response time from addition of flagellin to the production of S100A7c mRNA was longer than that for the induction of IL-8 but was comparable to the kinetics of S100A7c expression in KCs observed by others after stimulation with various cytokines (1, 49). The exploration of the cause for this relatively slow response exceeds the scope of our present study. However, our data argue against a role of autocrine activation of S100A7c production by IL-1 and IL-8, because blockade of the former did not inhibit flagellin-induced S100A7c expression and addition of the latter did not induce the expression of S100A7c when added to KCs.

Our data on the regulation of S100A7c extend the range of inducers of S100A7c in KCs. Previously, the

cytokines IL-1β, tumor necrosis factor-α, IFN-γ (1), IL-6, IL-17, IL-20, IL-22, IL-24, and oncostatin M (49), as well as late states of KC differentiation (53), have been shown to enhance the expression level of S100A7c. The role of flagellin in the control of S100A7c therefore needs to be further evaluated in combination with other factors, many of which may be highly relevant in the context of cutaneous wounds. Unfortunately, the mouse is not a suitable model system for the investigation of S100A7 regulation because the mouse has lost the *S100A7* genes during evolution (50). The murine gene most closely related to *S100A7* is *S100A15*, the ortholog of which has been lost during human evolution (50). Although *S100A15* may fulfill a role in the mouse equivalent to that of *S100A7* in humans, the regulatory mechanisms of both genes are likely to differ (18).

It is remarkable that the presence of the inducer of S100A7c, flagellin, distinguishes *E. coli* from the nonflagellated bacteria of the commensal skin microflora (54, 55). The specific response of KCs to a flagellated bacterium by induction of S100A7c and hBD-2 expression may exemplify a previously unappreciated ability of the innate immune system to discriminate between potentially harmful microbes and commensal bacteria. In line with this concept, S100A7c has been shown to exert virtually no bactericidal activity against the skin-resident bacterium *Staphylococcus epidermidis* (1). Further studies are necessary to determine whether the antimicrobial defense of the skin is adapted to specifically respond to and attack opportunistic bacteria while preserving the resident microflora. FJ

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4. Chapter II: Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking antimicrobial activities of RNase 5 and RNase 7

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Keywords: Ribonuclease, ribonuclease inhibitor, antimicrobial activity, stratum corneum, serine protease.

Abbreviations: RI, ribonuclease inhibitor; RNase, ribonuclease; KCs, keratinocytes; SC, stratum corneum; CFUs, colony-forming units; qRT-PCR, quantitative real-time PCR; MES, 2-(N-morpholino) ethanesulfonic acid; Ig, immunoglobulin; DEPC diethylpyrocarbonate.

Abstract

The antimicrobial defense of the skin is partially mediated by RNase 7, an abundant ribonuclease of the stratum corneum (SC). Here we investigated the expression and regulation of members of the RNase A family and of the endogenous RNase inhibitor (RI) protein in epidermal keratinocytes (KCs). RT-PCR screening revealed that KCs expressed not only RNase 7 but also RNase 5, which was previously shown to kill the yeast *Candida albicans*, as well as RNase 1, RNase 4 and RI. The mRNA and protein levels of RNase 5, RNase 7 and RI increased during KC differentiation. When RNase 5 and RNase 7 were incubated with RI *in vitro*, not only their ribonucleolytic activities but also their antimicrobial activities were strongly suppressed. Immunochemical analyses revealed that SC contains RNase 5 whereas RI was not detectable. Unlike recombinant RNase 5, recombinant RI was degraded when exposed to SC extract. The addition of aprotinin prevented the degradation of RI, indicating that serine proteases of the SC cleave RI. Taken together, this study adds RNase 5 to the list of antimicrobial factors present in the SC and suggests that proteases indirectly contribute to the defense function of the SC by releasing the RI-mediated inhibition of RNase 5 and RNase 7.

Introduction

The fact that the surface of human skin contains strong ribonuclease (RNase) activity is well known (Tabachnick and Freed, 1961; Steigleder and Raab, 1962). However, the physiological role of the skin ribonuclease activity and its control has not yet been characterized in depth. Recently, RNase 7 was isolated from human stratum corneum (SC), and was shown to exert antimicrobial activity (Harder and Schröder, 2002).

RNase 7 is a member of the human RNase A superfamily, which is encoded by a cluster of thirteen genes located on chromosome 14q11.2. Only eight genes (RNases 1 to 8) of this family encode for proteins which are catalytically active to varying degrees against standard RNA substrates (Dyer and Rosenberg, 2006). In addition to their ribonuclease activity, some members of this family such as RNase 2 (eosinophil-derived neurotoxin; EDN), RNase 3 (eosinophil cationic protein; ECP), RNase 5, the above-mentioned RNase 7 and RNase 8 have antimicrobial properties as well (Domachowske *et al*., 1998; Lehrer *et al*., 1989; Hooper *et al*., 2003; Harder and Schröder, 2002; Rudolph *et al*., 2006), indicating a role in the innate immune defense. RNase 7 is active especially against *Enterococcus faecium, Pseudomonas aeruginosa*, and *Pichia pastoris*, and to a lesser degree against *Propionibacterium acnes* and *E. coli* (Harder and Schröder, 2002; Huang *et al*., 2007). It has been suggested that the antimicrobial activity of RNase 7 is due to pore formation and disruption of the bacterial membrane and is independent of the ribonuclease activity (Huang *et al*., 2007). RNase 5, also known as angiogenin, has been implicated in blood vessel formation (Fett *et al.*, 1985), but a recent study revealed an antimicrobial activity of RNase 5 especially against *Candida albicans* (Hooper *et al*., 2003). RNase

5 is secreted by reconstructed human epidermis *in vitro* (Rendl *et al.*, 2001), however, its contribution to the skin defense is unknown.

Ribonuclease inhibitor (RI) is a horseshoe-shaped leucine-rich repeat protein (Kobe and Deisenhofer, 1993) which constitutes about 0.1% of the total protein in the cytosol of mammalian cells (Leland and Raines, 2001). Via its concave cavity, RI binds at a 1:1 stoichiometry to several members of the RNase A superfamily (Shapiro and Vallee, 1987; Maeda *et al*., 2002; Iyer *et al*., 2005; Johnson *et al*., 2007; Shapiro *et al*., 1986; Papageorgiou *et al*., 1997). RI has been shown to inhibit the enzymatic activity of RNases as well as the antiretroviral activity of RNase 2 (Domachowske and Rosenberg, 1997) and the angiogenic activity of RNase 5 (Shapiro and Vallee, 1987). By contrast, the killing activity of RNase 3 towards microfilaria and trypanosomes is not blocked by RI (Hamann *et al*., 1990; Molina *et al*., 1988).

Here we have investigated the regulation of RNase-RI interactions in the outer layers of the epidermis. We show that *in vitro*, the ribonuclease and antimicrobial activities of RNase 5 and RNase 7, which are both expressed by KCs and present in the SC, are sensitive to RI. Despite the fact that, like RNase 5 and RNase 7, RI is also up-regulated during terminal KC differentiation, it is virtually absent from normal SC. Co-incubation of RI and SC extracts revealed the existence of SC proteolytic activity able to degrade RI. We propose that by degrading RI, proteases of the SC contribute to the antimicrobial defense of the skin.

Materials & Methods

Cell culture

Human primary KCs prepared from neonatal foreskin were obtained from Clonetics (San Diego, CA) and cultured in serum-free keratinocyte growth medium (KGM, Clonetics) as described previously (Rendl *et al*., 2002). Third passage KCs were cultured in 12-well tissue culture plates (Corning Incorporated, Corning, NY) and used at a confluence of 60-70% (designated as proliferating) or maintained for 4 days after reaching confluence (designated as differentiated) by replacing the medium every 24 hours. The differentiation state of KCs was confirmed by determination of the differentiation marker filaggrin. Cultures were grown in triplicates. For *in vitro* stimulation recombinant interleukin (IL)-1α and IL-6 (R&D Systems, Minneapolis, MN, USA) were used.

RNA isolation, reverse transcription and PCR

RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNAs were synthesized as described previously (Abtin *et al*., 2008). Briefly, RNA was reverse-transcribed with murine leukemia virus reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA) and oligo dT primers (Roche Diagnostics, Basel, Switzerland). cDNA sequences of the genes under investigation were obtained from the GenBank. Primers were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA). The following forward (F) and reverse (R)

intron-spanning primers were synthesized by VBC Genomics (VBC Genomics, Vienna, Austria):

RNase 1: F, 5′- GATTGCAGAAACTGGCCTTC-3′; R, 5′-CTGGGGGAACTGTCTGAGTC-3′; RNase 2: F, 5′-CTGAACCCCAGAACAACCAG-3′; R, 5′-GAGCCCAGGTAAACTGTGGA-3′; RNase 3: F, 5′-AACAACCAGCTGGATCAGTTC-3′; R, 5′-CCACTGAGCCCTCGTAAACT-3′; RNase 4: F, 5′-AGAAGCGGGTGAGAAACAAA-3′; R, 5′-AGTAGCGATCACTGCCACCT-3′; RNase 5: F, 5′-AGAAGCGGGTGAGAAACAAA-3′; R, 5′-TGTGGCTCGGTACTGGCATG-3′; RNase 6: F, 5′-ATACACACAGGGCTCGAAGG-3′; R, 5′-GGACACACTGGTCCCCATAG-3′; RNase 7; F, 5′-GAGTCACAGCACGAAGACCA-3′; R, 5′-GGCTGCATGTGCTGAATTT-3′; RNase 8; F, 5′-CAGTGGTTTAAAACTCAGCATG-3′; R, 5′-ACTATGTAAGGTGTGTTCAGGT-3′; RI; F, 5′-CATCAGCTCTGCACTTCGAG-3′; R, 5′-CAAGAGGTTGTCGCTGAGGT-3′; B2M: F, 5′-GATGAGTATGCCTGCCGTGTG-3′; R, 5′-CAATCCAAATGCGGCATCT-3′; ALAS: F, 5′-CCACTGGAAGAGCTGTGTGA-3′; R, 5′-ACCCTCCAACACAACCAAAG-3′; human beta defensin-2 (hBD-2): F, 5′-ATCAGCCATGAGGGTCTTGT-3′; R, 5′-GAGACCACAGGTGCCATTTT-3′.

PCRs were performed in a volume of 50 µl containing 0.2 µl Red Hot® DNA polymerase (ABgene, Hamburg, Germany), 5 µl 10x reaction buffer IV (ABgene), 3 µl $MgCl_2$ (25 mM), 4 µl dNTPs (2.5 mM), 2.5 µl of each primer (10 µM) and 31.8 µl H_2O. The PCR included 5 minutes 94°C for initial denaturing followed by 38 cycles of 1 minute at 94°C, 45 seconds at 60°C, 45 seconds at 72°C and a final extension step at 72°C for 5 minutes. A negative control (omitting cDNA) and a genomic DNA control PCR were performed for each primer pair. Amplicons were subjected to electrophoresis in a 1.5% agarose gel containing ethidium bromide.

Quantitative real-time PCR (qRT-PCR) was performed by the LightCycler technology using the Fast Start SYBR Green I kit for amplification and detection (Roche Diagnostics). In all assays, cDNA was amplified using a standardized program (10 minutes denaturing step and 55 cycles of 5 seconds at 95°C; 15 seconds at 65°C, and 15 seconds at 72°C; melting point analysis in 0.1°C steps; final cooling step). Each LightCycler capillary was loaded with 1.5 µl DNA master mix, 1.8 µl $MgCl_2$ (25 mM), 10.2 µl H_2O, and 0.5 µl of each primer (10 µM). Relative quantification of target gene expression and amplification efficiencies were performed using a mathematical model as described previously (Pfaffl, 2001). The expression of the target gene was normalized to the expression of the housekeeping genes 5-aminolevulinic acid synthase (ALAS) and/or β-2-microglobulin (B2M). Similar target mRNA quantifications were obtained with either housekeeping gene as standard. All qRT-PCRs were performed in triplicate. The specificity of PCR reactions was confirmed by sequencing of the PCR products. For the identification of RNase 1 variants, PCR products were cloned into a pCR®2.1-TOPO® plasmid (Invitrogen) according to the manufacturer's instructions and clones with different insert lengths were sequenced.

Quantification of RNase 5 and RNase 7

Culture supernatants of KCs or SC extracts were depleted by centrifugation of insoluble cell fragments and stored at -20°C until analysis. The concentration of RNase 5 was determined by ELISA (R&D Systems) according to the manufacturer's instructions.

The concentration of RNase 7 was determined with an in-house RNase 7 ELISA, which has been developed using affinity-purified goat RNase 7 antibodies as will be described elsewhere[1].

[1] Könten, B., Gläser, R., Schröder, J.M., and Harder, J. (2008). RNase 7 contributes to the cutaneous defense against *Enterococcus faecium*. unpublished.

Immunohistochemical analysis

Normal human skin was kindly provided by the Department of Plastic Surgery, Medical University of Vienna, Austria. Immunohistochemical analysis was performed on paraffin-embedded, formalin-fixed skin sections (4 µm) using the indirect immunoperoxidase staining technique. Briefly, endogenous peroxidase activity of sections were blocked by 0.3% H_2O_2/PBS (pH 7.5) for 15 minutes, washed with PBS and then blocked for 30 minutes with 10% goat serum (PAA, Linz, Austria) diluted in 2% BSA/PBS. A polyclonal rabbit IgG anti-porcine RI antibody (kindly provided by J. Hofsteenge, Friedrich Miescher-Institut, Basel, Switzerland) was used. This antibody cross-reacts with human RI (Blazquez *et al*., 1996). Before immunolabeling with anti-RI or a rabbit IgG isotype control (Santa Cruz Biotechnology, Santa Cruz, CA) sections were pre-treated in ChemMate™ Target Retrieval Solution (Dako Cytomation, Glostrup, Denmark) in a microwave oven for 2 x 5 minutes at 500 W. The anti-RI antibody and/or rabbit IgG isotype control (both at 2 µg/ml) were diluted in 2% BSA/PBS and applied for 1 hour on sections at room temperature. After washing in PBS, slides were incubated with biotinylated goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA) for 30 minutes, washed in PBS and then exposed to StreptABComplex/HRP (Dako Cytomation) for 30 minutes. The AEC

(aminoethylcarbazole) substrate system (Dako Cytomation) was used as the chromogen. Sections were counter-stained with hematoxylin.

Ribonuclease activity assay

Recombinant RNase 5 (R&D Systems), purified RNase 7 (Harder and Schröder, 2002) or SC extract were pre-incubated with RI in 30 mM Tris (pH 7.5) containing 30 mM NaCl in a total volume of 15 µl for 10 minutes at 37°C. Afterwards 1.5 µg of total RNA were added to the reactions and incubated for 5 minutes at 37°C. Then RNA loading buffer (Fermentas, Glen Burnie, MD) was added and the mixture was heated for 5 minutes at 65°C, cooled on ice and subsequently electrophoresed through a 1% agarose gel containing ethidium bromide.

Ribonuclease activity of RNase 5 was blocked by pre-incubation with DEPC or benzopurpurin B (both from Sigma-Aldrich, Vienna, Austria) in 10 mM sodium phosphate buffer (pH 7.4) in a total volume of 15 µl for 1 hour at 37°C.

Antimicrobial activity assay

C. albicans ATCC 90028 and a vancomycin-resistant *E. faecium* clinical isolate were cultured overnight in tryptic soy broth (TSB; Fluka, Buchs, Switzerland) with agitation at 37°C, subcultured the next day in fresh TSB and then grown to optical densities of 1.0 at 600 nm for *E. faecium* or at 450 nm for *C. albicans*, respectively. Cells were washed twice with PBS and diluted to 10^4 colony-forming units (CFUs) per ml in 10 mM sodium phosphate buffer (pH 7.4) containing 1% (v/v) TSB.

Recombinant human RNase 5 (> 95% purity) was purchased from R&D Systems and natural RNase 7 was purified from heel SC as described previously (Harder and

Schröder, 2002). Recombinant human RI was purchased from Ambion (Warrington, UK). The antimicrobial activity was tested using a microdilution assay system. Tubes were pre-incubated with 2% BSA/PBS for 1 hour at 37°C and were then rinsed with 10 mM sodium phosphate buffer (pH 7.4) containing 1% (v/v) TSB. Since RI is provided with a storage buffer containing 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM dithiothreitol and 50% (v/v) glycerol, the same buffer without RI was used in control reactions. For inhibitory activity experiments, RNase 5 or RNase 7 were pre-incubated with buffer alone or with RI in 10 µl volumes of 10 mM sodium phosphate buffer (pH 7.4) for 1 hour at 37°C. 100 µl of the above-mentioned *C. albicans* or *E. faecium* suspensions were then added and incubated for 3 hours at 37°C with agitation. Afterwards, 20 µl aliquots were plated on Luria-Bertani agar or Sabouraud-Dextrose agar for *E. faecium* and *C. albicans*, respectively. Plates were incubated overnight at 37°C. On the following day the number of CFUs was determined. The growth inhibitory activities of the substances were calculated as follows: (CFUs after incubation with substance) / (CFUs after incubation without substance) x 100, which represents the percentage of remaining CFUs after treatment.

Antimicrobial activity and calculation of growth inhibition of recombinant RNase 5 towards *P. aeruginosa* ATCC 27853, methicillin-resistant *St. aureus* ATCC 33592 (MRSA), *E. coli* ATCC 35218 and a clinical isolate of group A *Streptococcus* (GAS) were performed as described above except that GAS was grown in TSB supplemented with 10 % heat-inactivated fetal calf serum. For the determination of CFUs, GAS was plated on Columbia agar, and *P. aeruginosa, St. aureus* and *E. coli* were plated on Luria-Bertani agar.

To test whether RNase 5 antimicrobial activity is blocked by DEPC or benzopurpurin B, RNase 5 was pre-incubated in 10 µl volumes of sodium phosphate buffer (10 mM, pH 7.4) for 1 hour at 37°C with increasing concentrations of DEPC or

benzopurpurin B. 100 µl of the above-mentioned *C. albicans* suspension was then added and incubated for 3 hours at 37°C with agitation. The growth inhibitory effect was calculated as described above.

SC extraction and incubation with RI

SC obtained from heels of healthy volunteers was extracted with 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 7.5 (Sigma-Aldrich). A ratio of 25 mg SC to 200 µl buffer was used. SC was homogenised in 1.5 ml tubes (Eppendorf, Hamburg, Germany) with a micropestle (Eppendorf) on ice (1-2 minutes) and then incubated for 1 hour rotating at 4°C. Thereafter, tubes were centrifuged with 15000 *g* for 20 minutes at 4°C. Subsequently, the supernatant was aliquoted and stored at -20°C until analysis. Sterility was verified by plating extracts on blood agar. Protein concentrations of SC extracts were measured by the bicinchoninic acid method (BCA; Pierce, Rockford, IL).

10 µg SC extract were co-incubated with 20 units (U) recombinant human RI or 50 ng recombinant RNase 5 (R&D Systems) at 37°C with agitation. The final reaction volume was 30 µl. Aliquots of SC were pre-incubated for 90 minutes at 37°C with the following protease inhibitors: complete protease inhibitor cocktail (EDTA free), leupeptin, aprotinin (all from Roche Diagnostic), EDTA pH 7.5 (Merck, Darmstadt, Germany) and GM6001 (Calbiochem, Inc., La Jolla, CA, USA).

Western blot analysis

For analysis of RI expression, KC monolayer cultures were lyzed in 1% Nonidet P-40 (Igepal CA-630) (Sigma-Aldrich), and insoluble cell debris was removed by

centrifugation. The epidermis of skin biopsies was separated by dispase II (30 min 37°C; Roche Diagnostic) from the dermis. Afterwards epidermis and/or heel scales were quick-frozen with liquid nitrogen, ground in a mortar and suspended in 100 mM MES pH 7.5 and left rotating for 1 hour at 4°C. Thereafter, samples were centrifuged (15000 *g* for 20 minutes at 4°C) and the supernatant was aliquoted. The protein concentration was measured by the BCA method (Pierce). Western blot analysis was performed as described previously (Mildner *et al*., 2006). Equal loading of protein lysates was confirmed by Ponceau staining of the membrane. Rabbit anti-RI antibody was used for detection of RI protein (dilution 1:3000). A peroxidase-conjugated goat anti-rabbit IgG antibody (dilution 1:10000, Pierce) was used as a secondary antibody. Mouse anti-caspase-14 (dilution 1:1000, MBL, Naka-ku Nagoya, Japan) was used for the detection of the proform and the active form of caspase-14 with a secondary peroxidase-conjugated sheep anti-mouse IgG antibody (dilution 1:10000, Amersham, Buckinghamshire, UK). For RNase 5 detection a polyclonal goat antibody (R&D Systems) and a secondary peroxidase-conjugated rabbit anti-goat (dilution 1:10000, Dako Cytomation) antibody were used. The membranes were developed using the Chemiglow reagent (Alpha Innotech, San Leandro, CA) according to the manufacturer's instructions.

Results

Epidermal KCs express RNases 1, 4, 5 and 7 as well as RI

To provide the basis for a better understanding of the potential roles of RNase–RI interactions in the skin, we determined the mRNA expression profile of RNases 1 to 8 and of RI in cultured epidermal KCs. RNases 1, 4, 5 and 7 as well as RI were detected by reverse transcription-PCR (RT-PCR) whereas other RNases were not. RNases 4, 5 and 7 were expressed in both proliferating (data not shown) and in differentiated KCs (Figure 1a). Differentiated KCs also expressed RNase 1 splice variants 2 (lower band, GenBank acc. no. NM198234) and 4 (upper band, GenBank acc. no. NM002933), which differ in the 5´-untranslated region but code for the same protein. For RNase 4, splice variant 2 (GenBank acc. no. NM002937) and for RNase 5, splice variant 1 (GenBank acc. no. NM001145) could be amplified (Figure 1a, upper panel). The identity of the PCR products was determined by direct sequencing and the integrity of the assays for RNases 2, 3, 6 and 8, which were not detected at the cDNA level, was confirmed by PCR amplification using genomic DNA as a template (Figure 1a, lower panel). The expression of RNases 9 to 13 was not investigated, since they do not have all elements to support ribonuclease activity (Dyer and Rosenberg, 2006).

Focusing on RNases with antimicrobial activities (Harder and Schröder, 2002; Hooper *et al*., 2003), we investigated the regulation of RNase 5 and RNase 7 during KC differentiation. By quantitative real-time PCR (qRT-PCR), we found that the expression of RNase 5 and RNase 7 mRNAs were up-regulated 4- and 9-fold, respectively, in differentiated cultures compared to proliferating KCs (Figure 1b, c). The concentrations of secreted RNase 5 and RNase 7 proteins were determined by

enzyme-linked immunosorbent assay (ELISA). Culture medium conditioned by differentiated KCs for 24 hours contained approximately 4- and 6-fold higher levels of RNase 5 and RNase 7, respectively, than medium conditioned by proliferating cells (Figure 1d, e). Since the cell biomass, estimated by protein quantification, was elevated by only 1.7-fold (data not shown), these data show that the production of RNase 5 and RNase 7 was up-regulated during differentiation of KCs. The absolute concentration of secreted RNase 5 was smaller than that of RNase 7 (Figure 1d, e). Similarly, the amount of soluble RNase 5 that we could extract from SC of human heels, *i.e.* 57 ± 38 pg per mg SC, was much smaller than the amount of RNase 7 that has been extracted from SC of different donors, *i.e.* up to 6000 pg per mg SC (Harder and Schröder, 2002).

RI is expressed in the epidermis but is absent in SC

We next investigated the expression of RI in human skin (n=5) by immunohistochemistry. RI was expressed in all viable layers of the epidermis, with the strongest expression being detectable in the uppermost layers of the epidermis. By contrast, RI immunoreactivity was not detectable in the SC (Figure 2a).

The expression of RI during KC differentiation *in vitro* was investigated by qRT-PCR and Western blot analysis. RI was up-regulated at both the mRNA (Figure 2b) and protein level (Figure 2c) in differentiated versus proliferating KCs.

RI blocks ribonuclease activity of SC as well as the antimicrobial activity of RNase 5 and RNase 7

In accordance with a previous report (Probst *et al.*, 2006), we readily detected ribonuclease activity in SC extracts. Addition of recombinant human RI virtually abrogated this activity, suggesting that the RNases of the SC can be bound and neutralized by RI (Figure 3a). Moreover, RI blocked the ribonuclease activity of recombinant RNase 5 and purified RNase 7 from SC *in vitro,* confirming the functionality of our proteins (Figure 3b).

Next we tested whether RI is also able to interfere with the antimicrobial activities of RNase 5 and RNase 7 against *C. albicans* and *E. faecium*, respectively (Hooper *et al*., 2003; Harder and Schröder, 2002). In the absence of RI, antimicrobial activity of RNase 5 and RNase 7 strongly reduced the colony-forming units (CFUs) of *C. albicans* and *E. faecium*, respectively. Pre-incubation with RI strongly suppressed the antimicrobial activity of both RNases, whereas addition of RI alone had no effect on either microbe (Figure 4a, b). These results demonstrate, for the first time, that RI not only blocks ribonuclease activity but also suppresses the antimicrobial activity of RNase 5 and RNase 7.

The antimicrobial effect of RNase 5 is blocked by chemical inhibitors of its ribonuclease activity

Since RI interacts with a large portion of the surface of target RNases (Papageorgiou *et al*., 1997) and not specifically with the catalytic centre, the blockade of antimicrobial activities of RNase 5 and RNase 7 may be mediated by the interference with the RNase catalytic activity or by sterical blockade of a bactericidal

protein domain. The antimicrobial activity of RNase 7 is mediated by a cluster of lysine residues and does not depend on catalytic activity (Huang *et al*., 2007). Amino acid sequence alignment showed that the residues critical for the antibacterial activity of RNase 7 are not conserved in RNase 5 (Figure S1). Therefore, we hypothesized that RNase 5 utilizes a different mechanism, possibly involving its ribonucleolytic activity to kill *Candida*. RNase 5 was pre-incubated with two chemical inhibitors of its ribonuclease activity, namely diethylpyrocarbonate (DEPC) (Shapiro *et al*., 1987) and benzopurpurin B (Jenkins and Shapiro, 2003). As expected, both substances inhibited the degradation of RNA by RNase 5 in a dose-dependent manner (Figure 5a, b). In close correlation with this blockade, both DEPC and benzopurpurin B also inhibited the candida-cidal activity of RNase 5 (Figure 5c, d), indicating that the antimicrobial effect of RNase 5 depends on its ribonucleolytic activity.

To further characterize the antimicrobial potential of RNase 5, we also tested *Pseudomonas aeruginosa, Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA)*, Enterococcus faecium* and group A *Streptococcus* (GAS) for their sensitivity towards RNase 5 treatment. However, among the microorganisms investigated, RNase 5 was only effective at killing *C. albicans* (Figure S2). To evaluate whether enhancement of expression of RNase 5 might contribute to the control of its antimicrobial activity, KCs were stimulated with heat-inactivated *C. albicans* or with the proinflammatory cytokines, interleukin (IL)-1α and IL-6. Neither treatment altered the expression of RNase 5 in KCs (Figure S3). Similarly, the expression of the RNase 5 inhibitor, RI, was not changed by proinflammatory stimuli (Figure S3).

RI is degraded by SC extract

Since immunohistochemistry suggested that RI, although present in the viable epidermal layers, was absent from SC (Figure 2a), we extracted proteins from the total epidermis and from SC alone and performed Western blot analysis. RI was detected in lysates of the epidermis but, confirming the immunohistochemistry data, was absent in SC extracts (Figure 6a). The integrity of SC extract was confirmed by Western blot analysis of caspase-14, which was readily detectable in its mature form (Figure. 6a) (Fischer *et al*., 2004).

The fact that only the viable layers of the epidermis but not the SC contained RI indicated the existence of a mechanism to eliminate RI during conversion of KCs into corneocytes. To investigate this hypothesis, recombinant human RI was co-incubated with SC extracts and then analyzed by Western blot. Under these conditions RI protein was degraded, with an intermediate cleavage product of RI being detectable (Figure 6b). Incubation of RI alone with the extraction buffer demonstrated stability of the protein under assay conditions. To investigate which types of enzymes are responsible for RI degradation, SC extracts were pre-treated with various protease inhibitors (*i.e.* aprotinin for serine proteases, leupeptin for thiol proteases, EDTA and GM6001 for metalloproteases, and a protease inhibitor cocktail (EDTA free) for serine and cysteine proteases) and then analyzed for their ability to degrade RI. The inhibitor cocktail as well as aprotinin and EDTA inhibited proteases from degrading RI (Figure 6b). Notably, EDTA was not effective at a concentration, that is considered sufficient to block metalloproteases, but only at a concentration, at which EDTA also interferes with the activity of some serine proteases (Beynon and Bond, 2001). The metalloprotease-specific inhibitor, GM6001, showed virtually no inhibition. This inhibition profile suggested that serine proteases are mainly accountable for RI

degradation by SC extracts. In contrast to RI, recombinant RNase 5 was stable when co-incubated with SC extracts (Figure S4).

Discussion

In this study, we have explored the expression of all functional members of the RNase A superfamily, as well as of RI in epidermal KCs and propose that differential breakdown of RI during SC formation contributes to the antimicrobial activity of the skin surface. Our expression screening showed that 4 members of the RNase A family are expressed by KCs. Two of these RNases, namely RNase 5 and RNase 7, act as antimicrobial proteins whereas no such activity has been reported for RNase 1 and RNase 4. This study is the first to demonstrate the presence of RNase 5 in human SC and thereby establishes another antimicrobial factor of the skin defense against microbes. Similar to RNase 7, RNase 5 was up-regulated during differentiation. However, proinflammatory or microbial inducers of expression could thus far be identified for RNase 7 only (Harder and Schröder, 2002). The rate of production of RNase 7 was approximately 5 times higher than that of RNase 5 in cultured KCs, and much higher amounts of RNase 7 than RNase 5 could be extracted from human SC, indicating that RNase 7 contributes more to the ribonucleolytic and antimicrobial activities of the skin surface. Nevertheless, it is conceivable that the local concentration of RNase 5, within distinct SC microcompartments may be sufficient for effective antimicrobial activity.

Importantly, the antimicrobial activity spectra of RNase 5 and RNase 7 appear to differ significantly. In particular, *C. albicans* is killed by low concentrations of RNase 5 (Hooper *et al*., 2003); this study) but much less effectively by RNase 7 (Harder and Schröder, 2002) whereas several bacteria are killed by RNase 7 (Harder and

Schröder, 2002) and not by RNase 5 (this study). The relative contribution of RNase 5 to the innate immune defense of the skin, which is mediated by a plethora of peptides and other substances (Schröder and Harder, 2006), remains to be determined in future studies.

Our study provides the first characterization of endogenous RI in the epidermis and, importantly, extends its range of functions to the inhibition of RNase antimicrobial activities. Both the candida-cidal activity of RNase 5 and the enterococcus-cidal activity of RNase 7 were effectively blocked by recombinant RI. Previous reports have shown that RI suppresses the ribonuclease activity, which is low as compared to other members of the RNase A family, and the angiogenic activity of RNase 5 (Shapiro and Vallee, 1987; Maeda *et al*., 2002; Iyer *et al*., 2005; Johnson *et al*., 2007; Shapiro *et al*., 1986; Papageorgiou *et al*., 1997). Two small chemical substances, DEPC and benzopurpurin B, which inhibit the catalytic activity of RNase 5, also suppressed its antimicrobial activity. The binding site of benzopurpurin B on RNase 5 has been determined (Jenkins and Shapiro, 2003) whereas the various RNase 5 subsites, likely to be targeted by DEPC via modification of lysine and histidine residues, have not been investigated for their individual contribution to the inhibitory effect of DEPC. Therefore, it appears likely that RI suppresses the antimicrobial function of RNase 5 by inhibiting its catalytic centre. However, further studies using point-mutated variants of RNase 5 are required to substantiate this hypothesis.

Although the antimicrobial activity of RNase 7 does not depend on ribonucleolytic activity (Huang *et al*., 2007), our data demonstrate that it is suppressed by RI. There are two possible explanations for this finding: (1) It is possible that RI, which has a horseshoe-like shape and binds RNases within its concave cavity, masks the amino acid residues responsible for antimicrobial action. (2) The interaction of RI with

RNase 1 has recently been shown to induce conformational changes of RNase 1 (Johnson *et al*., 2007). Since the interaction of RI and RNase 7 is likely to occur in a similar manner, a conformational change might also abrogate the antimicrobial activity of RNase 7.

The finding that RI is expressed strongly in the uppermost layers of the epidermis but is absent in SC and the fact that SC contains ribonuclease activity and antimicrobial activity of RNase 7 (Harder and Schröder, 2002), indicated that a physiological mechanism exists to prevent inhibition of skin surface ribonuclease functions. In support of this hypothesis, the results of our co-incubation experiments using recombinant RI and SC extract showed degradation of RI. Our finding that aprotinin blocked the degradation of RI indicates that this breakdown is mediated by serine proteases such as SCTE (stratum corneum tryptic enzyme, kallikrein5) or SCCE (stratum corneum chymotryptic protease, kallikrein7). EDTA, which scavenges divalent cations, also blocked the reaction, however, only when it was used at a concentration higher than that required to block metalloproteases but sufficient to also inhibit some serine proteases (Beynon and Bond, 2001). Further studies are necessary to determine the molecular identity of the RI-degrading protease(s).

The proteolysis of RI by SC proteases establishes a role of proteases in the antimicrobial defense function of the skin. Conceptually, this control mechanism resembles the control of the pro-apopototic DNase CAD/DFFB by the inhibitor ICAD/DFFA. ICAD inhibits CAD unless it is cleaved by the cysteine protease caspase-3 (Sakahira *et al.*, 1998). It remains to be investigated whether proteolysis of RI enhances the breakdown of cellular RNAs during differentiation-associated programmed cell death of KCs. Irrespective of this potential role in cellular remodelling, breakdown of RI appears to facilitate the activities of RNase after corneocyte formation. A more direct dependence on proteases of protein activation in

the SC has been demonstrated for cathelicidin, which is converted to the antimicrobial peptide LL-37 by the action of the serine proteases kallikrein 5 (SCTE) and kallikrein 7 (SCCE) (Yamasaki *et al*., 2006). The processing of cathelicidin by these proteases does not require a distinct stimulus and leads to the constitutive presence of LL-37 on the skin surface (Yamasaki *et al*., 2006). Similarly, the proteolytic degradation of RI is likely to occur in a constitutive manner. Taken together, the disturbance of SC proteolytic activities, either caused by genetic defects or environmental influences, may compromise antimicrobial skin defence.

In summary, this study identifies RNase 5 as a component of the innate immune system of the skin and reveals a novel level of regulation of antimicrobial RNases in human SC, *i.e.* the removal of their inhibitor by proteases.

Figures

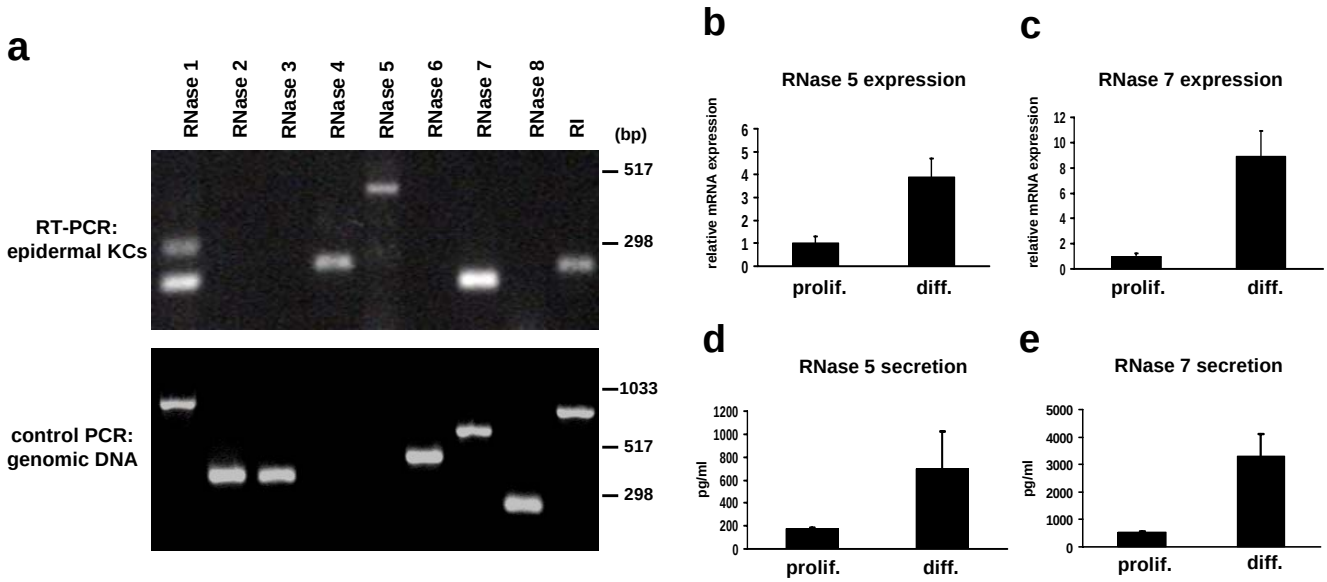

Figure 1. KCs express RNases 1, 4, 5 and 7 as well as RI.

Total RNA was isolated from proliferating (prolif.) and differentiated (diff.) human KC cultures and reverse-transcribed to cDNA. (a) cDNAs of differentiated KCs were amplified by PCRs specific for RNases 1 through 8 and for RI. Amplicons were separated on a 1.5% agarose gel containing ethidium bromide. To confirm the efficiency of each PCR, reactions with the same primer combinations were performed on genomic DNA. In these control reactions, exonic and intronic sequences of all genes except RNases 4 and 5, in which the primer annealing sites are separated by a long intron, were successfully amplified (lower panel). RNase 5 (b) and RNase 7 (c) expression in KCs was determined by qRT-PCR. Relative gene expression levels were normalized to the expression of the housekeeping gene ALAS. The mean values of the expression levels relative to the expression level in proliferating cells are displayed. Secretion of RNase 5 (d) and RNase 7 (e) of proliferating and differentiated KCs was determined by ELISAs. Data represent the mean ± SD. bp, base pair.

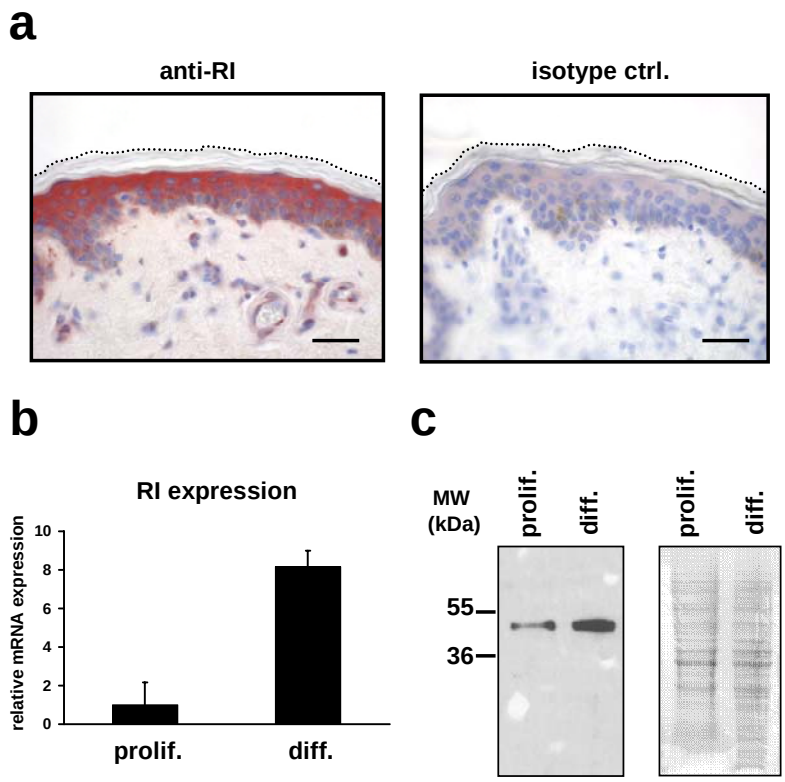

Figure 2. RI is expressed in the epidermis and in cultured KCs.

(a) RI was detected by immunohistochemistry in normal human skin. An isotype antibody was used as negative control (ctrl.). The outer border of the SC is marked by a dotted line. Bars=40 µm. (b) qRT-PCR of RI in proliferating (prolif.) and differentiated (diff.) KCs. Relative gene expression levels were normalized to the expression of the housekeeping gene ALAS. Data represent the mean ± SD. (c) Western blot analysis of RI in proliferating and differentiated KCs (left panel). Ponceau staining of the membrane served as loading control of protein lysates (right panel). MW, molecular weight; RI, ribonuclease inhibitor.

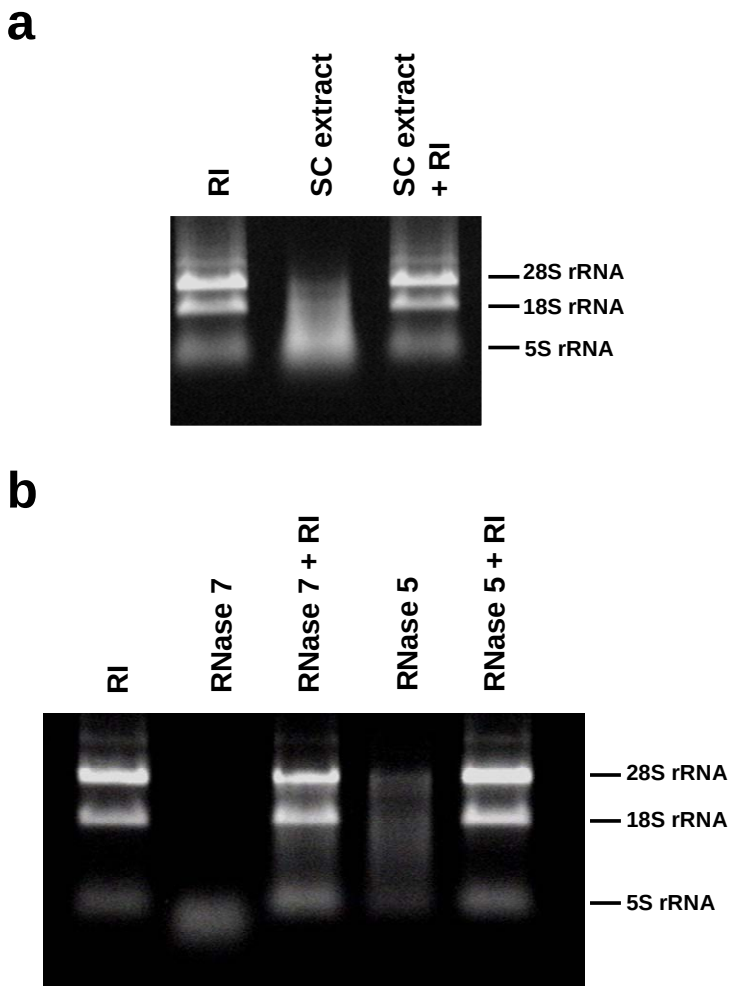

Figure 3. Ribonuclease activities of SC extract, RNase 5 and RNase 7 are blocked by RI.

(a) SC extract (30 ng), (b) RNase 5 (25 ng) and RNase 7 (5 ng) were pre-incubated with or without RI (30 U for SC extract and 40 U for RNase 5 and RNase 7). Subsequently, the mixtures were incubated with total RNA (1.5 μg) prepared from KCs as described in the Materials and Methods section. Thereafter samples were electrophoresed through an RNase-free agarose gel containing ethidium bromide. Positions of the 28S, 18S, 5S rRNA bands are indicated. U, units.

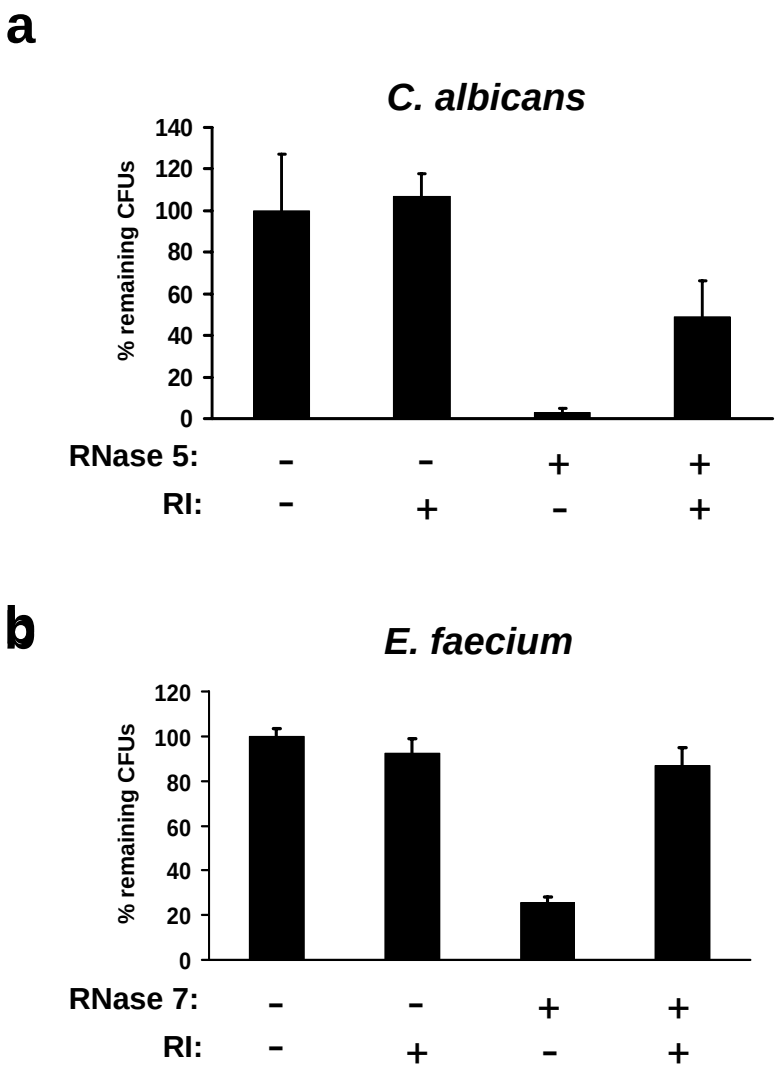

Figure 4. RI inhibits antimicrobial activity of RNase 5 and RNase 7.

(a) RNase 5 (0.2 µM) or (b) RNase 7 (0.33 µM) were incubated with and without RI (40 and 20 U for RNase 5 and RNase 7, respectively) and then tested for their ability to inhibit the growth of *C. albicans* or *E. faecium*, respectively. In addition, *C. albicans* or *E. faecium* were treated with buffer and RI only. The results are displayed as percent of remaining CFUs in relation to untreated controls (100%). Data represent the mean of triplicates ± SD. CFU, colony-forming units.

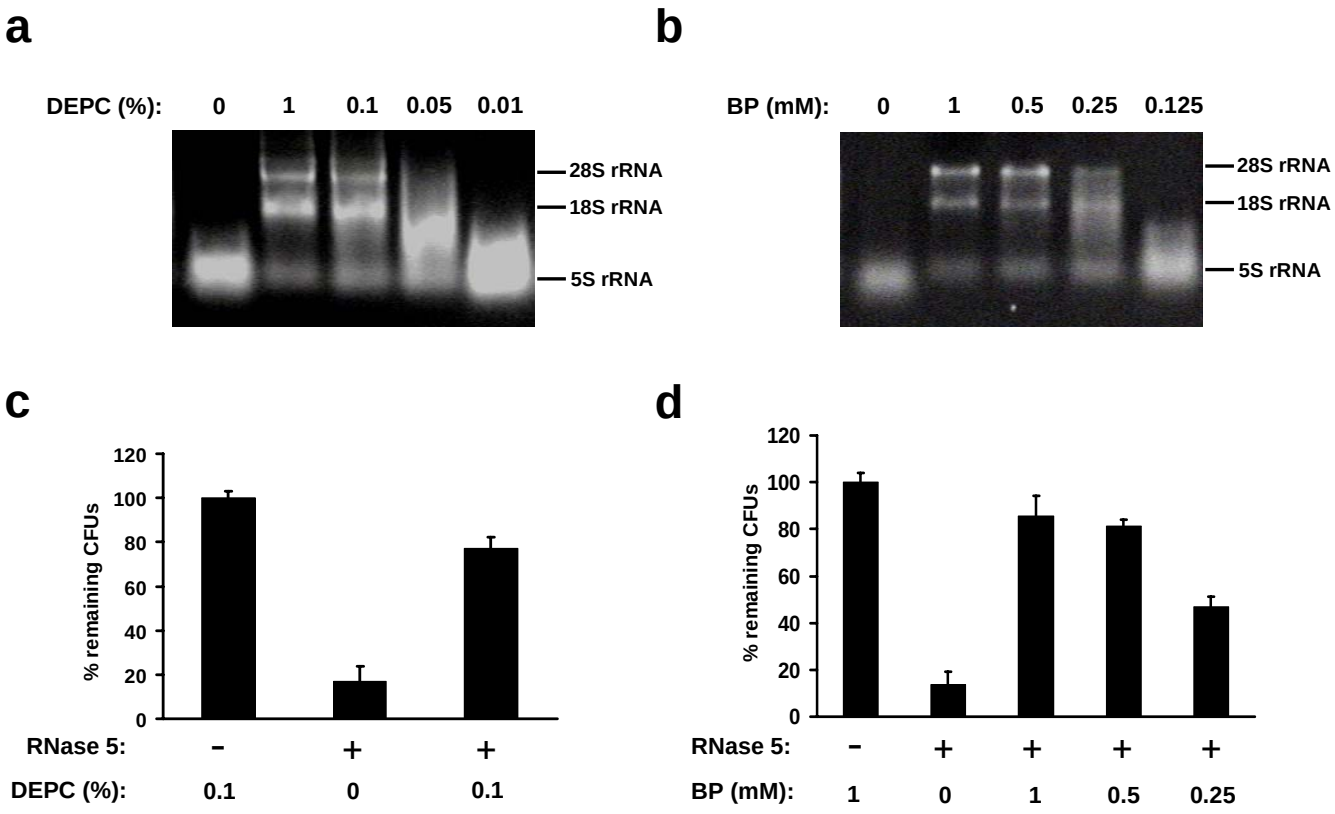

Figure 5. Antimicrobial effect of RNase 5 depends on its ribonuclease activity.

(a) Ribonuclease activity of RNase 5 (0.6 µM) was tested in the presence of diethylpyrocarbonate (DEPC) or (b) benzopurpurin B (BP) as described in the Materials and Methods section. Positions of the 28S, 18S, 5S rRNA bands are indicated. (c) RNase 5 (0.2 µM) was pre-incubated with DEPC or (d) BP and thereafter tested for its ability to inhibit the growth of *C. albicans*. The results are displayed as percent of remaining CFUs in relation to only DEPC or BP treated controls (100%). Data represent the mean of triplicates ± SD.

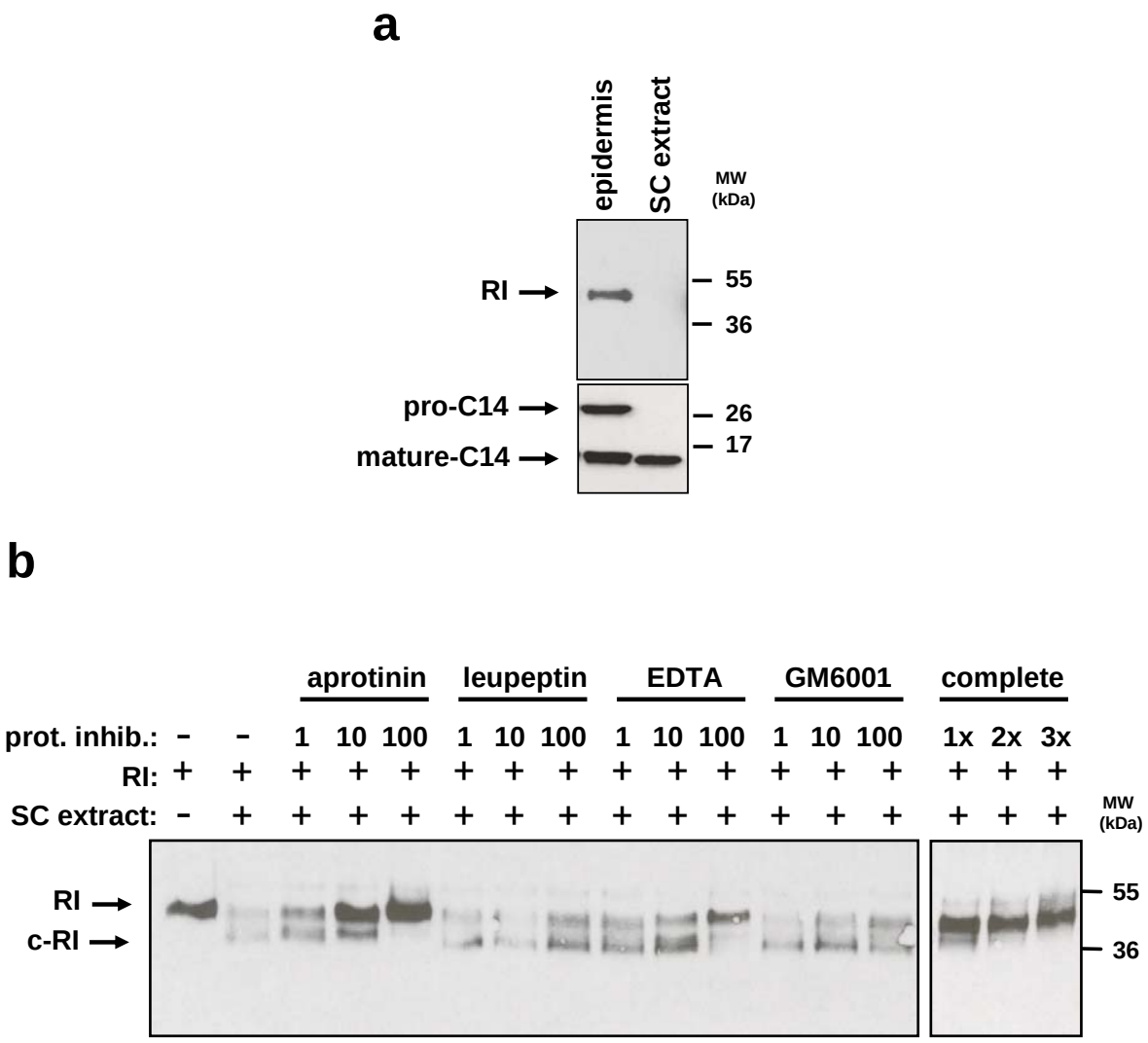

Figure 6. RI is absent in the SC and is degraded by SC extract.

(a) Epidermis and SC were extracted as described in the Materials and Methods section and subjected to Western blot analysis for RI and caspase-14 (C14). Positions of a molecular weight (MW) marker are indicated on the right. Note that the SC contains only the mature form of caspase-14 (Fischer *et al*., 2004) whereas both pro-caspase-14 (pro-C14) and mature caspase-14 are present in total epidermis. (b) SC extract were pre-incubated with increasing concentrations (1 to 100 µM) of the protease inhibitors aprotinin, leupeptin, EDTA, GM6001 or complete protease inhibitor cocktail, EDTA free (complete; 1x to 3x) and subsequently incubated with RI, as described in the Materials and Methods section. RI was detected by Western blot analysis. prot. inhib., protease inhibitor. c-RI, cleaved RI protein.

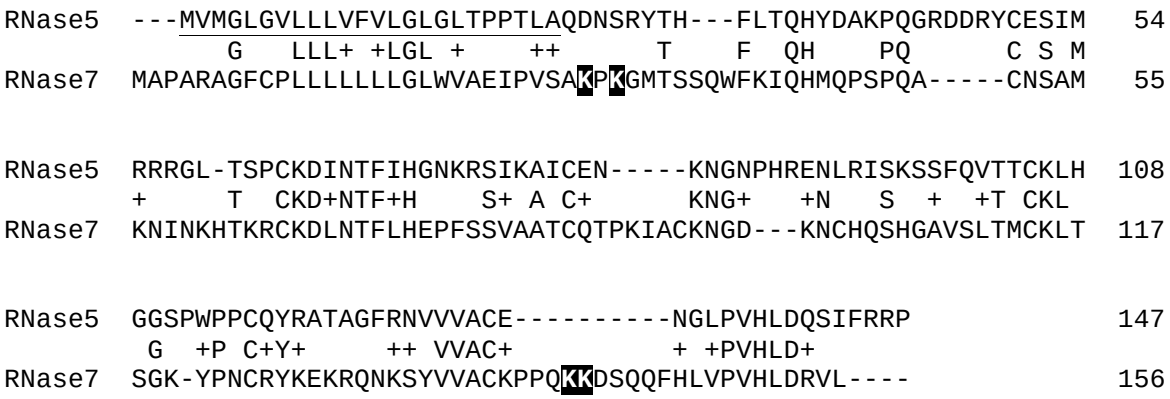

Figure S1. Amino acid sequence alignment of RNase 5 and RNase 7.

Residues critical for the antimicrobial activity of RNase 7 (Huang *et al*., 2007) are shown as white letters on black background. The signal peptide of RNase 5, which is missing in the mature protein, is underlined.

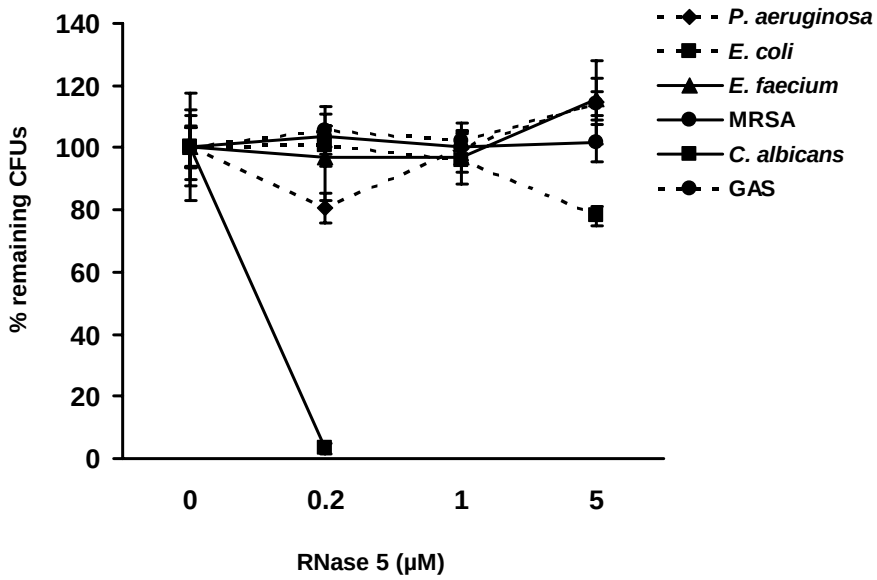

Figure S2. Antimicrobial activity of RNase 5 against potential skin pathogens.

Antimicrobial activity of increasing concentrations of RNase 5 was tested against Gram-positive (*E. faecium,* GAS and MRSA) and Gram-negative (*P. aeruginosa* and *E. coli*) bacteria and the yeast *C. albicans* . Data represent the mean of triplicates ± SD.

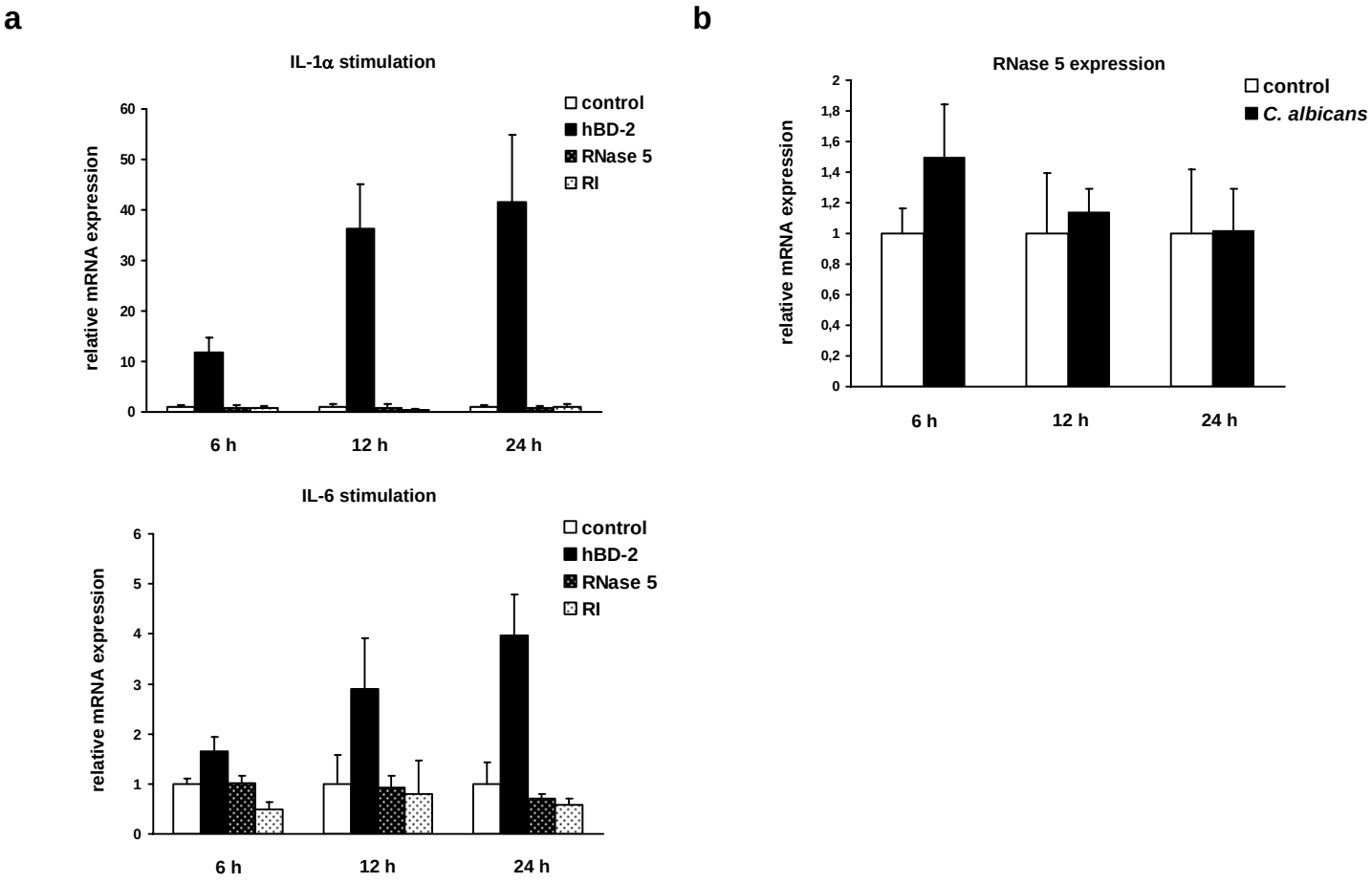

Figure S3. RNase 5 and/or RI are not induced by IL-1α, IL-6 or *C. albicans*.

Proliferating KCs were stimulated for 6, 12 and 24 hours (h) with (a) IL-1α or IL-6 (both at 10 ng/ml) and (b) with heat-inactivated *C. albicans* (10^7 CFUs/ml). After stimulation RNA was isolated and the relative gene expressions of RNase 5, RI and/or human beta defensin-2 (hBD-2) were determined by qRT-PCR. Relative gene expression levels were normalized to the expression of the housekeeping gene ALAS. Data represent the mean ± SD. The induction of hBD-2 in (a) served as a control response to IL-1α and IL-6 stimulation.

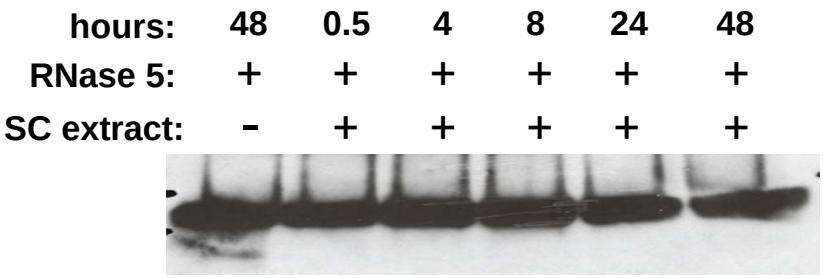

Figure S4. RNase 5 is not degraded by SC extract.

RNase 5 (50 ng) was incubated with and without SC (10 µg protein) extract for the indicated times and detected by Western blot analysis.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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5. Discussion

5.1. The role of flagellin in S100A7c expression by KCs.

S100A7c has been found to be expressed in patchy pattern in the epidermis (Gläser et al., 2005; Schröder and Harder, 2006), indicating that it may be up-regulated by external substances entering the epidermis through microwounds. However, distinct exogenous molecular triggers of S100A7c have remained elusive. Our demonstration that flagellin induces S100A7c strongly supports the concept that the expression of S100A7c is indeed positively up-regulated in response to bacteria. The finding that flagellin but no other bacterial components trigger S100A7c expression makes physiological sense, because the most potent antimicrobial activity of S100A7c is directed to *E. coli*, a flagellated bacterium (Gläser et al., 2005). Our results show that S100A7c is induced by concentrations of flagellin that are only slightly higher than those necessary for the induction of hBD-2, another antimicrobial peptide. Although it is difficult to estimate the concentration of flagellin on the skin surface, it is likely that any focal growth of flagellated bacteria will be associated with sufficiently high concentration of flagellin. Nevertheless, the relevance of flagellin in the induction of S100A7c and hBD-2 remain to be investigated *in vivo*.

The potent *E. coli*-cidal activity of S100A7c might explain the inability of *E. coli* in colonizing healthy human skin. Indeed, an important *in vivo* function of S100A7c has demonstrated that *E. coli* is effectively killed on human skin and that this killing activity was reduced by a neutralizing antibody to S100A7c *in vivo* (Gläser et al., 2005). Gene-deficient murine models have demonstrated that lack of a single antimicrobial peptide makes mice more susceptible to infections. For example, mice deficient in the expression of CRAMP (the mouse homologue to the human cathelicidin) were more susceptible to skin infections caused by group A *Streptococcus* (GAS) (Nizet et al., 2001). These mice were also

susceptible to urinary tract infections caused by uropathogenic *E. coli* (Chromek et al., 2006).

A key result was the demonstration that flagellin expression by *E. coli* strain was associated with the regulation of S100A7c. We show that a flagellin-deficient *E. coli* strain (ΔFliC) was unable to up-regulate the expression of S100A7c compared to wild-type strain. The finding that flagellin induces S100A7c expression and the data observed with the flagellin-deficient *E. coli* strain strongly suggest that flagellin is the only relevant *E. coli* component accountable for S100A7c stimulation in KCs.

With regard to possible mechanisms, we have identified the flagellin receptor TLR5 as an essential element of sensory and signaling system for *E. coli.* By using RNA interference technology, knock-down of TLR5 in KCs dramatically reduced responsiveness to *E. coli*, suggesting that the presence of *E. coli* is recognized by TLR5 and therefore a successful elimination is TLR5-dependent. In line with this concept, the significance of TLR5 for *E. coli* recognition was demonstrated in a murine model *in vivo* (Andersen-Nissen et al., 2007; Uematsu et al., 2006) where *Tlr5*$^{-/-}$ mice were more susceptible to urinary tract infections caused by uropathogenic *E. coli* compared to wild-type mice (Andersen-Nissen et al., 2007).

Our data on the regulation of S100A7c extend the range of inducers of S100A7c in KCs. Previously, the cytokines IL-1β, TNF-α, IFN-γ (Gläser et al., 2005), IL-6, IL-17, IL-20, IL-22, IL-24 and oncostatin M (Boniface et al., 2007), as well as late states of KC differentiation (Martinsson et al., 2005) have been shown to enhance the expression level of S100A7c. Therefore, the role of flagellin in the control of S100A7c needs to be further evaluated in the combination with other factors, many of which may be highly relevant in the context of cutaneous wounds. Unfortunately, the TLR5 knockout mouse (Uematsu et al., 2006) is not a suitable model system because mice lack a *S100A7* gene (Hahn et al., 2007). Since *S100A7* and *S100A15* have evolved from a common ancestral gene (Hahn et

al., 2007) which may have had antimicrobial activity and it is conceivable that S100A15 fulfils an equivalent role in the mouse as S100A7 in humans, the regulation of S100A15 may serve as a model to study the concept of cutaneous defense against bacteria. Ultimately, the antimicrobial function and the regulation of S100A7 remain to be tested in humans or primates.

In conclusion, the first part of the dissertation sheds light on the mechanism by which KCs sense and respond to the presence of *E. coli*. We show that a single bacterial component, *i.e.* flagellin is essential and sufficient to induce the up-regulation of the anti-bacterial defense protein S100A7c, which is able to kill *E. coli.* Since *E. coli* is to be considered a model for flagellated Gram-negative bacteria, including skin pathogens such as *Pseudomonas aeruginosa* (Schröder and Harder, 2006), our results provide important new insights into the cutaneous antimicrobial defense system.

5.2. SC proteolytic activity in establishing RNase-dependent antimicrobial functions.

The microbial defensive function of the skin is partly mediated by antimicrobial peptides such as human β-defensins, S100A7c, cathelicidin and also by RNase 7 a member of the RNase A superfamily (Schröder and Harder, 2006). In the second part of the dissertation, we explored the expression of all functional members of the RNase A superfamily as well as of RI in epidermal KCs. Our expression screening showed that 4 members of the RNase A family namly RNases 1, 4, 5 and the previously described RNase 7 are expressed by KCs. Two of this RNases *i.e.* RNase 5 and RNase 7 act as antimicrobial peptides, but until now no such activity has been described for RNase 1 and RNase 4.

This study is the first to demonstrate the presence of RNase 5 in human SC and thereby establishes another antimicrobial factor of the skin defense against microbes. The relative contribution of RNase 5 to the antimicrobial defense of the skin needs to be evaluated in further studies. The higher concentration of RNase 7 in SC compared to RNase 5 [4000 pg/mg SC (Harder and Schröder, 2002) vs. 60 pg/mg SC (our study)] suggests a prominent role in skin defense for RNase 7. Nevertheless, it is conceivable that local concentrations of RNase 5 might be sufficiently high for effective antimicrobial activity; especially against *C. albicans* which is sensitive to RNase 5 in very low micromolar concentrations (Hooper et al., 2003). However, RNase 5 is a multifunctional protein with ribonuclease, microbicidal and angiogenic properties but its key function in the skin has to be investigated in future studies.

Our study provides the first characterization of endogenous RI in the epidermis and, importantly, extends its range of functions to the inhibition of RNase antimicrobial activities. RI suppresses ribonuclease activities of RNases (Iyer et al., 2005; Johnson et al., 2007), but also contributes to intracellular redox homeostasis (Monti et al., 2007). The finding that RI is expressed strongly in the uppermost layers of the epidermis but is absent in SC and the fact that SC contains ribonuclease and antimicrobial activity indicate that a physiological mechanism may exists to prevent inhibition of skin surface ribonuclease and probably antimicrobial functions. In support with this hypothesis, co-incubation of recombinant RI with SC extract led to degradation of RI. This degradation could be blocked by the serineprotease inhibitor aprotinin. Furthermore, it needs to be considered RI can also be predisposed to degradation, at least within the intracellular environment, by oxidation of its thiol groups (Blazquez et al., 1996). Oxidation of RI at the skin surface may contribute to the inactivation of RI.

The significance of RI degradation was underlined with an *in vitro* experiment where the antimicrobial functionality of RNase 5 and RNase 7 were assessed in the presence of

RI. Interestingly, RI not only blocked the ribonuclease activity of these RNases, but also strongly suppressed their antimicrobial activity as well, indicating that presence of active RI in SC would interfere with the natural innate immune defense of skin-derived RNases. The inhibition of antimicrobial action by RI might be explained by conformational interaction of enzyme·inhibitor complex. RI has a horseshoe-shape structure and binds RNases within its concave cavity. Hence, it is likely that RI covers residues (active sites) responsible for antimicrobial action may lead to inactivity of this function. Although it has been reported that ribonuclease activity of RNases is not essential for their antimicrobial properties (Dyer and Rosenberg, 2006; Huang et al., 2007), it is possible that RI, covers active sites for both functions. Alternatively or in addition, the recently reported ability of RI to induce conformational changes of RNase 1 upon formation of a complex with this protein (Johnson et al., 2007) might suggest that conformations of RNase 5 and RNase 7 which are incompatible with antimicrobial activity, may be induced by RI.

The proteolysis of RI by SC proteases establishes an indirect role of proteases in the antimicrobial defense function of the skin. A direct dependence on proteases of protein activation in the SC has been demonstrated for cathelicidin, which is converted to the active antimicrobial peptide LL-37 by the action of the serine proteases kallikrein 5 and kallikrein 7 (Yamasaki et al., 2006). This suggests a two steps processing for an efficient antimicrobial activity of cathelicidin. The first step is on transcriptional level where the cathelicidin gene is transcribed and then translated to its protein precursor hCAP18, the second step appears on post-translational level with the enzymatic modification of the precursor to the active antimicrobial LL-37. A similar mechanism might be considered for the activity of RNase 7 and/or RNase 5 where RI is degraded on post-translational level by the enzymatic activity of SC proteases on human skin.

Taken together, these studies help us to better understand the finely regulated balance of SC enzymatic activities and its functionality. The disturbance of SC proteolytic activities,

either caused by genetic defects or environmental influences, may compromise antimicrobial skin defense.

6. References

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7. Appendix

7.1 Abstract in German (Kurzfassung)

Antimikrobielle Peptide sind ein wichtiger Teil des angeborenen Immunensystems der Haut. Epidermale Keratinozyten exprimieren eine Vielzahl verschiedener antimikrobielle Peptide, die eine schützende Barriere zu eindringenden Mikroorganismen bilden. Es wurde gezeigt, dass speziell das antimikrobielle Peptide S100A7c (Psoriasin) höchst effektiv gegen den humanpathogenen Keim *Escherichia coli (E. coli)* wirkt. Der genaue Mechanismus, der für die Regulation dieses antimikrobiellen Peptids in epidermalen Keratinozyten verantwortlich ist, war jedoch bis dato unklar. Deshalb untersuchten wir den Einfluss von verschiedenen mikrobiellen Bestandteilen auf die Expression von S100A7c. Wir konnten nachweisen, dass ausschließlich Flagellin, ein Ligand für Toll-like Rezeptor (TLR) 5, die Expression von S100A7c in primären humanen Keratinozyten stark induzierte, während alle weiteren TLR-Liganden keine Wirkung zeigten. Weiters konnten wir zeigen, dass hitzeinaktivierte Kulturüberstände von *E. coli* die mRNA- und Proteinproduktion von S100A7c stark induzierten. Im Gegensatz dazu hatte ein flagellin-defizienter *E. coli* Stamm (ΔFliC) keine induzierende Wirkung. Nun wollten wir untersuchen, ob auch hier die Signalweiterleitung über den TLR5 erfolgt. Durch Transfektion von TLR5 spezifischen siRNAs konnte die Expression dieses Rezeptors in Keratinozyten nahezu vollständig unterdrückt werden. Nach darauffolgender Stimulation mit Flagellin oder hitzeinaktivierte Kulturüberstände von *E. coli*, konnten wir keine Induktion von S100A7c mehr feststellen. Wir konnten daher durch diese Serie von Experimenten nachweisen, dass das antimikrobielle Peptid S100A7c in primären humanen Keratinozyten über TLR5 Aktivierung induziert wird, und dass Flagellin der bakterielle Bestandteil ist, der für diese Induktion notwendig ist.

Ein weiteres antimikrobielles Peptid, das von humanen Keratinozyten produziert wird ist RNase 7. Diese RNase trägt nicht nur zur Oberflächenribonukleasetätigkeit der menschlichen Haut bei, sondern ist vielmehr auch ein wichtiger Faktor für die antimikrobielle Abwehr an der Hautoberfläche. Mittels RT-PCR konnten wir zeigen, dass zusätzlich zu RNase 7, auch andere RNasen (RNasen 1, 4 und 5) sowie ein RNase-Inhibitor (RI) von humanen Keratinozyten produziert werden. RNase-Inhibitoren sind endogene Proteine, die die Aktivität von RNasen blockieren können. *In vitro* Experimente zeigten, dass dieser RI, sowohl die Ribonukleaseaktivität von Stratum corneum (SC) RNasen, als auch die antibakterielle Wirkung von RNase 7 und RNase 5 unterdrücken konnte.

In vivo konnten wir eine starke Expression von RI in den granulären Schichten, jedoch nicht in der verhornten Schicht der Epidermis nachweisen. Weiters konnten wir zeigen, dass SC Extrakte in der Lage waren rekombinant hergestellten RI abzubauen. Dies konnte durch Zugabe von Aprotinin, welches die Aktivität von Serinproteasen inhibiert, verhindert werden. Diese Ergebnisse deuten stark auf das Bestehen physiologischer Mechanismen zum Abbau von RI im SC hin, wodurch sowohl die Ribonukleaseaktivität als auch die antimikrobielle Aktivität von RNasen an der Hautoberfläche gewährleistet werden kann.
Zusammengefasst, konnten wir mit diesen Studien neue Einblicke in die Wirkungs-mechanismen und Regulation von antimikrobiellen Peptiden in der menschlichen Haut geben. Weiters konnten wir einen neuen wichtigen Aspekt der proteolytischen Aktivität von SC in der Haut demonstrieren.

7.2 Abbreviations

CCL	C-C chemokine ligand
CF	Cystic fibrosis
CFU	colony-forming units
CRAMP	cathelicidin-related antimicrobial peptide
DNA	deoxyribonucleic acid
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	Ethylendiamintetraaceticacid
GAS	group A Streptococcus
hBD	human β-defensins
hCAP-18	human cationic antimicrobial protein with a molecular size of 18 kDa
HNP	human neutrophil peptides
IFN	interferon
IL	interleukin
kDa	kilo Dalton
LD_{90}	lethal dose that achieves a CFU reduction of 90%
LPS	lipopolysaccaride
LRR	leucine-rich repeat
LTA	lipoteichoic acid
MDP	muramyl dipeptide
mRNA	messenger RNA
NLR	(NOD)-like receptor
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern
PMA	phorbol-myristate-acetate

PRR	pattern recognition receptors
RI	ribonuclease inhibitor
RNA	ribonucleic acid
RNase	ribonuclease
SC	stratum corneum
siRNA	small interference RNA
TLR	Toll-like receptor
TNF	tumour necrosis factor
VDR	vitamin D receptor
VDRE	vitamin D response element
ΔFliC	flagellin-deficient

7.3 Curriculum vitae (CV)

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List of publications

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