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Review

Antimicrobial skin peptides and proteins

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Abstract. Human skin is permanently exposed to microorganisms, but rarely infected. One reason for this natural resistance might be the existence of a 'chemical barrier' consisting in constitutively and inducibly produced antimicrobial peptides and proteins (AMPs). Many of these AMPs can be induced *in vitro* by proinflammatory cytokines or bacteria. Apart from being expressed *in vivo* in inflammatory lesions, some AMPs are also focally expressed in skin in the absence of inflammation. This suggests that non-inflammatory stimuli of endogenous and/or exogenous origin can also stimulate AMP synthesis without inflammation. Such mediators might be ideal 'immune stimulants' to induce only the innate antimicrobial skin effector molecules without causing inflammation.

Key words. Innate immunity; antimicrobial peptides; antimicrobial proteins; skin infection; keratinocytes; psoriasis; atopic dermatitis.

Introduction

As a barrier organ human skin is always in contact with the environment and is covered with a characteristic microflora [1]. Resident microorganisms, which surprisingly are present in rather stable numbers and composition, always grow as small colonies at the surface of the horny layer (stratum corneum) and within the uppermost stratum corneum layers. The composition varies qualitatively and quantitatively, depending on localisation, between 10^2 and 10^7 microorganisms/cm^2.

With respect to the capability of microorganisms to divide under optimal conditions (nutrient-rich conditions, higher temperature, humidity, ions) within 20 min, it might be an enigma that healthy body surfaces usually do not show excessive microbial growth and no signs of infection. This unexpected phenomenon might be explained by considering skin as a defense organ, where particular strategies have evolved to protect the skin from infection. One of the most important elements of this strategy is the existence of an intact physical barrier consisting in the stratum corneum in skin and mucus in mucosa. Both desquamation and secretion of mucus lead to a permanent renewal of these body surfaces and simultaneous elimination of the microorganisms adhering to these layers. Infiltration of microorganisms into the living epidermis will be inhibited by the lipid barrier present in the uppermost parts of stratum corneum [2, 3]. Although it has been suggested for a long time that the physical barrier is the sole component protecting skin from infection, there are a number of hints that this is not the case. Bacteria produce a number of enzymes that can degrade both lipids and proteins and therefore should make them capable of overcoming the physical barrier. Another strategy to protect the skin from infection would be phagocytosis of the invading microorganisms. Although it is commonly believed that active elimination of microorganisms is possible only by professional phagocytes, there does exist ultrastructural evidence that keratinocytes have the capacity to phagocytose *C. candida albicans* [4]. In contrast to skin keratinocytes, where phagocytosis of microorganisms represents a rare event, in mucosa cells phagocyto-

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sis of microorganisms is a more common phenomenon [5]. Despite these findings it is doubtful that active phagocytosis of microorganisms by keratinocytes is of major importance for maintaining skin integrity, because phagocytosis is a long-lasting process and thus efficacy is very low. All of these observations led to the hypothesis that, apart in addition to the physical barrier, a 'chemical barrier' of the skin should also exist. This could consist in chemicals produced in the uppermost parts of the skin that control growth of bacteria at body surfaces and inhibit infection. Because healthy skin does not contain any blood-derived leukocytes, such as neutrophils, which contain a number of bactericidal compounds [6], epithelial cells might be the source of 'chemical barrier' compounds.

There is ample evidence, that in plants, which do not contain any adaptive immune system, mainly antimicrobial peptides and proteins (AMPs) such as defensins and thionins represent the effector molecules (reviewed in [7]). These are often produced in the uppermost, environment-exposed layers of various plant organs [8], suggesting that these molecules may play a role as effector of the epithelial defense system to protect surfaces from infection. Apart from plants, invertebrate epithelia produce AMPs, as demonstrated in *Drosophila melanogaster*, where contact with microbes produces local induction of AMPs [9]. This observation favours the hypothesis that epithelial production of AMPs represents a general phenomenon which suggests effector molecules in an epithelial defense system. Since 1987, findings show that vertebrates might also use antimicrobial peptides as part of a 'chemical defense system' to protect skin from infection. Zasloff isolated from the skin of the African clawed frog *Xenopus laevis* a broad spectrum antimicrobial peptide he termed 'magainin' [10], which is also stored as an active, processed peptide in large granules within the neuroepithelial glands of the skin and is secreted upon stimulation with noradrenalin [11].

Based on these observations in frog skin and findings from insects and plants, it is no surprise that human skin can also produce AMPs. Indeed, in the past 10 years a number of skin-derived AMPs have been discovered in human skin material using a biochemical approach and table 1 summarizes the skin-derived AMPs discussed in this review, together with their major biological properties.

Table 1. Skin-derived AMPs.

Name	Antimicrobial activity			Chemotactic for	Induced under inflammatory conditions
	Gram+	Gram–	Fungi		
Lysozyme	+	+	–	?	–
Dermcidin	+	+	+	?	–
Psoriasin	(+)	+	?	T cells, neutrophils	+
RNase 7	+	+	+	?	+
hBD-1	(+)	+	?	T cells, dendritic cells	–
hBD-2	(+)	+	+	T cells, dendritic cells, mast cells	+
hBD-3	+	+	+	T cells, dendritic cells, monocytes	+
LL-37	+	+	+	neutrophils, monocytes, T cells, mast cells	+

Lysozyme, a human healthy skin-derived antimicrobial protein.

The first antimicrobial protein found in human skin was lysozyme [12, 13], which originally was discribed as 'bacteriolytic activity' in nasal secretions by Alexander Fleming [14], suggesting that human nasal epithelial glands also secrete antimicrobial compounds. In skin lysozyme is mainly located in the cytoplasm of epidermal cells in granular layers and malpighian cells [12, 15]. Pilosebaceous follicle cells and hair bulb cells as well as all parts of eccrine sweat glands were also positive for lysozyme.

Although lysozyme is mainly a Gram-positive bacteria (e.g. *S. aureus*)-killing antimicrobial peptide [16] it is also active against Gram-negative bacteria, e.g. *Escherichia coli* [17] or *Pseudomonas aeruginosa* [18], suggesting that it might function in healthy skin to control growth of bacteria. However, the contribution of lysozyme to cutaneous defense is still unclear because lysozyme is expressed exclusively in the cytoplasm, and immunoreactive lysozyme is absent in the stratum corneum [12] and skin washing fluid [19].

Antimicrobial peptides of sweat

The eccrine sweat gland is one of the major cutaneous appendages, and is a secretory as well as an excretory organ [20]. Its principal function is thermoregulation during exposure to a hot environment or physical exercise. In addition to this function, sweat may have also a role as an innate defense system controlling skin bacterial flora by releasing antimicrobial peptides.

Dermcidin

Dermcidin appears to be the principal sweat antimicrobial peptide that is constitutively produced exclusively

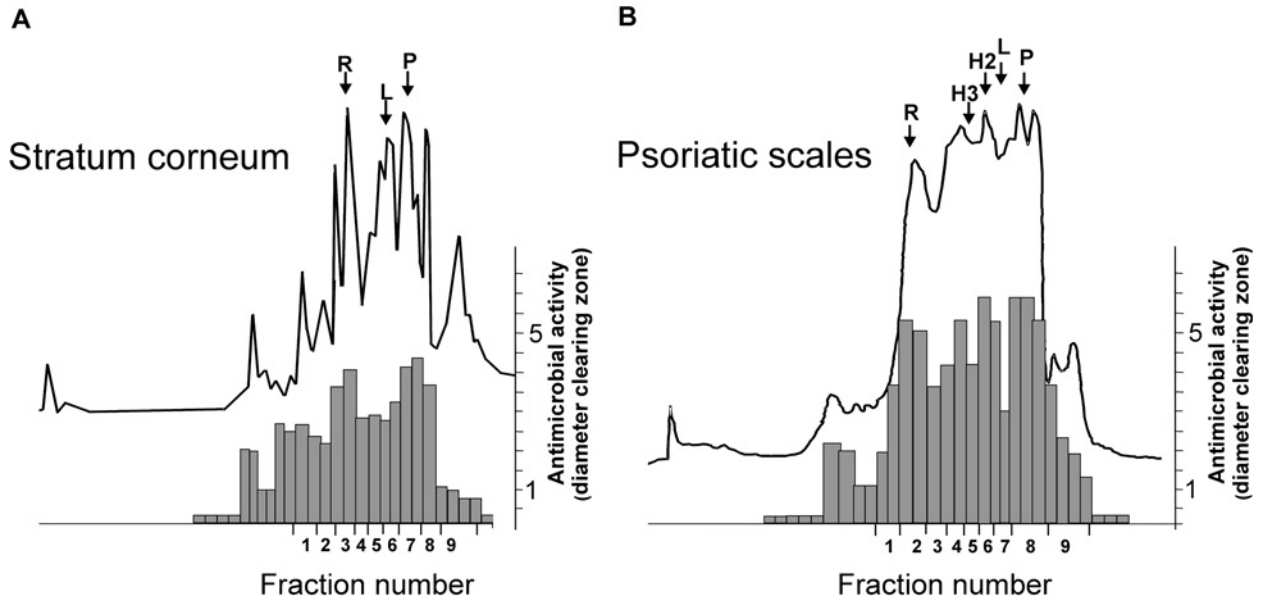

Figure 1. RP-HPLC of a healthy stratum corneum (*A*) and lesional psoriatic scale extracts (*B*). Heparin-bound material of extracts obtained from 100 g of pooled healthy stratum corneum or from 7 g of lesional psoriatic scales were separated by preparative wide-pore RP-8-HPLC and 10-µl aliquots of each fraction were tested for *E. coli*-cidal activity in the radial-diffusion assay system. Bars represent the diameter of the clearing zone in the assay system. Note the difference in the antimicrobial activity pattern and the increased activity in psoriatic scale extracts, despite the use of 14-fold less amounts of material. H2, hBD-2; H3, hBD-3; L, lysozyme; P, psoriasin; R, RNase7.

by eccrine gland cells [21, 22]. It is expressed as a 9.3-kDa precursor, that is proteolytically cleaved, resulting in dermcidin 1 (DCD-1), a 47-aa-containing peptide that shows antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *E. coli* as well as *C. albicans* at low micromolar concentration (1–10 µg/ml) [22]. Its activity is not affected by low pH and increased salt concentrations, conditions that could occur in sweat *in vivo* [22]. In sweat, concentrations of 1–10 µg/ml DCD-1 – depending on the donor – were found [22].

Patients with atopic dermatitis have recurrent bacterial or viral skin infections, an observation that led to the hypothesis that patients with atopic dermatitis may have a reduced amount of DCD peptides in sweat. Using surface-enhanced laser desorption ionisation time-of-flight mass spectrometry and enzyme-linked immunosorbent assay (ELISA) it could be shown that the amount of several DCD-derived peptides in sweat of patients with atopic dermatitis is significantly reduced [23]. Furthermore, atopic dermatitis patients with a history of bacterial and viral skin infections were found to have significantly less DCD in their sweat. In support of these findings, in healthy subjects, sweating leads to a reduction of viable bacteria on the skin surface, but this does not occur in patients with atopic dermatitis [23], indicating that reduced expression of DCD in sweat of patients with atopic dermatitis may be important.

Cathelicidin LL-37 in sweat

Apart from dermcidin, the human cathelicidin LL-37 is also expressed in eccrine glands and duct cells [24]. 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 of the ducts – in contrast to dermcidin, which is located in the secretory glands, but not in the ducts [24]. Interestingly, among traces of the CAP-18-precursor and traces of the mature LL-37 a predominant 14-kDa form of LL-37 was found in sweat, as shown by Western blot analyses [24]. In another study it was shown that enhanced antimicrobial activity exists in normal human sweat and is the consequence of processing of LL-37 to previously unknown, naturally occurring cathelicidin peptides [25]. High-performance liquid chromatography (HPLC) separation of sweat samples from different individuals showed remarkable consistency in the elution profile of antimicrobial molecules. Analyses of HPLC fractions by immunodetection against LL-37 suggested that some of these less abundant, yet apparently potent, antimicrobial molecules were similar to LL-37. Investigation of antimicrobial activity and identification of new peptides generated from synthetic LL-37 showed that further processing occurs by a serine protease present in sweat. The resulting 31, 30 and 20 residues containing LL-37 peptide fragments exhibited enhanced antimicrobial activity against skin pathogens such as *S. aureus* and *C. albicans* [25].

RNase 7 is a major AMP of healthy skin

Analyses of healthy person's stratum corneum extracts for AMPs revealed strong activity for both Gram-negative and Gram-positive bacteria [our unpublished results]. Attempts to identify the principal AMPs by biochemical approaches revealed a 14.5-kDa protein [26]. Amino acid sequence analyses and molecular cloning of the corresponding complementary DNA (cDNA) showed that this protein, RNase 7, is similar to members of the RNase A superfamily, among them, RNase 2 [eosinophil-derived neurotoxin (EDN)], RNase 3 [eosinophil-derived cationic protein (ECP)] and RNase 5 (angiogenin) [27, 28]. Electrospray-ionisation mass spectrometry (ESI-MS) analyses revealed the presence of four disulfide bridges [26]. RNase 7 is a highly basic protein (pI: 9.80) that represents one of the major heparin-binding proteins in healthy skin-derived stratum corneum extracts. We were able to isolate 4–8 µg/g stratum corneum [26]. In contrast, from psoriatic-scale material 10–25 µg/g RNase 7 could be recovered [29], suggesting that RNase 7 might be inducible, despite the high level of constitutive expression in healthy skin. Indeed, the proinflammatory 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 keratinocytes. Apart from primary cytokines, primary keratinocytes treated with heat-killed bacteria also induced RNase 7 [26]. As ye—t, the molecular mechanism of bacteria-mediated RNase 7 induction in keratinocytes remains unclear.

RNase 7 is a broad-spectrum antimicrobial protein

Natural skin-derived RNase 7 exhibits a broad spectrum of antimicrobial activity against both Gram-negative bacteria (*E. coli* and *P. aeruginosa*), and Gram-positive bacteria (*Propionibacterium acnes, S. aureus*) and the yeast *C. albicans*. Remarkably, the number of colony-forming units decreases by five orders at concentrations in the low micromolar range. It is of particular interest that for unknown reasons RNase 7 is extremely effective at killing a vancomycin-resistant strain of *Enterococcus faecium* already at 20 nM concentrations [26]. Thus – on a molar base – RNase 7 represents one of the most potent and efficacious human antimicrobial proteins known so far.

RNase 7 is not the only member of the RNase A family that shows antimicrobial activity: it has been demonstrated that the eosinophil-derived RNase ECP exhibits antimicrobial activity against *S. aureus* and *E. coli* with activity in the low micromolar range – similar to that of RNase 7 [30]. Also, human angiogenin (RNase 5) is active at low micromolar concentrations against *Streptococcus pneumoniae* and the yeast *C. albicans* [27]. As yet there is no evidence that stratum corneum obtained from healthy donors also contains these other RNases at concentrations sufficient to be microbiocidal – if at all.

The relative high abundance of RNase 7 in skin, its inducibility by contact with bacteria and its preference for killing Gram-positive *Enterococci* may indicate a major role of this AMP in preventing skin infection by Gram-positive gut bacteria.

Psoriasin (S100A7), an *E. coli*-cidal protein of healthy skin

Certain species of bacteria die rapidly on the surface of human skin, whereas other species do not [31]. This distinction can be readily demonstrated by exposure of the fingertips to either *E. coli* or *S. aureus* in a humid atmosphere for 30 min; the surface of a petri dish filled with nutrient agar is then touched with the bare fingertips, and the dish is incubated overnight. A *S. aureus* print of the fingertips is produced, but no viable *E. coli* are transferred [19]. This surprising observation led to the hypothesis that healthy human skin might constitutively produce a defense chemical that could preferentially and very effectively control the growth of *E. coli*. Such factor would also explain why *E. coli* is rarely found to colonise skin [1].

Biochemical analyses of *E. coli*-killing activity in extracts of a healthy person's stratum corneum identified psoriasin (S100A7) as a principal *E. coli*-cidal antimicrobial protein in healthy skin stratum corneum extracts [19]. Reversed-phase (RP)-HPLC analyses of a healthy person's washing fluid (fig. 2) together with ESI-MS analyses of HPLC fractions revealed psoriasin (MW: 11.366; the N-acetylated, terminal methionine missing form of S100A7c) as an additional principal protein constituent [19], indicating

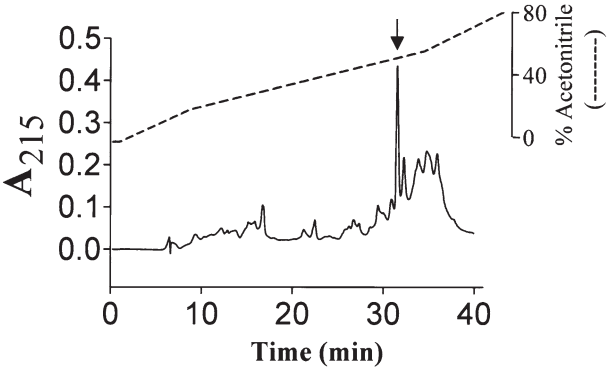

Figure 2. RP-HPLC of healthy skin washing fluid. Pooled washing fluid obtained from forearm skin of 50 persons by rinsing 2 cm² of each person with 2 ml of buffer was concentrated using protein-free Amicon YM2 filters and separated with an increasing acetonitrile gradient at a micropore C2C18-RP-HPLC column. Aliquots of each fraction were analysed by ESI-MS. The major absorbing peak at 215 nm indicated by the arrow revealed an exact mass of 11,366 Da, corresponding to the N-terminally acetylated, methionine-missing sequence of psoriasin, as shown by MS/MS analyses of tryptic digests. Reproduced courtesy of [19].

that psoriasin is released to the body surface. A detailed biochemical analysis of (pooled) healthy stratum corneum-derived psoriasin, however, revealed psoriasin to be heterogeneous with the 11.366-Da form as the predominant psoriasin form in these extracts [19].

Sequencing of the human S100 gene cluster has identified five copies of S100A7-like genes (S100A7a – S100A7e) [32], thus showing evidence of gene duplication during primate evolutionary history. We found that only the S100A7c variant (as 11.366-Da form) can be recovered from skin-washing fluid, suggesting that it is released from keratinocytes. In contrast, various other, not-yet-characterized psoriasin variants and post-translationally modified and mutated psoriasin forms could be isolated only from skin extracts [19], suggesting that these are stored within the keratinocytes.

Recently, in an effort to identify psoriasis-associated genes, a novel gene, named S100A15, with high homology to the S100A7 was discovered, which is also overexpressed in psoriasis [33]. Like psoriasin, S100A15 *in situ* hybridisation experiments revealed staining of epidermal layers of psoriatic skin, suggesting a similar function as psoriasin. As yet, however, its biological function is not clear. When we looked at ESI-MS analyses of crude psoriasin preparations for ions corresponding to calculated masses for S100A15 protein and its modified forms, we found no clear evidence for a high abundance of the protein – if any – in healthy stratum corneum extracts. ESI-MS analyses yielded several ions that could correspond to truncated forms of S100A15, but this observation needs further confirmation [unpublished results].

Psoriasin is a focally expressed AMP

In skin, psoriasin is focally expressed (fig. 3) [19] and released from keratinocytes, particularly in areas where high bacterial colonization is well documented, such as the uppermost parts of hair follicles (fig. 3A) and nose skin (fig. 3C). On the other hand, dry areas of the skin, such as lower leg skin (fig. 3B) show a rather 'patchy' staining for psoriasin. It is interesting to note that apart from keratinocytes, sebocytes, the lipid-secreting cells of sebaceous glands, also showed immunoreactive psoriasin (fig. 3C), suggesting that psoriasin is possibly also secreted together with lipids. Indeed, when healthy skin is first washed with buffer and then with acetone (to extract skin lipoids), psoriasin can be recovered from both aqueous and acetone extracts [19]. These data indicate that the highly hydrophobic psoriasin is also stored in the lipid layer of healthy skin.

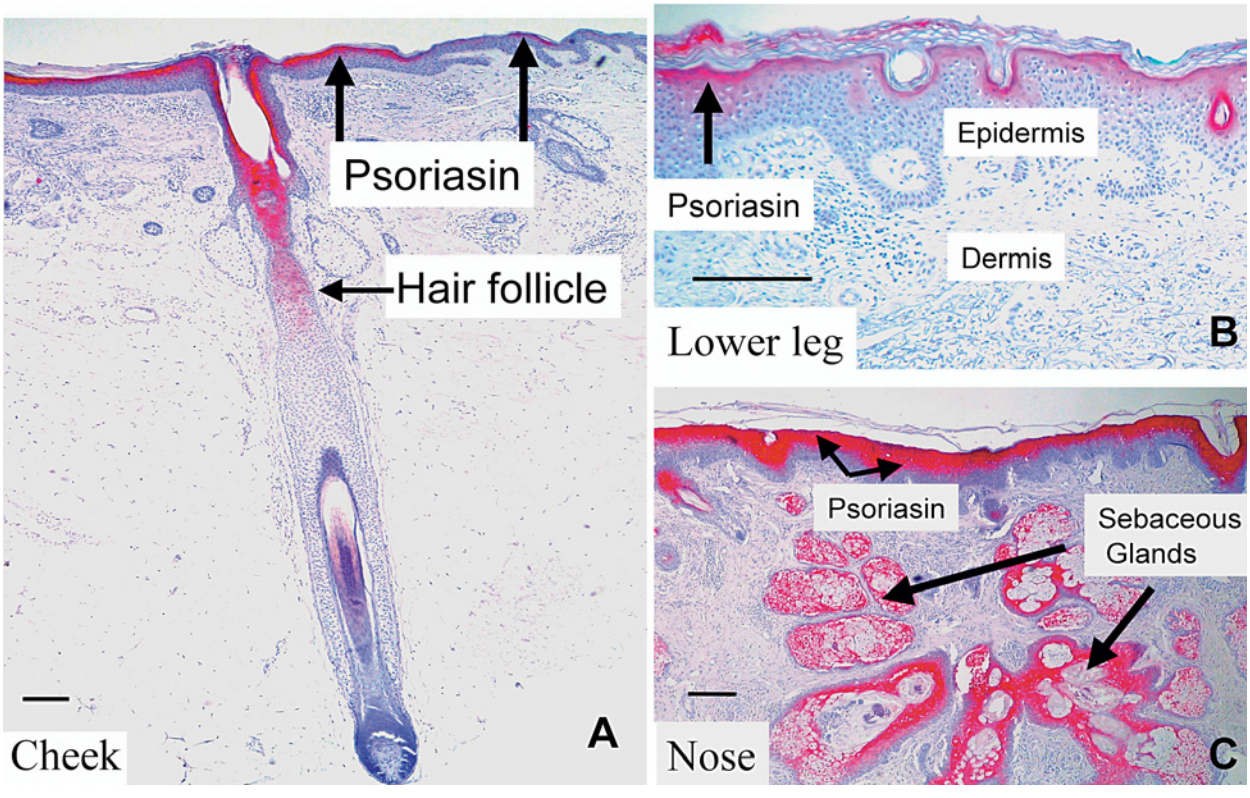

Figure 3. Immunohistochemical analyses reveal psoriasin to be expressed focally. There is strong staining (red) in the uppermost epidermal layers of the hair follicles and surrounding epidermis of the cheek (*A*), focal expression in the uppermost parts of the lower leg epidermis (*B*) and strong immunoreactivity in the suprabasal keratinocytes of the epidermis and in the sebaceous glands (SG) of nose skin (*C*). Note the absence of psoriasin in eccrine sweat glands (ES). Scale bars, 200 µm. Reproduced courtesy of [19].

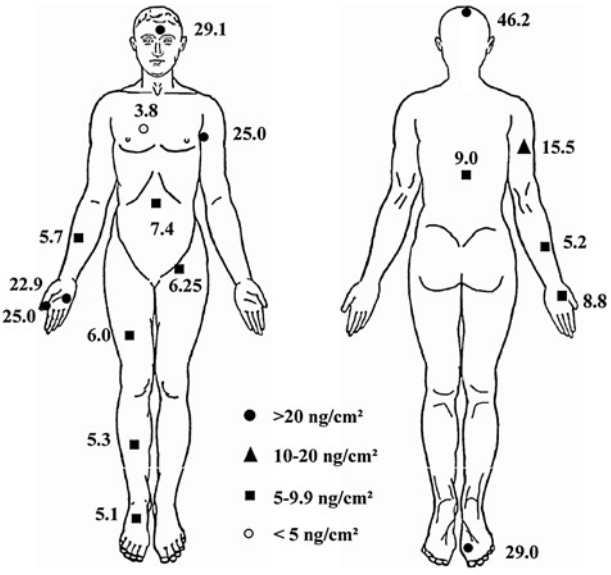

Figure 4. Psoriasin is secreted *in vivo* on the body surface. Standardized areas of various body locations on healthy volunteers were rinsed with buffer to determine the concentration of psoriasin present on the skin. Data represent the median concentration of psoriasin/cm^2 in all people for each body site. Reproduced courtesy of [19].

The focal expression of psoriasin seen upon immunohistochemistry indicates that local concentrations of psoriasin released to the skin surface may also depend on the body site. Indeed, in support of figure 3B, only small amounts of psoriasin were found at areas with dry skin, and high amounts at places where bacterial colonization mainly occurs (fig. 4) [19].

Psoriasin, the principal *E. coli*-cidal AMP of healthy skin

In vitro, psoriasin shows antimicrobial activity preferentially against *E. coli*, with a LD_{90} (lethal dose that achieves a CFU reduction of 90%) near 0.5 µM [19]. Whereas there is also bactericidal activity for *P. aeruginosa* and *S. aureus* at much higher concentrations (LD_{90} >30 µM), psoriasin is far less bactericidal for the commensal *Staphylococcus epidermidis* [19]. Psoriasin antimicrobial activity can be observed at neutral and acidic pH, as well as at increased concentrations of salt [19], as has been seen at healthy skin surfaces and upon conditions, when sweat is evaporating. Inhibition of psoriasin by a specific and neutralizing monoclonal antibody *in vivo* and *in vitro* confirmed that psoriasin is the principal *E. coli*-bactericidal component of healthy skin [19]. Pretreatment of healthy skin with neutralizing psoriasin antibodies also resulted in an increase of commensals, suggesting that a permanent psoriasin 'film' at skin surfaces might also contribute to growth control of the commensal flora.

Unlike defensins and cathelicidin LL-37, psoriasin seems to kill *E. coli* by a different mode of action, because ultrastructural analyses of psoriasin-treated *E. coli* revealed no signs of perforation. Interestingly, the antimicrobial activity of psoriasin, which exists in aqueous solution as a non-covalent dimer that contains two half Zn^{2+}-binding sites and two Ca^{2+}-binding EF-hands per molecule [34], 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 psoriasin kills *E. coli* [19]. This hypothesis is supported by experiments, which show that the Zn^{2+} chelator TPEN also kills *E. coli*, and that TPEN and psoriasin have additive effects [19]. The exact mechanism by which psoriasin kills *E. coli*, however, is yet unknown. It has been speculated that Zn^{2+} deprivation ultimately affects Zn^{2+}-dependent enzymes; Zn^{2+} as well as $Cu^{2+}$are essential transition metal ions for functional *E. coli* superoxide dismutase [35]. This enzyme has high sensitivity to chelators of divalent cations [36] and is almost exclusively synthesized in the aerobic stationary phase in the periplasm of many Gram-negative bacteria [37], suggesting that these bacteria have a particular need to defend themselves against oxidative damage by endogenously generated reactive oxygen species [38]. Zn^{2+} as well as Cu^{2+} compartimentalisation could, therefore, be a critical step in bacterial defense in oxidative stress; psoriasin might exploit compartmentalisation in its antimicrobial activity.

A few other studies indicated involvement of S100 proteins in innate host defense; calprotectin, a non-covalent heterodimer of S100A8 and S100A9, shows Zn^{2+}-sensitive, *C. albicans*-selective biostatic activity [39, 40]. Furthermore, a short C-terminal peptide fragment of S100A12 has Gram-negative bacteria-killing activities [41].

Many S100 proteins are expressed in epithelial tissues (reviewed in [42]), supporting the hypothesis that these proteins are also involved in epithelial defense.

Inducible epithelial antimicrobial peptides

Although a number of inducible antimicrobial peptides have been found in plants, in insects and also in cattle (reviewed in [43, 44]), it is not clear whether human epithelia produce inducible AMPs. The first inducible AMPs found in cattle were the β-defensins 'tracheal antimicrobial peptide TAP' [45] and structurally very similar 'lingual antimicrobial peptide, LAP' [46]. These peptides were discovered in the course of asking why mammalian mucosa, despite hosting a constant epithelium-specific microbial flora, is normally not infected. Both TAP and LAP can be induced in cultured bovine tracheal epithelial cells by heat-killed bacteria, bacterial lipopolysaccharide or TNF-α [45, 46]. *In situ* hybridisation studies revealed that LAP mRNA is widely expressed in numerous epithelia at a low level, but upregulated in infected skin and

lung [47]. These studies promised that inducible AMPs, in particular β-defensins, could also exist in humans, although until 1997 no experimental evidence was available and although a non-inducible human β-defensin, hBD-1, had already been discovered (as a trace peptide from human hemofiltrate obtained from patients with end-stage renal disease) [48].

With the observation that in cattle inducible epithelial peptide antibiotics are only detectable in inflammatory lesions, we hypothesised that a rich source of human epithelial inducible antimicrobial peptides should be inflammatory tissue material. The non-infectious inflammatory skin disease psoriasis is characterised by inflammatory lesions and hyperproliferation of epidermis, resulting in massive production of scales [49]. Surprisingly, patients with psoriasis rarely suffer from skin infections [50]. Therefore, it was suggested that within the skin lesions, antimicrobial peptides are generated, and therefore psoriatic scales might represent a rich source of human-inducible AMPs. Indeed, several novel human inducible AMPs could be purified from this human material, among them the human β-defensins hBD-2 and hBD-3 (reviewed in [29]).

Human β-defensin-2, hBD-2, the first human inducible defensin

When acidic lesional psoriatic scale extracts were separated by heparin affinity chromatography followed by preparative RP-HPLC, it is a striking observation that, when compared with heel stratum corneum extracts, psoriatic scale extracts – despite the 14-fold lower amount of scale material – show much more bactericidal activity in all HPLC fractions and a different activity pattern (fig. 1). This observation supports the idea that lesional psoriatic scales contain various inducible AMPs not present or not present in such amounts in healthy stratum corneum.

In order to selectively isolate AMPs (which strongly bind to bacteria) from psoriatic scales, a scale extract was applied to an *E. coli* affinity column generated by linking *E. coli* to sepharose [51]. By RP-HPLC of *E. coli* affinity column-bound material, we then identified as a principal bactericidal component a β-defensin-like 4328-Da peptide eventually termed hBD-2 [51]. HBD-2 is – like other defensins – a cationic peptide. Although the shape and charge distribution of hBD-2 are similar to other defensins, X-ray analyses revealed that an additional α-helical region distinguishes this defensin topologically from other mammalian α- and β-defensin structures. HBD-2 forms non-covalent dimers with a quarternary octameric arrangement [52].

The mature 41 residues containing hBD-2- peptide represents one of the major constituents of heparin-binding proteins in lesional psoriatic scale extracts [29]. There is no evidence so far for the presence of truncated forms of hBD-2 in psoriatic scale extracts [our unpublished results], although hBD-2 is susceptible towards cathepsins [53]. A crude estimate indicated amounts of 10–50 µg/g psoriatic scales, suggesting concentrations of 2–10 µM in skin [29], which is in agreement with estimated concentrations of hBD-2 in IL-1α-induced epidermal cultures, where concentrations of 3.5–16 µM were estimated [54]. Given that the aqueous intercellular space between the keratinocytes in the epidermis is small and hBD-2 is expressed locally, it is very likely that local concentrations of hBD-2 are much higher *in vivo* than the minimum bactericidal concentrations calculated from *in vitro* studies [29, 54].

hBD-2 is a preferentially Gram-negative bacteria-killing AMP

Natural, psoriatic scale-derived hBD-2 shows preferential antimicrobial activity against Gram-negative bacteria such as nonmucoid and mucoid strains of *P. aeruginosa* and *E. coli* (LD_{90}, 10 µg/ml), less activity against *C. albicans* (LD_{90}, 25 µg/ml) and only bacteriostatic activity against *S. aureus* at 100 µg/ml [51]. In insect cells, generated recombinant hBD-2 showed bacterial killing of *E. coli, E. faecalis, P. aeruginosa* and *S. aureus* [29, 54–56]. Synthetic hBD-2 exhibited antimicrobial activity against various periodontal bacteria [57, 58]. 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 [55–60], suggesting that hBD-2 will be unable to kill bacteria in serum or at skin surface covered with evaporated sweat. Some bacteria, e.g. *Burkholderia cepacia*, are surprisingly resistant to hBD-2 [61]. Most likely, alteration of the bacterial membrane structure is important. It has been suggested that the spirochete *Treponema denticola* is resistant to hBD-2 because it lacks lipopolysaccharide (LPS) in the membrane [62]. But other reasons could also be important: group A *Streptococci* (GAS) producing streptococcal inhibitor of complement (SIC) have been reported to be resistant towards a number of cationic antimicrobial peptides, including hBD-2 by binding of SIC towards AMPs [63].

HBD-2 is a locally induced AMP

Epidermis of human skin comprises multiple keratinocyte layers in various differentiation stages. Both the stratum corneum and the granular layer consist of terminally differentiated keratinocytes involved in superficial formation of the physical barrier [64] and contain so-called lamellar bodies, which represent lipid-rich secre-

tory granules. HBD-2 immunoreactivity is localized to the uppermost layers of the epidermis and/or stratum corneum. On a subcellular level, hBD-2 is stored in lamellar bodies of stimulated keratinocytes of the spinous layer of the epidermis, suggesting that hBD-2 is released with the lipidlike contents of lamellar bodies [65]. Interindividual and site-specific differences in intensity of immunostaining were observed, and the pattern of peptide localization was seen to be rather focal [66] – similar to the staining pattern of psoriasin [19], suggesting that hBD-2 is locally induced. HBD-2 is also seen in the stratified epithelia of the oral cavity as well as in gingival cells. Also, here it is predominantly localized in suprabasal stratified epithelium and in upper epithelial layers [67]. Whereas hBD-2 transcript expression is frequently higher in non-keratinised or ortho-keratinised epithelium, immunostaining for hBD-2 is more intense in hyperkeratinised epithelium [68]. Upon culture of oral epithelial cells, hBD-2 peptide is seen only in differentiating involucrin-positive cells upon stimulation with proinflammatory cytokines or bacterial products [67].

Human β-defensin-3

Because hBD-2 is not an antibiotic for Gram-positive bacteria, it has been hypothesised that keratinocytes may produce other inflammation-induced antimicrobial compounds that are active against *S. aureus*. In one study, hBD-3 was identified in lesional psoriatic scales using a biochemical approach, and based on protein sequence data, it was cloned from keratinocytes [69]. Following the hypothesis that all defensin genes are located on chromosome 8p22-23 and analysing the DNA sequence from a contig containing the hBD-2 gene also turned up the hBD-3 gene [70]. Two other, independent studies used bioinformatics to identify the hBD-3 gene [70–72].

HBD-3 is a highly basic antimicrobial peptide (pI 10.08). When cation exchange-HPLC analyses are performed with psoriatic scale extracts, hBD-3 elutes just before RNase 7, which represents the lastly eluting protein in a salt gradient [our unpublished results]. Unlike hBD-2 electrophoretic separation of hBD-3 in Tricine-urea, SDS polyacrylamide gel electrophoresis (PAGE) reveals a single band at 9 kDa corresponding to a noncovalent dimer, as confirmed by nuclear magnetic resonance (NMR), analyses [69, 73]. The connectivity of the cysteine bridges in most of the defensins known so far has been predicted, but not experimentally proven. Recent analyses of chemically synthesized topological analogs of hBD-3 with predefined disulfide connectivities, including the (presumably) native β-pairing, have revealed similar antibiotic properties for differently folded hBD-3 species. Interestingly, hBD-3 species with substitutions of all Cys residues still are antimicrobially active, suggesting that for antimicrobial activity the disulfide bridges in hBD-3 are not important [74].

HBD-3 is a broad-spectrum peptide antibiotic

In contrast to hBD-2, HBD-3 is a broad-spectrum antimicrobial peptide. Investigations with synthetic material revealed salt-insensitive and potent activity against many potentially pathogenic Gram-negative and Gram-positive bacteria and fungi, including methicillin-resistant strains of *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) [69]. 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 [75]. Synthetic hBD-3 has been reported to efficiently kill *Burkholderia cepacia* [72]. However, a systematic study that tests bactericidal properties of synthetic hBD-3 against Gram-positive cocci and Gram-negative fermentative and nonfermentative rods showed that all strains were highly or intermediately susceptible to hBD-3, except *B. cepacia* [76]. The mechanism by which hBD-3 kills *S. aureus* is not yet known. 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 (fig. 5). The morphological effects [69] resemble those seen when *S. aureus* is treated with penicillin [77].

HBD-3 exhibits chemotactic activity on monocytes and CCR6-transfected HEK 293 cells [72, 74]. This activity

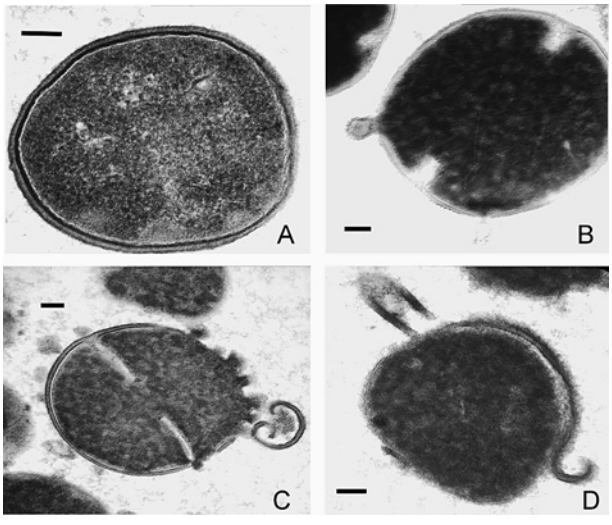

Figure 5. Morphology of hBD-3-treated *S. aureus*. Transmission electron micrographs of *S. aureus* incubated in 10 mM phosphate buffer for 2h (*A*) or treated with synthetic hBD-3 for 30 min (*B*) or 2 h (*C* and *D*) are shown. Bars represent 0.1 µm. Reproduced courtesy of [69].

strongly depends on the topology of the disulfide bridges in hBD3. The absence of any disulfide bridge abolishes the chemotactic activity of hBD-3, suggesting that a defined three-dimensional (3D) structure stabilized by disulfide bonding is required for productive binding to and activation of CCR6 receptor. In contrast to chemotatic activity, the bactericidal activity of hBD-3 remains unaffected by the absence of any disulfide bridge [74].

HBD-3 mRNA is expressed throughout the epithelia of many organs and in some non-epithelial tissues. Transcripts were found in skin, tonsils, gingival keratinocytes, esophagus, trachea, placenta, adult heart, skeletal muscle and fetal thymus [69, 71, 72].

The human cathelicidin hCAP-18/LL-37

Cathelicidins are bipartite molecules with an N-terminal cathelin domain (= cathepsin L inhibitor) and an antimicrobial C-terminal domain. Humans apparently have only one cathelicidin gene, originally found to be expressed in bone marrow [78–80]. Its product, hCAP-18, is present as one of the major proteins in the secondary (specific) granules of neutrophils [81]. This protein is stored as a precursor that requires additional processing to yield its C-terminal antimicrobial peptide, LL-37. Although the neutrophil is the major cellular source of hCAP-18, it is also expressed in other blood cells that are involved in inflammatory and immune responses, including natural killer (NK) cells, $\gamma\delta$T cells, B cells and monocytes [82]. In addition, this gene is widely expressed in skin, epithelia of the airways, mouth, tongue, esophagus, intestine, cervix and vagina, in epididymis and in testis (reviewed in [83]).

Upregulation of this human cathelicidin gene has been demonstrated in inflammatory skin disorders, whereas in normal skin no induction was found [84]. *In situ* hybridization and immunohistochemistry showed the transcript and peptide to be located in keratinocytes throughout the epidermis of the inflammatory regions [84]. On a subcellular level, lamellar granules were identified to store hCAP-18/LL-37 [85]. The content of lamellar bodies is released within 30 min after disruption of the permeability barrier [86], a timing consistent with antimicrobial peptide production and release following injury. A further study showed that LL-37 is expressed in healthy skin in eccrine glands and ductal epithelium [24].

LL-37 is a broad-spectrum AMP

LL-37 represents a highly cationic peptide with a +6 net charge at neutral pH. It is a membrane-active amphipathic α-helical peptide that shows a wide spectrum of antimicrobial activity. In *vitro* it 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 [87]. Although in a previous report yeast species of *Candida* appear to be resistant to LL-37 [87], a recent study reported *Candida*-cidal activities of LL-37 [88].

Unlike many defensins, LL-37 is active against several bacteria in high salt conditions [87]. LL-37 is not highly selective to prokaryotic cells, whereas tripomastigotes of the protozoan parasite *Trypanosoma cruzi* and peripheral leukocytes are susceptible *in vitro* to micromolar concentrations of this peptide, this cytotoxic activity is inhibited by serum components [89].

Apart from the C-terminal hCAP18 peptide LL-37 the cathelin domain of hCAP18 is also antimicrobially active as demonstrated with recombinant cathelin, which shows growth inhibition of *S. aureus* and *S. epidermidis* (MIC of 32 μM) [90].

Investigations of hCAP18 processing in neutrophils indicate that the propeptide is cleaved to generate the antimicrobially active peptide LL-37 in exocytosed material of these cells, liberated by proteolytic processing coincident with degranulation and secretion [91]. Experimental evidence shows that proteinase 3 is solely responsible for processing the hCAP18 propeptide after exocytosis in neutrophils [91]. Further studies of hCAP18 have shown cleavage of epididymal-derived hCAP18 in seminal plasma by the prostate-derived protease gastricsin (pepsin C) in the presence of vaginal fluid of low pH generating antimicrobially active ALL-38, suggesting that processing of hCAP18 by gasticsin at low pH represents a mechanism to prevent infection after sexual intercourse [92]. The detection of proteolytically processed cathelicidin in skin keratinocyte extracts indicates also that keratinocytes have the enzymatic machinery necessary for generation of hCAP18 C-terminal peptides. Analysis of stratum corneum from normal and virally infected skin has shown that cathelicidin present in this compartment is processed to a mature 5-kDa form that is presumably LL-37 or a smaller processed cathelicidin form such as KS-30 or RK-31 [25, 85, 93].

The *in vivo* relevance of cathelicidins in cutaneous host defense has been demonstrated in a mouse model. Mice deficient in the expression of the cathelicidin CRAMP (the mouse homolog to the human LL-37) were more susceptible to skin infections caused by group A *Streptococcus* (GAS). GAS mutants resistant to CRAMP resulted in more severe skin infections in normal mice [94].

Possible immune functions of skin-derived AMPs

Several reports suggest that a number of peptides and proteins exhibit not just antimicrobial but also immuno-

regulatory properties (for reviews see [95, 96]). β-Defensins were shown to be chemotactic for dendritic cells and memory T cells by using the chemokine receptor CCR6, which is shared by the chemokine MIP3α/LARC/CCL20 [97]. The β-Defensins hBD-1 and hBD-2 are selectively chemotactic only for CCR6-expressing cells, including iDCs and resting memory CD4CD45RO, as well as some CD8 T lymphocytes. Although the dose necessary to elicit half-maximum responses in these cells was found to be nearly 20-fold higher than the dose of the natural CCL-20, which is in the nanomolar range, this finding might be of high relevance *in vivo*, because hBD-2 represents one of the major peptides in inflamed skin such as psoriatic lesions, where it is present at micromolar concentrations [29]. Apart from being an attractant for immature dendritic cells [74], hBD3 is also chemotactic for monocytes, which do not express CCR6, suggesting it uses an additional chemotaxin receptor. On the basis of usage of chemokine receptors and the tertiary structural similarities between defensins and chemokines [98], the defensins were considered to be 'microchemokines' which act on cells of the adaptive immune system [99]. Thus, β-defensins might also contribute to regulation of host adaptive immunity against microbial invasion under inflammatory conditions.

Defensins have also considerable immunological adjuvant activity, and linkage of β-defensins (or selected chemokines) to an idiotypic lymphoma antigen has yielded potent antitumor vaccines [96, 99]. The functional overlap between defensins and chemokines is reinforced by reports that some chemokines have antimicrobial activities [100]. However, although showing similarity in activity and overall tertiary structure, the evolutionary relationship between defensins and chemokines remains to be determined.

In contrast to β-defensins, LL-37/hCAP-18 is chemotactic for neutrophils, monocytes and T cells in the micromolar range, but not for dendritic cells. Thus, these antimicrobial peptides have distinct, host-target cell spectra. The chemotactic activity of LL-37 is mediated by formyl peptide receptor-like 1. The capacities of β-defensins and cathelicidin LL-37 to mobilize various types of phagocytic leukocytes, immature dendritic cells and lymphocytes provide evidence for their participation in alerting, mobilizing and amplifying innate and adaptive antimicrobial immunity of the host (reviewed in [101]).

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 (reviewed in [102]). By promoting re-epithelialisation of healing skin, LL-37 could play a role in repair of damaged tissue. High levels of LL-37 are present upon wounding, and an anti-LL-37 antibody inhibits re-epithelialisation. Moreover, decreased levels of LL-37 are associated with chronic wounds such as chronic ulcers [103]. LL-37 could contribute to cutaneous wound vascularisation as well, because application of LL-37 results in angiogenesis in two model systems. This peptide stimulates proliferation of cultured human umbilical vein endothelial cells (HUVECs) and causes endothelial sprouting in a hamster model system, biological activities that appear to be mediated by interaction of LL-37 with FPRL1 expressed on endothelial cells [104].

Regulation of AMP expression in skin

Some antimicrobial peptides such as hBD-1 are virtually constitutively expressed in skin keratinocytes. HBD-1 mRNA and hBD-1 immunoreactivity were found to be consistently expressed in skin samples from various body sites and were localized to the suprabasal keratinocytes, sweat ducts and sebaceous glands of human skin [66, 105], suggesting that hBD-1 represents a rather constitutively expressed antimicrobial peptide. The expression of hBD-1 in suprabasal, more differentiated areas of the skin (malpighian layer, stratum corneum) suggest that differentiation regulates hBD-1-expression. *In vitro* differentiation of keratinocytes can be induced by increasing the concentration of Ca^{2+}, conditions which led to upregulation of hBD-1 gene expression [106–108]. In contrast to many other antimicrobial peptides, gene expression of hBD-1 is not markedly induced by proinflammatory cytokines such as IFN-γ, IL-1β or TNF-α, or by bacteria such as *P. aeruginosa* [108].

HBD-2 is induced by differentiation, IL-1 and *P. aeruginosa*

In contrast to hBD-1, hBD-2 expression is markedly increased in the skin surrounding inflamed regions, but is not detectable in adjacent noninflamed regions, supporting the hypothesis that hBD-2 is inducibly expressed *in vivo*. HBD-2-induction in skin keratinocytes only occurs in differentiated cells. Whereas basal cell layers of the epidermis and a basal cell carcinoma cell line (KB) always lack hBD-2-immunoreactivity [66], in cultured primary keratinocytes conditions that facilitate differentiation – such as culture of keratinocytes in the presence of high concentrations of Ca^{2+}– increases background hBD-2 expression [108, 109], suggesting that sufficient differentiation is a prerequisite of hBD-2 production in skin.

Genomic analyses of the hBD-2 promoter region revealed several putative transcription factor binding sites, including nuclear factor kappa B (NFκB), activator protein 1 (AP-1), AP-2 und NF-IL-6, which are known to be involved in the induction and regulation of inflammatory responses [60, 110]. For induction of hBD-2 in skin ker-

atinocytes and respiratory tract epithelial cells under inflammatory conditions, IL-1α, IL-1β or *P. aeruginosa* have proven to be the most effective *in vivo*-relevant stimuli, whereas in these cells other bacteria and cytokines such as TNF-α or IL-6 have little or no ability to induce hBD-2 [51, 54, 108, 111–113]. Recently, the Th1 cell-derived cytokine IL-22 has been identified as a potent stimulus for hBD-2 and hBD-3 expression in keratinocytes, where expression of the IL-22 receptor was upregulated by IFN-γ [114].

Toll-like receptors: major pathogen-recognition-receptors for hBD-2-induction?

The induction of host antimicrobial molecules following binding of pathogen components to pathogen pattern recognition receptors such as CD14 and the Toll-like receptors (TLRs) is a key feature of innate immunity. Epithelia represent important environmental interfaces where LPS recognition pathways may be important in inducing hBD-2. Indeed, in tracheobronchial epithelial cells, hBD-2 is induced by a LPS preparation, ultimately activating NFκB through a CD14-dependent mechanism [111]. This might be possible also in skin keratinocytes, although this is still an open question. Induction of hBD-2 via TLR-4 was seen in some studies [115–118], whereas others observed no hBD-2-induction in keratinocytes with LPS [112]. In other studies, no TLR4 expression was seen in normal keratinocytes, but it was seen in HaCaT keratinocytes [119, 120]. TLR-2 may also mediate hBD-2 induction. HEK293 cells transfected with TLR-2 respond to stimulation with bacterial lipoprotein by hBD-2 production [121]. In one study, commercial LPS preparations could stimulate epidermal keratinocytes to produce hBD-2 and IL-8, and the LPS response was inhibited with monoclonal antibody (mAb) specific for TLR2, but not for CD14 or TLR4. Repurified LPS and lipid A did not stimulate epidermal keratinocytes, whereas peptidoglycan from Gram-positive bacteria and yeast cell wall particle induced β-defensin-2 and IL-8 production. Thus, epidermal keratinocytes express functional TLR2, but not CD14 or TLR4. And the 'LPS' response of epidermal keratinocytes shown in several previous studies was suggested to be mediated by TLR2-dependent recognition of non-LPS bacterial components contaminating commercial LPS preparations [122].

IL-1 is the major endogenous inducer of hBD-2

The expression of hBD-2 in skin keratinocytes was previously found to be IL-1 dependent [113]. Only the IL-1R antagonist inhibited the hBD-2 expression in epidermal culture that has been stimulated either with supernatants of LPS-treated mononuclear cells or LPS alone, suggesting that keratinocyte-derived IL-1 may also contribute to the induction of hBD-2. Thus, induction of hBD-2 may predominantly occur through IL-1 signalling [112]. *In vivo* the indirect way via mononuclear phagocyte-derived IL-1 appears to be the most relevant induction pathway in cutaneous inflammation, because keratinocytes can only produce the inactive IL-1β precursor, and the pool of stored IL-1α is not released [113].

Direct bacterial induction of hBD-2 by a novel pathway?

A number of reports indicate that bacteria can also directly stimulate epithelial cells for production of hBD-2 – a pathway that might represent an ancient, cytokine-independent pathway possibly also used by invertebrates and plants. In oral epithelial cells (or cell lines) of different origin, hBD-2-transcription is induced directly by various bacteria (and its cell wall products), e.g. *P. aeruginosa, E. coli, S. aureus, S. epidermidis* and *Fusobacterium nucleatum* (an oral commensal bacterium), but not or far less by *Streptococcus pyogenes* and *Porphyromonas gingivalis* (an periodontal pathogen) [123–125]. In a study with respiratory tract epithelial cells a clinical isolate of a mucoid phenotype of *P. aeruginosa* turned out to be the most powerful inducer of hBD-2 in these cells [60] and in primary skin keratinocytes [our unpublished results]. The observation that only this mucoid *P. aeruginosa* strain induces hBD-2 at a low bacteria/epithelial cell ratio support the hypothesis that apart from known common TLR-activating ligands such as LPS, peptidoglycan and flagella filament structural protein, most likely other, yet-to-be-determined 'pathogen-associated molecular patterns (PAMPs)', are responsible for hBD-2 induction. Kinetic analyses of bacteria-induced hBD-2 production indicate involvement of multiple distinct signalling pathways. One study has revealed that NFκB is neither essential nor sufficient for hBD-2 induction. E.g. hBD-2 regulation by *F. nucleatum* occurs via p38 and Jun-N-terminal kinase (JNK), while phorbolester (which is a powerful β-defensin inducer in keratinocytes [108]) induces hBD-2 via the p44/42 extracellular signal-regulated kinase pathway [124].

Growth factors are major hBD-3 inducers

Although hBD-3 represents an inducible β-defensin, its expression is regulated in a different manner than expression of hBD-2. In skin-derived keratinocytes as well as in gingival keratinocytes, hBD-3 mRNA is moderately induced by TNF-α, whereas INF-γ is a more powerful hBD-3-inducing cytokine [69, 126, 127]. Growth factors of major importance in wound healing, insulin-like growth factor-I (IGF-I) and transforming growth factor-α (TGF-α), induce the expression of hBD-3 in human keratinocytes. This induction occurs by transactivation of the

epithelial growth factor (EGF) receptor [112]. In this process the EGF receptor is activated by membrane-bound ligands such as TGF-α released through a metalloprotease-dependent process, which may lead to a rapid localized activation of the receptors by low ligand concentrations. Interestingly, proinflammatory cytokines such as IL-1 and IL-6 may modulate the transactivation of the EGF receptor, leading to a further increased expression of hBD-3 during inflammation, even though these cytokines did not induce hBD-3 expression directly [112].

Like β-defensins in cattle and hBD-2, hBD-3 is also induced when epithelial cells come into contact with bacteria [69, 72, 128]. This induction occurs at various levels, suggesting dependency on the virulence state, a hypothesis supported by the observation that a mucoid clinical isolate of *P. aeruginosa*, as seen for hBD-2 [60] and RNase-7 [26], is the strongest microbial inducer of hBD-3 [69].

Downregulation of β-defensin expression

Whereas several studies reveal mechanisms of β-defensin induction in epithelial cells by various stimuli, there is only little information about downregulation of hBD induction. Whereas in one study LPS-dependent hBD-2 induction in a human airway cell line can be inhibited by corticosteroids [129], another study failed to identify inhibitory effects of dexamethason upon hBD-2 induction in bronchial epithelial cells, but observed inhibition of hBD-3 induction [130]. In skin keratinocytes *all-trans* retinoic acid dose-dependently downregulated hBD-2, hBD-3 and hBD-4 induction by various stimuli, suggesting that these therapeutics may have yet unrecognised side effects by downregulating innate epithelial defense effector molecule expression [108].

Regulation of cathelicidin LL-37 expression

The cathelicidin hCAP18/LL-37 also represents an inducible AMP that is produced by skin keratinocytes *in vivo* upon wounding and in inflammation [84, 103]. In an *in vitro* model, the highest hCAP18 levels are attained at 48 h post-injury, declining to pre-injury levels upon wound closure [103]. Apart from infiltrating neutrophils, hCAP18 is detected in the epithelium migrating over the wound bed, suggesting that hCAP18/LL-37 induction in keratinocytes depends on wound-healing processes [103]. In support of this hypothesis, it was found that IGF-I induces the expression of hCAP-18/LL-37 in cultured human keratinocytes [131].

Another major inducer of hCAP-18/LL-37 might be 1,25-Dihydroxyvitamin D3. 1,25-Dihydroxyvitamin D3 and three of its analogs induced expression of hCAP-18/LL-37 [132]. The induction was observed in immortalized keratinocytes and several other cells. It occurred via a consensus vitamin D response element (VDRE) in the hCAP-18/LL-37 promoter that was bound by the vitamin D receptor (VDR). Interestingly, induction of the hCAP-18/LL-37 homolog in murine cells was not observed. This was due to the absence of the VDRE in the murine hCAP-18/LL-37 homolog promoter [132].

Implications of AMPs in skin diseases

The observation that AMPs are expressed in the uppermost parts of the epidermis in skin may suggest a protective function of these effector molecules as part of a 'chemical barrier' in skin infection and inflammation. 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. On the other hand, it is possible that overexpression of AMPs in skin may lead to increased protection against skin infection. 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 [50]. Our biochemical analysis of antimicrobial peptides in psoriatic scale extracts have shown the presence of hBD-2, RNase 7 and psoriasin as principal antimicrobial proteins. Moreover, hBD-3 and several other as yet not structurally characterized antimicrobial peptides were identified (fig. 1) [29].

In contrast to psoriasis, patients with atopic eczema often suffer from skin infection with *S. aureus*. In acute and chronic lesions of these patients, a decreased expression of hBD-2, hBD-3 and LL-37 has been observed [127, 133]. It is believed that the expected induced expression of antimicrobial peptides in inflamed atopic lesions is inhibited as a result of elevated Th2 cytokines in atopic skin. Indeed, it could be shown that Th2-cytokines such as IL-4 and IL-13 suppress the cytokine-mediated induction of hBD-2 and hBD-3 [127, 133]. The lack of these inducible antimicrobial peptides, however, could be also interpreted by the lack of major inducers in skin, such as IL-1β, or the rather selective endogenous 'defensin inducer' IL-22 [114].

Skin-derived antimicrobial peptides may also play a role in other skin diseases. In lesional and perilesional epithelium of patients with acne vulgaris, a marked upregulation of hBD-2 has been observed that might be the consequence of inflammation [134]. An upregulated expression of hBD-2 was also seen in superficial folliculitis [135]. The expression of LL-37 in keratinocytes of patients with condyloma acuminatum and verruca vulgaris was found to be increased, suggesting a role of this cathelicidin in cutaneous papillomavirus infection [93]. Furthermore, LL-37 is able to inactivate vaccinia virus, which has implications for eczema vaccinatum [136].

Decreased levels of antimicrobial peptides and proteins have been found to be associated with burns and chronic wounds. In skin, hBD-2 is only produced by keratinocytes, suggesting that lack of keratinocytes in chronic ulcera or dead keratinocytes, as in burn wounds, may result in a lack of hBD-2 [137, 138]. This suggests a host defense defect within the burn wound and a therapeutic role for antimicrobial peptides in the management of burn wounds [138]. Indeed, a transient cutaneous adenoviral gene therapy with hCAP-18/LL-37 was found to be effective for the treatment of burn wound infections [139].

These observations allow us to speculate that other recurrent skin infections may be associated with a dysregulation of antimicrobial peptide and protein production caused by lack of induction, inhibition by Th2 cytokines or by pathogenic bacteria that may either inactivate these peptides [140] or interfere with induction.

General conclusions and future directions

Findings of the past 10 years clearly show, that apart from the physical defense shield with the formation of stratum corneum, skin epidermal cells also have the capacity to mount a 'chemical barrier'. This barrier includes preformed compounds present at the uppermost layers of the skin (including the stratum corneum), as well as newly synthesised antimicrobial peptides and proteins that are produced within the living epidermis upon stimulation. The stimulus could be wounding, which may include disruption of the physical barrier, or contact with pathogenic bacteria or bacterial products and/or endogenous proinflammatory cytokines and growth factors.

A number of peptides and proteins with antimicrobial activity of different protein families have been described in skin. The relative importance of each in protecting skin from infection or actively defending against infection is currently speculative. One would expect that skin-derived antimicrobial peptides *in vivo* should be present in amounts that have been seen to be microbicidal *in vitro*. This has been demonstrated in part by *in vivo* experiments in mice for cathelicidins [94] and in humans for psoriasin [19].

Although there is currently no report describing the relative abundance of currently known antimicrobial peptides and proteins of different classes in healthy and inflamed skin, a rough estimate of proteins present in skin extracts after RP-HPLC by silver staining, together with ESI-MS analyses for verification of its structure, indicates, that in healthy skin-derived stratum corneum extracts psoriasin represents the most abundant AMP, followed by RNase 7 and lysozyme (fig. 6a). Whereas hBD-2 was isolated only in low amounts, hBD-3 as yet wasn't detected. hCAP-18/LL-37 as well as proteolytic fragments thereof have not yet been identified in the extract, suggesting that LL-37 and its fragments represent rather a trace AMP in healthy

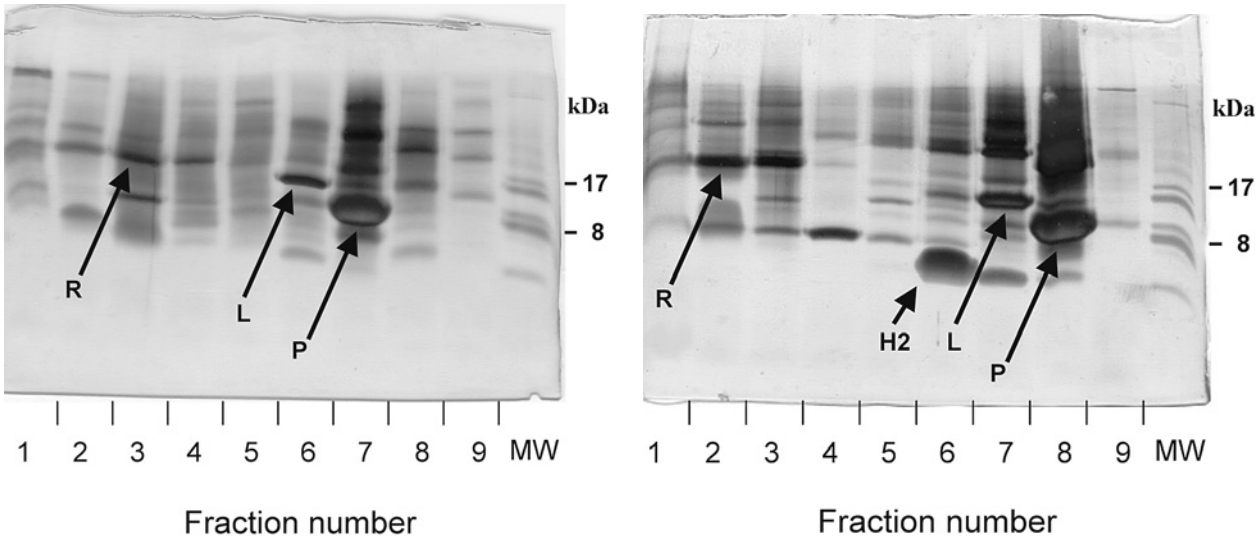

Figure 6. SDS-PAGE analyses of proteins in extracts of stratum corneum and psoriatic scales, after RP-HPLC. 10 µl-aliquots of HPLC fractions (as shown in fig. 1) were analysed in a Tricine/SDS/urea-PAGE-system [69], and proteins and peptides were silver stained. Note in healthy pooled stratum corneum extract (*A*) psoriasin (P), RNase 7 (R) and lysozyme (L) as predominant antimicrobial proteins, confirmed by ESI-MS-analyses (data not shown). The 4-kDa band of a hydrophobic peptide eluting behind psoriasin does not show identity with the cathelicidin LL-37 and belongs to an unrelated peptide. In psoriatic scale extracts (*B*) psoriasin (P) represents again the most abundant antimicrobial protein. The second most abundant antimicrobial peptide is HBD-2 (H2), followed by lysozyme (L) and RNase 7 (R). Again, in HPLC fractions where LL-37 or truncated products would be expected, neither silver-stained bands nor ESI-MS analyses revealed a peptide that would correspond to LL-37.

skin that is present in amounts only detectable with sensitive immunochemical methods. The faint band of a 4-kDa peptide, which is reproducibly seen upon RP-HPLC fractions with elution time corresponding to LL-37, shows upon ESI-MS analyses an exact mass of 4037, which does not fit with the mass of LL-37 or any mass of the LL-37 fragments found in sweat [25], suggesting that it belongs to a different peptide. HPLC analyses of psoriatic scale extracts with sensitive silver staining of protein bands (fig. 6b) and identification of the currently known AMPs by ESI-MS analyses revealed again psoriasin as the most abundant AMP, now followed by hBD-2 and RNase 7, lysozyme, HNP-1-3 and elafin [141]. Although HNP-1-3 may originate from infiltrating neutrophils (leukocytes, which are very common in active psoriasis lesions), the absence of myeloperoxidase and lactoferrin in these extracts make it possible that HNP-1-3 also originates from keratinocytes [141]. HBD-3 can also be detected, but it is not found as a major peptide [141]. Again, LL-37 has not been identified with these methods [141]. Thus, one may conclude that host defense of healthy human skin is mediated, in part, by a 'chemical barrier', which consists in several AMPs that are expressed at different parts of the epidermis, which comprises various keratinocyte layers with multiple states of differentiation. Some of these AMPs are constitutively produced in the uppermost areas of the epidermis, where they are stored in lamellar bodies and then secreted upon final differentiation (being now present at the surface of fully differentiated keratinocytes that finally will become stratum corneum).

Psoriasin, the most abundant and secreted antimicrobial protein of healthy skin, is bactericidal by essential trace element zinc deprivation [19]. We suspect that at skin surfaces, microbial growth is mainly controlled by essential trace element deprivation, in particular also iron (by siderophore-binding lipocalin 2) [131], and physical removal from body surfaces by desquamation.

In microwounds, which can occur from physical stress by disruption of the physical barrier, the skin may use an (inducible) antimicrobial defense strategy with induction of keratinocyte-derived AMPs but not proinflammatory mediators, as outlined in figure 7. On intact skin with an intact physical barrier, bacterial growth will be controlled by bacteriostatic and bactericidal compounds (e.g. psoriasin and RNase 7) at the skin surface (left side of fig.7A). Once the physical barrier is disturbed (middle portion of fig. 7A), bacteria (or bacterial products) have access to living epidermal keratinocytes. These may now produce and secrete inducible antimicrobial peptides, which, when present in sufficient amounts or activity, would stop infection – without inflammation. This model, however, would implicate the existence of microbial products that induce antimicrobial peptides without inducing proinflammatory mediators – a hypothesis not yet proven by published reports. However, our own, unpublished observations indicate the existence of such compounds.

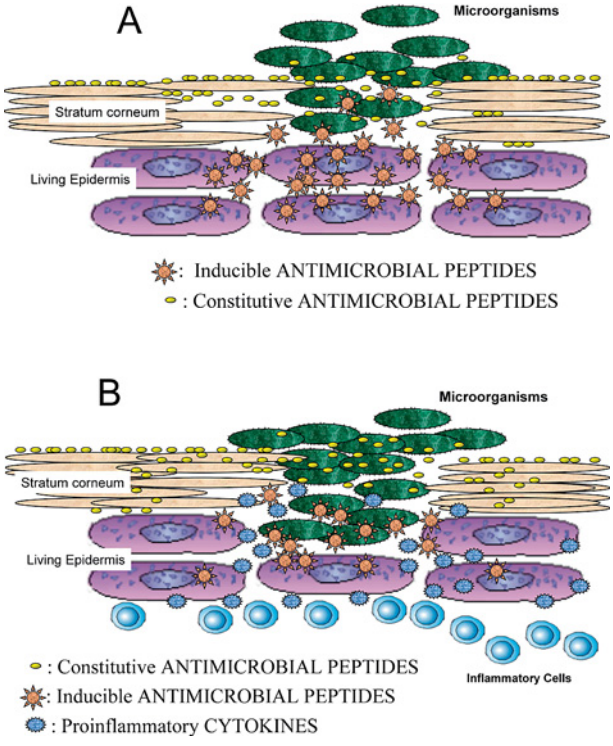

Figure 7. Putative model of a protective function of keratinocyte-derived antimicrobial peptides for microwounds to prevent inflammation. Bacterial growth on intact skin with an intact physical barrier will be controlled by bacteriostatic and bactericidal compounds at the skin surface (*A*, left). Once the physical barrier is disturbed [e.g. loss of stratum corneum (*A*, center)], bacteria (or bacterial products) have access to living epidermal keratinocytes, which may now produce and secrete inducible antimicrobial peptides. When microorganisms induce proinflammatory cytokines and come into contact with keratinocytes and immune cells in deeper areas of the skin, inflammatory cells are recruited, and inflammation begins (*B*).

In the case of extensive wounding of the epithelium or a high load of pathogenic bacteria, which cannot be controlled by epidermis-derived AMPs, inflammatory reactions would be initiated by epidermal recognition of pathogenic bacteria, which may mediate the production of proinflammatory cytokines, initially by keratinocytes, and then followed by immune cells, eventually resulting in leukocyte tissue infiltration of the skin (fig. 7B). The model shown in figure 7A would occur without any visible signs and might therefore never even have attracted notice.

The focal expression of various AMPs in healthy skin without any visible signs of inflammation suggests that, indeed, conditions may exist which cause AMP induction in the absence of proinflammatory cytokines or growth factors produced during wound healing and that microbes present at skin surfaces may facilitate antimicrobial peptide induction without inflammation. A re-

cent observation that a number of probiotic bacteria, including *E. coli* strain Nissle 1917, induce the expression of hBD-2 in Caco-2 intestinal epithelial cells supports this hypothesis. Therefore, the beneficial effects of probiotic bacteria may come from their ability to induce AMPs [142]. It is intriguing to speculate about bacterial components that solely induce AMPs without causing inflammatory reactions. Such compounds could initiate epithelial induction of AMPs and would be ideal 'immune stimulants' that could be used for artificial stimulation of AMP synthesis in various epithelia. In consequence, in such epithelia one would expect increased resistance towards infection.

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