Old Herborn University Seminar Monograph 28:
The epidermis of man: co-existing with commensals.
Editors: Peter J. Heidt, Pearay L. Ogra, Michael Zasloff, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 93-108 (2015).

THE DISCOVERY OF HUMAN EPITHELIAL ANTIMICROBIAL PEPTIDES

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SUMMARY

All higher organisms are living in an environment, which is laden with incredibly large numbers of microorganisms that potentially can harm them. Further, each organism has at its surface a more or less specific commensal microflora, which is characteristic for each species and its anatomical sites. Despite the enormous numbers of microorganisms at body surfaces, infections are a rather rare event. In humans we had explained this unexpected phenomenon until ecently by the presence of humoral and cellular components of the adaptive immune system. This, however, cannot explain, why organisms lacking an adaptive immune system naturally resist infections and therefore this indicates the presence of another, more ancient innate defence system. By addressing the question, why invertebrates resist infections and why cattle epithelia, which are permanently in contact with microbes, are not infected upon wounding, a number of antimicrobial peptides have been discovered using biochemical approaches. These discoveries led to the hypothesis that also human epithelia (skin, gut, lung, genitourinary tract) should produce such antimicrobial compounds for innate defence against infection. This was one of the key questions in the author's team, for which we found some answers by the use of classical biochemical analyses with modern analytical equipment and reasonable antimicrobial read-out systems. It is not the aim of this review to give an overview and discussion of the role of the human peptide antibiotics, which were isolated in our laboratory. Instead, I will give some information how we came to these discoveries, giving more an overview about the stories behind the discovery stories.

INTRODUCTION

Although all higher organisms are living in an environment laden with potentially pathogenic microbes, infections of the skin are rather rare and mostly occurring upon disruption of the epidermal barrier or upon wounding. At a first glance it seems to be clear why this is the case: we are equipped with a very effective adaptive immune system with its phagocytes and selective antigen-recognizing and antibody-producing immune cells. When thinking about this explanation in more detail, we will recognize several discrepancies: healthy skin does not contain any professional phagocytes, which permanently eliminate microbes from the skin surface and animals (including invertebrates) and plants, which both have no adaptive immune system and

mostly no phagocytes, have to solve the same problem to protect their body surface from infection. How is this possible? A plausible explanation for this, in principle unexpected, phenomenon is the presence of factors which limit the growth of microbes and/or the presence of antibiotic factors, in particular antimicrobial peptides (AMPs). When such factors are located at the body surface, these should effectively control invasion of microbes and inhibit infection. The important question, how an adaptive immunesystem-lacking organisms (like invertebrates) protect themselves from microbial infection has been addressed by pioneering work of the group of Hans Boman in silkworm larvae (*Steiner* et al., 1981) and the group of Jules Hoffmann in the fruitfly (*Fehlbaum* et al., 1994). Both groups identified insect antimicrobial peptides like cecropins or drosomycin and others. These findings prompted search for the existence of AMPs in vertebrates, where textbook knowledge told us that antimicrobial defence was achieved, apart from the lytic complex of activated complement, by phagocytes as professional effector cells of the adaptive immune system. As bactericidal effector molecules, reactive oxygen species, generated from the myeloperoxidase-hydrogen peroxide-halogenide system, as well as nitric oxide seemed to be solely relevant. Not before Robert Lehrer and co-workers observed that apart from these short-living inorganic compounds also protein-like bactericidal and fungicidal activity is associated with phagocytes, it became clear that also vertebrates are preventing microbial infection via peptides called "defensins" (*Ganz* et al., 1985).

Utilizing the *Xenopus laevis* oocyte system to study RNA expression in eukaryotes, Michael Zasloff wondered why incisions made in the frog's skin did not cause any infections despite the fact that freshly surgically treated animals were put into a microbially contaminated laboratory tank. This surprising observation led to the hypothesis that frog skin contains an antibiotic principle, which protects it from microbial infection. As antibiotic compound, a peptide has been characterized which was termed "Magainin" (*Zasloff*, 1987). Magainin is the first discovered vertebrate AMP produced by epithelial cells. With the finding that frog skin contains antibiotic peptides it was suggested that also epithelia of other vertebrates, such as cattle, could have the capacity to produce AMPs. Subsequently a structurally unrelated epithelial antimicrobial peptide (AMP) has been discovered in cattle trachea ("tracheal AMP, TAP") (*Diamond* et al., 1991). The hypothesis that grazing led to many small wounds on the tongue epithelium without infection, led to the discovery of another AMP belonging to the so-called beta-defensin family, the "lingual antimicrobial peptide, LAP" (*Schonwetter* et al., 1995). Subsequently another structurally related epithelial beta-defensins has been discovered in cattle gut epithelia ("enteric beta-defensin, EBD") (*Tarver* et al., 1998). These previous observations suggested that also human epithelia, in particular the skin, should also have the capacity to produce similar AMPs. Interestingly, in the midst of the 90's all efforts failed to identify human orthologs of LAP, TAP and EBD in human epithelia, including the skin by using a cloning strategy.

Recent microbiome studies revealed that healthy skin is colonized by a huge number of different genera and species of bacteria and fungi (*Findley* et al., 2013). Further, different skin habitats are hosting different microbial species, e.g. in the moist, rather mucosal areas of the aero-digestive tract and urogeni-

tal tract other species are living than in rather dry and/or lipid-rich areas like the skin of the face, scalp or lower legs.

So the questions come up: what shapes the composition of the cutaneous microbiome at the different skin habitats, why is the number of microbes at the skin surface relatively constant, and why are some microbes present and others not although in principle the growth conditions should be optimal.

THE DISCOVERY OF THE FIRST HUMAN INDUCIBLE PEPTIDE ANTIBIOTIC, HUMAN BETA-DEFENSIN-2 (HBD-2): A HISTORICAL SUMMARY

The major scientific focus of our research in the 90's has been the role of neutrophils and eosinophils in cutaneous inflammation, in particular psoriasis (PS) and atopic dermatitis (AD). In particular, the mediators and cytokines which cause skin infiltration by neutrophils in PS or eosinophils in AD, were in our focus. After we had discovered that the major neutrophil attractants in psoriatic lesions (which we had isolated and purified from lesional psoriatic scale material) are chemotactic cytokines (*Schröder* et al., 1992) [originally termed MONAP or ANAP and today termed as the chemokines interleukin 8 (CXCL8) and Gro-α (CXCL1)], we were interested to know whether, apart from these chemokines, additional neutrophil chemo-attractants are of relevance in psoriasis lesions. With the use of a monoclonal antibody, which recognized an epitope common in both, IL-8 and Gro-α, an affinity-column was generated and lesional psoriatic scale extracts were analysed for remaining PMN-chemotactic activity in the through flow. These findings indicated, that apart from the chemokines IL-8 and Gro-α no other PMN-chemoattractant is present in lesional PS-skin extracts. To recover the affinity-bound IL-8 and Gro-α we stripped the affinity column with acidic glycine-buffer and performed a reversed phase (RP) high performance liquid chromatographic (HPLC) analysis with the stripped material. To our surprise, the major UV-absorbing peaks came not from both expected chemokines, but from unknown proteins. The major UV-absorbing peak gave upon SDS-PAGE analysis a single, silver-stained 15k-band, which stained in the upper area rather brownish and in the lower area rather dark grey (*Schröder*, 2010). Further analyses indicated that the material was not pure and consisted of two components. We had one component identified as lysozyme. The other was unknown. A special HPLC-column allowed separation of lysozyme from the unknown protein, which, after performing SDS-PAGE analysis in urea-containing buffer, now gave a single band at 4k and not 15k, which suggested that this protein forms a tetramer. Although this protein lacked PMN-chemotactic activity, we performed N-terminal sequencing by Edman-degradation. We had not found the proposed amino acid (AA) sequence in a 1992 data bank and there was no similarity to any protein. By chance, I had read a paper about epithelial antibiotics (*Schonwetter* et al., 1995), where the frog skin-derived AMP Magainin showed exactly the same N-terminal three amino acid (AA)-containing peptide motif Gly-Ile-Gly as the 4k-peptide. To check whether we possibly found a human ortholog of Magainin, we re-analysed the Edman degradation raw data and found at two positions a blank (instead

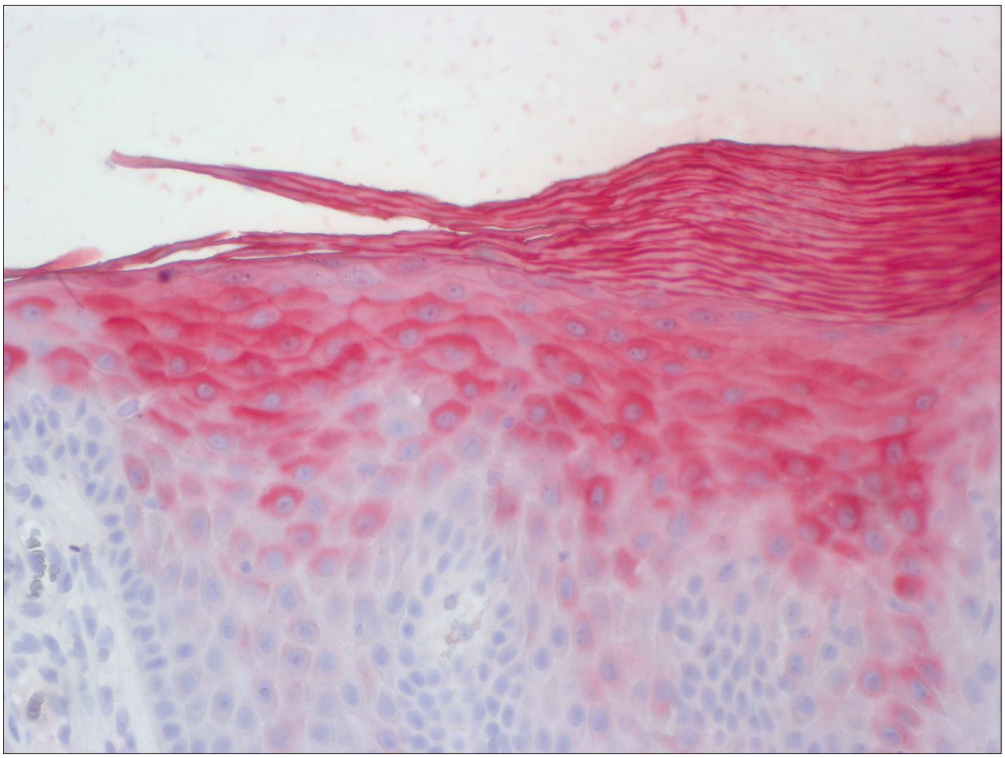

Figure 1: Human beta-defensin-2 is strongly expressed in lesional psoriatic skin. Note the presence of immunoreactive (i)hBD-2 mainly in the upper, granular layers of lesional psoriatic skin, where it is located within granules. In the uppermost granular layer intracellular staining seems to be missing, suggesting release. In the stratum corneum, released ihBD-2 seems to stick to the corneocyte surface.

of the computer-proposed AA). Cysteines in an AA sequence give a blank upon Edman degradation. We therefore inserted Cys residues in the proposed AA sequence at these positions and now found similarity to bovine beta-defensins, suggesting that this peptide is a human beta-defensin. We created the name "human beta-defensin-2 (hBD-2), because another, in blood filtrates found peptide, was termed hBD-1. We then established a radial-diffusion antimicrobial assay (RDA) system as it has been optimized by Bob Lehrer's group (*Steinberg* and *Lehrer*, 1997) to check whether hBD-2 is an AMP. The use of agarose instead of agar (as used for testing of antibiotics in medical microbiology) was essential for detecting antimicrobial activity of hBD-2 (*Harder* et al., 1997) and other cationic peptide antibiotics, confirming recommendations repeatedly reported by Bob Lehrer's group.

INITIAL STUDIES ON THE ROLE OF HBD-2 IN SKIN PHYSIOLOGY AND INFLAMMATION

hBD-2 has been discovered in extracts of lesional psoriatic scale material (*Harder* et al., 1997). Stratum corneum extracts obtained from the heel did not

or nearly not contain hBD-2. This suggests that hBD-2 needs to be induced. Numerous studies reported that hBD-2 is exclusively produced by epithelial cells, like keratinocytes and others (*Schröder* and *Harder*, 2006). Immunohistochemistry analyses show a characteristic staining pattern with strong intracellular expression of hBD-2 in the uppermost stratum granulosum cells, where it is located within the lamellar bodies (Figure 1). The next apical stratum granulosum cell layer now lacks immunoreactive hBD-2, which suggests a release (as lamellar bodies are depleted in that cell layer) of hBD-2 and now localized at the surface of corneocytes.

Although hBD-2 was originally found to be an AMP for *E. coli* (*Harder* et al., 1997), later on various Gram-negative bacteria and the yeast *Candida albicans* were seen to be killed, whereas for *S. aureus* only bacteriostatic activity was seen at high hBD-2-concentrations (*Harder* et al., 2000). Thus, we hypothesized that hBD-2 might be a rather Gram-negative bacteria-directed peptide antibiotic of the skin. The observation that hBD-2 is absent in healthy skin suggested that it needs to be induced. Because it has been isolated from the scales obtained from psoriasis skin lesions and because psoriasis is an inflammatory skin disease where pro-inflammatory cytokines play an important role, TNF-α and IL-1β were tested and found to be powerful hBD-2-inducers in keratinocytes.

Previous studies on induction of beta-defensins in cattle have shown that also heat-inactivated bacteria are able to induce AMPs in tracheal epithelial cells (*Diamond* et al., 1991). This observation prompted us to test the hypothesis of hBD-2-induction in keratinocytes by heat-killed bacteria. Because our research focus was on the role of neutrophils and eosinophils in inflammatory skin diseases and not in microbiology, we had to look for bacteria. Fortunately, the department of dermatology has had that time a microbiology laboratory for diagnostic investigations. When asking for any bacterium we could use in bacterial stimulation experiment, we got a laboratory strain of *Pseudomonas aeruginosa*, which had been isolated from a leg ulcus.

Indeed, this heat-inactivated *P. aeruginosa* strain was found to be able to induce hBD-2 in keratinocytes (*Harder* et al., 1997), suggesting that generally heat-inactivated bacteria are inducing hBD-2 in keratinocytes and, as seen later on, in other epithelial cells. We became aware, however, that this seems not to be a general fact: after we had published our results, someone let us know that they could not reproduce the bacterial stimulation of hBD-2. The strain they had used was a laboratory strain of *Pseudomonas aeruginosa*, PA01. This strain in our hands also failed to induce hBD-2 suggesting that the clinical *Pseudomonas aeruginosa* strain we had accidentally used, should bear some unique characteristics. When re-culturing this ulcus-derived strain, we saw a slimy growth, which was not seen with the laboratory *Pseudomonas* strain PA01. In order to characterize the factor(s) responsible for hBD-2 induction, we also analysed bacteria-free culture filtrates and found strong activity, which apparently was released from the bacteria. A likely candidate bacterial "microbe-associated molecule, MAM" was lipopolysaccharide, which would activate keratinocytes via TLR-4. For keratinocytes there are contradictory studies about the TLR-4 expression in normal keratinocytes, and a recent study suggests that TLR-4 is expressed only in pro-inflammatory cytokine-activated keratinocytes (*Terhorst* et al., 2010). In

addition, studies claiming that LPS is an inducer of hBD-2 in keratinocytes showed that concentrations of 10-100 µg/ml of a LPS-preparation were necessary. These concentrations are nearly 10,000-100,000-fold higher than those able to activate macrophages via TLR-4 for IL-1-production, suggesting that the active principle is a contaminant in the LPS-preparation. This hypothesis is supported by the fact that synthetic LPS is inactive as hBD-2 inducer.

FLAGELLIN IS THE PRINCIPAL *PSEUDOMONAS AERUGINOSA* DERIVED HBD-2-INDUCER

We first followed the hypothesis that *Pseudomonas aeruginosa* laboratory strains should be able to produce and release the hBD-2-inducer. Numerous experiments with different culture conditions eventually revealed that *P. aeruginosa* culture filtrates contain maximum hBD-2-inducing activity, when the bacteria were grown: a) at low nutrient availability conditions, and b) at stationary growth conditions. At these culture conditions the bacteria grow as slimy colonies, similar as we had seen with our clinical *P. aeruginosa* isolate. All attempts to purify the active principle failed. We often ended up, however, with rhamnolipids, which are biosurfactants released from biofilm-forming *P. aeruginosa*. To further study the role of rhamnolipids, which are by themselves not able to induce hBD-2 (*Gerstel* et al., 2009), various *P. aeruginosa* strains were treated with rhamnolipids and supernatants analysed for hBD-2-content. Surprisingly, all *P. aeruginosa* strains released hBD-2-inducing activity, except a flagellin (Fln)-knock out-strain, suggesting that flagellin is the hBD-2 inducer. This could be confirmed and it was seen that Fln induces hBD-2 at a half maximum effective dose of 5 ng/ml (*Gerstel* et al., 2009), a finding that implicates attention for Fln as possible hBD-2-inducing contaminant when using partially purified bacterial MAM-preparations for cell culture experiments.

HBD-2 IS A CHEMOKINE AND CHEMOKINES ARE ANTIMICROBIAL PEPTIDES

The major scientific focus at the time that we had discovered hBD-2 were still the chemotactic cytokines, which since 1992 are termed chemokines. At a Keystone conference on chemokines in the winter of 1996-1997 I had a private scientific conversation with J.J. Oppenheim, a well-known chemokine scientist, on the role of alpha-defensins as leukocyte chemotactic factors. When I told him that we had discovered in psoriasis scale material a new defensin which was a beta-defensin, he speculated that this could be, like the alpha-defensin HNP-1, also a leukocyte chemo-attractant acting as a chemokine. Although initial tests with chemokine-receptor transfected HEK293 cells failed to support his hypothesis, a CCR6-HEK293 transfectant responded by chemotaxis towards psoriatic scale-derived hBD-2 in a dose-dependent manner. This finding indicates that beta-defensins use a chemokine receptor although there is no sequence homology with the only chemokine lig-

and for CCR6, LARC (CCL20) (*Yang* et al., 1999). Although the concentration of hBD-2 needed for half maximum chemotaxis is much higher than that of CCL20, it is likely to be relevant *in vivo* because the amounts of hBD-2 produced within the skin for direct antimicrobial defence are within the µ *M*-range whereas tissue CCL20 concentrations are in the n*M*-range. Due to the limited availability of natural hBD-2 (which was purified from psoriatic scale extracts), hBD-2 has been chemically synthesized. Initially synthetic hBD-2, however, was far less potent than the psoriasis-derived hBD-2 in chemotactic activity, although antimicrobial properties of natural and synthetic hBD-2 did not differ. The reason for this discrepancy are connections of the three disulphide-bridges, which have a defined connectivity in the natural hBD-2 and which represent a mixture of all theoretically possible variants in synthetic hBD-2. In the case of the beta-defensin hBD-3 it was shown that only the natural isomer is chemotactically active and binds to the chemokine receptor. CCR6 is highly expressed in dendritic cells (DC) and memory T cells. So hBD-2 *in vivo* may, through CCR6, recruit immature DCs and memory T cells to sites of microbial invasion in the skin and mucosa (*Yang* et al., 1999).

The observation that the chemokine receptor CCR6 is a target of a beta-defensin led to the hypothesis that also chemokines could be antimicrobial peptides. This was proven for CCL20 and several others including the IFN-γ-inducible chemokines MIG (CXCL9), IP-10 (CXCL10), as well as SDF-1 (CXCL12). Interestingly neither IL-8 (CXCL8) nor RANTES (CCL5) were antimicrobially active (*Icard* et al., 1986). The high concentrations (10 µg/ml) necessary to elicit antimicrobial activity would explain that an unbiased biochemical approach so far failed to identify chemokines as AMPs and makes it unlikely that chemokines represent AMPs *in vivo*.

DISCOVERY OF PSORIASIN/S100A7 AS THE PRINCIPLE SKIN FACTOR PREVENTING *E. COLI* INFECTION

More than 30 years ago medical students had to perform an experiment in a medical microbiology course where they dipped fingers of one hand in a *S. aureus* suspension and fingers of the other hand in an *E. coli* suspension. After incubation in a moist atmosphere they made a fingerprint on agar plates and investigated bacterial growth after some time. They found, to their surprise, that clear fingerprints were seen with *S. aureus*, but nothing was seen with *E. coli*. The conclusion drawn from this observation was that finger surfaces contain factors that kill *E. coli*, but not *S. aureus*. Surprisingly, this experiment has been done by generations of medical students but never the question was addressed why and how solely *E. coli* is killed. It was therefore our aim to understand the molecular basis of these findings. Because originally these experiments have been done with fingers, the whole hand was incubated in buffer-containing gloves and thereafter the washing fluid was analysed for *E. coli*-cidal activity. As principal *E. coli*-cidal component the S100 protein psoriasin (S100A7) was identified (*Gläser* et al., 2005). *In vitro* dose-response studies revealed an LD90, which is well in the range found at the skin surface (*Gläser* et al., 2005). To investigate whether psoriasin is an

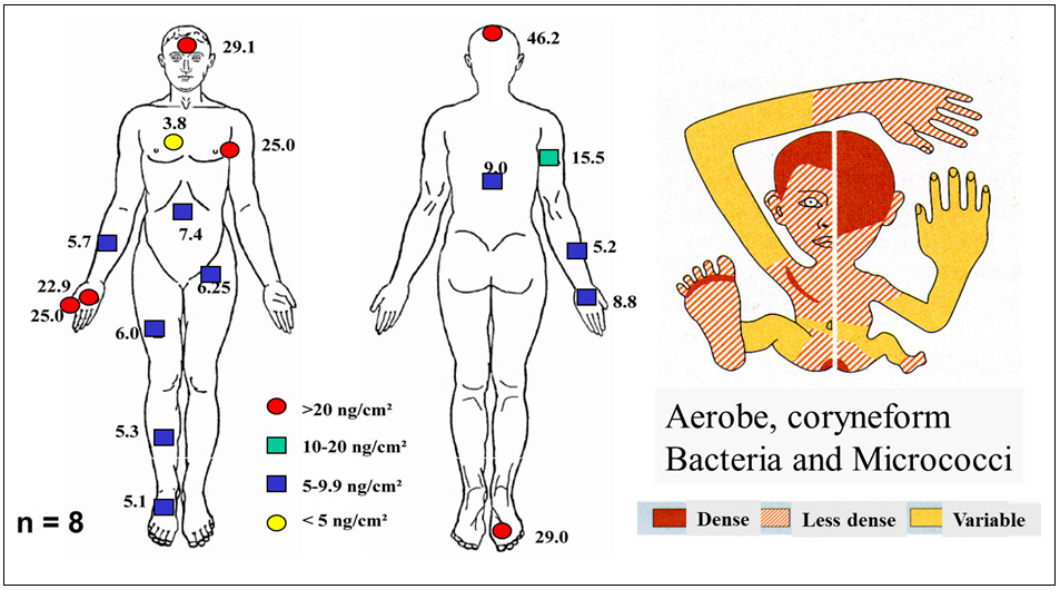

Figure 2: Psoriasin is secreted *in vivo* on the body surface. Standardized areas of various body locations on healthy volunteers were rinsed with 10 mM sodium phosphate buffer, pH 7.4, to determine the concentration of psoriasin present on the skin by ELISA. Note the presence of highest amounts of psoriasin in areas, where bacterial densities are highest (adapted from: Gläser et al, 2005).

AMP *in vivo* capable of killing *E. coli* on the skin surface, we applied *E. coli* psoriasin antibody or an irrelevant antibody. As result it was shown that psoriasin antibodies increased survival of *E. coli* on the skin surface (*Gläser* et al., 2005). Thus, psoriasin contributes to the skin's marked resistance towards infection by the gut bacterium *E. coli* although additional, yet not identified factors at the skin surface, play an additional role in defence. We were interested to see where psoriasin is located in healthy skin. Immunohistochemistry revealed unexpectedly a patchy staining pattern only in the uppermost keratinocyte layers (stratum granulosum) of healthy skin. Sometimes patches within the stratum corneum layer showed marked psoriasin staining, but not living epidermis areas beneath. This could be interpreted as local, temporary induction of psoriasin, maybe two weeks before taking the on defined forearm skin areas in the absence or presence of a neutralizing biopsies. In other words, psoriasin seems to be induced locally at the skin surface. This suggestion was confirmed by analysis of the local psoriasin amounts present at different anatomical skin sites (Figure 2). A markedly increased psoriasin amount was seen at some anatomical locations such as the scalp, hands, feet, axilla and face. On the other side, at rather dry anatomical sites such as forearm or lower leg, psoriasin amounts were rather low (*Gläser* et al., 2005). Anatomical areas with high amounts of psoriasin are highly colonized by microbes. Therefore, it was tempting to speculate that the local skin microflora, or in general bacteria, induce psoriasin in the ecological niches where they are colonizing. Immunohistochemical analyses supported this suggestion: here we saw strong psoriasin staining in the lower

parts of hair follicles, exactly where high microbe densities are present. To further test the hypothesis that bacteria induce psoriasin in skin keratinocytes, *E. coli* culture media were applied to healthy donor's skin, which increased psoriasin levels in washing fluid (*Gläser* et al., 2005). This suggests that bacterial culture supernatants contain a psoriasin-inducing component (which was identified as flagellin) and not, as commonly suggested, bacterial LPS (*Abtin* et al., 2008). It is important to note that ng/ml-amounts of flagellin are sufficient to induce psoriasin or hBD-2. Therefore, the high amounts of LPS preparations (10-100 µg/ml) necessary to elicit AMP-induction in keratinocytes (or other epithelial cells) and the absence of the LPS-receptor TLR4 suggests that commercially available LPS-preparations (phenol-extracts) commonly used could be contaminated by ng-amounts of flagellin.

The rather preferential killing of *E. coli* by psoriasin suggests that it could represent an important defence effector molecule of the genito-urinary system at locations where *E. coli* contamination is a principal risk. Most commonly, urinary tract infections are due to uropathogenic *Escherichia coli*, which are classically thought to migrate from the gut to the bladder and then subsequently undergo highly specialized adaptations to increase their pathogenicity within the bladder (*Schaeffer*, 2013). Since *E. coli* permanently challenges the healthy female reproductive tract it should have an effective epithelial defence system to inhibit *E. coli* survival at these locations and its migration to the bladder. A recent study analysed *E. coli*-cidal factors in vaginal secretions of healthy women and identified psoriasin as the principal *E. coli* killing antimicrobial (*Mildner* et al., 2010), which is constitutively expressed in vulva, vaginal, and ectocervical epithelium but not in endocervical epithelium.

The human mouth is an area colonized by a high variety of microbes and challenged by microbes contaminating food. Despite these permanent threats, the human tongue is highly resistant against microbial colonization and infection by oral intake. *E. coli* acts as an indicator organism for the microbiological quality of food and beverages. Despite daily exposure of *E. coli* strains from animal reservoirs through the mouth, lingual infections with *E. coli* (which is not a member of the oral microflora) are rather rare. This suggests the presence of *E. coli*-cidal factors in the mouth. Biochemical analyses of tongue tissue-extracts identified again psoriasin (S100A7) as a dominating antimicrobial component of the healthy human tongue (*Meyer* et al., 2008). The highest psoriasin expression was found in the anterior part of the tongue with decreasing expression posteriorly. Since the anterior part of the tongue has first contact with microbes and is more vulnerable to surface trauma, it is plausible that this lingual region requires additional protection by high expression of antimicrobial proteins such as psoriasin. Psoriasin seems to be stored and rapidly released to the surface with minimal adherence to the most superficial epithelial cells, as suggested by high psoriasin levels found in the rinsing fluids of human tongues. Interestingly repeatedly rinsed standardized areas of the human tongue of healthy volunteers could not reduce the psoriasin concentration, which might indicate a high psoriasin production in the upper lingual epithelium, possibly as effective defence response towards to permanent microbial and/or inflammatory stress.

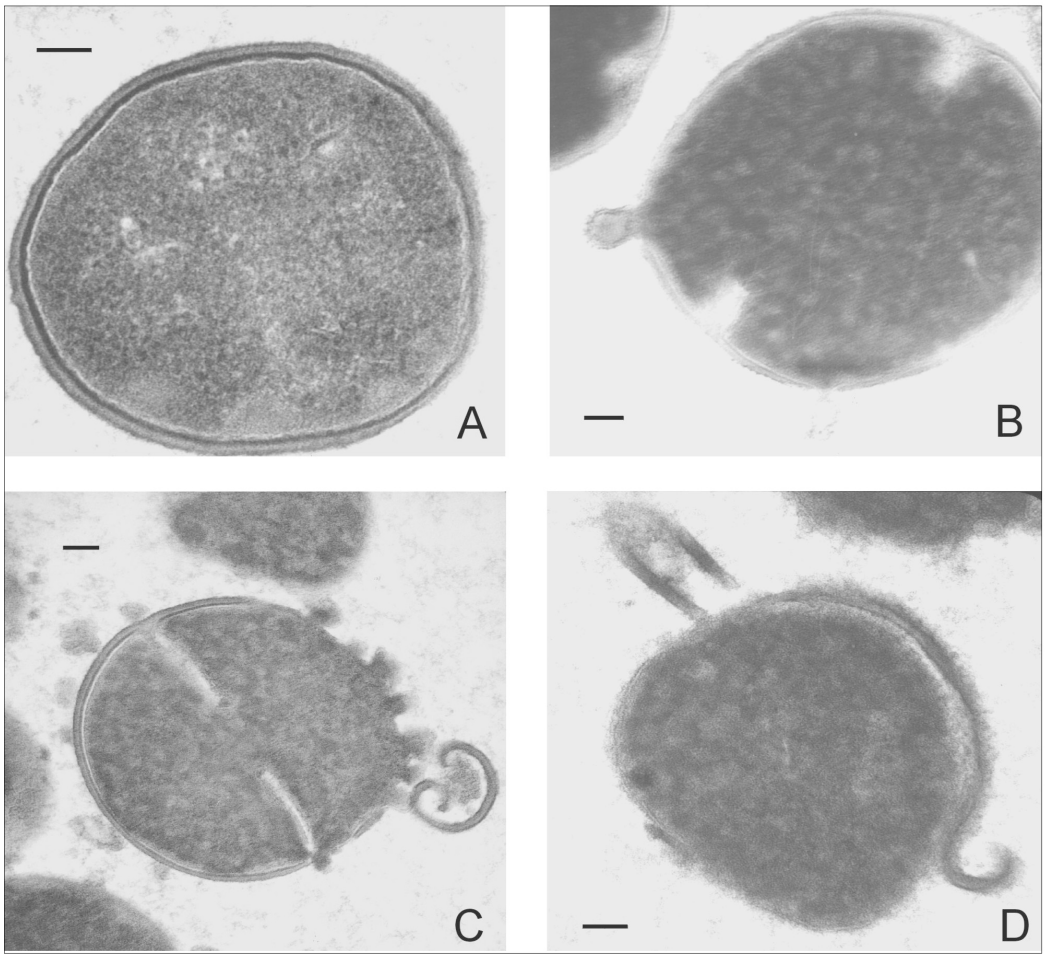

Figure 3: Morphology of hBD-3-treated *S. aureus*. Transmission electron micrographs of *S. aureus* (10^8 cells/ml) incubated in 10 mM phosphate buffer for 2h (A) or treated with synthetic hBD-3 (500µg/ml) for 30 min (B) or 2h (C and D) are shown. Bars represent 0.1µm (from: Harder et al, 2001).

THE DISCOVERY OF HUMAN EPITHELIAL ANTIMICROBIAL PEPTIDES CAPABLE OF KILLING *STAPHYLOCOCCUS AUREUS*

The observation that hBD-2 is selectively killing Gram-negative bacteria and *Candida albicans* but not *S. aureus* (*Harder* et al., 2000) prompted us to search for human *S. aureus*-killing AMPs. With the idea that a *S. aureus*-killing AMP should bind first to *S. aureus*, we generated affinity columns where *S. aureus* has been covalently coupled. These columns indeed bound *S. aureus*-killing peptides, which we suspected in lesional psoriatic scale extracts, the source of hBD-2. Purification and subsequent structural analyses gave a new, 5,054 Da peptide, which was termed "human beta-defensin-3, hBD-3" due to its structural similarity to beta-defensins (*Harder* et al., 2001). Unlike hBD-2, hBD-3 is a broad-spectrum AMP. Although it has been dis-

covered as *S. aureus*-killing factor, it showed activity against various Gram-positive and Gram-negative bacteria as well as *Candida albicans* with minimal bactericidal concentrations at sub-micromolar concentrations (*Harder* et al., 2001). It is killing *S. aureus* by interacting with the lipid II-system, similar as penicillin, resulting in very similar ultrastructural changes (Figure 3; *Harder* et al., 2000) Although hBD-3 is inducible by mucoid *P. aeruginosa* strains (*Harder* et al., 2001), its major induction pathway is EGF-receptor-dependent (*Sørensen* et al., 2005), making this AMP important in wound healing processes where the missing physical skin barrier makes the wound highly vulnerable towards infection. Main cellular sources of hBD-3 are epithelial cells, although also muscles contained hBD-3-transcripts (*García* et al., 2001). Apart from being a broad-spectrum antibacterial peptide, hBD-3 has antiviral activity: several studies report expression of hBD-3 in common warts, molluscum contagiosum and human papilloma virus infections of the skin and mucosa (*Meyer-Hoffert* et al., 2008, 2010). This induction, which seems to be independent of NFκB, possibly occurs TLR-3-dependently, as it has been seen by other antiviral peptides like HD-5.

SYSTEMATIC SEARCH FOR OTHER SKIN-DERIVED AMPS

hBD-2 and hBD-3 have been originally discovered and purified by following the hypothesis that these should bind to targeted bacteria with the use of bacteria-coated affinity columns. To perform the analyses in a more general biochemical systematic way, we optimized conditions for AMP-extraction, separation and purification. Various frustrating pitfalls to obtain some AMPs from skin specimens in the last twenty years prompted me to publish a successful way to purify natural human skin-derived AMPs (*Schröder*, 2010). Several aspects needed special consideration: the read-out system should allow the detection of cationic antimicrobial peptides. Therefore instead of agar, always low electro endosmotic agarose had to be used because agar acts as cation exchanger, binding all cationic AMPs, which leads to false-negative results (*Schröder*, 2010). Biochemical techniques had to be established, which allowed sufficient separation as well as structural characterization of AMPs. We used the strategy to first extract AMPs from stratum corneum (SC), because preliminary experiments revealed SC as rich source of antimicrobially active factors. Extraction conditions needed optimization with ethanol-containing acidic buffers with volatile acids (to perform ESI-MS-analyses without problems). Enrichment of cationic AMPs was possible by heparin-affinity chromatography (because heparin is a weak cation-exchanger). The low antimicrobial activity in the effluent confirmed that the majority of antimicrobial activity came from cationic AMPs and not from anionic compounds such as fatty acids.

By reversed-phase (RP) high performance liquid chromatography (HPLC), a procedure, which allowed separation according to hydrophobicity, we separated heparin-bound material. Then aliquots of HPLC fractions were analysed for antimicrobial activity. Using different bacterial species (which could have been cultured at different conditions) and different radial-diffusion-test conditions (aerobic, anaerobic, neutral pH, low pH, low or high ionic strength, presence or absence of nutrients) a

wide variety of broad-spectrum AMPs or bacteria-specific AMPs could be identified.

Final purification to homogeneity needs methods which allow separation of contaminants by utilizing the different physicochemical properties of AMPs and contaminants, e.g. by using cation-exchange-HPLC followed by narrow pore RP-HPLC (*Schröder*, 2010).

THE DISCOVERY OF RNASE 7, HEALTHY SKIN'S PRINCIPAL AND BROAD-SPECTRUM AMP

The discovery of high amounts of AMPs like hBD-2 and hBD-3 in lesional psoriatic scale material supports the hypothesis of psoriasis skin lesions being infected less frequently by bacteria and fungi than one would have expected. Thus, induction of AMPs at inflammatory conditions, such as psoriasis lesions, was observed which might help to better understand the resistance of psoriatic lesions towards infection.

It, however, does not give a reasonable explanation why healthy skin shows rare infections, because these inducible AMPs are absent. With the hypothesis that also healthy skin should contain antimicrobial factors, we have chosen the stratum corneum from a healthy person as substrate to test our hypothesis. Indeed SC-extracts contained high titre antimicrobial activity against several Gram-negative and Gram-positive bacteria.

Using *E. coli* and *S. aureus* as test organisms in our antimicrobial readout-system, we could purify the 14.6 kDa protein RNase-7 (R7) as principal AMP of healthy donor's SC (*Harder* and *Schröder*, 2002). R7, by keratinocytes and various other epithelial cells, is produced constitutively (*Harder* and *Schröder*, 2002). It is active against many Gram-negative and Gram-positive bacteria as well as fungi. By yet unknown reasons enterococci are extremely sensitive towards R7, suggesting that it serves as special AMP killing gut-derived Gram-positive bacteria.

R7 possesses RNase-activity and represents the principal RNase of human skin, which let molecular biologists require bearing of gloves for molecular biology studies to protect RNA from degradation. Unlike one would have expected, antimicrobial activity of R7 does not depend on its RNase-activity, as site-directed mutagenesis has revealed (*Huang* et al., 2007). Artificial membrane studies with R7 and some truncated R7-peptides indicated that membrane disruption, as it is seen with diverse AMPs like defensins, is not R7's mode of bactericidal action (*Huang* et al., 2007).

The high amounts of R7 present in SC extracts and the nearly complete inhibition of *S. aureus* antimicrobial activity by R7 antibodies (*Simanski* et al., 2010) suggests that R7 is an important component of the skin's antimicrobial defence system against *S. aureus*. Indeed, determination of R7 transcripts in travellers returning with *S. aureus* positive skin infections relative to levels in controls revealed higher transcript levels in unaffected control subjects, compared with unaffected skin of case patients. No such association was present for hBD-2 or hBD-3 (*Zanger* et al., 2009).

Recent studies reveal an important role of R7 in protecting the urinary tract from infection (*Spencer* et al., 2011). The urothelium of the lower urinary tract and kidney cells produce

RNase 7. Regulation of its antimicrobial activity depends, similarly as seen in the skin (*Abtin* et al., 2009), on an endogenous inhibitor, ribonuclease inhibitor (RI) which forms in healthy epithelium a stable complex with R7, inhibiting its antimicrobial activity. Upon infection, RI is destroyed by proteolysis, liberating now antimicrobially active R7 and thus defines a unique regulatory pathway that may affect how RNase 7 maintains urinary tract sterility (*Spencer* et al., 2014).

STRATUM CORNEUM: AN UNIQUE SOURCE OF HUMAN PEPTIDE ANTIBIOTICS

With the discovery of several human antimicrobial peptides to be more or less specifically targeting different microbes, it seemed to be worth to test the hypothesis that skin (and stratum corneum) contains a huge number of AMPs, which act against different bacteria with differences in its preference to kill them. There are a number of bacteria and fungi, which are found to have the skin as habitat. Some of them are growing in an environment which is rather humid, others are growing in lipid-rich or in rather dry skin areas. Further, some are growing at rather aerobic conditions; others have their habitats in the deep, rather anaerobic, areas of the stratum corneum.

Therefore, it would be interesting to test a standard stratum corneum extract for antimicrobial activity against different bacteria. Such an analysis, which we had performed with psoriasis scale extracts in previous studies (*Harder* and *Schröder*, 2005), revealed that each bacterium shows a rather microbe-characteristic antimicrobial activity profile of HPLC fractions of a healthy person's stratum corneum extract. It was seen that in these unbiased studies that stratum corneum contains a high number of factors (characterized by its different retention times upon RP-HPLC analyses), which kill either *E. coli* or *P. aeruginosa* (different factors). With this observation one is tempting to speculate that this might be one reason why the skin is well protected from infection by the soil- and water-born bacterium *P. aeruginosa* (as well as the gut bacterium *E. coli*).

On the other side, there have been seen only very few fractions containing *S. aureus*-killing activity. This might be a reason why *S. aureus* skin-infections are not so rare. Using different microbes (skin commensals and skin pathogens) as targets in the antimicrobial read-out system, one should be able to identify with this strategy all major skin-derived antimicrobial compounds, which are produced by either healthy or inflamed skin and thus giving a profile of the antimicrobial potential of healthy and inflamed skin.

OUTLOOK

The skin is a habitat of numerous microbes, which shows an anatomical site-dependent colonization at the skin surface as well as in deeper stratum corneum layers (*Zeeuwen* et al., 2012). This is difficult to be explained solely by the presence of, until now discovered, skin-derived AMPs because their activity profile does not cover all of them. In addition, we have conditions,

which could dramatically affect activity of AMPs, such as local salt concentration, the pH and the redox potential (which would affect the presence or absence of di-sulphide bridges in e.g. defensins). Furthermore, it is possible that AMPs are generated *in situ* by cleavage of bigger skin proteins by specific enzymes which were released by bacteria. This would represent a versatile, rather microbe (enzyme)-specific defence system of the skin. Testing this hypothesis with identification of peptide fragment size and cleavage site will only be possible by analysing skin-derived material.

There is no reasonable explanation why healthy skin's microbiota, is as it has been identified (*Grice* et al., 2009), and why so many differences in its composition have been found when different anatomical sites were analysed. One would postulate that in addition to host-derived AMPs, which should be present in the stratum corneum, also microbe-derived AMPs and other, rather specifically acting antimicrobial compounds of microbial origin, contribute to the composition of the microflora. Challenging these questions by detailed analyses of host-derived protein fragments as antimicrobials and skin bacteria-derived antibiotic compounds could be a new way not only to understand what shapes the skin microflora, it could also be an innovative way for the discovery of novel antibiotics which are permanently present at our skin surface and thus are optimized for the human skin surface during evolution.

ACKNOWLEDGEMENT

The work was supported by several grants of the Deutsche Forschungsgemeinschaft.

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