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Cutaneous Biology

Dermcidin is constitutively produced by eccrine sweat glands and is not induced in epidermal cells under inflammatory skin conditions

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Summary

Background Antimicrobial peptides (AMPs) are important effector molecules of innate immunity, protecting epithelial surfaces of multicellular organisms. In human skin two classes of AMPs—the β-defensins and the cathelicidins—are produced by keratinocytes primarily under inflammatory conditions. In contrast, dermcidin (DCD), a recently discovered AMP with broad-spectrum activity, is expressed in eccrine sweat glands and transported via sweat to the epidermal surface.

Objectives To investigate whether DCD expression is induced under inflammatory conditions in epidermal keratinocytes.

Methods Lesional skin of the inflammatory skin diseases atopic dermatitis, psoriasis and lichen planus was analysed by immunohistochemistry using a polyclonal anti-DCD antiserum. We also examined whether DCD RNA expression is induced in cultured human keratinocytes, fibroblasts, melanocytes and melanoma cells.

Results Whereas DCD was constitutively expressed in eccrine sweat glands of all skin biopsies, we found that, independent of the type of the inflammatory skin lesion, DCD protein expression was not induced in human epidermal keratinocytes. In contrast, β-defensin 2 was expressed in epidermal keratinocytes of inflammatory human skin, but not in keratinocytes of healthy human skin. Upon stimulation of the cultured cells with 12-*O*-tetradecanoyl-phorbol-13-acetate, tumour necrosis factor-α, lipopolysaccharide or H_2O_2, DCD mRNA expression was not detected in primary keratinocytes, fibroblasts and melanocytes, but was detected in MeWo and SKMEL28 melanoma cells.

Conclusions These results indicate that, unlike human cathelicidins and β-defensins which are inducible peptides that primarily function in response to injury and inflammation, DCD is exclusively part of the constitutive innate defence of human skin. By modulating surface colonization, DCD may help to prevent local and systemic invasion of pathogens.

Key words: antimicrobial peptide, dermcidin, eccrine glands, inflammation, sweat

Epithelia of multicellular organisms are under constant microbial assault.[1] Antimicrobial peptides (AMPs) are important effector molecules of the innate immune defence protecting epithelial barriers of plants, insects, amphibians and mammals. AMPs show broad-spectrum antimicrobial activity against a wide range of pathogens including bacteria, fungi and enveloped viruses.[2] Apart from being natural antibiotics, recent evidence suggests that AMPs additionally play a crucial role as signalling molecules in linking innate and adaptive immune responses.[3–5]

Two classes of AMPs have been studied extensively in mammalian skin: β-defensins and cathelicidins. β-Defensins are expressed in human keratinocytes and along the epithelial lining of the urogenital,

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respiratory and alimentary tracts. Human β-defensin (HBD)-1 is expressed at low amounts constitutively in human skin, while HBD-2 and HBD-3 are produced primarily in response to injury and inflammation.[6–8] The sole human cathelicidin, LL-37, is expressed by neutrophils, mast cells and epithelia of the respiratory and gastrointestinal tract. LL-37 can be found as a component of wound fluid and is expressed in epidermal keratinocytes in inflammatory conditions such as psoriasis, subacute lupus erythematosus and nickel contact dermatitis.[9,10]

Dermcidin (DCD) is a recently discovered AMP with broad-spectrum activity and no homology to other known AMPs. DCD expression is restricted to human skin where it is expressed in eccrine sweat glands, secreted into sweat and transported to the epidermal surface. DCD expression could not be observed in epidermal keratinocytes of healthy human skin.[11] Full-length DCD consists of 110 amino acid residues with an N-terminal 19 amino acids signal peptide characteristic for secreted proteins. In eccrine sweat different processed DCD-derived C-terminal peptides of 48 amino acid residues (DCD-1L), 47 amino acid residues (DCD-1) and shorter fragments can be detected by surface-enhanced laser desorption/ionization technology.[12] DCD-1 shows antimicrobial activity against pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Candida albicans* under *in vitro* conditions resembling human sweat. Recently, it has been described that a small percentage of breast cancer cells can express DCD RNA.[13] Furthermore, it was shown that after oxidative stress induction different types of tumour cells produce proteolytically processed DCD peptides different from DCD-1 and with different functional capabilities.[14–18]

As other AMPs such as LL-37 and HBD-2/3 are induced during inflammation and injury, in this study we investigated whether DCD expression is induced in human keratinocytes under inflammatory conditions. We therefore analysed skin biopsies of patients with different inflammatory skin disorders (atopic dermatitis, psoriasis, lichen planus) by immunohistochemistry for DCD expression and compared it with the expression of HBD-2. In addition, reverse transcription–polymerase chain reaction (RT–PCR) experiments were carried out to determine whether DCD mRNA could be induced in cultured human keratinocytes, melanocytes, fibroblasts or melanoma cells by stimulation with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), tumour necrosis factor (TNF)-α, lipopolysaccharide (LPS) or H_2O_2.

Materials and methods

Patients and skin biopsies

Normal skin samples were obtained from 4-mm punch biopsies of healthy volunteers or from excess tissue from unrelated surgical procedures [skin samples from seven healthy individuals, mean age 38·1 years; sites included palms, extremities, trunk, scalp and buccal mucosa (one biopsy)]. Lesional skin was obtained from 4-mm punch biopsies from three patients with acute atopic dermatitis and five patients with chronic atopic dermatitis (mean age 38·5 years; sites included palms, extremities and trunk), six patients with psoriasis (mean age 37·8 years; sites included palms, extremities, trunk and scalp) and six patients with lichen planus (mean age 49·7 years; sites included extremities, trunk, scalp and buccal mucosa). Biopsies were fixed in formalin and embedded in paraffin.

Immunohistochemistry

A polyclonal antiserum to DCD-1 (amino acid residues 63-109 of full-length DCD) was obtained by immunizing a rabbit with a DCD-1 peptide–keyhole limpet haemocyanin conjugate. The antiserum was purified by affinity chromatography using the DCD-1 peptide immobilized on cyanogen bromide-activated Sepharose. DCD antiserum without immune-affinity purification showed nonspecific staining. For immunostaining of DCD, the same protocol was used as described by Schittek *et al.*[11] except that a 1 : 5000 dilution of immune-affinity purified polyclonal rabbit antiserum to DCD-1 was used. Negative controls were established with the use of preimmune serum or only secondary antibody.

For immunostaining of HBD-2, the paraffin sections were blocked with normal horse serum, followed by incubation with a 1 : 2000 dilution of rabbit polyclonal anti-HBD-2 antibody (Biologo, Kronshagen, Germany) and a biotinylated antirabbit IgG (Vector, Burlingame, CA, U.S.A.). Sections were then washed and developed as outlined above.

Culture and stimulation of cells

Keratinocytes, melanocytes and fibroblasts were isolated from neonatal foreskin after routine circumcision and cultured as already described.[19] The melanoma cell line MeWo was grown in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 2 mmol L^{-1} L-glutamine and 100 µg mL^{-1} penicillin–streptomycin.

For stimulation of the cells, keratinocytes, melanocytes and fibroblasts were incubated with 0·1% H_2O_2 for 60 min or 3·5 h. The melanoma cell line MeWo was incubated with 0·03% H_2O_2 (30 min) or 0·1% H_2O_2 (30 or 60 min) or with TPA (10 ng mL^{-1}) for 30 min. After two washes with Hank's balanced saline solution (HBSS) cells were cultured for 30 or 60 min in their respective growth media, trypsinated and after two further washes cell pellets were stored at −80 °C until RNA extraction. Alternatively, the cells were incubated with 0·1 µg mL^{-1} LPS of *Salmonella typhimurium* (Sigma, St Louis, MO, U.S.A.) and/or 10 ng mL^{-1} human recombinant TNF-α (Sigma) for 1 h. After two washes with HBSS cells were cultured for 3·5 h in their respective media, trypsinated and pellets stored at −80 °C as outlined above.

Reverse transcription–polymerase chain reaction

RNA isolation, cDNA synthesis and RT–PCR for DCD and glyceraldehyde-3-phosphate dehydrogenase were carried out as described earlier.[11]

Results

Immunohistochemical analysis of dermcidin and human β-defensin-2 expression in healthy and inflammatory human skin

Immunohistochemical staining of biopsies from healthy human skin with an immune-affinity purified DCD

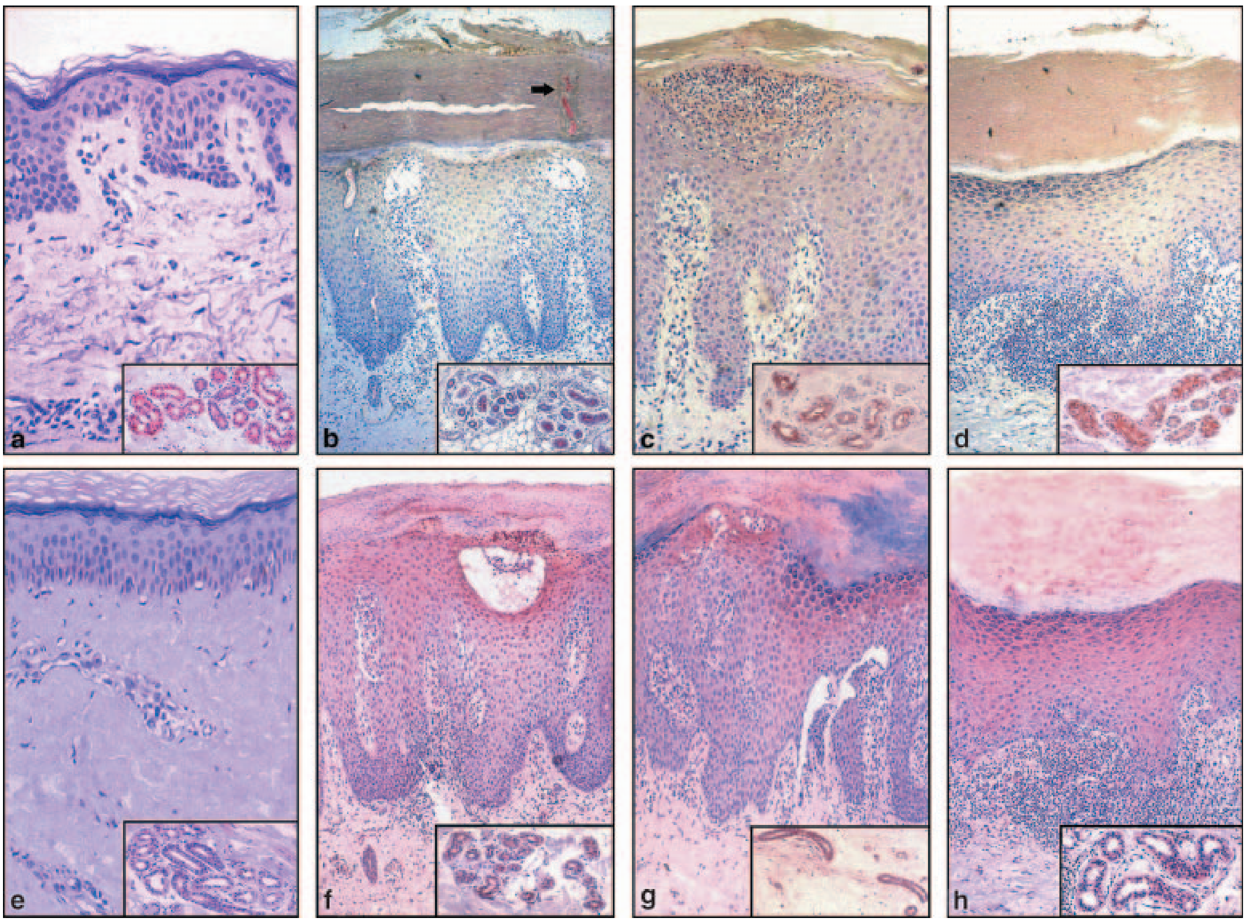

Figure 1. Immunohistochemical analysis of skin biopsies from healthy individuals or from patients with inflammatory skin lesions. Immunohistochemistry for dermcidin (DCD) (a–d) and human β-defensin-2 (e–h) protein expression. (a,e) Healthy human skin without signs of inflammation; (b,f) skin biopsy of a patient with atopic dermatitis (palmar); (c,g) skin biopsy of a patient with psoriasis; (d,h) skin biopsy of a patient with lichen planus. Original magnifications: (a,c,e) ×50; (b,d,f,g,h) × 25; insets with eccrine sweat glands: (a,c,d,e,h) ×25; (b,f,g) ×12·5. The arrow in (b) shows a DCD-positive lumen of an eccrine pore in the stratum corneum.

antiserum revealed strong expression of DCD in the secretory coils of eccrine sweat glands (six of six biopsies) (Fig. 1a). Immunoreactivity was observed in dermal and epidermal portions (acrosyringium) of sweat ducts and the staining was strongest in the apical/luminal portions of the eccrine glands. Epidermal keratinocytes and dermal fibroblasts of healthy human skin did not show DCD expression. DCD antiserum without immune-affinity purification showed nonspecific staining of the stratum corneum in addition to staining of eccrine sweat glands (data not shown).

Next, we analysed whether DCD is expressed in keratinocytes of inflammatory human skin *in vivo*. Immunohistochemical analysis of skin biopsies from patients with atopic dermatitis, psoriasis and lichen planus showed expression of DCD in eccrine sweat glands in almost all cases (eight of eight biopsies in atopic dermatitis, six of six biopsies in psoriasis, five of six biopsies in lichen planus). However, as in normal human skin DCD expression could not be observed in epidermal keratinocytes, melanocytes or dermal fibroblasts regardless of the type of inflammatory skin disorder (Fig. 1b–d).

HBD-2 is known to be induced in inflammation. As a positive control, we therefore conducted immunohistochemical analysis of HBD-2 expression in healthy and inflammatory human skin. Immunoreactivity was not detected in healthy human skin (Fig. 1e), whereas HBD-2 expression was observed in epidermal keratinocytes of the stratum granulosum and to a lesser extent in the stratum spinosum from patients with atopic dermatitis, psoriasis and lichen planus. In addition, eccrine sweat glands from all skin biopsies showed weak expression of HBD-2 (Fig. 1f–h).

Reverse transcription–polymerase chain reaction analysis of dermcidin expression in human skin cells after stimulation with inflammatory signals

In order to determine whether DCD gene expression is induced in skin cells *in vitro* we incubated primary cultures of melanocytes and keratinocytes with 0·1% H_2O_2, LPS (0·1 μg mL^{-1}) and/or TNF-α (10 ng mL^{-1}). Stimulation of these cells with H_2O_2 or with LPS and/or TNF-α did not induce DCD mRNA (Fig. 2a). Fibroblasts could also not be stimulated to express DCD. Furthermore, stimulation of melanocytes, keratinocytes or fibroblasts with TPA (10 ng mL^{-1}) did not induce DCD mRNA expression (data not shown). This indicates that DCD expression is restricted to sweat glands and is not induced by oxidative stress or inflammatory stimuli in primary skin cells.

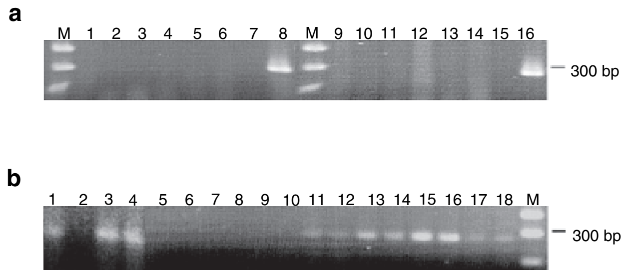

Figure 2. Reverse transcription–polymerase chain reaction (RT–PCR) analysis of dermcidin mRNA expression after stimulation with inflammatory signals. (a) Cultured human melanocytes (lanes 1–7) and keratinocytes (lanes 9–15) were stimulated with 0·1% H_2O_2 (60 min) (lanes 2, 10), 0·1% H_2O_2 (3·5 h) (lanes 3, 11), or for 60 min with 0·1 μg mL^{-1} lipopolysaccharide (LPS) (lanes 4, 12), 10 ng mL^{-1} tumour necrosis factor (TNF)-α (lanes 5, 13) or 0·1 μg mL^{-1} LPS and 10 ng mL^{-1} TNF-α (lanes 6, 14). Lanes 1, 9, negative control cells cultured in media without stimulation; lanes 7, 15, PCR-negative controls; lanes 8, 16, PCR-positive controls; lane M, 100-bp marker (MBI Fermentas, St Leon-Rot, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was successfully amplified from all samples except the PCR-negative control. (b) Stimulation of melanoma cell line MeWo (60 min) with 0·1 μg mL^{-1} LPS (lane 1), 0·1 μg mL^{-1} LPS and 10 ng mL^{-1} TNF-α (lane 3) or 10 ng mL^{-1} TNF-α (lane 4). Incubation (30 min) with 10 ng mL^{-1} 12-*O*-tetradecanoyl-phorbol-13-acetate (lanes 11, 12), 0·1% H_2O_2 (lanes 15, 16), 0·03% H_2O_2 (lanes 17, 18) or 0·1% H_2O_2 (60 min) (lanes 13, 14). Negative control cells were cultured in media without stimulation (lanes 2, 9, 10). Lanes 6, 8, 10, 12, 14, 16, 18 after 35 cycles RT–PCR, lanes 1–5, 7, 9, 11, 13, 15, 17 after 40 cycles RT–PCR. Lanes 5–8, PCR-negative controls; lane M, 100-bp marker. GAPDH was successfully amplified from all samples except the PCR-negative control.

As it has been described that DCD expression is induced in tumour cells,[14] we determined whether DCD gene expression can be induced in skin tumour cells. We stimulated the melanoma cell line MeWo with 0·03% or 0·1% H_2O_2, TPA (10 ng mL^{-1}), LPS (0·1 μg mL^{-1}) and/or TNF-α (10 ng mL^{-1}). DCD expression was induced by all stimuli in the melanoma cell lines MeWo (Fig. 2b) and SKMEL28 (data not shown). This shows that melanoma cells are able to express DCD after stimulation.

Discussion

Skin provides the epithelial barrier between the body and the environment. It is colonized by a variety of microorganisms, both harmless commensals and potential pathogens. Thus, effective defence mechanisms are essential for protection of this barrier. AMPs significantly contribute to the epithelial defence of multicellular organisms.[20]

DCD is a novel AMP that has been shown to be secreted into eccrine sweat.[11] We showed that DCD is constitutively expressed in eccrine sweat glands of all healthy human skin biopsies analysed. We next raised the question whether the expression of DCD is induced in epidermal keratinocytes under inflammatory conditions. By immunohistochemistry we showed that DCD expression is not induced in epidermal keratinocytes in the inflammatory skin disorders atopic dermatitis, psoriasis and lichen planus. However, as in normal skin, lesional skin showed DCD expression in the sweat glands in almost all cases. Additionally, we conducted RT–PCR experiments on cultured human keratinocytes, fibroblasts and melanocytes after *in vitro* stimulation with the inflammatory mediators TNF-α and LPS and oxidative stress by H_2O_2. RT–PCR revealed no induction of DCD mRNA upon stimulation of primary human skin cells.

Our results reveal DCD as part of the constitutive cutaneous defence. Constantly secreted, sweat-derived DCD represents a constitutive antimicrobial 'preservative' on top of the epithelial sheets of the skin, analogous to the AMP-rich biofilm overlying the mucosal linings inside the human body. Thus, DCD contributes to the epithelial defence by modulating the surface colonization rather than by responding to injury and inflammation as observed with the inducible peptides HBD-2 and -3 or human cathelicidin LL-37. Modulating the colonization could include two functions: (i) effects against microbial overgrowth on the skin surface, and (ii) preventing colonization of pathogenic microorganisms, thereby establishing a host-friendly resident flora. Further investigations to define the antimicrobial spectrum of DCD will elucidate the contribution of DCD to surface colonization.

The eccrine sweat gland is one of the major cutaneous appendages and until recently only its role in thermoregulation was appreciated.[21] The presence of DCD indicates that eccrine sweat plays a role in epithelial defence mechanisms. Recently, a second AMP, human cathelicidin LL-37, has been detected in eccrine sweat.[22] Although in very limited concentration, LL-37 may contribute to the defence mechanisms mediated in sweat by acting synergistically with DCD and potential other AMPs in sweat. Accordingly, HBD-1 and HBD-2 have been localized by immunohistochemistry in sweat glands and ducts.[23,24] In our studies we observed weak expression of HBD-2 in eccrine sweat glands in healthy human skin and expression of HBD-2 in sweat glands and keratinocytes in skin biopsies of patients with psoriasis, atopic dermatitis and lichen planus. We did not detect HBD-2 expression in healthy human skin, as described earlier.[6,23] It has recently been described that the amount of HBD-2 is significantly decreased in lesions from patients with atopic dermatitis.[10] As we did not quantify the amount of HBD-2 protein using immunohistochemistry, it might be that the level of HBD-2 expression is different between the inflammatory disorders we analysed.

We observed DCD mRNA induction in the melanoma cell lines MeWo and SKMEL28. It has already been described that tumour cells can generate two overlapping proteolytically processed DCD peptides: (i) survival-promoting peptide Y-P30 derived from the 5′-terminus (amino acid residues 20–49), and (ii) human cachexia-associated protein PIF (proteolysis inducing factor, amino acid residues 20–39). Y-P30 is synthesized by oxidatively stressed neuronal cell lines and retinoblastoma cells, providing a survival advantage to other cells.[14] PIF is a sulphated proteoglycan and has been identified as a cancer cachectic factor, produced by prostate[15] or gastrointestinal cancer cells[16] and provoking muscle wasting after experimental administration in mice.[17,18] It was recently described that DCD is expressed in 10% of invasive breast carcinomas and that DCD expression in these cells promotes cell growth and survival.[13] This indicates that different types of tumour cells can express DCD mRNA and are able to generate DCD-derived peptides with functional activities different from those of the DCD-1 peptide (derived from the 3′-end of the full-length protein) found in human sweat.

In conclusion, expression of the novel AMP DCD is not induced in epidermal keratinocytes, melanocytes and dermal fibroblasts in the inflammatory skin disorders atopic dermatitis, psoriasis and lichen planus. Being expressed constitutively in eccrine sweat, DCD functions primarily in modulating the colonization of the skin rather than responding to injury and inflammation when epithelial integrity is lost. By its unique mode of delivery, sweat-derived DCD contributes to the first line of defence by providing a protective shield that overlies the keratinized epithelial skin.

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