Human β Defensin-1 and -2 Expression in Human Pilosebaceous Units: Upregulation in *Acne Vulgaris* Lesions

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A rich residential microflora is harboured by the distal outer root sheath of the hair follicle and the hair canal – normally without causing skin diseases. Although the basic mechanisms involved in the development of inflammation during *acne vulgaris* remain unclear, microbial agents might play an important role in this process. In this study we have analyzed by *in situ* hybridization and immunohistochemistry the expression patterns of two antimicrobial peptides, human β defensin-1 and human β defensin-2, in healthy human hair follicles as well as in perilesional and intralesional skin of *acne vulgaris* lesions such as comedones, papules, and pustules. Strong defensin-1 and defensin-2 immunoreactivity was found in all suprabasal layers of the epidermis, the distal outer root sheath of the hair follicle, and the pilosebaceous duct. Marked defensin-1 and defensin-2 immunoreactivity was also found in the sebaceous gland and in the basal layer of the central outer root sheath including the bulge region. The majority of acne biopsies displayed a marked upregulation of defensin-2 immunoreactivity in the lesional and perilesional epithelium – in particular in pustules – and a less marked upregulation of defensin-1 immunoreactivity. The upregulation of β-defensin expression in *acne vulgaris* lesions compared to controls suggests that β-defensins may be involved in the pathogenesis of *acne vulgaris*. *J Invest Dermatol* 117:1120–1125, 2001

The hair canal, distal outer root sheath (ORS) of the hair follicle, and the pilosebaceous duct constitute major ports of entry for microbial invasion in humans and harbour a rich residential microflora such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Demodex folliculorum*, and *Malassezia furfur*, which, however, do not normally cause disease (Paus, 1997; Paus *et al*, 1999). The distal ORS and the pilosebaceous duct are also characterized by many features of immunologic activity such as classical and nonclassical major histocompatibility complex class I expression, intercellular adhesion molecule-1 expression, and the presence of intraepithelial Langerhans cells and perifollicular macrophages (Paus, 1997; Paus *et al*, 1999; Christoph *et al*, 2000). This area of the pilosebaceous unit is also a hot spot in the development of *acne vulgaris* lesions.

Hypercornification of the distal ORS and the pilosebaceous duct in concert with increased sebum production and abnormalities of the microbial flora are considered to be major factors in the pathogenesis of *acne vulgaris* (cf. Cunliffe and Simpson, 1998). The role of microbial agents is still not clear, however. *P. acnes* is likely to be the major organism as selective antibiotic studies reveal that only antibiotics that suppress *P. acnes in vivo* are associated with

clinical benefit. Furthermore there is some relationship between *P. acnes* resistance and clinical failure (Eady *et al*, 1989; 1993; Bojar *et al*, 1994). Although many *acne vulgaris* patients respond well to antibiotic drugs, there is little relationship between the numbers of bacteria on the skin surface and the severity of acne (Leyden *et al*, 1975; Cove *et al*, 1983). Colonization of the pilosebaceous duct is a feature of established comedones and early inflammatory lesions (Leeming *et al*, 1985; 1988). Why some colonized ducts become inflamed and others do not is uncertain. Variation in the microenvironment of the duct could be important. Such microenvironmental variations are likely to influence the production and activity of inflammatory mediators (Holland *et al*, 1978; 1998). *P. acnes* produces many likely candidates for inflammation such as lipases, neuramidases, phosphatases, and proteases (Greenman *et al*, 1983). One alternative hypothesis might be that *acne vulgaris* patients suffer from a dysregulation of the production of innate and specific antimicrobial peptides.

More than 500 antimicrobial peptides have been described in plants, insects, amphibians, and mammals, with broad-spectrum activity against bacteria, fungi, and viruses, representing an integral part of innate immunity (Ganz, 1999; Boman, 2000). Mammalian defensins are a family of cationic antimicrobial peptides 28–42 amino acids long containing three disulfide bonds. They have been divided into two subtypes, the α-defensins and the β-defensins (Diamond and Bevins, 1998). The α-defensins are found in neutrophil granules or in the paneth cells of the small intestine (Ganz and Lehrer, 1998). The two β-defensins so far identified, human β defensin-1 (hBD1) and human β defensin-2 (hBD2), are produced in various epithelia (Harder *et al*, 1997; Valore *et al*,
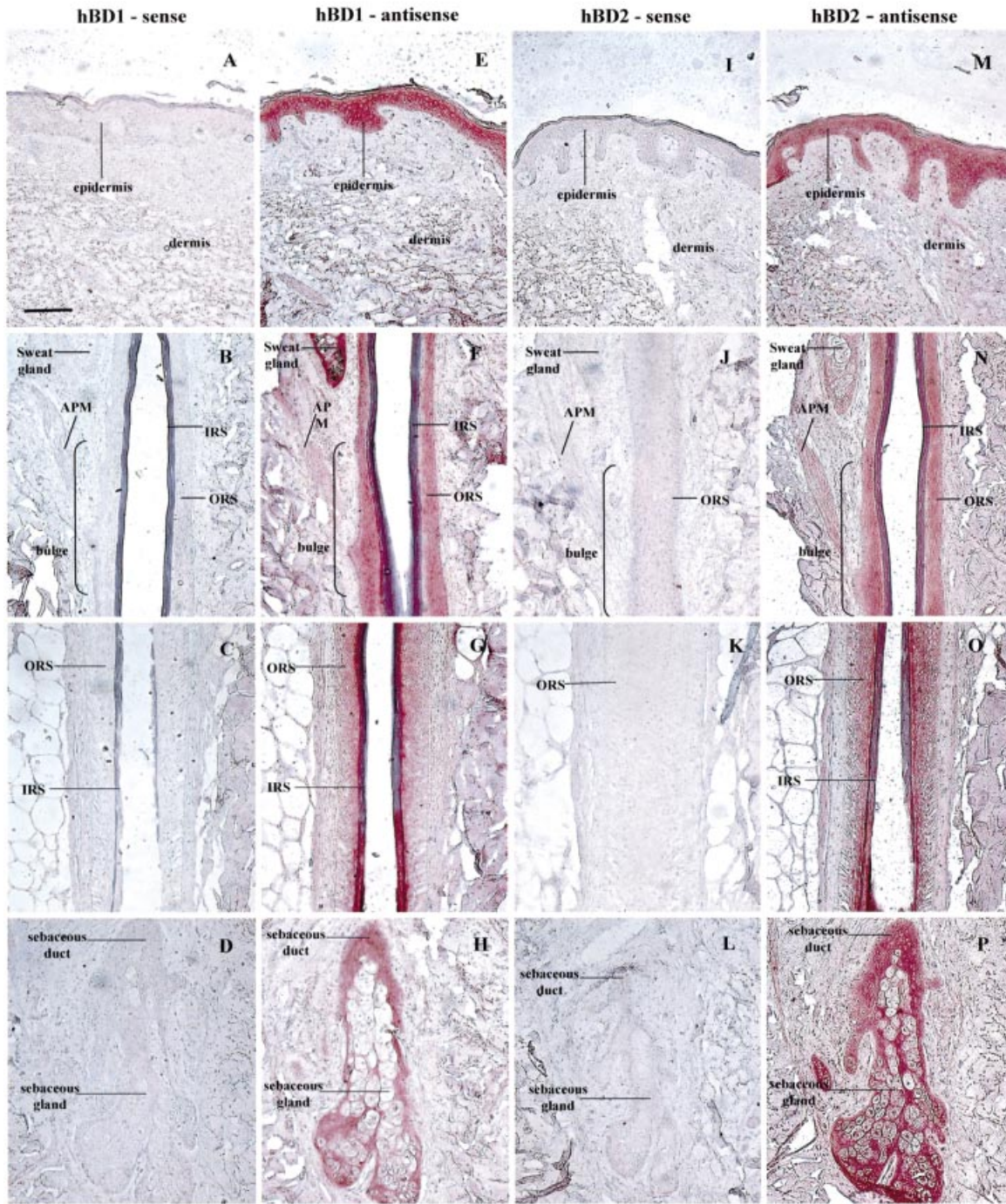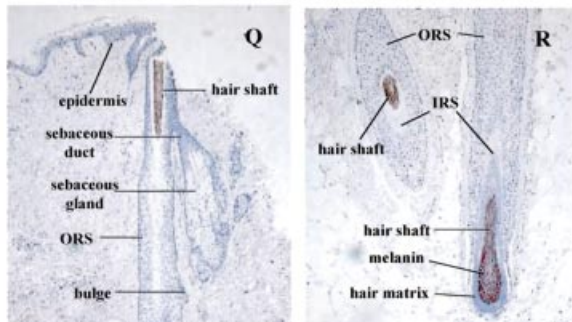
Negative Controls (Immunohistochemistry)

1998). hBD1 was first isolated from human blood filtrates and hBD2 was first isolated from human psoriatic scale (Bensch *et al*, 1995; Harder *et al*, 1997). Recent studies have shown hBD1 and hBD2 expression in the skin by the polymerase chain reaction and *in situ* hybridization (Fulton *et al*, 1997; Harder *et al*, 1997).

In view of the proposed role for ductal *P. acnes* in *acne vulgaris* (Holland *et al*, 1978; 1998) we have studied the expression patterns of hBD1 and hBD2 in human terminal hair follicles and in *acne vulgaris* lesions compared to healthy pilosebacous follicles.

MATERIALS AND METHODS

Tissue specimens Scalp skin containing terminal hair follicles was collected from 10 individuals undergoing cosmetic surgery (facelifts). Biopsies were fixed in 10% formalin for 8 h prior to embedding in paraffin wax. Biopsies of normal pilosebaceous follicles were taken from the backs of five healthy volunteers and compared to lesional skin biopsies taken from the backs of 11 *acne vulgaris* patients. Biopsies were taken of blackheads (*n* = 4), whiteheads (*n* = 1), papules (*n* = 2), and pustules (*n* = 4). All biopsies were fixed in 4% paraformaldehyde for 2.25 h and embedded in Paraplast embedding medium (Paraplast X-tra, Sigma). Ethical approval was obtained from all patients and all patients had been off acne therapy for 4 wk. No patient had previously received oral isotretinoin.

In situ hybridization Riboprobes for *in situ* hybridization were prepared using hBD1 and hBD2 plasmids, which were generated by reverse transcription polymerase chain reaction (Fulton *et al*, 1997; Ali *et al*, 2001). The plasmids were linearized for *in situ* hybridization by digestion with apropriate restriction enzymes and antisense and sense probes were prepared from the linearized fragments (Ali *et al*, 2001). *In situ* hybridization was performed as follows: 4 μm paraffin sections were cut onto superfrost slides (BDH). Sections were deparaffinized, rehydrated, and transferred to RNase-free water. Tissue sections were microwaved in RNase-free preheated 10 mM citrate buffer solution, pH 6.0, for 10 min, transferred quickly and cooled in RNase-free water, and washed in RNase-free phosphate-buffered saline (PBS), pH 7.2, for 5 min. The sections were prehybridized for 3 h at 42°C and then hybridized at 42°C. Sections were subsequently washed in 2 × sodium citrate/chloride buffer (SSC) for 10 min. Following stringency washes to 0.1 × SSC/50% formamide at 42°C, sections were incubated in buffer 1 with 1% normal sheep serum, 0.3% Triton X-100 for 30 min at room temperature. Antidigoxigenin antibody linked to alkaline phosphatase (Boehringer) was applied at 1:500 dilution in buffer 1 (100 mM Tris-HCl, 150 mM NaCl) with normal sheep serum, 0.3% Triton X-100 for 90 min at room temperature. After rinsing in buffer 1 twice for 5 min and buffer 2 (50 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl$_2$) for 5 min sections were treated with 5-bromo-4-chloro-3-indolylphosphate/ Nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma) overnight at room temperature. The reaction was stopped in buffer 3 (10 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid). Slides were then air dried and mounted in Aquamount (BDH).

Immunohistochemistry Localization of hBD1 and hBD2 immunoreactivity (IR) was carried out using two sets of specific rabbit-anti-human polyclonal antibodies. The first set of anti-hBD1 and anti-hBD2 antibodies were a kind gift from Dr. T. Ganz, Los Angeles, CA (Liu *et al*, 1998; Valore *et al*, 1998). The second set of anti-hBD1 and anti-hBD2 antibodies was generated in our laboratory. The specificity of these antibodies was determined by dot blot analysis (Ali *et al*, 2001). Five micron sections were cut on a rotary microtome (Biocut, Reichert-Jung) and placed on electrostatically charged slides (Superfrost Plus, BDH). All sections were baked for a minimum of 30 min at 60°C. Sections were de-waxed in xylene and rehydrated. IR was detected using a standard avidin–biotin complex peroxidase method (Vectastain Universal Elite ABC kit, Vector Laboratories). Endogenous peroxidase

activity was blocked with 3% hydrogen peroxide in ice-cold methanol (−20°C) for 10 min and rinsed in PBS. Non-specific binding was blocked with a 1:5 dilution of normal horse serum in PBS for 10 min. Sections were incubated with a 1:1500 dilution of either anti-hBD1 or anti-hBD2 antibody overnight at 4°C. Slides were washed twice in Tris-buffered saline (pH 7.6). The secondary biotinylated antibody (Vectastain Universal Elite ABC kit, Vector Laboratories) was applied at room temperature for 45 min. Sections were again washed twice in Tris-buffered saline before application of the streptavidin horseradish peroxidase tertiary antibody, 30 min at room temperature. The reaction product was visualized using diaminobenzidine (Liquid DAB substrate pack, BioGenex) and sections were counterstained with Mayer's hematoxylin.

RESULTS

In this study we have investigated the patterns of hBD1 and hBD2 mRNA and protein expression in terminal scalp hair follicles as well as in normal pilosebaceous follicles and *acne vulgaris* lesions from the back.

HBD1 and hBD2 mRNA is expressed in terminal scalp hair follicles We have analyzed sense and antisense staining of hBD1 (**Fig 1A–H**) and hBD2 (**Fig 1I–P**), respectively. hBD1 (**Fig 1A–D**) and hBD2 (**Fig 1I–L**) sense controls only displayed nonspecific staining confined to the stratum corneum (**Fig 1I**) and in the distal, highly differentiated inner root sheath (IRS) (**Fig 1B, C**). Strong homogeneous staining for hBD1 mRNA expression was detected in the basal and suprabasal layers of the epidermis (**Fig 1E**) as well as in the distal outer root sheath (ORS) (**Fig 1F**). In contrast, the proximal ORS showed strong hBD1 mRNA expression only in the upper suprabasal layers (**Fig 1G**). Strong hBD1 mRNA expression was also found in the less differentiated, proximal IRS (**Fig 1G**) as well as in the sebaceous gland and pilosebaceous duct (**Fig 1F, H**). Similarly, homogeneous hBD2 mRNA expression was observed in all layers of the epidermis (**Fig 1M**) and the distal ORS (**Fig 1N**). In addition, hBD2 mRNA expression was also observed in the suprabasal layers of the proximal ORS (**Fig 1O**) and in the sebaceous gland and duct (**Fig 1N, P**). No hBD1 or hBD2 mRNA was detected in the hair matrix or the dermal papilla (DP) (not shown).

HBD1 and hBD2 IR in terminal scalp hair follicles and pilosebaceous follicles In contrast to the mRNA expression patterns, strong hBD1 (**Fig 2A, C**) and hBD2 (**Fig 2B, G**) IR was only seen in the suprabasal layers of the epidermis, the distal ORS, sebaceous gland, and sebaceous duct. Unlike the epidermis and the distal part of the hair follicle, marked hBD1 (**Fig 2A**) and hBD2 (**Fig 2B**) IR was found on basal layer keratinocytes in the isthmus and bulge area of the ORS. Weaker hBD1 (**Fig 2A, C**) and hBD2 (**Fig 2B, G**) IR was also present in the central and proximal regions of the ORS as well as in the proximal portion of the IRS, whereas no hBD1 or hBD2 IR was detected in the hair matrix or the DP.

We found consistent expression patterns in all biopsies but some variations in the levels of hBD1 and hBD2 mRNA and protein expression between the 10 different sex- and age-matched patients (hBD1: very strong IR, two; moderate, six; weak, two patients; hBD2: very strong IR, two; moderate, seven; weak, one patient).

HBD1 and hBD2 IR in acne lesions (back skin) In both comedones (**Fig 2D**) and papules (**Fig 2E**) strong hBD1 IR was localized in the suprabasal layers of both lesional and perilesional epithelium, the pilosebaceous duct, the sebaceous gland, and the

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Figure 1. hBD1 and hBD2 *in situ* hybridization. hBD1 sense control (*A–D*) and hBD2 sense control (*I–L*) were compared to the corresponding hBD1 antisense staining (*E–H*) and hBD2 antisense hybridization (*M–P*). Selected skin compartments were compared: epidermis (*A, E, I, M*), the distal IRS and ORS including the bulge region (*B, F, J, N*), the proximal IRS and ORS (*C, G, K, O*), and the sebaceous gland and duct (*D, H, L, P*). Purple, nonspecific staining is found in the sense controls in the stratum corneum (*I*) and in the IRS (*B, C*). Homogeneous, strongly brown, specific antisense staining for both defensins is homogeneously present in all layers of the epidermis (*E, M*) and the distal ORS including the bulge area (*F, N*). Slight hBD2 antisense hybridization is found on the arrector pili muscle (APM). Strong hBD1 and hBD2 antisense hybridization is only detected in the upper suprabasal layers of the proximal ORS (*G, O*) and in the sebaceous gland and duct (*H, P*). (*Q, R*) Negative controls for hBD1 and hBD2 IR. Negative controls that were incubated without the primary antibody show no IR in any key compartment of the hair follicle (*H, P*).
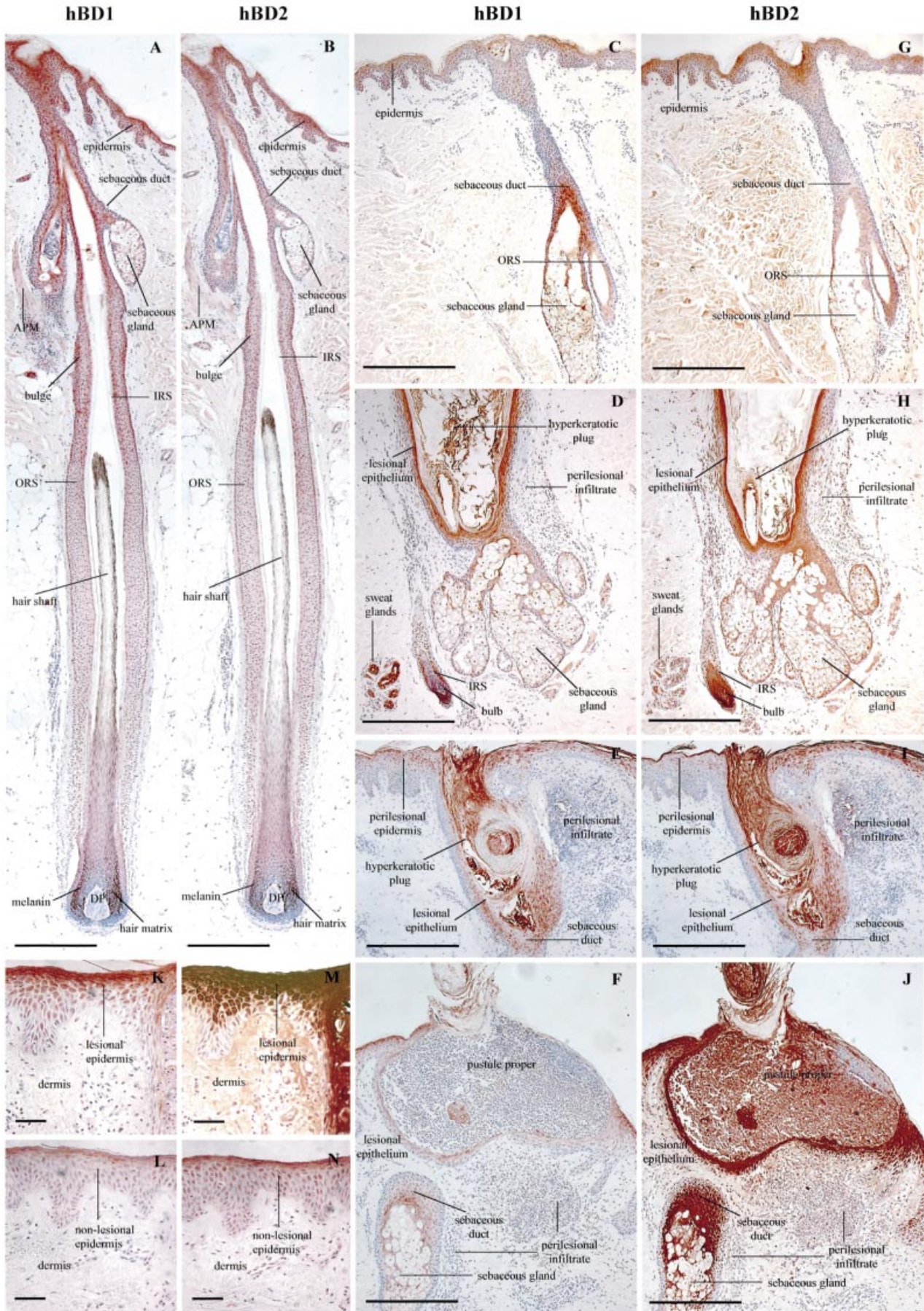
follicular IRS. Pustules show hBD1 IR in the suprabasal lesional and perilesional epithelium and the pilosebaceous duct (**Fig 2F**) whereas little hBD1 IR was seen in the inflammatory area of the pustule. Compared to healthy controls and the interlesional epithelium of the same patient, six of 11 *acne vulgaris* patients showed an upregulation of hBD1 IR in comedones or papules.

Strong hBD2 IR is seen in the comedonal plug, the suprabasal layers of the epidermis and lesional epithelium, the IRS, pilosebaceous duct, and gland in all comedones (**Fig 2H**) and papules (**Fig 2I**). Compared to healthy follicles and other lesions, the strongest hBD2 upregulation has been found in and around pustules (in contrast to the interlesional epithelium of the same patient) including the area of maximal inflammation, the suprabasal perilesional epidermis and lesional epithelium as well as the pilosebaceous duct and gland (**Fig 2J**).

HBD1 and hBD2 IR in lesional *versus* nonlesional skin   The comparison of defensin IR in lesional and nonlesional epithelium on the same section from each *acne vulgaris* patient revealed substantially stronger hBD1 IR in lesional epithelium (**Fig 2K**) compared to nonlesional epithelium (**Fig 2L**). Furthermore, we found substantially stronger hBD2 IR in lesional epithelium (**Fig 2M**) compared to nonlesional epithelium (**Fig 2N**).

DISCUSSION

In this study we have investigated the mRNA and protein expression patterns of hBD1 and hBD2 in healthy human pilosebaceous units as well as in *acne vulgaris* lesions. We found strong constitutive mRNA and protein expression of both β-defensins in the distal ORS surrounding the hair canal and in the pilosebaceous duct of the hair follicle. This finding is consistent with the concept that these regions are highly exposed to microbial invasion as well as to the physiologic skin microflora. In contrast, hair follicle compartments that are rarely exposed to microbial invasion such as the proximal ORS and IRS as well as the hair follicle bulb show only very weak hBD1 and hBD2 expression. Therefore, the patterns of defensin expression shown here suggest that hBD1 and hBD2 may play a key role in protecting the pilosebaceous unit from microbial invasion.

Although hBD1 has been reported to be constitutively expressed and not upregulated under inflammatory conditions in oral mucosa (Mathews *et al*, 1999), we found a moderate upregulation of hBD1 IR in most *acne vulgaris* lesions compared to non-lesional skin of the same patient and also compared to pilosebaceous follicles from healthy back skin controls. The functional significance of this is not clear. It is interesting to note that there was strong upregulation of hBD1 in the proximal IRS of a small pilosebaceous follicle adjacent to one comedo (**Fig 2D**). A moderate to strong upregulation of hBD2 IR was seen in all types of acne lesion. It is important to note that these observations are based on a limited sample number from 11 *acne vulgaris* patients. An increase of hBD2 expression has also been found, however, in other inflammatory conditions such as psoriasis and mastitis (Schonwetter *et al*, 1995; Stolzenberg *et al*, 1997; cf. Schroder and Harder, 1999).

There is increasing evidence that pro-inflammatory cytokines (such as interleukin-1β, tumor necrosis factor-α) and bacterial lipopolysaccharides can upregulate β-defensins (Diamond *et al*, 1996; Harder *et al*, 1997; Stolzenberg *et al*, 1997). Therefore, the observed upregulation of β-defensins in *acne vulgaris* lesions is most probably a secondary response to the marked perilesional infiltrate. In this context it is interesting to note that there are marked differences between hBD1 and hBD2 expression in the perilesional follicle in **Figs 2(D)** and **2(H)**: increased expression of hBD1 is restricted to the IRS of the follicle whereas hBD2 is strongly increased throughout the proximal follicle including the me-senchymally derived DP. These preliminary findings suggests that inflammatory cytokines may be able to upregulate β-defensin expression in both the distal and proximal follicle. It is important to note that this is only based on one bulb of a lesional pilosebaceous unit, however, and many more would need to be studied. This would be very difficult in lesional biopsies as we only observed one hair follicle bulb in the 11 acne biopsies studied.

Finally, in the normal facelift and back skin biopsies that have been investigated in this study we detected substantial variations of the hBD1 and hBD2 IR intensity between sex- and age-matched patients, between face and back skin, as well as between different hair follicle types (terminal hair follicles and pilosebaceous follicles). In further studies it will be interesting to investigate whether good responders to antibiotic anti-acne treatment differ in their β-defensin levels and/or activity from bad responders.

In conclusion, this study provides evidence that human β-defensins are expressed in human hair follicles and that their expression is upregulated in *acne vulgaris* lesions.

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Figure 2. hBD1 and hBD2 IR in human hair follicles and *acne vulgaris* lesions. (*A*), (*B*) hBD1 and hBD2 IR in terminal hair follicles (scalp). Strong hBD1 (*A*) and hBD2 (*B*) IR is found in the suprabasal layers of the epidermis and the distal ORS; strong basal expression is seen in the bulge area. Strong β-defensin expression is found in the sebaceous gland and duct. Weaker expression is present in the suprabasal layers of the central and proximal ORS and in the proximal IRS. No hBD1 or hBD2 IR is detected in the hair matrix or the DP. (*C*) hBD1 IR – Pilosebaceous hair follicles (back skin). Moderate hBD1 IR is seen in the suprabasal epidermal layers, the distal ORS, and the sebaceous gland and duct. (*D*) hBD1 IR – Comedo (back skin). Strong hBD1 IR is found in the hyperkeratotic plug, the suprabasal layers of the lesional epithelium, the pilosebaceous duct, the sebaceous gland, and the proximal follicular IRS. (*E*) hBD1 IR – Papule (back skin). Strong hBD1 IR is present in the hyperkeratotic plug, suprabasal layers of the epidermis, and lesional epithelium, including the pilosebaceous duct and sebaceous gland. (*F*) hBD1 IR – Pustule (back skin). Virtually no hBD1 IR is found in the inflammatory area of the pustule. Moderate IR is present in the suprabasal layers of the epidermis, the lesional epithelium, and the pilosebaceous duct and sebaceous gland. (*G*) hBD2 IR – Pilosebaceous hair follicle (back skin). Moderate hBD2 IR is detected in the suprabasal epidermal layers, the distal ORS, the IRS, the sebaceous gland, and duct. (*H*) hBD2 IR – Comedo (back skin). Strong hBD2 IR is found in the suprabasal layers of the lesional epithelium and the sebaceous gland and duct. Note that the proximal follicular IRS and the bulb show strong hBD2 IR. (*I*) hBD2 IR – Papule (back skin). Strong hBD2 IR is present in the hyperkeratotic plug and the upper suprabasal layers of the epidermis and lesional epithelium including the pilosebaceous duct. (*J*) hBD2 IR – Pustule (back skin). Intense hBD2 IR is detected in the inflammatory area of the pustule, the suprabasal layers of the perilesional epidermis and lesional epithelium, the pilosebaceous duct, and sebaceous gland. (*K*), (*L*) hBD1 IR – Lesional epithelium compared to nonlesional epithelium of the same patient. Intense hBD1 IR is seen in the lesional epithelium (*K*) of an *acne vulgaris* patient compared to significantly weaker hBD1 IR in nonlesional epithelium (*L*) of the same patient in the same section. (*M*), (*N*) hBD2 IR – Lesional epithelium compared to nonlesional epithelium of the same patient. Intense hBD2 IR is found in the lesional epithelium (*M*) of an *acne vulgaris* patient compared to significantly weaker hBD2 IR in nonlesional epithelium (*N*) of the same patient in the same section.

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