An Intrinsic Antibiotic Mechanism in Wounds and Tissue-Engineered Skin

To the Editor

Despite the loss of epidermal barrier function and subsequent exposure to environmental microorganisms, superficial epidermal wounds in vertebrates usually heal without major complications, suggesting the existence of a functional antimicrobial mechanism during wound healing. This general observation provides the basis for testing a hypothesis for the existence of a functional antimicrobial mechanism, which is active at cutaneous surfaces. By using standard immunohistochemical techniques, we have investigated expression of human β defensin-2 (hBD-2) in normal and lesional human skin. hBD-2 is a member of the defensin family of antimicrobial peptides, and has microbicidal activity to Gram negative bacteria and Candida albicans (Schröder and Harder, 1999). Moreover, hBD-2 has been shown to provide a link between innate and adaptive immunity (Yang *et al*, 1999). hBD-2 was first isolated from psoriatic scales (Harder *et al*, 1997), and is strongly expressed by psoriatic keratinocytes at sites of epidermal inflammation (Liu *et al*, 1998; **Fig 1*a***). Although hBD-2 was not detected in the intact adult human epidermis (**Fig 1*b***) or in the neonatal cornified foreskin epidermis (**Fig 1*c***), it was also clearly detectable in the mucosal epithelium of the neonatal foreskin (**Fig 1*d***), a finding that may well explain why mucosal surfaces rarely develop clinically relevant infections, despite the absence of a stratum corneum. Most notably, this study shows that hBD-2 is induced in the regenerating epidermis of acute surgical wounds (**Fig 1*e***), suggesting that keratinocyte activation in response to loss of epidermal barrier function involves induction of an intrinsic antibiotic mechanism. Of special interest is the observation that hBD-2 is expressed in the epidermis of Apligraf (**Fig 1*f***), a tissue-engineered human skin equivalent (HSE) produced from neonatal human foreskin. This product consisting of living fibroblasts anchored in a bovine type I collagen lattice and living keratinocytes forming a stratified epidermis has been developed for the treatment of wounds (Wilkins *et al*, 1994) and has beneficial effects in patients with chronic venous leg ulcers (Falanga *et al*, 1998).

Unlike the other defensin genes, the hBD-2 gene contains several binding sites for the nuclear transcription factor NF-κB (Liu *et al*, 1998), which mediates responses to lipopolysaccharide (LPS) and proinflammatory cytokines. Therefore, we determined if hBD-2 expression is upregulated in the HSE following a topical bacterial challenge or exposure to LPS. Topical treatment with 0.5 μg per ml (5 ng per cm^2) and 1 μg per ml (10 ng per cm^2) purified bacterial LPS (from *E. coli* serotype 0127:B8; Sigma-Aldrich, Bucks, Switzerland) for 24 h caused a strong, concentration-dependent, and statistically significant ($p < 0.05$) upregulation of hBD-2 expression (30.9 ± 14.6 and 59.1 ± 4.5; data represent fold change *versus* untreated HSE ± SEM). Recent studies have suggested that a close interaction between CD14 and TLR4 participates in LPS signaling, leading to nuclear translocation of NF-κB (Jiang *et al* 2000); however, to our knowledge, neither CD14 nor TLR-4 has been found to be expressed in keratinocytes. Therefore, a CD14/TLR-4 independent LPS response mechanism may exist in keratinocytes. Alternatively, the possibility that a contaminant in the used LPS preparation might be responsible for the strong induction of hBD-2 in Apligraf cannot be excluded. Nevertheless, the hBD-2 induction in keratinocytes appears to be the result of pro-inflammatory processes, since other known chemical mediators of skin inflammation, e.g., phorbol 12-myristate 13-acetate and sodium lauryl sulfate, also enhanced hBD-2 mRNA expression in the HSE (data not shown). To investigate the effect of bacterial challenge on hBD-2 mRNA expression in Apliraf *E. coli* K12 bacteria grown over night in TY medium, pelleted, washed twice, and resuspended in DMEM/HAM-F_{12} (1:1) cell culture medium, were applied topically onto the epidermal surface of the HSE

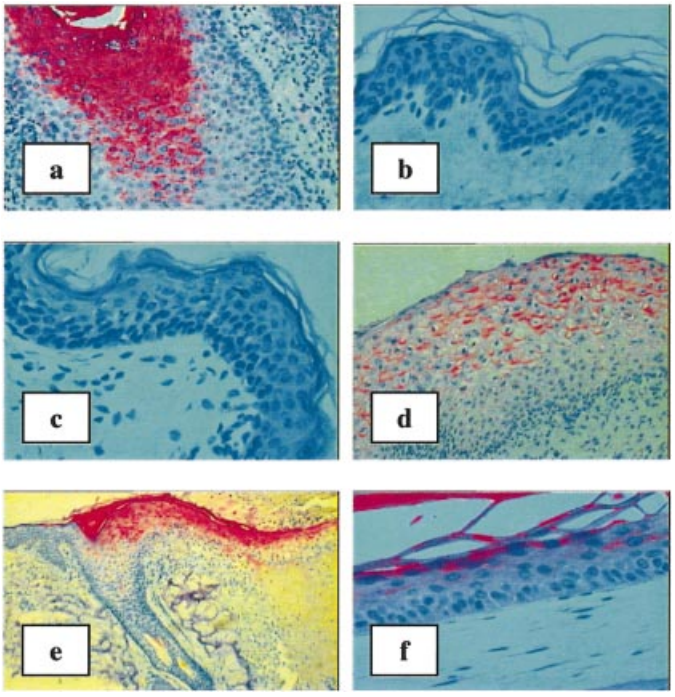

Figure 1. Immunostaining of hBD-2 in paraffin sections of human skin samples. Tissue samples were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline (PBS) pH 7.4 and then embedded in paraffin according to the standard procedures. Five micrometer sections were dewaxed, rinsed in PBS pH 7.4 and incubated for 1 h in blocking solution (PBS containing 1% bovine serum albumin and 0.1% tween 20) prior to incubation with rabbit anti-hBD-2 polyclonal IgG (kindly provided by Professor Thomas Ganz; Department of Medicine and Pathology, UCLA School of Medicine, Los Angeles, CA; diluted 1:500 in blocking solution) for 1 h at room temperature. Sections were subsequently reacted with biotinylated goat antirabbit polyclonal antiserum (BioGenex, San Ramon, CA) and streptavidine-alkaline phosphate complex (BioGenex), each 30 min at room temperature. Staining was performed with Fast Red chromogen (Sigma; F-4648) according to the instructions of the supplier. Sections were counterstained with Mayer's Haematoxylin. (*a*) Psoriatic lesion; (*b*) normal adult human skin; (*c*) cornified neonatal human foreskin; (*d*) neonatal human foreskin mucosa; (*e*) edge of a normally healing noninfected wound 3 d following injury; and (*f*) Apligraf. Immunostaining is indicated by a deposition of red color.

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(approximately 10^6 bacteria per cm^2). Bacterial challenge resulted in a weak but significant ($p < 0.05$) upregulation of hBD-2 mRNA expression (2.0 ± 0.4 after 24 h; 3.0 ± 0.7 after 48 h; data represent fold change *versus* untreated HSE ± SEM) as revealed by standard Real Time RT-PCR analysis. The observation that nonvirulent bacteria are much weaker inducers of hBD-2 than virulent bacteria, such as mucoid Pseudomonas aeruginosa (Harder *et al*, 2000), may provide a rationale for the fact that only a weak induction of hBD-2 was achieved, although a rather high cell number of *E. coli* K12 bacteria was applied topically.

To investigate if hBD-2 expression in the epidermis has functional relevance, we have soaked Apligraf with *E. coli* growth medium and subsequently exposed its surface to *E. coli* followed by a 24 h incubation at 37°C. Bacterial colonies did not grow on the epidermal surface of the intact HSE, although colonies clearly formed on the dermal surface exposed to bacteria following mechanical separation of the epidermal layer.

In conclusion, the findings of this study suggest that wound healing involves induction of an intrinsic antibiotic mechanism, which is also functional in epidermis of tissue-engineered skin.

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Coincident PTCH and BRCA1 Germline Mutations in a Patient with Nevoid Basal Cell Carcinoma Syndrome and Familial Breast Cancer

To the Editor

Nevoid basal cell carcinoma syndrome (NBCCS, OMIM no. 109400) is an autosomal dominant disease that is associated with various developmental anomalies and different types of neoplasms (Gorlin, 1987). Major manifestations are multiple basal cell carcinomas (BCC), odontogenic keratocysts, palmar and plantar dyskeratotic pits, and intracranial calcification (Evans *et al*, 1993; Shanley *et al*, 1994). In addition, rib and vertebral malformations, epidermal cysts, macrocephaly, facial anomalies, ovarian fibromas, and cerebellar medulloblastoma are associated with this syndrome. NBCCS results from germline mutations in the human homolog of the *Drosophila* segment polarity gene *patched* (*PTCH*) (Hahn *et al*, 1996; Johnson *et al*, 1996). *PTCH* maps to 9q22.3 and codes for a transmembrane protein (Ptch) that functions as receptor for the hedgehog family of signaling molecules (Wicking *et al*, 1999). So far, more than 60 different *PTCH* germline mutations have been found in NBCCS patients. There appears to be no genotype-phenotype correlation (Wicking *et al*, 1997).

Here, we report on a 78-y-old woman who has undergone multiple operations for BCC of the face and trunk since 1979. In total, more than 300 BCC have been resected. She has also been repeatedly operated on jaw keratocysts. At the age of 74 y, she developed a carcinoma of the left breast that was treated by mastectomy and lymphadenectomy. Histology showed multifocal manifestation of invasive ductal carcinoma without regional lymph node metastases or distant metastases [pT2m, pN0 (0/17), pM0, G2]. Estrogen and progesteron receptors were negative. At present, the patient is without recurrent breast carcinoma but still suffers from multiple BCC of the face, neck, and trunk. Her family history is remarkable as it does not only show NBCCS in both of her daughters but also reveals a marked clustering of breast carcinomas and other malignancies (**Fig 1**).

Breast carcinomas are not typically associated with NBCCS (Evans *et al*, 1993; Shanley *et al*, 1994). We therefore decided to investigate our patient for *PTCH* mutation as well as for mutation of the hereditary breast and ovarian cancer gene *BRCA1* (Miki *et al*, 1994). The patient gave her informed consent to both analyses. All 22 coding exons of *PTCH* were examined by single strand confirmation polymorphism analysis followed by direct sequencing. A heterozygous transition mutation (c. 3394T>C, nucleotide numbering according to GenBank accession no. U59464) was found in the patient's constitutional DNA (**Fig 2*a***). This mutation translates into an exchange of serine to proline at codon 1132 (S1132P). The identical mutation was detected in one of the patient's BCC (**Fig 2*a***). We additionally investigated this BCC for loss of heterozygosity (LOH) at three microsatellite markers from 9q22.3 (*D9S287*, *D9S197*, *D9S280*). LOH analysis was carried out on DNA purified from microdissected tumor areas. While *D9S197* and *D9S280* were constitutively homozygous, *D9S287* demonstrated LOH in the BCC (**Fig 2*b***).

Mutational analysis of the entire *BRCA1* coding region was performed by denaturing high pressure liquid chromotography and DNA sequencing (Arnold *et al*, 1999). The patient carried a heterozygous germline mutation within exon 11, i.e., an insertion of 31 bp at nucleotide 848 (c. 848ins817–847, nucleotide numbering according to GenBank accession no. U14680) (**Fig 2*c***). This mutation results in a frameshift and introduces a premature stop codon at position 247. The same mutation was found in the patient's daughter (data not shown).

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