Human β-Defensin-2 Production in Keratinocytes is Regulated by Interleukin-1, Bacteria, and the State of Differentiation

Alice Y. Liu, Delphine Destoumieux, Annie V. Wong, Christina H. Park, Erika V. Valore, Lide Liu, and Tomas Ganz

Departments of Medicine and Pathology, UCLA School of Medicine, Los Angeles, California, U.S.A.

Intact human epidermis resists invasion by pathogenic microbes but the biochemical basis of its resistance is not well understood. Recently, an antimicrobial peptide, human β-defensin-2, was discovered in inflamed epidermis. We used a recombinant baculovirus/insect cell system to produce human β-defensin-2 and confirmed that at micromolar concentrations it has a broad spectrum of antimicrobial activity, with the striking exception of *Staphylococcus aureus*. Immunostaining with a polyclonal antibody to human β-defensin-2 showed that the expression of human β-defensin-2 peptide by human keratinocytes required differentiation of the cells (either by increased calcium concentration or by growth and maturation in epidermal organotypic culture) as well as a cytokine or bacterial stimulus. Interleukin-1α, interleukin-1β, or live *Pseudomonas aeruginosa* proved to be the most effective stimuli whereas other bacteria and cytokines had little or no ability to induce human β-defensin-2 synthesis. In interleukin-1α-stimulated epidermal cultures, human β-defensin-2 first appeared in the cytoplasm in differentiated suprabasal layers of skin, next in a more peripheral web-like distribution in the upper layers of the epidermis, and then over a few days migrated to the stratum corneum. By semiquantitative Western blot analysis of epidermal lysates, the average concentration of human β-defensin-2 in stimulated organotypic epidermal culture reached 15–70 μg per gram of tissue, i.e., 3.5–16 μM, well within the range required for antimicrobial activity. Because of the restricted pattern of human β-defensin-2 distribution in the epidermis, its local concentration must be much higher. Defensins and other antimicrobial peptides of inflamed epidermis are likely to play an important antimicrobial role in host defense against cutaneous pathogens. *Key words: antimicrobial peptides/innate immunity/skin infections. J Invest Dermatol 118:275–281, 2002*

Epithelia that cover body surfaces constitute a barrier to infection by surrounding microbes. Skin in particular is constantly exposed to injury and challenged by environmental microorganisms. In normal hosts, however, the deeper layers of skin remain free of infections, suggesting that skin has an innate ability to fight invading microbes. Interesting clues to epidermal innate immunity have been provided in lower vertebrates by the example of the highly exposed aquatic organisms. Fish (Cole *et al*, 2000) and amphibian skin (Simmaco *et al*, 1998) are known to be rich sources of numerous and diverse antimicrobial peptides that are believed to play an essential role in the defense against the numerous aqueous microbes (Goraya *et al*, 2000). Despite important structural differences, human skin displays a protective ability comparable to that observed in the skin of these lower vertebrates, and indeed, recent studies have demonstrated the presence of antimicrobial peptides in human skin (reviewed by Gallo and Huttner, 1998).

Antimicrobial peptides are found in both the plant and animal kingdoms, where they are regarded as key elements of innate immunity (Borregaard *et al*, 2000). These gene-encoded antibiotics are small and cationic molecules characterized by an amphipatic structure (Martin *et al*, 1995). They are able to inactivate a wide spectrum of microorganisms, mainly by forming pores or other disruptive structures that permeabilize microbial membranes. The most common human antimicrobial peptides belong to the α- and β-defensin families, which differ from one another by the spacing and connectivity of their six cysteine residues (Ganz and Weiss, 1997). α-Defensins are found in storage granules of specialized cell types such as neutrophils or the small intestine Paneth cells, whereas β-defensins are characteristic of epithelial tissues. Human β-defensin-1 (hBD-1) is highly expressed in the kidney, and to a lesser extent in pancreas, salivary gland, airway epithelia, female urogenital system, and placenta (Zhao *et al*, 1996; Valore *et al*, 1998). Human β-defensin-2 (hBD-2) expression is reported in skin, urogenital tract, trachea, and lung (Harder *et al*, 1997; Bals *et al*, 1998; Hiratsuka *et al*, 1998; Liu *et al*, 1998).

hBD-2 was originally isolated from the desquamated scales of psoriatic skin (Harder *et al*, 1997), suggesting that this defensin may be involved in cutaneous defense and inflammation. To investigate

Manuscript received May 22, 2001; revised September 24, 2001; accepted for publication October 15, 2001.

Reprint requests to: Dr. Alice Y. Liu, Departments of Medicine and Pathology, UCLA School of Medicine, Los Angeles, CA 90095-1690. Email: tganz@mednet.ucla.edu

Abbreviations: hBD-1, human β-defensin-1; hBD-2, human β-defensin-2.

the role of hBD-2 in skin immunity, we examined a model of human epidermis for hBD-2 gene expression and protein production.

MATERIALS AND METHODS

Production and purification of recombinant hBD-2 Recombinant hBD-2 (rhBD-2) was produced in recombinant baculovirus-infected insect cells according to the procedure previously described for hBD-1 (Valore *et al*, 1998). Total RNA isolated from human small airway epithelial cells was used as a template for reverse transcription and polymerase chain reaction (RT-PCR). The oligonucleotides hBD-2-UP, 5′-GGGATCCGCTCCCAGCCATCAGCCATG-3′, and hBD-2-DW, 5′-AGCGAATTCAGCTTCTTGGCCTCCTCATG-3′, specific for hBD-2 cDNA amplification, were used as upstream and downstream primer, respectively. The PCR product was cloned into the pBacPAK9 transfer vector (Clontech, Palo Alo, CA). After double-strand DNA sequencing, the selected clone was cotransfected into the insect *Spodoptera frugiperda* Sf21 cells with *Bsu* 361-digested BacPAK6 viral DNA. The recombinant baculovirus was isolated and tested for rhBD-2 production, and the producing clones were selected to infect *Trichoplusia ni* Hi5 cells. The culture media were harvested 96 h after infection and subjected to cation exchange on a CM Macro-Prep ion exchange resin (Biorad). Elution was performed with 5% acetic acid (20 min, 4°C). The lyophilized eluate was finally purified by reverse phase high performance liquid chromatography (HPLC) on a 4.6 × 250 mm Vydac C_{18} column (Separations Group, Hesperia, CA) where it eluted at 37% acetonitrile. Peptide purity was monitored on a silver-stained acid-urea polyacrylamide gel (Valore *et al*, 1998).

Mass spectrometry measurements by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) Twenty nanograms of HPLC-purified rhBD-2 (1 μl) were loaded on a thin layer of α-cyano-4-hydroxycinnamic acid crystals (Sigma, Aldrich) made by fast evaporation of a saturated solution in 80% acetonitrile. The droplets were allowed to dry before introduction into the mass spectrometer. MALDI-TOF-MS was carried out at the UCLA Mass Spectroscopy Facility on a Voyager reverse phase mass spectrometer (PerSeptive BioSystems, Framingham, MA) operating in a positive linear mode.

Production of polyclonal antibodies against hBD-2 Two milligrams of HPLC-purified rhBD-2 were coupled to ovalbumin (Sigma, Aldrich) in a 1:1 mass ratio after activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce, Rockford, IL). The purified conjugate was used to immunize rabbits by six intradermal injections at 30 d intervals with 400 μg of the ovalbumin-coupled hBD-2. The immune sera were validated in enzyme-linked immunoassay and in Western blots against rhBD-2, and used as anti-hBD-2 polyclonal antibodies.

To test the specificity of anti-hBD-2 serum, we antigen-blocked it with excess rhBD-2. Briefly, 200 μl of serum was mixed with 10 μg of rhBD-2 for 2 h, diluted to 1 ml total with phosphate-buffered saline (PBS), and ultracentrifuged at 174,000*g* for 1 h to remove precipitate. Mock-extracted serum was prepared in the same manner substituting diluent for rhBD-2.

Microbial strains and antimicrobial assays The microbial strains used in this study, including the Gram-negative bacteria *Escherichia coli* ML35p (ampicillin-resistant strain) and *Pseudomonas aeruginosa* ATCC 9027, the Gram-positive *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *and S. aureus* ATCC 6341, and the fungus *Candida albicans* 820, were kindly provided by Robert I. Lehrer (UCLA, School of Medicine, Los Angeles, CA). Peptide activity was assayed according to the previously described procedure (Porter *et al*, 1997). Briefly, microorganisms were grown to exponential growth phase, and adjusted to an input concentration of 5×10^6–10^7 cells per ml for *S. epidermidis, S. aureus, S. agalactiae,* and *P. aeruginosa*, or 10^5–5×10^5 cells per ml for *E. coli* ML35p and *C. albicans*. The microbial suspensions were then incubated with 0–50 μM rhBD-2, under low-salt (10 mM Na phosphate, pH 7.4, 0.03% TSB), or high-salt (low-salt with 100 mM NaCl added) conditions. After a 1 h incubation at 37°C, the suspensions were plated on agar plates to quantitate surviving microbes and were incubated overnight at 37°C until colonies became visible.

Cell and tissue cultures

Keratinocytes Pooled normal human neonatal epidermal keratinocytes were obtained from Clonetics (Walkersville, MD) and propagated in KGM serum-free medium (Clonetics) at 37°C in a CO_2 incubator. Cells were passaged by trypsinization. Calcium concentration of KGM medium (0.15 mM) was altered by addition of $CaCl_2$ to 1.3 mM.

Organotypic epidermal cultures EpiDerm System organotypic cultures were obtained from MatTek (catalog #201-AF-AB, Ashland, MA). The cultures grown from normal human epidermal keratinocytes on collagen-coated Millicell CM membranes (0.45 μm pores, 24-well size) were placed in 12-well plates at the air–liquid interface with 1.2 ml EPI-201-AB-AF medium (1.8 mM Ca^{2+}) provided by MatTek. Cultures were incubated at 37°C in a CO_2 incubator and medium was changed every 2 d.

Stimulated cultures

Cytokine and endotoxin stimulation Cultures of keratinocytes and epidermis were stimulated by the addition of 10–100 ng per ml recombinant human cytokines [interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor α (TNF-α)], purchased from R&D Systems (Minneapolis, MN) and Peprotech (Rocky Hill, NJ), or *E. coli* 055:B5 lipopolysaccharide (LPS) (Sigma, Aldrich) into the culture medium. The LPS stimulation was also performed in the presence of 5% fetal bovine serum. Stimulation of keratinocytes was performed in eight-well glass chamber slides from LAB-TEK (Naperville, IL), whereas organotypic cultures were stimulated in a 12-well plate by replacing the medium under the membrane inserts. Keratinocytes were maintained for 5 d, and epidermis for up to 6 d after stimulation.

Microbial stimulation Heat-killed (100°C, 20 min) or live microorganisms in stationary growth phase were washed with PBS and resuspended in EPI-201-AB-AF culture medium at a concentration of 10^6 cells per ml. Ten microliters of each microbial suspension were spread onto the air-exposed surface of the epidermal culture and the treated cultures were incubated for another 48 h. EPI-201-AB-AF medium in which bacteria were grown overnight was filtered (0.2 μm) and spread on epidermal cultures in the same assay conditions to test the effect of bacterial secretions alone, compared to fresh EPI-201-AB-AF medium. After challenge, all cells and tissues were incubated at 37°C in a CO_2 incubator, collected at various times, and treated for protein/RNA extraction as described below.

Immunohistochemistry, light, and epifluorescence microscopy Immunochemical staining was performed as described previously (Valore *et al*, 1998). Briefly, cells and tissues were fixed in 3.7% formaldehyde in PBS. For immunoperoxidase staining, sectioned tissues (4–6 μm sections) and permeabilized cells were treated for endogenous peroxidase inactivation (0.1 M periodic acid followed by 0.02% sodium borohydride). Specimens were then blocked and incubated overnight with primary antibody. Anti-hBD-2 primary antibody was used at a 1:500–1:1000 dilution. In some experiments, hBD-2-extracted anti-hBD-2 antibody, or another antidefensin antibody (anti-HNP1-3, Ganz *et al*, 1985) raised in the same manner against a similar but not crossreacting antigen, were used as negative controls at the same dilution. Anti-human keratinocyte transglutaminase antibody (mouse monoclonal IgG2a, clone B.C1), obtained from Biomedical Technologies (Stoughton, MA) was used at a 1:20 dilution. For peroxidase detection, the specimens were washed and incubated overnight with 0.4 μg per ml horseradish-peroxidase-conjugated goat antirabbit IgG secondary antibody (Pierce) and developed in 0.6 mg per ml diaminobenzidine. For alkaline phosphatase (AP) detection, we used 1:2000 dilution of AP-conjugated goat antirabbit IgG (Pierce), and developed in Fast Red TR/Naphthol AS-MX with levamisole (Sigma). For fluorescence detection, Texas-Red-conjugated goat antirabbit IgG antibody, or fluorescein-conjugated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA), or fluorescein-conjugated horse antimouse IgG antibody (Pierce) were used at a concentration of 5 μg per ml. After a 90 min incubation, specimens were mounted in Vectriol (Vector Laboratories) for observation by epifluorescence microscopy.

Washes were made in 0.05% Tween 20, 500 mM NaCl, 20 mM Tris–HCl pH 7.5, or PBS. Antibody dilution buffers included the addition of 1% gelatin or 3% bovine serum albumin (BSA, Sigma). All steps were performed at room temperature.

Protein extraction, electrophoresis, and Western blot analysis Organotypic cultures were separated from the inert support membranes and homogenized in lysis buffer [130 mM glycine, 2% sodium dodecyl sulfate (SDS), 7.7% glycerol in 70 mM Tris–HCl, pH 8.8] by repeated passage through a 26 gauge needle. Media conditioned by the cultures were adjusted to lysis buffer conditions. Samples in lysis buffer were boiled for 5 min and loaded directly onto a 16.5% SDS-tricine polyacrylamide gel (Schagger and von Jagow, 1987). Transfer to

Immobilon-PSQ membranes (Millipore, Bedford, MA) was performed for 1 h at 0.18 A in 0.05 M sodium borate, pH 9.0, with 20% methanol and 0.05% SDS, and immunodetection was performed as described earlier (Valore *et al*, 1998), with anti-hBD-2 antibodies used at a 1:1000 dilution, or antihuman keratin 10 antibody (mouse monoclonal IgG1 tissue culture supernatant; DAKO DE-K10, Denmark) at a 1:200 dilution.

RNA extraction and northern blot analysis Total RNA was extracted from Epiderm cultures using Trizol reagent (BRL, Life Technologies). Ten to 15 μg of each sample were loaded for RNA electrophoresis on a 1% agarose gel containing 17% formaldehyde and transferred to Hybond-N filter membranes (Amersham). A cDNA probe for hBD-2 was generated by PCR amplification of hBD-2 cDNA with the oligonucleotides hBD-2-UP and hBD-2-DW described above and subsequent radiolabeling using the Prime-It RmT labeling kit (Stratagene). The membranes were hybridized at 52°C for 12 h with the [^{32}P]-labeled probes fragment in a solution containing 50% formamide, 5 × sodium citrate/chloride buffer (SSC), 8 × Denhardt's solution, 0.05 M sodium phosphate pH 6.5, 0.1% SDS, and 100 μg per ml denatured salmon sperm. The filters were washed in 1 × SSC, 0.1% SDS at 65°C followed by autoradiography. After stripping with 0.1% SDS at 100°C, the membranes were rehybridized in the same conditions with a probe corresponding to [^{32}P]-labeled β-actin cDNA (MTN control probe, Clontech).

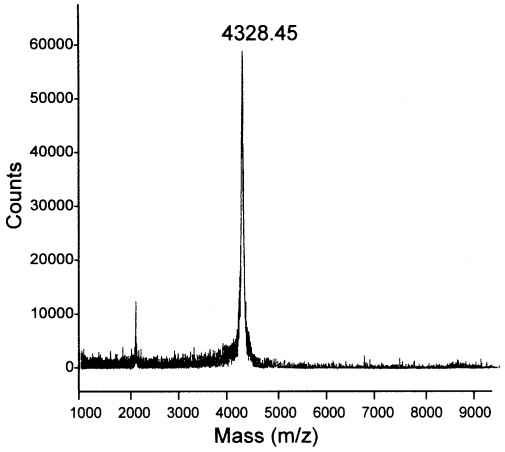

Figure 1. Recombinant hBD-2 analysis. MALDI-TOF spectrum of the rhBD-2 after purification by reversed phase HPLC. Masses are given as MH^+ values.

RESULTS

Activity of recombinant hBD-2 Recombinant hBD-2 produced by baculovirus expression in Hi5 insect cells was isolated from the culture medium (see *Materials and Methods*) and subjected to MALDI-TOF-MS for purity control and molecular mass measurement. A single peptide species at $MH^+ = 4328.45$ was identified in the sample (**Fig 1**), in agreement with the calculated mass (4328.18 Da). After purification to homogeneity, the production yield for rhBD-2 was estimated at 1 mg per liter of culture medium.

The activity of the recombinant peptide was then assayed in the range of 0–30 μM against the yeast *C. albicans* and the bacteria *E. coli* ML35p, *P. aeruginosa*, *S. agalactiae*, *S. epidermidis*, and *S. aureus*. Antimicrobial activity was detected against all six tested strains after 1 h incubation of microorganism with rhBD-2 (**Fig 2**). A dose-dependent microbicidal effect was observed for hBD-2, starting at concentrations from 1 to 3 μM, against all the tested strains with the exception of *S. aureus* where rhBD-2 was at most bacteriostatic in the range of concentrations tested. The microbicidal effect was reduced (*S. epidermidis*, *E. coli* ML35p) or changed to a static effect (*S. agalactiae*, *C. albicans*) under high-salt concentrations (100 mM NaCl). Data for *P. aeruginosa* are only given under high-salt conditions as the bacteria did not grow in low-salt medium.

Induction of hBD-2 production in keratinocyte cultures Immune serum to hBD-2 was used in immunohistochemistry experiments to analyze hBD-2 expression in human primary keratinocytes induced with cytokines or bacterial endotoxin. Cultures stimulated with 100 ng per ml IL-1α or IL-1β demonstrated intense hBD-2 staining in a rare subpopulation of cells (**Fig 3*A*, *C***), whereas no immunoreactivity could be detected in cultures stimulated with the same concentrations of TNF-α or LPS (**Fig 3*F*, *G***). Addition of fetal bovine serum to LPS stimulation did not induce detectable amounts of hBD-2 protein (data not shown). Nonstimulated keratinocytes used as controls did not show reactivity with the anti-hBD-2 serum.

Localization of hBD-2 protein to differentiated keratinocytes In stimulated keratinocyte cultures, hBD-2 protein was not detected uniformly in all cells. Cells staining for hBD-2 protein were rare, large, polygonal cells in areas of higher cell density and multiple cell layers (**Fig 3**). We hypothesized that these cells were the more differentiated keratinocytes in the culture. To test this

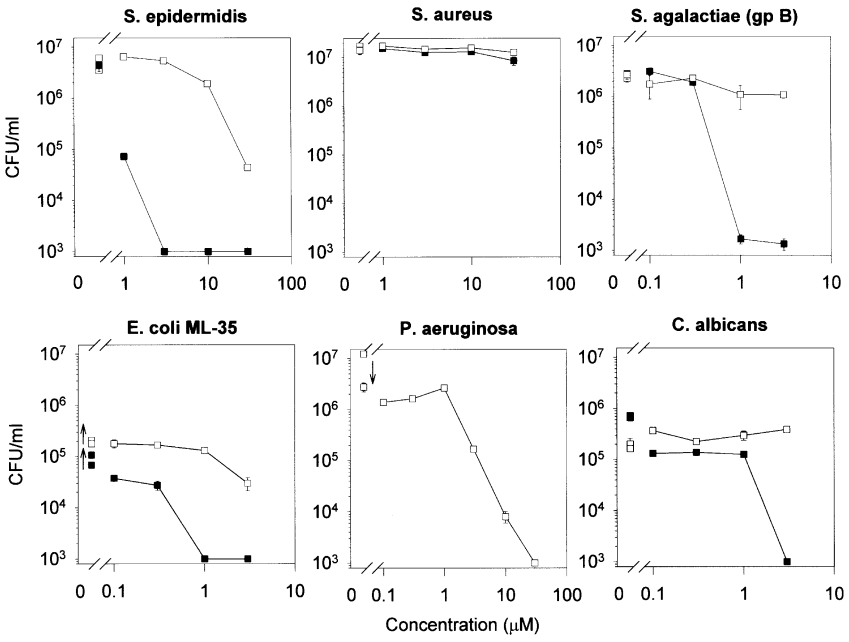

Figure 2. Antimicrobial activity of rhBD-2 against *S. epidermidis*, *S. aureus*, *S. agalactiae*, *E. coli* ML35p, *P. aeruginosa*, and *C. albicans*. The number of CFU was determined after a 1 h incubation with rhBD-2. Experiments made in low-salt or high-salt concentrations are indicated by *black* and *open squares*, respectively. Controls containing peptide solvent only (0.01% acetic acid) are also shown for incubation times of 0 and 1 h. If CFU concentrations changed significantly during incubation with solvent only, *arrows* indicate whether the CFU increased or decreased compared to inoculum. *Error bars* indicate standard deviations of the mean of triplicate experiments.

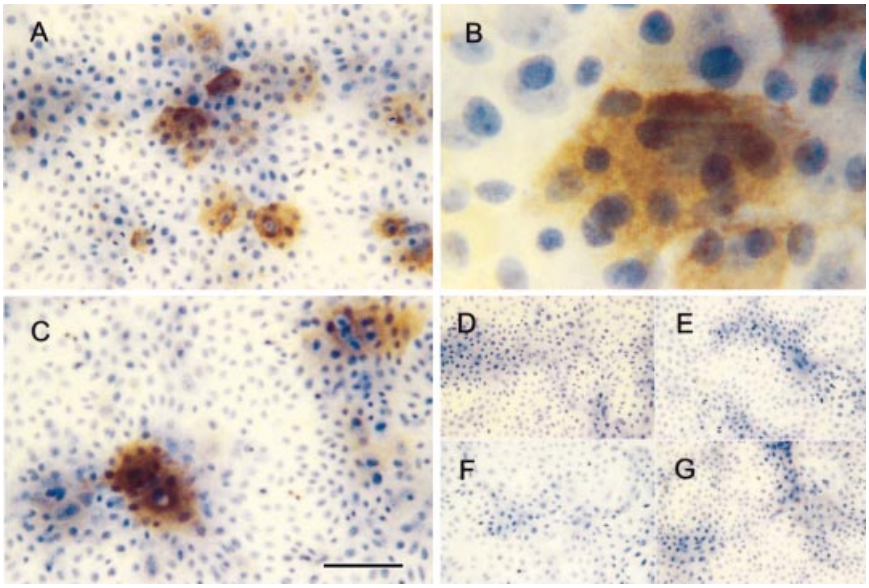

Figure 3. Immunostaining of stimulated keratinocyte cultures with anti-hBD-2 antibody. Keratinocytes stimulated for 3 d with 100 ng per ml IL-1α (*A*, *B*), 100 ng per ml IL-1β (*C*), 100 ng per ml TNF-α (*F*), or 100 ng per ml LPS (*G*) were grown and fixed on cytoslides and immunostained using anti-hBD-2 polyclonal antibodies with peroxidase detection (brown staining product). IL-1α-stimulated keratinocytes stained with preimmune serum (*D*) and unstimulated keratinocytes stained with anti-hBD-2 (*E*) are shown for comparison. Cells were photographed under light microscopy with a 10× objective except for *panel B*, which was photographed under 40×. *Panels D–G* are shown half size. *Scale bar*: (*A*, *C*) 100 μm; (*B*) 25 μm.

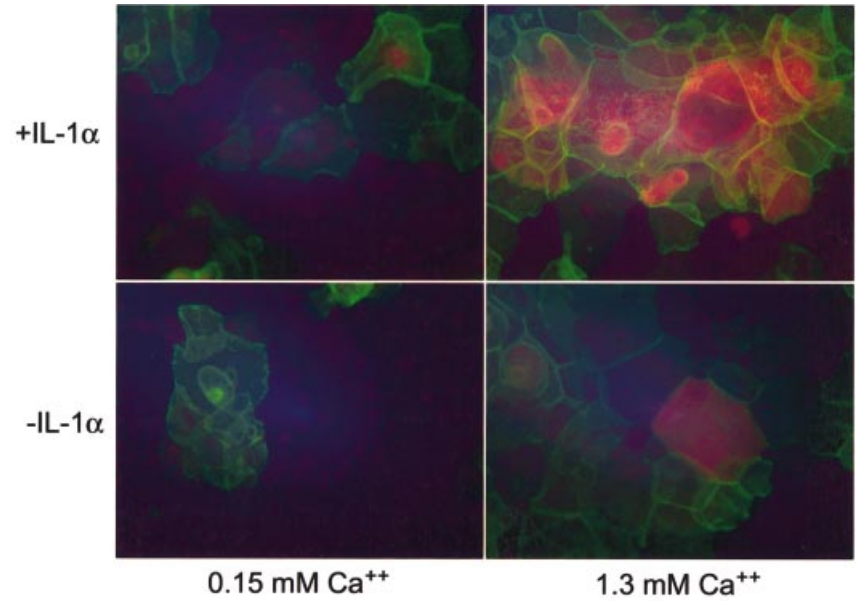

Figure 4. The effect of calcium-induced differentiation of keratinocytes on hBD-2 and tranglutaminase expression. Keratinocytes were cultured in low calcium (0.15 mM) and high calcium (1.30 mM) concentrations with 100 ng per ml IL-1α or control medium for 5 d. Fluorescein staining (green) was used for transglutaminase detection and Texas Red staining (red) for hBD-2 detection. Immunostained cells were photographed under epifluorescence microscopy.

hypothesis, we stimulated keratinocyte cultures grown at lower density with IL-1α in the presence of low (0.15 mM) and high (1.3 mM) calcium concentrations to induce keratinocyte differentiation. Cultures were stained for both hBD-2 and keratinocyte transglutaminase, a membrane associated protein found in differentiated layers of epidermis (Thacher, 1989), and then were photographed by epifluorescence microscopy. Cells displaying transglutaminase staining were more numerous at high calcium concentration (**Fig 4**). Similarly, increase in calcium concentration increased the number of keratinocytes staining for hBD-2 in culture induced with IL-1α. The induced hBD-2 staining was localized in the transglutaminase-positive keratinocytes.

Production of hBD-2 in keratinocyte organotypic culture We next examined hBD-2 production in a model of epidermal differentiation, a keratinocyte organotypic culture created by growing nontransformed human keratinocytes on an inert membrane at the air–liquid interface. Under these conditions, the keratinocytes differentiate into an epidermis-like culture demonstrating a basal layer, a granular layer, and a stratum corneum (**Fig 5*A***), and expressing markers of epidermal differentiation (Klausner *et al*, 1998). Keratinocyte organotypic cultures were stimulated with 100 ng per ml IL-1α, and hBD-2 expression was analyzed by immunohistochemistry on epidermis sections at various times after stimulation (**Fig 5**). After 2 d of induction, hBD-2 was seen in all layers of the culture except for the basal layer (less differentiated cells). The lack of basal cell staining is particularly well seen in **Fig 5(*D*)**. In the lower cell layers, hBD-2 staining appeared to be cytoplasmic, whereas in upper cell layers, staining appeared at the membrane or the exterior of the cell. At 4 d of induction, hBD-2 was detected in the upper keratinocytes of the culture and in the stratum corneum. Finally, at 6 d of induction, hBD-2 was located predominantly in the stratum corneum. Similar results were found with 20 ng per ml IL-1α and 100 ng per ml IL-1β stimulation (data not shown). Noninduced organotypic cultures demonstrated minimal to low levels of hBD-2 staining.

Organotypic culture lysates were analyzed by Western blot for hBD-2 protein expression. Immunoreactivity in the extracts was found to be associated with a protein with the same size as rhBD-2

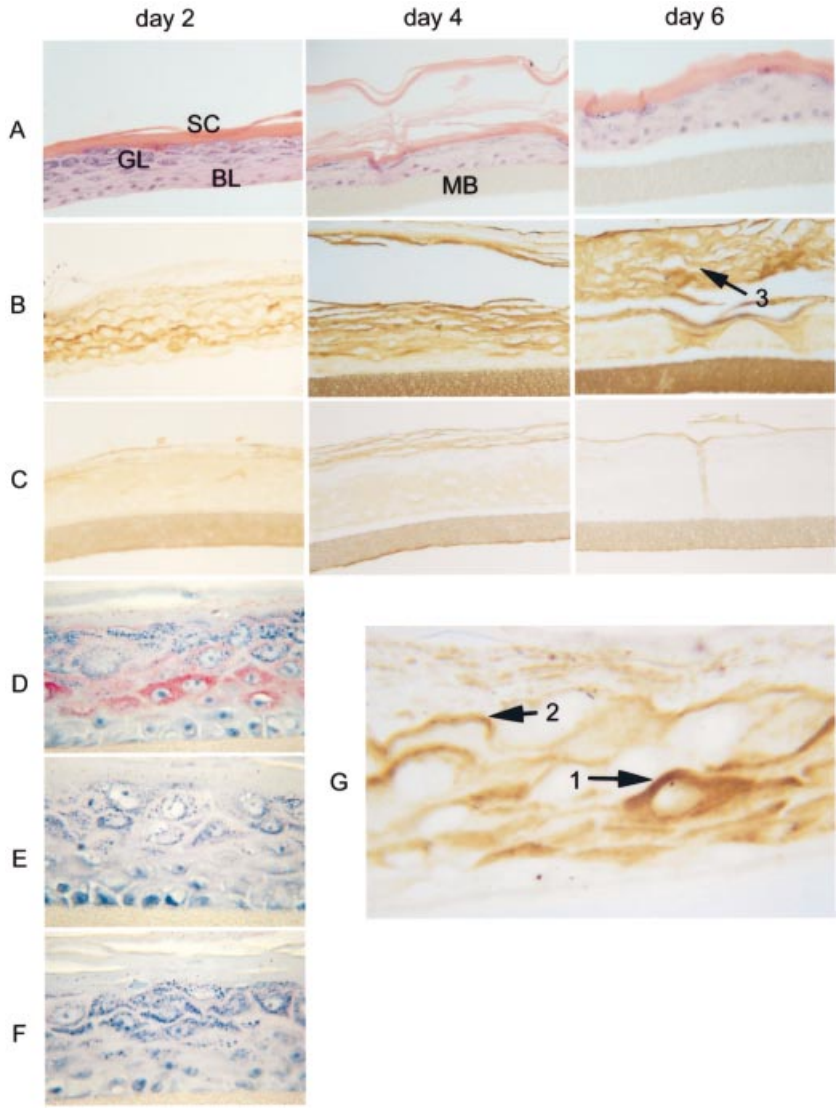

Figure 5. hBD-2 expression in IL-1α-induced epidermal cultures. Epidermal cultures were observed at 2, 4, and 6 d after stimulation with 100 ng per ml IL-1α. (*A*) Sections of IL-1α-induced epidermal cultures were stained with hematoxylin and eosin. BL, GL, SC, and MB stand for basal layer, granular layer, stratum corneum, and support membrane, respectively. In some sections, the membrane was lost during processing. (*B*) IL-1α-induced epidermal cultures were immunoperoxidase stained for hBD-2 and developed in diaminobenzidine, with no counterstain. Adjacent sections treated identically except for preimmune serum substituted for anti-hBD-2 did not show staining (data not shown). (*C*) Sections of noninduced epidermal cultures stained as in (*B*). (*D*) Sections prepared as in (*B*) were immunostained for hBD-2, developed with Fast Red TR/Naphthol AS-MX, and counterstained with hematoxylin. (*E*) Same as (*D*) but anti-hBD-2 antibody was blocked with excess hBD-2. (*F*) Same as (*D*) but antibody to human neutrophil defensins hNP1–3 was substituted for anti-hBD-2. (*G*) Higher magnification of an IL-1α-induced epidermal culture from day 2, immunoperoxidase stained for hBD-2. Numbers indicate the various locations of hBD-2: (1) in the cytoplasm of a suprabasal keratinocyte; (2) at the cell periphery in the granular layer keratinocytes; and (3) in the stratum corneum at day 6 of stimulation.

(**Fig 6**). The immunoreactive band observed in the IL-1α-induced culture is almost undetectable in the noninduced culture, whereas both samples show identical immunoreactivity to the control keratin-10 antibodies. By comparison to standards, the amount of hBD-2 per lane of stimulated organotypic culture after 2 d appears to be between 1 and 5 ng, i.e., 15–70 μg per gram of tissue. Assuming that the density of the epidermal tissue is 1 g per ml, the concentration of hBD-2 in the stimulated culture can be estimated at 3.5–16 μM. From a faint hBD-2 signal detected by Western blot of conditioned medium from induced culture, the total amount of hBD-2 found in the medium is about 1/5–1/10 of the amount detected in the tissue.

We next analyzed the effect of IL-1α stimulation (10 ng per ml) on hBD-2 mRNA expression in the organotypic cultures. Total RNA extracted from the epidermal cultures was analyzed by northern blot using the ^{32}P-labeled hBD-2 cDNA as a probe. After stimulation, there is an increase in hBD-2 mRNA concentration over 48 h, with detectable hybridization signal present already at 6 h (**Fig 7*a***). As the hybridization signal with hBD-2 mRNA intensifies over the 48 h time-course, the observed size decreases, consistent with poly-A shortening seen with stable mRNA maturation. RNA from noninduced cultures used as a control did not display hybridization with the hBD-2 cDNA probe. Secondary hybridization with a ^{32}P-labeled probe corresponding to β-actin and used to normalize the signals showed in contrast a decrease in signal intensity over time with no changes in electrophoretic behavior.

We next tested the effect of live microorganisms on hBD-2 expression by spreading them on the air-exposed Epiderm surface. After 48 h of infection, only the *P. aeruginosa* strain was able to induce a significant increase in hBD-2 mRNA concentration in the epidermis (**Fig 7*b***). In samples challenged with other microbes, hBD-2 mRNA concentrations were similar to sterile medium control.

DISCUSSION

We have prepared a recombinant version of hBD-2, the recently identified epidermal antimicrobial peptide, and demonstrated its *in vitro* antimicrobial activities. We then showed the dependence of hBD-2 expression in keratinocytes on the dual factors of differentiation and cytokine or microbial stimulation. We have also shown that the concentration in stimulated epidermis reaches levels necessary for antimicrobial activity.

Baculovirus expression of rhBD-2 allowed us to obtain 1 mg recombinant peptide per liter of initial cell culture medium after a one-step HPLC purification procedure. This yield is comparable to that obtained previously for other human defensins expressed in the same system (Porter *et al*, 1997; Valore *et al*, 1998). MALDI-TOF analysis of rhBD-2 demonstrated that this expression system and purification procedure were adapted to the preparation of a pure

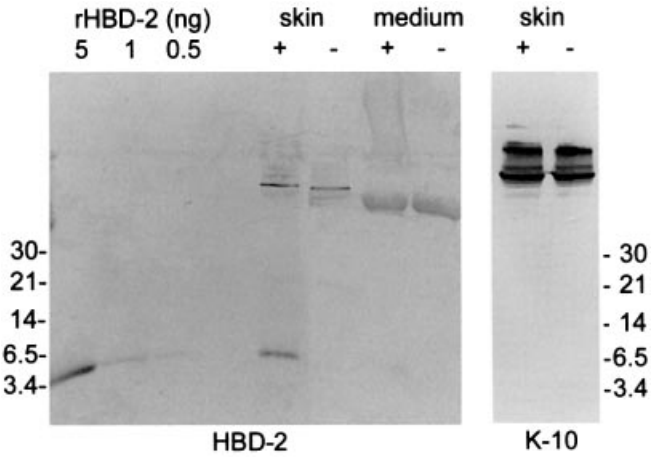

Figure 6. Western blot analysis of hBD-2 expression in epidermal cultures induced with IL-1α. Epidermal cultures were stimulated with 20 ng per ml IL-1α for 2 d, and then homogenized in lysis buffer as described. Extracts originating from 75 μg epidermal culture, or aliquots of conditioned media corresponding to 30 μg of epidermal culture, were loaded into the indicated lanes. The indicated amounts of rhBD-2 were used as standards. Samples were separated on nonreducing SDS-tricine polyacrylamide gel and analyzed by Western blot for hBD-2 or human keratin-10. Stimulated and nonstimulated cultures are indicated with (+) and (–), respectively. The locations and sizes (kDa) of molecular weight markers are shown. Note that uncharacterized high molecular weight protein bands crossreacting with anti-hBD-2 antibody are observed in both skin extracts and media but they are not affected by IL-1α stimulation.

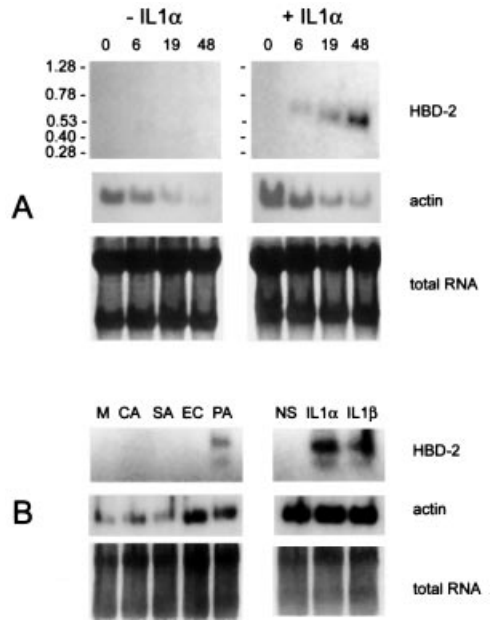

Figure 7. Northern blot analysis of hBD-2 expression in stimulated epidermal cultures. (*A*) IL-1α stimulated cultures (10 ng per ml in culture medium) were analyzed for hBD-2 expression at 0, 6, 19, and 48 h after stimulation. Total RNA samples were loaded at 15 μg per lane. (*B*) Cultures were stimulated by spreading 10 μl of live microbes (10^6 per ml) or culture medium onto the epidermal surface. *Lanes M*, *SA*, *EC*, *PA*, and *CA* contain lysates of epidermis exposed to fresh medium, *S. aureus*, *E. coli* ML35p, *P. aeruginosa*, and *C. albicans*, respectively. Positive controls were exposed to IL-1α or IL-1β in the culture medium; in the negative control (NS) only the saline solvent was added to the culture medium. Total RNA was extracted after 48 h and loaded at 12 μg per lane. Blots were successively hybridized with ^{32}P-labeled hBD-2 and β-actin cDNA.

folded rhBD-2 displaying the correct primary sequence (as indicated by a mass measurement in perfect agreement with the calculated mass). The activity spectrum of rhBD-2 covered a broad range of microorganisms including bacteria (both Gram-negative and Gram-positive) and fungi identified as human pathogens. Except for one strain (*S. aureus*) this antimicrobial activity was shown to be microbicidal at concentrations > 1 μM. As also reported by Bals *et al* (1998), we observed that rhBD-2 was less active at high-salt concentrations. However, substantial rhBD-2 antimicrobial activity remains at salt concentrations (100 mM) higher than those found in human sweat (20–60 mM) (Lentner, 1981), which is in equilibrium with the salt concentration in upper keratinocyte layers.

We used polyclonal antibody to rhBD-2 to study hBD-2 expression in skin at the peptide level. We observed that the cytokines IL-1α and IL-β induced hBD-2 synthesis in keratinocyte and epidermal cultures. The findings were confirmed at the mRNA level in epidermal cultures. In this system, IL-1α stimulation induces a rapid appearance of hBD-2 mRNA (within the first 3 h), which then tends to slowly accumulate (up to 48 h) as indicated by the decreasing size of the mRNA on northern blots (consistent with the known shortening of the poly-A tail in stable maturing mRNAs). This rapid increase in mRNA concentration, together with the identification of NF-κB-like binding motifs in the hBD-2 gene promoter region (Liu *et al*, 1998), suggests transcriptional control of hBD-2 expression by IL-1α and IL-β (activators of NF-κB) in skin, and is in agreement with the expression of hBD-2 in inflammatory skin disorders but not in normal skin (Harder *et al*, 1997).

Although TNF-α has been reported to stimulate hBD-2 expression in keratinocyte cultures (Harder *et al*, 1997) and respiratory epithelia (Harder *et al*, 2000), in our hands this cytokine did not induce keratinocytes to produce hBD-2 peptide. We also did not detect LPS induction of hBD-2, which had been reported in oral mucosa (Mathews *et al*, 1999). The lack of response to LPS is not due to the absence of LPS-binding protein as addition of serum did not induce hBD-2 expression. It is possible that *in vivo* LPS that penetrates deep into the skin may activate hBD-2 expression through macrophages or Langerhans cells serving as intermediates for LPS detection and production of cytokines that stimulate hBD-2 expression in differentiated keratinocytes.

Although LPS did not induce hBD-2 expression in our conditions, epidermal cultures responded to live microbes. Induced expression of hBD-2 was evidenced by northern blots, showing a high responsiveness of epidermal cultures to *P. aeruginosa*. The activation of hBD-2 expression may be mediated by *P. aeruginosa* exotoxins or secretion system. Indeed, some Gram-negative bacteria such as *Helicobacter pylori* are able to activate NF-κB in epithelial cells by their type IV secretion system (for review see Naumann, 2000). Interestingly, a recent study has demonstrated that *P. aeruginosa* type IV pilus was necessary to activate host genes in infected epithelial cells whereas LPS had only very limited effect (Ichikawa *et al*, 2000). Such type IV secretion system functioning only in live bacteria could be responsible for the activation of hBD-2 transcription. The lack of response of organotypic epidermal keratinocytes to LPS and most tested bacteria points to the potential importance of other cell types (macrophages, dendritic cells, mast cells, etc.) in initiating cutaneous responses to these agents. IL-1 produced by the latter cells could serve as a potent host defense activation signal for keratinocytes.

In IL-1α-stimulated primary keratinocyte cultures, hBD-2 was selectively detected in large and polygonal cells that we identified as possible differentiated keratinocytes. We confirmed that cultures induced to differentiate with high calcium concentrations produced higher quantities of hBD-2 in response to IL-1α stimulation. The cellular colocalization of hBD-2 and transglutaminase [used as a reporter of differentiated keratinocytes (Thacher, 1989)] indicates that hBD-2 expression is favored in the more mature keratinocytes.

This finding parallels the localization of hBD-2 to the differentiated suprabasal layers of skin in IL-1α-stimulated epidermal cultures. Over time, hBD-2 migrates from the suprabasal layers to the stratum corneum. The localization of hBD-2 in the cell cytoplasm in lower layers and then at the keratinocyte periphery as cells move closer to the surface suggests that hBD-2 is secreted onto the cell membrane or into the intercellular space and finally concentrates in the dehydrated cells of the epidermal surface (days 4–6). Such a hypothesis is in agreement with the presence of a signal peptide in the hBD-2 precursor sequence (Harder *et al*, 1997). Compared to the amount of hBD-2 in stimulated epidermis, less hBD-2 was detected in the corresponding culture medium. This difference could be due to the efficient retention of hBD-2 within the epidermis. We cannot exclude the possibility that the inert membrane also absorbs a fraction of the secreted peptide, however, or that our system predominantly measures products secreted downward through the basal layer of the epidermis. Importantly, the calculated epidermal hBD-2 concentration of 3.5–16 μM falls within its antimicrobial range, and as the volume of distribution is probably restricted to the intercellular space, the local concentrations could be much higher. If so, hBD-2 could serve its antimicrobial function in skin by concentrating in spaces exposed to microbial invasion such as the intercellular space or the epidermal surface. It is important to note that the known chemotactic activity of hBD-2 for immature dendritic cells and memory T cells (Yang *et al*, 1999) requires much lower concentrations than its antimicrobial activity and thus the relevant chemotactic concentrations could be reached even quicker. The inducibility of hBD-2 production by IL-1 and live *P. aeruginosa* suggests a dynamic system that can be rapidly activated in response to infections.

In concert with hBD-2, antimicrobial peptides such as the cathelicidin hCAP18/LL-37 (Frohm *et al*, 1997), and to a lesser extent the β-defensin hBD-1 (Boe *et al*, 1999), were shown to be expressed in keratinocytes, and like hBD-2, hCAP18/LL-37 expression may be increased by inflammation. Additional antimicrobial activity was also attributed to other factors expressed in skin such as the antileukoprotease (Wiedow *et al*, 1998). Together, the antimicrobial peptides of human skin are likely to provide an effective shield from microbial infection.

We gratefully acknowledge the assistance of Drs John Sheasgreen, Joe Kubilus, and Mitch Klausner at the MatTek Corporation, Ashland, MA 01721, and Adam Mogil at the Human Tissue Research Center at UCLA. The work was supported in part by NIH Grants HL 46809 and AI 40248.

REFERENCES

Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M, Wilson JM: Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J Clin Invest* 102:874–880, 1998

Boe R, Silvola J, Yang J, Moens U, McCray PB Jr, Stenfors LE, Seljfelid R: Human beta-defensin-1 mRNA is transcribed in tympanic membrane and adjacent auditory canal epithelium. *Infect Immun* 67:4843–4846, 1999

Borregaard N, Elsbach P, Ganz T, Garred P, Svejgaard A: Innate immunity: from plants to humans [published erratum appears in Immunol Today 21 (4):191, 2000]. *Immunol Today* 21:68–70, 2000

Cole AM, Darouiche RO, Legarda D, Connell N, Diamond G: Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. *Antimicrob Agents Chemother* 44:2039–2045, 2000

Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, Gudmundsson GH: The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 272:15258–15263, 1997

Gallo RL, Huttner KM: Antimicrobial peptides: an emerging concept in cutaneous biology. *J Invest Dermatol* 111:739–743, 1998

Ganz T, Weiss J: Antimicrobial peptides of phagocytes and epithelia. *Semin Hematol* 34:343–354, 1997

Ganz T, Selsted ME, Szklarek D, Harwig SSK, Daher K, Bainton DF, Lehrer RI: Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* 76:1427–1435, 1985

Goraya J, Wang Y, Li Z, O'Flaherty M, Knoop FC, Platz JE, Conlon JM: Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*. *Eur J Biochem* 267:894–900, 2000

Harder J, Bartels J, Christophers E, Schroeder J-M: A peptide antibiotic from human skin. *Nature* 387:861–862, 1997

Harder J, Meyer-Hoffert U, Teran LM, Schwichtenberg L, Bartels J, Maune S, Schroder JM: Mucoid *Pseudomonas aeruginosa*, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol* 22:714–721, 2000

Hiratsuka T, Nakazato M, Date Y, Ashitani J, Minematsu T, Chino N, Matsukura S: Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochem Biophys Res Commun* 249:943–947, 1998

Ichikawa JK, Norris A, Bangera MG, Geiss GK, ''t Wout AB, Bumgarner RE, Lory S: Interaction of *Pseudomonas aeruginosa* with epithelial cells: identification of differentially regulated genes by expression microarray analysis of human cDNAs. *Proc Natl Acad Sci USA* 97:9659–9664, 2000

Klausner M, Neal P, Kubilus J: Initial results with Epi-201, a developing *in vitro* model of human epidermis. *J Invest Dermatol* 110:558, 1998 (Abstr.)

Lentner C: *Geigy Scientific Tables*, 8th edn. Basle: CibaGeigy, 1981

Liu L, Wang L, Jia HP, et al: Structure and mapping of the human β-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 222:237–244, 1998

Martin E, Ganz T, Lehrer RI: Defensins and other endogenous peptide antibiotics of vertebrates. *J Leukoc Biol* 58:128–136, 1995

Mathews M, Jia HP, Guthmiller JM, et al: Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect Immun* 67:2740–2745, 1999

Naumann M: Nuclear factor-kappaB activation and innate immune response in microbial pathogen infection [In Process Citation]. *Biochem Pharmacol* 60:1109–1114, 2000

Porter EM, vanDam E, Valore EV, Ganz T: Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infec Immunity* 65:2396–2401, 1997

Schagger H, von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379, 1987

Simmaco M, Mignogna G, Barra D: Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 47:435–450, 1998

Thacher SM: Purification of keratinocyte transglutaminase and its expression during squamous differentiation. *J Invest Dermatol* 92:578–584, 1989

Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB, Ganz T: Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest* 101:1633–1642, 1998

Wiedow O, Harder J, Bartels J, Streit V, Christophers E: Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. *Biochem Biophys Res Commun* 248:904–909, 1998

Yang D, Chertov O, Bykovskaia SN, et al: Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 286:525–528, 1999

Zhao CQ, Wang I, Lehrer RI: Widespread expression of beta-defensin HBD-1 in human secretory glands and epithelial cells. *FEBS Lett* 396:319–322, 1996