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A Model for Antimicrobial Gene Therapy: Demonstration of Human β -Defensin 2 Antimicrobial Activities *In Vivo*

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Abstract

We transfected host cells with an antimicrobial peptide/protein-encoding gene as a way to enhance host defense mechanisms against infection. The human β -defensin 2 (HBD-2) gene was chosen as a model because its protein does not require cell type-specific processing. Using a retroviral vector carrying HBD-2 cDNA, we treated several mouse or human cell lines and primary cell cultures including fibroblasts, salivary gland cells, endothelial cells, and T cells. All transduced cells produced detectable HBD-2. In *Escherichia coli* gel overlay experiments, secreted HBD-2 from selected cell lines showed potent antimicrobial activity electrophoretically identical to that of purified HBD-2. We then used a mouse model (nonobese diabetic/severely compromised immunodeficient [NOD/SCID]) to test HBD-2 antimicrobial activities *in vivo*. HT-1080 cells carrying HBD-2 or control vector were implanted subcutaneously into NOD/SCID mice to allow tumor formation. *Escherichia coli* was then injected into each tumor mass. Tumors were resected after 16 hr and homogenized for bacterial colony-forming unit analysis. Compared with control tumors, HBD-2-bearing tumors contained only 7.8 $\pm$ 3.3% viable bacteria. On the basis of this demonstration of HBD-2 *in vivo* antimicrobial activity, enhancement of antibacterial host defense by HBD-2 gene therapy may be feasible.

OVERVIEW SUMMARY

Augmentation of innate immunity through antimicrobial gene transduction has not been systematically investigated. The increasing list of newly discovered antimicrobial protein/peptides, gradual progress in gene therapy, as well as the search for alternative antimicrobial therapeutic modalities prompted our attempt to examine the concept of antimicrobial gene therapy. The present study was aimed to test the following, using HBD-2 as a model: (1) the use of a viral vector gene transduction approach, (2) the ability of a variety of cell types to produce functional antimicrobial peptides after gene transduction *in vitro*, and (3) the antimicrobial activity of exogenous HBD-2 *in vivo*. We found that a viral vector can effectively transduce a variety of cell types to secrete HBD-2. Using a novel approach with HBD-2-transduced tumor cells grown in NOD/SCID mice, we demonstrated, for the first time *in vivo*, the antimicrobial activity of HBD-2. The findings support the potential utility of antimicrobial gene therapy.

INTRODUCTION

WITH INCREASING RESISTANCE of bacteria to conventional antibiotics, attention has focused on alternative anti-infectious therapies. Antimicrobial peptides are promising new agents that have low susceptibility to microbial resistance mechanisms. Unlike conventional antibiotics, antimicrobial peptides are encoded by single genes and can be introduced into infected tissues by gene therapy approaches.

Antimicrobial peptides are widely distributed in plants and animals (Borreagaard *et al.*, 2000) and are found abundantly in many host defense systems, from plant seeds and arthropod hemolymph to human neutrophils and epithelia (reviewed by Ganz and Lehrer, 1999). In general, the peptides are thought to be active against a broad range of microbes at micromolar concentrations within a particular microenvironment of the host (Bevins *et al.*, 1999). The peptides preferentially form lytic pores on the phospholipid bilayers of microorganisms as opposed to eukaryotic host cells, depending on differences in the phospholipid composition of the surface membranes (Matsuzaki *et al.*, 1995; Latal *et al.*, 1997).

Most presently known antimicrobial peptides from the animal kingdom fall into one of three structural groups: (1) cysteine-rich *b*-sheet peptides, (2) *a*-helical, amphipathic molecules, and (3) proline-rich peptides. Molecules of the small *b*-sheet peptide families include *a*- and *b*-defensins, insect defensins, tachyplesins, protegrins, bactenecin dodecapeptides, and others. Defensins are small cationic, cysteine-rich peptides with a broad spectrum of antimicrobial activity against many gram-negative bacteria, gram-positive bacteria, fungi, and other microorganisms (reviewed by Miyasaki and Lehrer, 1998). The *b*-defensins were discovered in cattle as antimicrobial peptides of airway epithelial cells, and then in the leukocytes of cattle and chicks, but are not known to be present in human leukocytes (Miyasaki and Lehrer, 1998; Lehrer and Ganz, 2002). In epithelia of many other species, *b*-defensins are expressed constitutively or inducibly. Two human *b*-defensins, HBD-1 and HBD-2, have been found in many epithelia, but are particularly abundant in the urogenital tract and inflamed skin, respectively (Zhao *et al.*, 1996; Liu *et al.*, 1998, 2002; Valore *et al.*, 1998; Bevins *et al.*, 1999; Schroder and Harder, 1999; Shi *et al.*, 1999; Tunzi *et al.*, 2000; Dunsche *et al.*, 2001). More recently, HBD-3 and -4 were discovered (Garcia *et al.*, 2001; Harder *et al.*, 2001), and many additional human *b*-defensins are thought to exist on the basis of genomic surveys (Jia *et al.*, 2001).

The purpose of this study was to establish a system to test whether tissue cells such as fibroblasts can be transfected to secrete antimicrobial peptides and to enhance the innate immune mechanisms. HBD-2, a potent and well-characterized HBD, was chosen as a study model over other *a*-defensins, because it does not require specific posttranslational modification (other than the removal of the signal peptide) for its functionality. Many other defensins require activation by enzymes that are produced only by specialized cells, for example, neutrophils (Ganz *et al.*, 1993). We present herein an *in vivo* model system to test the possibility of future antimicrobial gene therapy.

MATERIALS AND METHODS

Cell cultures

The following cell cultures and cell lines were utilized: PA317 (ATCC CRL-9078); NIH 3T3 (ATCC CRL-1658) obtained from N.-H. Park (UCLA, Los Angeles, CA); L929 (mouse fibrosarcoma cell line, ATCC CCL-1); HT-1080 (human fibrosarcoma cell line, ATCC CCL-121); HSG, a human submandibular salivary gland cell line (Shirasuna *et al.*, 1981) from L. Bobek (State University of New York, Buffalo, NY); ECV304, a human endothelial cell line (Takahashi *et al.*, 1990) from H. Shau (UCLA, Los Angeles, CA); and Jurkat clone E6-1

(human T cell line, ATCC TIB-152) from A. Jewett (UCLA, Los Angeles, CA). Primary human gingival fibroblasts (HGFs) were derived from human gingival tissues resected from patients receiving routine periodontal surgeries at the Periodontal Clinic of the UCLA School of Dentistry. Procedures involving tissue sample collection were performed according to a protocol approved by the UCLA Medical Institutional Review Board. PA317, NIH 3T3, L929, and HGF cell were grown in Dul-becco's modified Eagle's medium (DMEM; Life Technologies/GIBCO-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS). HT-1080 cells were grown in minimum essential medium- α with 10% FBS. HSG cells were grown in DMEM-F12 (1:1 mixture, with GlutaMAX-I; Life Technologies) with 10% FBS. ECV304 and Jurkat cells were grown in RPMI 1640 (Life Technologies) with 10% FBS. All cell culture media included penicillin G (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (Fungizone, 0.25 mg/ml; Gemini Bio-Products, Calabasas, CA) except under conditions used for gel overlay experiments, described below.

Construction of retroviral vector

HBD-2 cDNA containing the entire coding region previously cloned into a baculoviral vector (Couto *et al.*, 1994) was released with *Bam*HI and *Eco*RI restriction enzymes and inserted into a multiple cloning site of a retroviral vector, pBabeNeo (Fig. 1) (Morgenstern and Land, 1990). The retroviral vector carrying the HBD-2 gene was designated pBabeNeoHBD-2 and the inserted HBD-2 sequence confirmed with a standard DNA sequencing method.

Transfection of packaging cells

Transfection with pBabeNeoHBD-2 or pBabeNeo vector alone into PA317 packaging cells was by the Lipofectin method (Life Technologies). Stable transfectants were selected with G418 (1 mg/ml; Life Technologies). The supernatants of the stable transfectants were collected and secreted HBD-2 was measured by enzyme-linked immunosorbent assay (ELISA), as described below. The supernatant of one clone, designated PA317-HBD-2, that showed the highest amount of secreted HBD-2, and the supernatant of another clone carrying pBabe-Neo vector alone, designated PA317-pBabe, were used to infect target cells.

Infection of target cells

PA317-HBD-2 or PA317-pBabe cells were grown to confluence. Medium was refreshed 1 day after cells became confluent. One to 2 days later, the supernatant was collected and used to infect target cells in the presence of Polybrene (8 mg/ml; Sigma, St. Louis, MO) for 3 hr, followed by adding more medium to dilute the Polybrene to 2 mg/ml, and the culture was incubated for 3 days. Afterward, cells were split under selection conditions, using G418, and 7-10 days later selected cells were pooled and subcultured in 12-well plates. Supernatants were tested for the presence of HBD-2. Single clones of transduced HT-1080 and L929 cells that expressed the highest amount of secreted HBD-2 were selected from among 20-30 clones randomly chosen from the pool and were used for all the experiments in the studies presented herein. For Jurkat cells, a cocultivation method was used, as the above-described method was not successful in transducing these cells. Confluent PA317-HBD-2 cells were treated with 10 mg/ml mitomycin C for 4 hr to inhibit cell division. Cultures were then washed several times with medium and Jurkat cells were added to the PA317-HBD-2 cells with fresh medium and incubated for 4-5 days in the presence of Polybrene (2 mg/ml). Jurkat cells were then removed from the virus-producing cells and selected with G418.

Enzyme-linked immunosorbent assay

The amount of HBD-2 secreted into the supernatant was determined by ELISA, using optimal concentrations of monoclonal anti-human HBD-2 antibodies as capturing antibodies, polyclonal rabbit anti-human HBD-2 antibodies (Liu *et al.*, 1998) as detecting antibodies, and

horseradish peroxidase (HRP)-labeled polyclonal goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL) as a second-step antibody. Bound HRP was visualized with fresh developing buffer containing 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD; Sigma), 0.01% H₂O₂, and 20 mM sodium citrate, pH 4.0. The developing reaction was stopped by the addition of 2.5 M sulfuric acid. Absorbance was determined at 490 nm with a microplate reader (Bio-Tek Instruments, Laguna Hills, CA) and concentrations were determined with DeltaSoft III software.

Western blot analysis

The supernatants were purified with Macro-Prep CM support system for weak cation-exchange support (Bio-Rad, Hercules, CA). The CM resin matrix equilibrated in 25 mM ammonium acetic acid (pH 6-7) was mixed with the supernatants and incubated with rotation for 2-3 hr at 4°C. The matrix was then washed 4 times with 25 mM ammonium acetic acid binding buffer. The bound proteins were eluted with 10% glacial acetic acid once and 5% acetic acid once, each for 5-20 min with a gentle mix at 4°C. The eluates were pooled, dried, and resuspended into 0.1% trifluoroacetic acid (TFA) in high-performance liquid chromatography (HPLC)-grade H₂O. For acid-urea polyacrylamide gel electrophoresis (AU-PAGE), the resuspended proteins from the supernatants were further purified and desalted in a ZipTipC18 chromatography system (Millipore, Bedford, MA) according to the manufacturer protocol. Peptides were eluted with 50% acetonitrile in 0.1% TFA.

Purified samples were loaded onto a 12.5% AU-polyacrylamide gel, electrophoresed, and blotted onto a membrane. Blots were then fixed in 0.05% glutaraldehyde in Tris-buffered saline (TBS) for 30 min, washed with H₂O, and incubated for 30 min at 37°C in 0.75% nonfat milk in phosphate-buffered saline (PBS). The blots were then incubated overnight with primary antibody rabbit anti-human HBD-2 (1:1000 dilution) (Liu *et al.*, 1998). Unbound antibodies were washed from the blots with 0.1% (w/v) bovine serum albumin (BSA) in TBS (wash buffer) and subsequently incubated with a 1:2000 dilution of alkaline phosphatase-conjugated polyclonal goat anti-rabbit secondary antibodies (Pierce). After removing the secondary antibodies with wash buffer, blots were developed with a developing solution containing nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as a chromogenic substrate (Cole *et al.*, 1999).

Northern blot analysis

Cellular RNA was isolated with RNA STAT-60 (Tel-Test B, Friendswood, TX). Fifteen micrograms of total RNA was size fractionated on a 1.5% formaldehyde-agarose gel, transferred to a nitrocellulose filter, and probed with a specific ³²P-labeled cDNA fragment of human HBD-2.

Antibacterial gel overlay assay

The gel overlay assay was performed as described by Lehrer *et al.* (1991). Briefly, sample peptides were separated by AU-PAGE, and the gel was neutralized by washing for 5 min in saline with 10 mM sodium phosphate, pH 7.4 (0.01 M PBS), and 0.01 N NaOH, and then by washing in 0.01 M PBS alone for 15 min. The gel was then placed on a plate (10 × 3 × 10 cm) containing a 10-ml solid layer of 1% agarose with 0.1% trypticase soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ) and 4 × 10⁶ *Escherichia coli* ML-35p (an HBD-2-sensitive strain; Liu *et al.*, 2002) and was incubated at 37°C for 3 hr to allow the HBD-2 in the gel to diffuse into the bacterial layer. The gel was then removed and the agarose was overlaid with a nutrient layer that contained 10 ml of 6% TSB in 1% agarose. After 18 hr of incubation at 37°C to allow visible bacterial growth, antibacterial activity was indicated by a clear zone (no bacterial growth).

In vivo antibacterial assay

Tumor cells (10^7 HT-1080 cells transduced with HBD-2 or pBabeNeo vector alone) in 0.2 ml of Hanks' balanced salt solution (HBSS) were injected subcutaneously, using a 26-gauge needle, into the right flank of the hind leg of approximately 5-week-old, male, severe combined immunodeficient mice (SCID, NOD.CB17-*Prkdc-scid/J*; Jackson Laboratory, Bar Harbor, ME). Each mouse received one inoculation of HT-1080 cells. Tumors were grown until they reached approximately 1.5 × 1.5 cm in size. In preliminary experiments (our unpublished observations), we found that tumors carrying HT-1080-HBD-2 cells grew slower than those carrying HT-1080-vector (control) cells in SCID mice, possibly because of an antitumor effect of HBD-2. Therefore, HT-1080-HBD-2 cells were inoculated approximately 1 week before the inoculation of control cells, such that all tumors reached a similar size at the time of bacterial injection. *Escherichia coli* ML-35p (10^3 or 10^5) grown to exponential phase were resuspended in 50 ml of PBS and injected into the tumor mass in SCID mice. After 16 hr, the mice were killed. Each tumor mass was resected aseptically, divided equally into four parts, and randomly selected for standard histological examination (one part), HBD-2 reverse transcription-polymerase chain reaction (RT-PCR; one part), and for recovery of viable bacteria (two parts). The resected tumor mass was homogenized and the supernatant of the homogenate was serially diluted in PBS and plated onto agar-medium plates for bacterial colony-forming unit (CFU) analysis. *Escherichia coli* ML-35p is an ampicillin-resistant laboratory strain, therefore, the agar-medium contained ampicillin to reduce the possibility of other bacterial contamination during any of the experimental procedures. In addition, some parallel experiments were performed in which sterile PBS alone was injected into the tumors and no bacteria were recovered from the resected tumors. Mice were maintained in the animal care center at the UCLA Division of Laboratory Animal Medicine. All procedures involving experimentation with and handling of mice followed the protocols approved by the UCLA Animal Research Committee.

Reverse transcription-polymerase chain reaction

To detect HBD-2 expression in tumors, an RT-for-PCR kit (Clontech, Palo Alto, CA) was used to synthesize cDNA from 1 mg of total RNA isolated from tumors. An appropriate amount of cDNA, specific primers for HBD-2 (Liu *et al.*, 1998) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from the RT-for-PCR kit; Clontech), and *Pfu* DNA polymerase (Stratagene, La Jolla, CA) were then used for PCRs according to the following protocol: 1 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C for 35 cycles; and 7 min at 72°C as the final step. Primer sequences were as follows: HBD-2 (sense primer, 5'-GGGGGATCCGCTCCCAGCCA TCAGCCATG-3'; antisense primer, 5'-AGCGAATTCAGCTTCTTGG CCTCCTCATG-3'; with an expected targeting size of 245 bp); hGAPDH (sense primer, 5'-TGAAGGTCGGAGTCAACGGA TTTGGT-3'; antisense primer, 5'-CATGTGGGCCATGAGGCTC ACCAC-3'; with a targeting size of 1041 bp). The HBD-2 sense and antisense primers contain *Bam*HI and *Eco*RI linkers at the 5' and 3' end, respectively, for the purpose of subcloning in previous experiments (Liu *et al.*, 1998). PCR products were size fractionated in a 1.5% agarose gel for visualization.

RESULTS

Detection of secreted HBD-2 from transduced cells

Supernatants from nontransduced and transduced cell types were analyzed by ELISA to assess the amounts of secreted HBD-2. Table 1 shows that all the cell types used in this study secreted various amounts of HBD-2 after transduction, whereas their nontransduced counterparts did not secrete any detectable HBD-2. This is consistent with our current understanding that HBD-2 is not expressed in the cell types utilized in this study. In addition, no secreted HBD-2 was detected from nontransduced NIH 3T3, HT-1080, and L929 cells stimulated with human

interleukin 1 β (IL-1 β ; our unpublished observations). Transduced NIH 3T3 and HT-1080 cells secreted HBD-2 in greater amounts than did other transduced cell lines and HGFs. Western blot analysis demonstrated that secreted HBD-2 from all the transduced cell lines and HGFs had approximately the same mass-to-charge ratio as the HPLC-purified HBD-2 (Fig. 2).

Detection of HBD-2 mRNA from transduced cells

To assess the expression of HBD-2 mRNA in transduced cells, Northern blot analysis was performed (Fig. 3). The relative HBD-2 mRNA expression levels among each transduced cell line appear to correlate with the secreted HBD-2 peptide from each cell line. Two major forms of HBD-2 mRNA are detected by the blot analysis, each of a size much larger than that of natural HBD-2 mRNA (336 bp) (Liu *et al.*, 1998). These could be the result of delayed and differential termination of transcription of the mRNAs generated from the juxtaposition of HBD-2 and viral sequences.

In vitro antimicrobial activity of secreted HBD-2 from transduced cells

Antimicrobial analysis of HBD-2 secreted from transduced cells was performed by the *E. coli* gel overlay method. NIH 3T3 and HT-1080 cells, which secreted greater amounts of HBD-2 than did the other cell lines, were selected for this study. As presented in Fig. 4, HBD-2 secreted from these transduced cell lines showed significant antimicrobial activity and was electrophoretically identical to purified HBD-2. There was a significant background antimicrobial effect as evidenced by the samples prepared from cell culture medium alone.

Antimicrobial activity of HBD-2 from transduced cells in vivo

Previous observations have shown that HBD-2, in purified form, is a salt-sensitive antimicrobial peptide (Bals *et al.*, 1998; Liu *et al.*, 2002). The *E. coli* gel overlay method used in the present studies assesses its antimicrobial activities under low-salt conditions. To test whether HBD-2 is functional in a physiological *in vivo* environment, we utilized a novel approach in which HT-1080 cells were implanted (subcutaneously) into SCID mice to form tumors. *Escherichia coli* bacteria were then injected into the tumor mass. Expression of HBD-2 in tumors was verified by RT-PCR (Fig. 5). Amplified HBD-2 cDNA fragments were present in the samples derived from tumors bearing HT-1080-HBD-2 cells but not in those from tumors carrying HT-1080-vector cells. Histological examination of tumors by standard hematoxylin and eosin staining revealed that there was no significant difference between tumors expressing HBD-2 and those carrying vector alone with respect to the architecture of the tumor and the adjacent tissues (data not shown).

The number of recovered *E. coli* bacteria from HBD-2-expressing tumors was only 0.3-15% (mean 6 SEM, 7.8 $\pm$ 3.3%) of that recovered from tumors carrying vector alone (Table 2 and Fig. 6). For statistical analysis, we computed the log ratio of the recovered *E. coli* counts from the HBD-2 group to those from the vector group for each experiment and used a *t* test to determine whether this was significantly different from the null result, that is, a ratio of 100% (Table 2). The HBD-2/vector ratio of CFU in the four experiments is significantly different from 100% (geometric mean, 3.8%; *p* 5 0.0375). There was some variation in tumor size measured after resection. Generally, HT-1080-HBD-2 cells formed a slightly smaller tumor mass than their vector-bearing counterpart despite the early inoculation. However, the slight variation of tumor size did not appear to affect the CFU analysis.

DISCUSSION

The present study has demonstrated *in vivo* antimicrobial activities of HBD-2 and established a model system for further investigation of antimicrobial gene therapy. The ultimate goal of this study is to introduce antimicrobial peptide genes into explanted human cells, or directly

into host tissues or organs to enhance host defense mechanisms against infection, particularly those of immunocompromised individuals.

Antimicrobial gene therapy faces at least two potential limitations: (1) the amount of antimicrobial peptides secreted by the chosen cell type, and (2) the spectrum of bacteria killed or inhibited by the antimicrobial peptide. As evidenced by our findings, fibroblasts appeared to express higher levels of HBD-2, whereas other cell types tested (ECV, HSG, and Jurkat) secreted minimal amounts of HBD-2. It is not certain whether these cells will express higher levels of HBD-2 when transduced by alternative methods. The difference in HBD-2 mRNA expression levels among these cell types could be the result of different gene copy number obtained in each cell type during transduction, or could be due to posttranscriptional regulation, the mechanism of which is largely unknown at present.

The spectrum of microbes susceptible to HBD-2 has been tested only in an *in vitro* setting with purified HBD-2 at relatively high concentrations, for example, the minimal inhibitory concentrations for HBD-2 are 4-62 mg/ml for *E. coli* and its antimicrobial activity is reduced at higher concentrations of sodium chloride (Bals *et al.*, 1998; Liu *et al.*, 2002). Our *in vivo* mouse model revealed that HBD-2 can effectively kill/inhibit bacterial growth in a physiological salt environment, suggesting that factors not tested *in vitro* may be involved in HBD-2 antimicrobial activity. Nonetheless, the magnitude of the *in vivo* antimicrobial effect is strongly dependent on HBD-2 concentration. We also inoculated L929 cells (vector or HBD-2 transduced), which secreted a lower concentration of HBD-2 compared with HT-1080 cells (Table 1), to form tumors in mice. The *in vivo* antimicrobial effect of this transduced cell line in SCID mice was also lower: only 35% reduction of bacterial growth relative to its control, suggesting that the concentration of HBD-2 is limiting. Therefore, to make antimicrobial gene therapy effective, higher expression levels of antimicrobial peptides must be achieved. In addition, to cover a broader spectrum of microbes, the expression of multiple antimicrobial peptides/proteins may be required.

Antimicrobial gene therapy could be useful for several important clinical problems: (1) preventing infection during wound healing; (2) protecting regenerated tissues/organs or prostheses from infection, especially in immunocompromised patients; and (3) reducing the need for conventional antibiotics that induce drug resistance. Extensive dermal tissue loss due to injuries or diseases traditionally requires tissue grafting and transplantation. The advent of tissue-engineering technology provides a promising future in restoring lost tissues. The combination of tissue engineering and antimicrobial gene therapy could potentially work hand-in-hand to make tissue repair more successful as infection is always a major risk factor during tissue restoration. In the case of burn injuries, HBD-2 is not detected in burn blister fluids (Ortega *et al.*, 2000), suggesting the susceptibility of these wounds to infection. One report (Schmid *et al.*, 2001) showed that cytokine-stimulated epidermis rich in HBD-2 was functionally competent in preventing the growth of *E. coli* in Apligraf—a tissue-engineered human skin equivalent *in vitro*, whereas bacteria clearly formed colonies on the dermal surface when the epidermis was removed. This finding, along with ours, further suggests the potential usefulness of dermal cells expressing HBD-2 to prevent dermal infection when there is a break in the epidermis.

The use of conventional antibiotics is currently the most effective means to control infections. However, systemic administration of antibiotics is often undertaken for localized infections, unnecessarily exposing uninfected sites to antibiotics. Although the side effects of conventional antibiotics are well known, little can be done in many clinical situations to avoid them. Local delivery of antibiotics to the infected site is problematic in that sustained effectiveness is difficult to achieve and the delivery system is usually technically demanding. Above all, the widespread development of antimicrobial resistance to conventional antibiotics poses a serious

problem. The discovery of natural antimicrobial peptides has opened a potential alternative to the use of conventional antibiotics. With the advent of gene therapy approaches, it may be possible to develop antimicrobial gene therapy. Although there are still many obstacles in gene therapy that need to be overcome (Romano *et al.*, 2000; Somia and Verma, 2000), antimicrobial gene therapy could eventually be one of many powerful gene therapy approaches for clinical problems. Limited, but promising, antimicrobial gene therapy approaches have been reported previously (O'-Connell *et al.*, 1996; Yarus *et al.*, 1996; Bals *et al.*, 1999a-c). Further exploration of the potential of antimicrobial gene therapy is warranted.

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REFERENCES

- BALS R, WANG X, WU Z, FREEMAN T, BAFNA V, ZASLOFF M, WILSON JM. Human *b*-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J. Clin. Invest* 1998;102:874–880. [PubMed: 9727055]
- BALS R, WEINER DJ, MOSCIONI AD, MEEGALLA RL, WILSON JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun* 1999a;67:6084–6089. [PubMed: 10531270]
- BALS R, WEINER DJ, MEEGALLA RL, WILSON JM. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. *J. Clin. Invest* 1999b;103:1113–1117. [PubMed: 10207162]
- BALS R, XIAO W, SANG N, WEINER DJ, MEEGALLA RL, WILSON JM. Transduction of well-differentiated airway epithelium by recombinant adeno-associated virus is limited by vector entry. *J. Virol* 1999c;73:6085–6088. [PubMed: 10364362]
- BEVINS CL, MARTIN-PORTER E, GANZ T. Defensins and innate host defence of the gastrointestinal tract. *Gut* 1999;45:911–915. [PubMed: 10562592]
- BORREGAARD N, ELSBACH P, GANZ T, GARRED P, SVEJGAARD A. Innate immunity: From plants to humans. *Immunol. Today* 2000;21:68–70. [PubMed: 10652463]
- COLE AM, DEWAN P, GANZ T. Innate antimicrobial activity of nasal secretions. *Infect. Immun* 1999;67:3267–3275. [PubMed: 10377100]
- COUTO MA, LIU L, LEHRER RI, GANZ T. Inhibition of intracellular *Histoplasma capsulatum* replication by murine macrophages that produce human defensin. *Infect. Immun* 1994;62:2375–2378. [PubMed: 8188361]
- DUNSCH A, ACIL Y, SIEBERT R, HARDER J, SCHRODER JM, JEPSEN S. Expression profile of human defensins and antimicrobial proteins in oral tissues. *J. Oral Pathol. Med* 2001;30:154–158. [PubMed: 11271630]
- GANZ T, LEHRER RI. Antibiotic peptides from higher eukaryotes: Biology and applications. *Mol. Med. Today* 1999;5:292–297. [PubMed: 10377520]
- GANZ T, LIU L, VALORE EV, OREN A. Posttranslational processing and targeting of transgenic human defensin in murine granulocyte, macrophage, fibroblast, and pituitary adenoma cell lines. *Blood* 1993;82:641–650. [PubMed: 8329717]
- GARCIA JR, KRAUSE A, SCHULZ S, RODRIGUEZ-JIMENEZ FJ, KLUVER E, ADERMAN K, FORSSMANN U, FRIM-PONG-BOATENG A, BALS R, FORSSMANN WG. Human *b*-defensin 4: A novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB J* 2001;15:1819–1821. [PubMed: 11481241]
- HARDER J, BARTELS J, CHRISTOPHERS E, SCHRODER JM. Isolation and characterization of human *b*-defensin-3, a novel human inducible peptide antibiotic. *J. Biol. Chem* 2001;276:5707–5713. [PubMed: 11085990]

- JIA HP, SCHUTTE BC, SCHUDY A, LINZMEIER R, GUTH-MILLER JM, JOHNSON GK, TACK BF, MITROS JP, ROSENTHAL A, GANZ T, MCCRAY PB JR. Discovery of new human *b*-defensins using a genomics-based approach. *Gene* 2001;263:211–218. [PubMed: 11223260]
- LATAL A, DEGOVICS G, EPAND RF, EPAND RM, LOHNER K. Structural aspects of the interaction of peptidyl-glycylleucine-carboxamide, a highly potent antimicrobial peptide from frog skin, with lipids. *Eur. J. Biochem* 1997;248:938–946. [PubMed: 9342250]
- LEHRER RI, GANZ T. Defensins of vertebrate animals. *Curr. Opin. Immunol* 2002;14:96–102. [PubMed: 11790538]
- LEHRER RI, ROSENMAN M, HARWIG SS, JACKSON R, EISENHAUER P. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* 1991;137:167–173. [PubMed: 1901580]
- LIU AY, DESTOUMIEUX D, WONG AV, PARK CH, VALORE EV, LIU L, GANZ T. Human *b*-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J. Invest. Dermatol* 2002;118:275–281. [PubMed: 11841544]
- LIU L, WANG L, JIA HP, ZHAO C, HENG HHQ, SCHUTTE BC, MCCRAY PB JR, GANZ T. Structure and mapping of the human *b*-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 1998;222:237–244. [PubMed: 9831658]
- MATSUZAKI K, SUGISHITA K, FUJII N, MIYAJIMA K. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry* 1995;34:3423–3429. [PubMed: 7533538]
- MIYASAKI KT, LEHRER RI. *b*-Sheet antibiotic peptides as potential dental therapeutics. *Int. J. Antimicrob. Agents* 1998;9:269–280. [PubMed: 9573496]
- MORGENSTERN JP, LAND H. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* 1990;18:3587–3596. [PubMed: 2194165]
- O'CONNELL BC, XU T, WALSH TJ, SEIN T, MASTRANGELI A, CRYSTAL RG, OPPENHEIM FG, BAUM BJ. Transfer of a gene encoding the anticandidal protein histatin 3 to salivary glands. *Hum. Gene Ther* 1996;7:2255–2261. [PubMed: 8953316]
- ORTEGA MR, GANZ T, MILNER SM. Human *b* defensin is absent in burn blister fluid. *Burns* 2000;26:724–726. [PubMed: 11024605]
- ROMANO G, MICHELL P, PACILIO C, GIORDANO A. Latest developments in gene transfer technology: Achievements, perspectives, and controversies over therapeutic applications. *Stem Cells* 2000;18:19–39. [PubMed: 10661569]
- SCHRODER JM, HARDER J. Human *b*-defensin-2. *Int. J. Biochem. Cell. Biol* 1999;31:645–651.
- SCHMID P, GRENET O, MEDINA J, CHIBOUT SD, OSBORNE C, COX DA. An intrinsic antibiotic mechanism in wounds and tissue-engineered skin. *J. Invest. Dermatol* 2001;116:471–472. [PubMed: 11231325]
- SHI J, ZHANG G, WU H, ROSS C, BLECHA F, GANZ T. Porcine epithelial *b*-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations. *Infect. Immun* 1999;67:3121–3127. [PubMed: 10338529]
- SHIRASUNA K, SATO M, MIYAZAKI T. A neoplastic epithelial duct cell line established from an irradiated human salivary gland. *Cancer* 1981;48:745–752. [PubMed: 7248901]
- SOMIA N, VERMA IM. Gene therapy: Trials and tribulations. *Nat. Rev. Genet* 2000;1:91–99. [PubMed: 11253666]
- TAKAHASHI K, SAWASAKI Y, HATA J, MUKAI K, GOTO T. Spontaneous transformation and immortalization of human endothelial cells. *In Vitro Cell. Dev. Biol* 1990;26:265–274. [PubMed: 1690702]
- TUNZI CR, HARPER PA, BAR-OZ B, VALORE EV, SEMPLE JL, WATSON-MACDONELL J, GANZ T, ITO S. *b*-Defensin expression in human mammary gland epithelia. *Pediatr. Res* 2000;48:30–35. [PubMed: 10879797]
- VALORE EV, PARK CH, QUAYLE AJ, WILES KR, MCCRAY PB JR, GANZ T. Human *b*-defensin-1: An antimicrobial peptide of urogenital tissues. *J. Clin. Invest* 1998;101:1633–1642. [PubMed: 9541493]

- YARUS S, ROSEN JM, COLE AM, DIAMOND G. Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. *Proc. Natl. Acad. Sci. U.S.A* 1996;93:14118–14121. [PubMed: 8943070]
- ZHAO C, WANG I, LEHRER RI. Widespread expression of *b*-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett* 1996;396:319–322. [PubMed: 8915011]

FIG. 1. Construction of pBabeNeoHBD-2. HBD-2 cDNA was inserted into the *Bam*HI and *Eco*RI sites of the multiple cloning site.

FIG. 2.

Western blot analysis of secreted HBD-2. **(A)** First two left lanes are HPLC-purified HBD-2 (8 and 4 ng). Other lanes represent HBD-2-transduced and nontransduced cells. **(B)** An independent experiment with transduced cells. **(C)** First two left lanes are HPLC-purified HBD-2 (8 and 4 ng). Other lanes are nontransduced (2) versus HBD-2-transduced (1) cell lines. The differences in the detected HBD-2 levels among the cell types in each blot do not exactly reflect the relative amounts of HBD-2 secreted. Quantitative measurements are presented in Table 1.

FIG. 3.

Northern blot analysis of HBD-2 mRNA. *Top*: Nontransduced (2) or HBD-2-transduced (1) cell lines were harvested and 15 mg of total RNA was used for analysis. *Bottom*: Corresponding ethidium bromide gel staining of ribosomal RNAs (18S and 28S).

FIG. 4.

Gel overlay analysis of HBD-2 antimicrobial activities. Cell culture supernatants (without supplemented antibiotics) from nontransduced (2) or HBD-2-transduced (1) NIH 3T3 (**A**) or HT-1080 (**B**) cells were purified and concentrated for analysis. Dark bands or zones indicate areas clear of bacterial growth (*E. coli*). Lanes 1 and 2, HPLC-purified HBD-2 (500 and 100 ng), indicated by the arrows; lanes 3, bactericidal activities of supernatant from nontransduced cells; lanes 5, bactericidal activity of HBD-2 secreted from transduced cells, indicated by the arrowheads; lanes 6, background bactericidal activities of cell culture medium alone. The cell culture medium alone was also treated with Macro-Prep CM support system and ZipTipC18, as were the sample supernatants; lanes 7 and 8, HPLC-purified HBD-2 (100 and 500 ng) mixed with the treated cell culture medium, like those seen in lanes 6.

FIG. 5. RT-PCR of HBD-2 or GAPDH expression in HT-1080 tumors. Vector, PCR product from control HT-1080 (pBabe) tumor; HBD-2, PCR product from HT-1080-HBD-2 tumor; Marker, *fX174* DNA-*Hae*III digest.

FIG. 6.

In vivo antimicrobial analysis for HBD-2, using NOD/SCID mice. **(A)** Agar plate on the left indicates the recovered bacteria from a tumor with HT-1080 cells transduced with pBabe vector alone; agar plate on the right shows bacteria recovered from a tumor with HT-1080-HBD-2 cells. Results are from experiment I in Table 2. **(B)** CFU analysis of *E. coli* recovered from tumors carrying either pBabe vector or HBD-2. Data are presented as means $\pm$ SEM of the HBD-2 group ($7.8 \pm 3.3\%$) compared with the vector group (100%), determined by the four independent experiments shown in Table 2 [column entitled "*E. coli* recovered (% of vector)"].

TABLE 1
SECRETION OF HBD-2 DETECTED BY ELISA^a

<i>Cell type</i>	<i>HBD-2 transduction</i>	<i>Secreted HBD-2 (ng/10⁶ cells/4 days)^b</i>
NIH 3T3	-	0
	+	78.0 ± 14.1
HGFs (A) ^c	-	0
	+	15.9 ± 2.5
HGFs (B) ^c	-	0
	+	17.9 ± 7.9
HSG	-	0
	+	0.7 ± 0.2
ECV304	-	0
	+	0.8 ± 0.3
Jurkat ^b	-	0
	+	<0.5
HT-1080	-	0 ^d
	+	25.2 ± 4.4 ^d
L929	-	0 ^d
	+	11.4 ± 3.8 ^d

^aTransduced HT-1080 and L929 cells are from a single clone, whereas the rest of the transduced cells are pooled. Data values represent means ± SEM of the results of at least three independent experiments performed in duplicate or triplicate assays.

^bCells were grown to 90-100% confluence (except Jurkat) and fresh medium was added and incubated for 4 days before supernatants were collected for ELISA. Cell number was measured after the supernatant was taken. An optimal number of Jurkat cells was seeded and allowed 4 days for accumulation of secreted HBD-2.

^cHGFs, Human gingival fibroblasts from two different donors, A and B.

^dData represents amounts of HBD-2 (ng) secreted per 10⁶ cells in 3 days.

Table 2
HBD-2 ANTIMICROBIAL ANALYSIS IN NOD/SCID MICE^a

<i>Experiment</i>	<i>n</i>	<i>E. coli inoculated/ tumor</i>	<i>E. coli recovered</i> ^b	<i>E. coli removed (% of vector)</i> ^c
I				
Vector	1	10 ⁵	5.5 × 10 ⁶	100
HBD-2	1	10 ⁵	1.5 × 10 ⁴	0.3
II				
Vector	3	10 ³	2.0 × 10 ⁷	100
HBD-2	3	10 ³	9.0 × 10 ⁵	4.5
III				
Vector	2	10 ³	3.6 × 10 ⁷	100
HBD-2	1	10 ³	4.1 × 10 ⁶	11.3
IV				
Vector	3	10 ³	1.4 × 10 ⁷	100
HBD-2	3	10 ³	2.1 × 10 ⁶	15.0

^aHT-1080 cells (vector or HBD-2) were injected into NOD/SCID mice to form tumors.

^bData are from one mouse, or represent means of two or three mice, studied in each group within each experiment. The difference between the vector and HBD-2 groups is statistically significant (*t* test, *p* < 0.05).