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The investigation of Defensins and LL-37 in human nasal and sinus mucosa

Fang Sheen-Yie, M.D.

Professor

Department of otolaryngology, National Cheng Kung University Hospital,
College of Medicine, National Cheng Kung University, 138 Sheng-Li Road,
Tainan, 70428 Taiwan.

Telephone: +886-6-2353535 ext. 5311
Fax: +886-6-2377404
Email: sheen@mail.ncku.edu.tw

E-Mail address: sheen@mail.ncku.edu.tw
Abstract

Human beta defensin (HBD)-2 and LL37, antimicrobial peptides, have been discovered to be produced by a number of epithelial cells. They are identified as the key elements in the innate host defense mechanism. Because little is known about the expression of HBD-2 and LL37 in human sinonasal tract, we conducted this study to investigate the expression of HBD-2 and LL37 mRNA gene by the reverse transcription polymerase chain reaction (RT-PCR) and localization of HBD-2 and LL37 peptide by immunohistochemistry in 12 human nasal inferior turbinates and maxillary sinus mucosa. The transcripts of the HBD-2 and LL37 gene were detected at a similar level in all human nasal tissues analyzed by RT-PCR. Using immunohistochemistry, HBD-2 peptide was predominately localized in surface epithelial cells. The transcripts of the LL-37 gene were detected in all human nasal tissues analyzed by RT-PCR. There is significant increase of LL-37 mRNA expression in nasal polyps as compared with the normal nasal mucosa. Using immunohistochemistry, LL-37 peptide was localized in surface epithelial cells and submucosal glands.

It suggests that HBD-2 and LL37 may be secreted by nasal mucosa and sinus mucosa, especially the inflammatory status. They all contributed to the nasal host defense mechanism.
Introduction

Defensins are small cationic proteins with broad-spectrum antimicrobial activity. They are identified as key elements in the innate host defense mechanism. In human, they are divided into two subfamilies, α- and β- defensins based on the pairing of the cystein residues in the disulfide bridges. Six α-defensins have been identified. Four of them, called human neutrophil peptide (HNP) 1 to 4, are produced by neutrophils. The other two, called human defensin (HD)-5 and HD-6, are produced by intestinal Paneth’s cells. Two human β-defensins have been described, HBD-1 and HBD-2. They are mainly produced by epithelial cells and indicated to play an important role in the mucosal defense mechanism. HBD-1 mRNA expression is detected predominately in kidney, pancreas, urogenital tract and to a lesser extent in trachea and lung epithelia, whereas HBD-2 appears to be expressed mainly in skin and the respiratory tract. HBD-1 is constitutively produced and not transcriptionally regulated by inflammatory agents. In contrast, the expression of HBD-2 gene is inducible by various proinflammatory agents such as cytokines and bacteria.

The nasal mucosa is potentially deposited with toxic or infectious pathogens. It depends on the normal local host defense mechanism to effectively eliminate these harmful pathogens. This includes the normal mucociliary function, epithelial integrity and antimicrobial substances in the lining fluid. If this defense mechanism fails, microorganisms can become adherent to epithelial cells and begin to colonize. Persistence of microbial colonization encourages an amplified host inflammation that will unselectively damage more mucosal tissues and lead to a vicious circle with progression and chronicity of the disease. Human nasal secretions contain a number of well-characterized antimicrobial products. Lately, the β-defensin family of antimicrobial peptides has been discovered to be produced by a number of epithelial
cells. It is possible that β-defensins also contribute to the antimicrobial activity of
human nasal secretions.

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HBD-1 mRNA expression was detected recently in healthy human inferior turbinate mucosa. This implicates HBD and LL37 may play an important role in the local host defense mechanism of the nasal mucosa. Still little is known about the expression of HBD-2 and LL37 in human sinonasal tract. In the present study, we investigated the expression of HBD-2 and LL37 mRNA gene by RT-PCR and localization of HBD-2 and LL37 peptides by immunohistochemistry.
Materials and Methods

**Tissue Preparations.** Inferior turbinates were obtained from 12 patients undergoing inferior turbinectomy and from nasal polyps, sinus mucosa of maxillary sinus. They have no history of recent infection or allergy. Each of the specimens was divided into two portions. One was frozen in liquid nitrogen and stored in -70°C for RNA isolation. The other was fixed in paraformaldehyde and then embedded in paraffin for immunohistochemistry.

**RT-PCR.** Total RNA was prepared by the Rneasy Total RNA Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was then synthesized directly from total RNA using the Ready-to-use First Strand cDNA Synthesis Kit (Maxim Biotech Inc., CA., USA) using oligo dT as a primer. The reaction mixture was then incubated at 37°C for 1 hour and heated to 90°C for 5 minutes to denature the reverse transcriptase. The generated cDNA was subjected to 35 cycles of PCR amplification on a DNA Thermal Cycler (Hybaid Omnigene, UK). For PCR amplification, 100μl reaction mixture contained 5μl cDNA solution, 100 pmol of each primer, 2 mM each dNTP, 1.5mM MgCl2, and 2.5U of Taq DNA polymerase. The amplification cycles consisted of denature, 94°C for 1 min; annealing, 55°C for 2 min; and extension, 72°C for 1 min. The primer used to amplify HBD-2 was designed on the basis of the published sequence [5]: sense (5’-CCA GCC ATC AGC CAT GAG GGT-3’) and antisense (5’-GGA GCC CTT TCT GAA TCC GCA-3’). β-actin PCR was performed in the same reaction to serve as an internal control with the following primers: sense (5’-AGC GGG CGC AAA TCG TGC GTG-3’) and antisense (5’-CAG GGT ACA TGG TGG TGG TGC C-3’). The PCR products were fractionated by agarose electrophoresis, stained with ethedium bromide and visualized under UV light. An amplified 255dp PCR product was extracted from
the gel and sequenced by ABI PRISM 377 DNA Sequencer (Applied Biosystems, CA., USA).

*Immunohistochemistry.* The paraffin sections were thinly sliced (4um thickness), and mounted on polylysine coated glass slides. After deparaffinization and rehydration, slides were microwave treated for antigen retrieval. Endogenous peroxidase was inhibited by immersing slides in 3% methanol/H$_2$O$_2$ for 10 min. To avoid non-specific binding, slides were incubated with 10% goat serum for 60 minutes. Slides were washed with phosphate buffered saline (PBS) and incubated with rabbit anti-HBD-2 antiserum (1/500 dilution, Alpha Diagnostic Inter., San Antonio, USA), overnight at 4°C. After rinsed with PBS, slides were incubated with biotinylated goat anti-rabbit immunoglobulin G (Dako, Glostrup, Denmark) at room temperature for 15min. Slides were then washed with PBS and treated with streptavidin-horseradish peroxidase reagent (Dako) at room temperature for 15min. After washed with PBS, the activity of peroxidase was detected using AEC Substrate Kit (Zymed, CA., USA) and then slides were counterstained with hematoxyline. Negative control was done with omitting primary antibody.
Results

Expression of HBD-2 Gene and LL-37 Gene. The expression of HBD-2 gene in human inferior turbinates was investigated using RT-PCR. The transcripts of the HBD-2 gene were detected at a similar level in all human nasal tissues analyzed. Primers for HBD-2 amplified a predicted size of 255 bp sequences in inferior turbinate tissues studied. The sequence of the amplified PCR product was computer-matched with the known cDNA sequence of HBD-2 available from EMBL/Genbank database, accession number Z71389. The expression of LL-37 gene in human nasal mucosa was investigated using RT-PCR. The transcripts of the LL-37 gene were detected in all human nasal tissues samples. Primers for LL-37 amplified a predicted size of 523 bp sequences in the inferior turbinate tissues studied. The sequence of the amplified PCR product was computer-matched with the known cDNA sequence of LL-37 available from EMBL/Genbank database, accession number Z38026. The mean densitometry index of LL-37 is $1.30 \pm 0.24$ in the nasal polyp group and $0.48 \pm 0.03$ in the normal nasal mucosa group (Fig. 2). There is a significant increase of LL-37 expression in the nasal polyp group as compared with the normal nasal mucosa group ($P = 0.005$).

Localization of HBD-2 Peptide and LL-37 Peptide. HBD-2 staining was localized in the nucleus and cytoplasm of the epithelial cells. Epithelial cells that stained strongly for HBD-2 were observed more often in the basal layer. Positive staining of LL-37 could be found in both nasal polyps and normal nasal mucosa tissues. It was localized in the cytoplasm of surface epithelial cells as well as the submucosal glands.
Discussion

HBD-2 has a broad antibacterial activity and is approximately 10-fold more potent than HBD-1. Recombinant HBD-2 demonstrated detectable bacterial killing against a number of Gram-negative and Gram-positive bacteria such as Pseudomonas aeruginosa, E. coli and Staphylococcus aureus. The synergic effects of this peptide with other antimicrobial substances such as lysozyme and lactoferrin make it more potent against infectious pathogens. We demonstrated the expression of HBD-2 mRNA by RT-PCR in human nasal mucosa. By immunohistochemistry, the localization of HBD-2 peptide is predominately in surface epithelial cells. It suggests that HBD-2 is secreted by nasal mucosa and plays an important role in nasal host defense mechanism. In the future the recombinant HBD-2 may act as a useful therapeutic agent during sinonasal infection.

LL-37, an antimicrobial peptide, is expressed in leukocytes, skin keratinocytes and epithelial cells in the lung, gastrointestinal tract and urogenital tract. Studies have showed that this endogenous cathelicidin plays an important role in the host defense. In this study, we investigated if LL-37 is expressed in human nasal mucosa under inflammatory and noninflammatory conditions, for the purpose of additional innate antimicrobial defense. We have detected the expression of LL-37 mRNA by RT-PCR in all specimen studied. There is significant increase of LL-37 mRNA expression in the nasal polyp group as compared with the normal nasal mucosa group. LL-37 peptide is localized in the surface epithelial cells as well as the submucosal glands. This implies that LL-37 is constitutively expressed in human
nasal mucosa and may play a key role in the nasal host defense like other antimicrobial substances in nasal secretions.

It has been noted that the concentration of LL-37 was increased in tracheal aspirates of mechanically ventilated newborn infants with pneumonia and systemic infection.\textsuperscript{12} Induction of LL-37 on both mRNA and protein level was also significant in human epidermis during inflammation.\textsuperscript{7} In addition, production of LL-37 was found to be induced after sterile incision and infection in skin.\textsuperscript{13} In this study, we found there was a significant increase of LL-37 mRNA expression in the nasal polyp group as compared with normal nasal mucosa. This is consistent with previous studies that expression of LL-37 was upregulated in inflammation status.

An increased immunoreactivity for lysozyme and lactoferrin was noted in maxillary mucosa from patients with recurrent or chronic sinusitis.\textsuperscript{14} The baseline nasal secretions in patients with recurrent sinusitis were relatively elevated with lysozyme and lactoferrin as compared with that in normal control subjects.\textsuperscript{15} These findings suggest that these antimicrobial peptides like LL-37 were upregulated during inflammation. The change in activity of these antimicrobial peptides may play a determinant role in the pathogenesis of sinonasal inflammatory diseases.

The most difference between HBD-1 and HBD-2 is that HBD-2 gene expression is inducible by bacteria and inflammatory cytokines. In cultured skin keratinocytes, the expression of HBD-2 gene was upregulated by tumor-necrosis factor-\(\alpha\), Gram-negative and Gram-positive bacteria, and yeasts. In cultured airway epithelia, the expression was also upregulated by IL-1\(\beta\). This pattern of expression was reflected in bronchoalveolar lavage study in vivo. HBD-2 peptide was detected in the bronchoalveolar lavage fluid, and its concentration was increased in bacterial pneumonia. It indicates that pathogens or inflammatory cytokines stimulate the biosynthesis of HBD-2 and the peptide’s release into the airway in response to
infection. In a recent study, nasal secretions collected from healthy volunteer donors were analyzed. HBD-2 was detected by Western blot in three of five donors. This confirms HBD-2 is secreted by the nasal mucosa. Further investigation is needed to determine how the expression of HBD-2 is during acute or chronic rhinosinuitis. The alternation in activity of HBD-2 may play a determinant role in the pathogenesis of rhinosinusitis.

References


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