Expression of hBD-2 induced by 23-valent pneumococcal polysaccharide vaccine, Haemophilus influenzae type b vaccine and split influenza virus vaccine

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Abstract. Human β-defensin-2 (hBD-2) is an antimicrobial peptide with high activity and broad spectrum activity. hBD-2 expression may be highly elevated by microorganisms and inflammation. We reported that the majority of common vaccines used, including 23-valent pneumococcal polysaccharide vaccine, Haemophilus influenzae type b vaccine and split influenza virus vaccine, could induce the expression of hBD-2 in epithelial cells. Among them, the 23-valent pneumococcal polysaccharide vaccine was effective at a lower concentration (0.5 μg/ml), while Haemophilus influenzae type b vaccine and split influenza virus vaccine were effective at the concentration of 1 μg/ml. However, bacteriostatic experiments revealed that the split influenza virus vaccine was capable of inducing the highest antimicrobial activity. The medium of the 23-valent pneumococcal polysaccharide vaccine treatment group had a higher antimicrobial activity than the medium of the Haemophilus influenzae type b vaccine treatment group. The transcriptional regulator of hBD-2, that is, the NF-κB subunit, had a high level of activity, while the normal epithelial cells showed barely detectable activity, indicating that these vaccines have potential for clinical application.

Introduction

Human defensins are a group of antimicrobial peptides that defend hosts against bacterial, fungal and viral infections (1). Human β-defensins (hBDs) are a subfamily of defensins distributed at various mucosal surfaces (2). Although multiple gene duplications were observed in the human genome, only hBD-1, hBD-2, hBD-3 and hBD-4 have been described in detail to date (3). hBD-2 is the first member of the human defensin family that has been observed to be highly induced in inflamed epidermal cells, such as airway epithelia and in psoriasis (4,5). Previous studies had demonstrated that hBD-2 had high antimicrobial activity and broad spectrum activity (6,7).

The epithelial surface of the lungs is exposed to a large number of potentially pathogenic microorganisms. Several defence mechanisms are required to protect against infection. The production of antimicrobial peptides is one of the most important and evolutionarily conserved mechanisms. hBD-1 and hBD-2 were found to be locally expressed in the lung. Previous studies indicated that hBD-2 expression was induced by inflammation, whereas hBD-1 serves as a defense mechanism in the absence of inflammation (5,8). Furthermore, by measuring the concentration of hBD-2 in the bronchoalveolar lavage fluid of cystic fibrosis patients, the decrease of β-defensins in advanced lung disease was found to lead to a secondary defect of the local host defense (9). Hence, hBD-2 in the lung is likely to provide an effective shield from microbial infection.

hBD-2 exhibits particularly effective capacity against Gram-negative bacteria and certain fungi (7,10). Gram-negative bacteria, receiving notable concern in recent years, show the feature of using a plethora of mechanisms against antibiotics, which are due to gene cassettes and integrons (11,12). This feature makes them more resistant to antibiotics. While antibiotic resistance has become an increasing global problem, the discovery and development of new antibiotics has declined (13). Infections due to antibiotic-resistant bacteria cause a major public health dilemma, which may lead to future serious medical and social problems. Moreover, hospital-acquired infections have become a major threat to patient safety, and are associated with prolonged hospital stays, higher healthcare costs and increased mortality (14-16). The emergence of antibiotic-resistant infections mainly caused by Gram-negative bacteria aggravates the situation (14,15,17,18). Thus, in this study, we intend to elevate endogenous hBD-2 protein expression in order to meet the emerging clinical challenge.

Since the discovery of natural antimicrobial peptides, much research has been carried out to improve the innate immune response, including the study of many gene-based therapies (19-24). However, to date, all these methods are under preliminary investigation, and no new clinical application has

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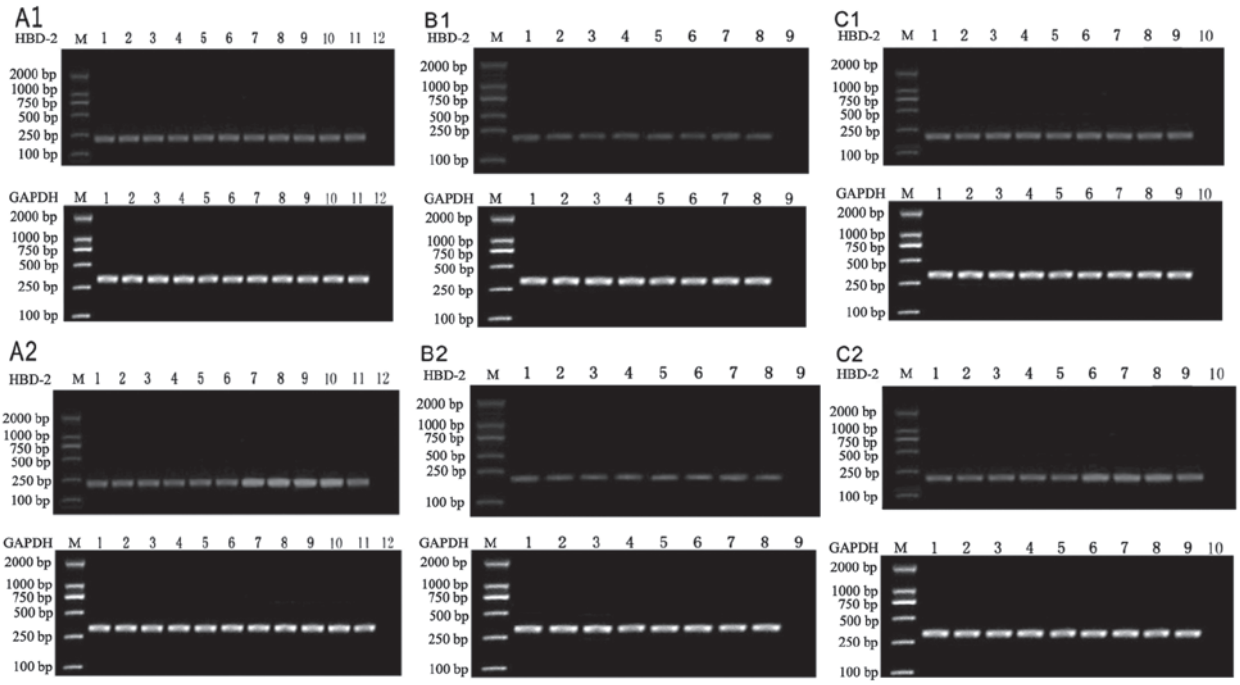

Figure 1. Analysis of mRNA expression for hBD-2 in epithelial cells treated with Streptococcus pneumoniae vaccine for (A1) 6 h and (A2) 12 h, Hemophilus influenzae vaccine for (B1) 6 h and (B2) 12 h and influenza virus vaccine for (C1) 6 h and (C2) 12 h. Lanes 1-10: without the vaccine; 0.00001, 0.00005, 0.0001, 0.005, 0.05, 0.5, 1, 2, 5 μg/ml of the vaccine; lane 11, ATP; lane 12, PBS; M, marker. hBD-2, human β-defensin-2.

been proposed. In this study, we found that killed vaccines, including the 23 valent pneumococcal polysaccharide vaccine, Haemophilus influenzae vaccine and split influenza virus vaccine, could serve as potential inducers of hBD-2. We propose that these vaccines, which already have proven biosafety records, may be used as a potential clinical approach.

Materials and methods

Primary cell culture and treatment. Human bronchial epithelia were surgically resected from patients without lung infection or tumors. The research was approved by the Ethics Committee of Tongji University, China. Primary tissues were digested by 0.125% trypsin with 0.01% EDTA, and cultured in Dulbecco's modified Eagle's medium/ nutrient mixture F12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For treatment, cells were plated in 6-well plates at a density of $1x10^5$/well. Treatment with 23-valent pneumococcal polysaccharide vaccine, haemophilus influenzae type b vaccine or split influenza virus vaccine was performed after the cells reached ~80% confluence. Two independent experiments were performed and each experiment consisted of at least triplicate samples.

RNA extraction and semi-quantitative RT-PCR. Following treatment, cells were lysed with 1 ml of TRIzol reagent. Total RNA was extracted according to manufacturer's instructions and dissolved in 30 μl H_2O with DEPC. Total RNA (2 μg) was used for semi-quantitative RT-PCR according to the manufacturer's instructions. GAPDH was selected as an internal control for normalization. For hBD-2 expression, the primers were designed according to the cDNA sequences (GeneBank accession no. NM_004942.2): the forward primer was 5'-CTT GTATCTCCTCTTCTCGTTCC-3' and the reverse primer was 5'-TTTCTGAATCCGCATCAGC-3'. For GAPDH expression, the forward primer was 5'-GTCGGTGTGAACGGATTT-3' and the reverse primer was 5'-ACTCCACGACGTACTC AGC-3'. cDNA (2 μl) was amplified for hBD-2 and GAPDH. PCR was performed in a 20-μl final volume containing 2 μl 10X reaction buffer, 0.2 mM dNTP, 2 mM $MgCl_2$ and 1.5 units TaqDNA polymerase. The cycle numbers that generated approximately half maximal amplification were determined by testing different cycle numbers of amplification for each gene, and were found to be 40 cycles for hBD-2 and 30 cycles for GAPDH. The PCR conditions were 94˚C for 15 sec, annealing at 61˚C for hBD-2 and 57˚C for GAPDH for 20 sec and extension at 72˚C for 30 sec. The PCR product (5 μl) was checked on a 2.0% agarose gel and visualized using ethidium bromide staining with UV illumination. The intensity of each band was analyzed using the BioSenSC300 system.

Enzyme-linked immunosorbent assay. The medium was collected at 6 and 12 h following treatment. After centrifugation at 3000 g for 20 min, the supernatant samples were removed. The manufacturer's instructions for enzyme-linked immunosorbent assay (ELISA) were followed. Briefly, the microtiter plates were coated by adding 100 μl of the samples. After 90 min incubation, the microtiter plates were agitated to remove the samples. Biotin-labeled antibody (100 μl) was added and the plates were then incubated at 37˚C for 60 min. After three washing steps with 0.01 M PBS, the plates were filled with with 100 μl avidin-biotin-peroxidase complex at 37˚C for 30 min. After extensive washing, the immunological reactions were revealed by 30-min incubation in the dark with 90 μl TMB Microwell substrate. TMB stop solution was added to stop the reaction. The optical density results were

converted into concentrations deduced from a calibration curve.

Electrophoretic mobility shift assay (EMSA). According to the core recognition site of NF-κB, the following oligonucleotides were synthesized for double-stranded probes: forward (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and reverse (3'-GCC TGG GAA AGT CCC CTC AAC T-5'). The annealed oligonucleotides were labeled with DIG dUTP by terminal transferase. The cellular extracts was incubated at room temperature for 20 min with 1 μg Poly(dI-dC) in a binding buffer containing 1X binding buffer, 2.5% glycerol, 0.05% NP-40, 100 mM $MgCl_2$ and 20 fM biotin end-labeled target DNA. DNA-protein complexes were separated from free DNA by electrophoresis in a non-denaturing polyacrylamide gel at 4˚C and electrophoretically transferred onto nylon membranes. The membranes were then dried in a drier at 70˚C for 1 h and blocked with blocking buffer for 15 min. After extensive washing, the membranes were incubated with substrate equilibration buffer for 5 min; subsequently substrate working solution was added and the samples were incubated for another 5 min. Finally, images of the membranes were captured on film cassette following exposure to X-ray for 20 min.

Bacteriostatic experiment. The concentrated medium of cells treated with 23 valent pneumococcal polysaccharide vaccine, Haemophilus influenzae vaccine and split influenza virus vaccine were collected. The round filter paper was immersed in the medium, PBS and clean medium, respectively. The spread plate method was used to measure the antimicrobial activity.

Measurement of the concentration of Ca^{2+}. Cells were plated in a 15x15-mm culture dish. After the cells reached ~90% confluence, the medium was replaced with D-Hanks buffer. An appropriate amount of Fluo-3/AM was added into the buffer at a final concentration of 5-10 μmol/l and incubated with the cells at 37˚C in a 5% CO_2 atmosphere for 60 min in the dark. Cells were then washed twice by PBS and once by D-Hanks buffer. Fluorescent images of the cells were visualized in D-Hanks buffer under a laser scanning confocal microscope at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical analyses. Data were presented as the means ± SD. Statistical analysis was performed using SPSS 11.0.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of hBD-2 mRNA in epithelial cells with various stimuli. To investigate the expression of hBD-2 mRNA in epithelial cells with different vaccine treatments, dose-effect experiments were performed (Fig. 1). As shown in Fig. 2, no marked upregulation was observed following 6 h of treatment. Another 6 h later, when the concentration of 23 valent pneumococcal polysaccharide vaccine was above 0.5 μg/ml, it enhanced the expression over 1.5-fold. The expression

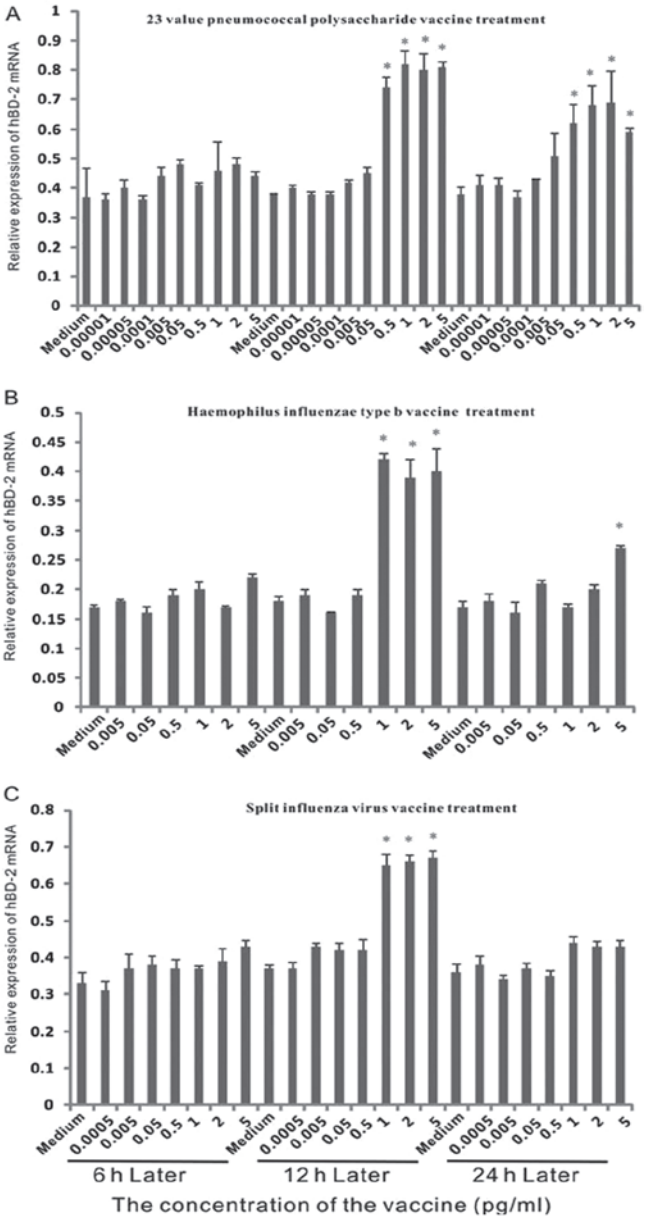

Figure 2. Expression of hBD-2 mRNA in epithelial cells treated with (A) 23 valent pneumococcal polysaccharide vaccine, (B) Haemophilus influenzae type b vaccine and (C) split influenza virus vaccine by semi-quantitative RT-PCR. The results are presented as normalized fold expression with respect to β-actin levels for the same sample. Cell samples were collected after 6 or 12 h of incubation with or without the vaccines. Asterisks indicate a significant difference between the non-treatment group and the treatment groups at the same time point ($P<0.05$). hBD-2, human β-defensin-2.

exhibited over 2-fold induction when cells were treated with greater than 1 μg/ml Haemophilus influenzae vaccine. For the split influenza virus vaccine, when the concentration was above 1 μg/ml, it stimulated the expression by greater than 1.5-fold.

At 24 h, the expression level of hBD-2 with 23 valent pneumococcal polysaccharide vaccine treatment was lower than at 12 h. In the Haemophilus influenzae vaccine treatment group, a significant difference was only observed at the concentration of 5 μg/ml. In the split influenza virus vaccine treatment group, no significant difference was detected. This indicated that the effect of vaccines on hBD-2 expression may be short-term.

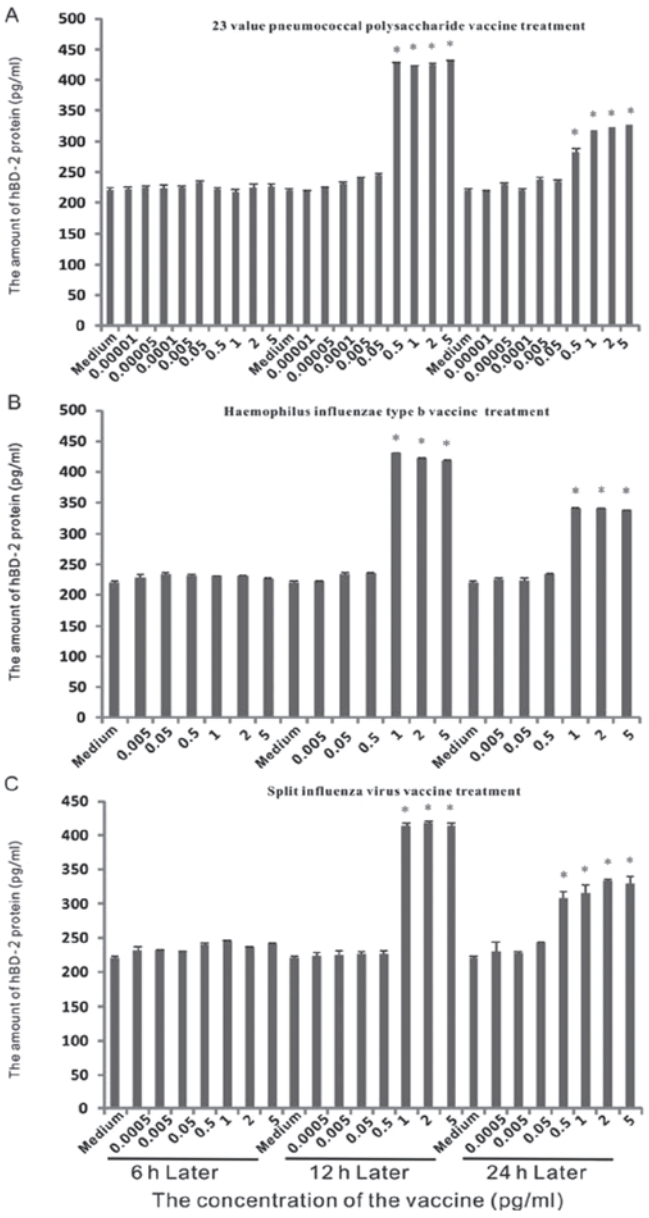

Figure 3. Expression of hBD-2 protein in the medium of the epithelial cells treated with (A) 23 valent pneumococcal polysaccharidevaccine, (B) Haemophilus influenzae type b vaccine and (C) split influenza virus vaccine by ELISA. The media of cells with or without treatment was collected after 6 or 12 h of incubation. Asterisks indicate a significant difference between the non-treatment group and the treatment groups at the same time point ($P<0.05$). hBD-2, human β-defensin-2.

Expression of hBD-2 protein in epithelial cells with various stimuli. To determine the concentration of protein, we performed ELISA with the medium of the dose-effect experiments mentioned previously (Fig. 3). After the first 6 h, no marked increase of protein occurred. Following 12 h of treatment, there was a ~2-fold induction by the 23 valent pneumococcal polysaccharide vaccine with concentrations of above 0.5 μg/ml. Similarly, a ~2-fold induction was observed when the Haemophilus influenzae vaccine or split influenza virus vaccine was used with concentrations above 1 μg/ml. At 24 h, the amount of hBD-2 protein was lower than at 12 h, but remained higher than that in the control group.

Figure 4. The activity of the NF-κB subunit measured by electrophoretic mobility shift assay (EMSA). Target DNA was labeled by biotin. The gel was visualized by exposing to X-ray. Lanes 1-4: the activity of NF-κB subunit in (1) normal epithelial cells and (2) epithelial cells treated with 23 valent pneumococcal polysaccharidevaccine, (3) Haemophilus influenzae type b vaccine and (4) split influenza virus vaccine.

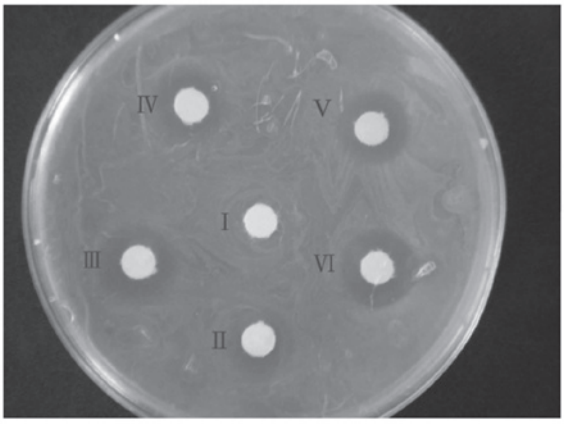

Figure 5. Bacteriostatic experiment. I, PBS; II, medium; III, ATP; IV, Haemophilus influenzae type b vaccine; V, 23 valent pneumococcal polysaccharidevaccine; VI, split influenza virus vaccine.

NF-κB involved in the signal pathway of vaccine-stimulation. Since NF-κB is a well-known mediator of induction of hBD-2 in epithelial cells, we tested the activity of the NF-κB subunit in normal epithelial cells and epithelial cells treated with the vaccines by EMSA (Fig. 4). Treatment with vaccines markedly increased the activity of the NF-κB subunit, while the activity of the NF-κB subunit was barely detected in normal epithelial cells.

Comparison of antimicrobial ability. To analyze the antimicrobial ability, concentrated medium was used for the bacteriostatic experiment (Fig. 5). The spots of inhibition were not clear for PBS and normal medium. The spot diameter was 12 mm with medium treated with 23 valent pneumococcal polysaccharide vaccine, 10 mm with medium treated with

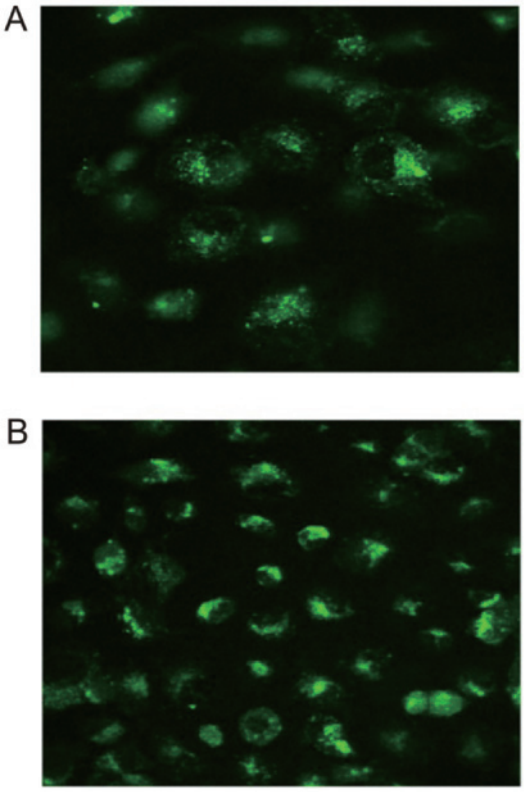

Figure 6. The concentration of Ca^{2+} in (A) normal epithelial cells and (B) epithelial cells treated with 0.5 μg/ml Streptococcus pneumoniae vaccine for 12 h.

Haemophilus influenzae vaccine and 19 mm with medium treated with split influenza virus vaccine.

Discussion

In this study, we have used vaccines, including 23 valent pneumococcal polysaccharide vaccine, Haemophilus influenzae vaccine and influenza virus vaccine, to elevate the expression of hBD-2. The expression level of hBD-2 is markedly low in the absence of inflammation (8). *In vivo*, the vaccines may significantly induce the expression of hBD-2 within the first 12 h at an appropriate concentration. After 24 h, the expression of hBD-2 declined, but it remained significantly higher than the control group in the 23 valent pneumococcal polysaccharide vaccine treatment group and in the Haemophilus influenzae vaccine treatment group. This indicated that the effect of vaccines may be short-term.

Several microorganisms are capable of inducing the expression of certain defensins (25-27). Besides microorganisms, LTB4, L12, L23, L27 and certain other immune factors also induce the expression of hBD-2 (24,28). However, using these microorganisms to study the regulation and function *in vivo* and *in vitro* often leads to the threat of a biological hazard. Our results showed that vaccines could also serve as inducers of hBD-2, but with improved biosafety, simplicity and inexpensiveness. Hence, vaccines may be an alternative to the use of microorganisms and may facilitate the study of hBD-2.

Functional binding sites for NF-κB and AP-1 were identified in the promoter of hBD-2 (29-31). Besides these, little was known about the mechanism of induction of hBD-2. Multiple signaling pathways are involved in the inducible expression of hBD-2 (26). Hence, our study set up a biosecure model that facilitates the study of the mechanism of inducible expression in epithelial cells. As the NF-κB subunit was activated by the vaccines in our model, this indicated that our model could simulate the *in vivo* defense mechanism against microorganisms. Moreover, our preliminary results showed that vaccines could increase the concentration of Ca^{2+} within epithelial cells, which is in line with a previous study that proposes that the increase of Ca^{2+} is necessary for the activation of NF-κB (Fig. 6) (32).

During our investigation, the medium of split influenza virus vaccine treatment was revealed to have the highest antimicrobial activity with the least amount of hBD-2. Certain other antimicrobial peptides induced by the specific vaccine may contribute to the variation of the antimicrobial activity. The 23 valent pneumococcal polysaccharide vaccine was more sensitive to stimulating hBD-2 expression. Further research is required to explain the variation in innate immune response to exogenous stimulation.

Although the pharmaceutical industry endeavors to develop effective therapies against Gram-negative bacteria, it is difficult to meet the clinical challenge (33). hBD-2, with high antimicrobial activity against Gram-negative bacteria, is regarded as an approach to solve the problem. Thus, the effectiveness of vaccine-induced hBD-2 should be investigated in future studies.

Acknowledgements

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