Human beta-defensin-3 (hBD-3) upregulated by LPS via epidermal growth factor receptor (EGFR) signaling pathways to enhance lymphatic invasion of oral squamous cell carcinoma

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Objective. In this study, the hypothesis that hBD-3 is upregulated by LPS via epidermal growth factor receptor (EGFR) signaling pathways to enhance metastasis in oral squamous cell carcinoma (OSCC) was tested.

Study design. hBD-3 expression in human tissue specimens was evaluated by RT-qPCR and immunohistochemical staining. The presence of hBD-3 peptide in the culture supernatants of each type of treated cells was evaluated by enzyme-linked immunosorbent assay. The chemotaxis response to LPS or hBD-3 protein of SCC-25 cells or siRNA-hBD-3 transfected cells were also measured by chemotaxis assay. Paired, 2-tailed Student t test and analysis of variance was used to assess the statistical significance between 2 groups or many groups.

Results. hBD-3 is highly expressed and associated with lymphatic invasion of OSCC. hBD-3 expression and EGFR phosphorylation were markedly upregulated when SCC-25 cells were treated with LPS. When SCC-25 cells were preincubated with EGFR inhibitor or TLR4-neutralizing Ab before LPS stimulation, a decrease in the expression of hBD-3 was observed. hBD-3 markedly enhanced cancer metastasis, and the chemotaxis response to LPS of SCC-25 cells was partly blocked by siRNA target hBD-3.

Conclusion. These findings indicate that hBD-3 is upregulated by LPS via EGFR signaling pathways to enhance lymphatic invasion of OSCC. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:616-625)

In the oral cavity, chronic inflammation has been observed at various stages of oral squamous cell carcinoma (OSCC). This inflammation could result from persistent mucosal or epithelial cell colonization by microorganisms. There is increasing evidence of the involvement of oral bacteria in inflammation that warrants further studies on the association of bacteria in the progression of OSCC.1 Toll-like receptor 4 (TLR4) is a member of membrane proteins Toll-like receptors (TLRs). TLR4 senses lipopolysaccharide (LPS), which is a major integral component of the outer membrane of gram-negative bacteria and is one of the most potent stimuli of inflammation. The activation of TLR4 by LPS stimulation results in the activation of multiple signaling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), which lead to the induction of proinflammatory and antimicrobial responses.2 Epithelial cells represent a barrier that displays expression of TLR4, and this barrier is usually adequate to restrain commensal microbes.2,3 Among the different signaling pathways induced by LPS, activation of epidermal growth factor receptor (EGFR) cascade has been identified in the respiratory epithelium, biliary epithelial cells, and human biliary carcinoma cells.3-5 Normally, many bacteria are symbiotic in the oral cavity. The association between high concentrations of some bacterial colonies in the oral cavity and oral cancer development has been
This finding suggests that bacterial factors may play an important role in the pathophysiology of OSCC, which implies some connection between LPS and OSCC development.

Human β-defensins (hBDs), including the well-known hBD-1-4 and cathelicidin LL37, are mainly produced by the epithelial cells of many organs, including the skin, lungs, kidneys, pancreas, uterus, eyes, and nasal and oral mucosa. hBD gene expression is inducible in response to various signals, such as bacteria, pathogen-associated molecular patterns (PAMPs), or proinflammatory cytokines. In human gingival epithelial cells, keratinocytes, and oral epithelial cells, it has been demonstrated that the presence of LPS or bacteria can induce the release of hBD-3 and induce the production of inflammatory cytokines. In an esophageal cell line, it has been shown that hBD-3 expression was induced solely via an EGFR-dependent pathway. It has been demonstrated that hBD-1-3 expression is absent in OSCC and EGFR signaling is important in oral cancer development. However, recently it has been reported that hBDs undergo spatiotemporal expression in oral dysplasia and at various stages of oral cancer, and hBD-3 uses CCR2 to recruit tumor-associated macrophages that enhance cancer progression.

Although the information gathered to date implies some connection between LPS and OSCC development, the precise mechanism(s) underlying hBD-3 expression in OSCC remains incompletely explored. As stated earlier, activation of an EGFR cascade induced by LPS has been identified in the respiratory epithelium, biliary epithelial cells, and human biliary carcinoma cells. This suggests a possible connection between LPS-triggered EGFR activation and hBD-3 expression in OSCC cells. To determine whether hBD-3 expression is involved in the mechanisms underlying the connection between LPS and OSCC development, we tested the hypothesis that LPS can enhance metastasis by upregulated hBD-3 expression in OSCC cells through EGFR activation.

MATERIAL AND METHODS

Cell culture and reagents

SCC-25 cell lines were purchased from ATCC (Manassas, VA, USA), derived from patients with OSCC, and were maintained in Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco Laboratories). LPS was provided by Sigma-Aldrich (St. Louis, MO). β-actin, anti-p-Tyr992-EGFR, and anti-EGFR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The TLR4- and TLR5-neutralizing Abs were from Serotec (Raleigh, NC). hBD-3 antibody was purchased from PeproTech (Rocky Hill, NJ). hBD-3 protein and siRNA-hBD-3 (hBD-3 small interfering RNA) were purchased from Santa Cruz (Santa Cruz, Inc.). Cetuximab was obtained from Merck Pharma (Merck Pharma, GmbH, Erbitux, Darmstadt, Germany).

Stimulation of cells

Cells were trypsinized and washed in complete cell culture medium. Then, 1.5 to 2.0 × 10⁶ cells were seeded in 2 mL of the appropriate medium in each well of a 6-well cell culture plate and incubated at 37°C with 10 μg/mL LPS. After incubation, the cell culture supernatants were collected for use in hBD-3 enzyme-linked immunosorbent assay (ELISA). The cells were trypsinized, washed in RNase-free phosphate-buffered saline (PBS) and kept at −80°C until RNA extraction and western blotting.

RT-qPCR analysis of hBD-3 and EGFR. The total cellular RNA was isolated from the cells by using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using total RNA (2 μg) and SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse-transcriptase–polymerase chain reaction (RT-PCR) was performed according to the procedure provided by the manufacturer. The following primers were used: hBD-3: 5′-tcataaggagctgccttacacct-3′, 5′-gactggatgaaaaggtgtgcttggtc-3′; EGFR: 3′-acacctcgctcgctgaatac-5′, 5′-agtggggt-agaactggaacctc-3′; β-actin: 5′-aactggaaggtgaaggtggtgtt-3′, and 5′-agtggggt-ggctcggtgga-3′. Samples were subjected to thermal cycling at 95°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds; and with a final extension at 72°C for 5 minutes. RT quantitative PCR (RT-qPCR) was performed using SYBR Green PCR Master Mix with 2 μL of cDNA and 200 nM of the upstream and downstream primers per 20 μL of reaction. Each sample was amplified in duplicate for every experiment. The gene for β-actin was amplified as an endogenous reference. Amplification was performed using 40 cycles of the following program: 94°C for 30 seconds, followed by 62°C for 30 seconds, and 72°C for 30 seconds. The mean efficiency of hBD-3 and β-actin amplification was 2.04 and 2.08, respectively. Reactions were run on an ABI 7900HT instrument (Carlsbad, CA, USA). Each qPCR was run in triplicate, and the experiment was repeated at least 3 times. Relative quantification of gene expression was determined by using the comparative Ct method. Results were normalized using the human β-actin housekeeping gene.
Analysis of hBD-3 in medium supernatants by ELISA

At the end of each experiment, concentrations of hBD-3 in cell medium supernatants were quantified using an ELISA method as described previously. The value of the optical density (OD) was read at 415 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The mean value was calculated from 3 repeated experiments.

Cell culture for cetuximab and TLR4-neutralizing Ab experiments

SCC-25 cells (1 × 10⁴) were seeded in 2 mL of the appropriate medium in each well of a 6-well cell culture plate. The EGFR antibody cetuximab was diluted in medium to final concentrations of 0.1 μg/mL, 1.0 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL. TLR4-neutralizing Ab (TLR4 Ab) and TLR5-neutralizing Ab (TLR5 Ab, as the isotype-matched control Ab) were diluted in medium to 10 μg/mL. Cells were pretreated with different concentrations of cetuximab, TLR4 Ab, or TLR5 Ab for 30 minutes before LPS stimulation in triplicate. At the times indicated, cells and supplements were harvested for hBD-3 detection or EGFR phosphorylation by ELISA or western blot analysis, respectively. The mean value was calculated from 3 repeated experiments.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in lysis buffer (25 mmol/L Tris-HCl, 300 mmol/L NaCl, 1 mmol/L CaCl₂, 1% Triton X-100 [pH 7.4], protease inhibitor [Complete Mini; Roche, Penzberg, Germany]). The whole-cell lysates were centrifuged at 10,000g for 20 minutes and the supernatants were collected. The protein concentration was determined by the bicinchoninic acid–based (BCA) Protein Assay kit (Pierce, Perbio Science France, Beacons, France). Proteins (30-50 μg per sample) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Membranes were probed with primary antibodies followed by secondary antibodies. All immunoblots were detected using an enhanced chemiluminescence system.

Tumor cell chemotaxis assay

Chemotaxis assay was performed in 24-well Millipore cell culture inserts from Millipore (Millipore Corporation, Billerica, MA), using an insert of 8-μm pore membranes as described previously. SCC-25 cells transfected with siRNA-hBD-3 or control vector were suspended in RPMI1640 at 2 × 10⁴/mL and added to the upper chamber. The lower chamber contained the chemotaxis buffer (RPMI1640 + hBD-3 protein) or LPS-treated SCC-25 cells transfected with siRNA-hBD-3 or control vector. After incubation for 24 hours for chemotaxis assays, cells on the lower surface of the membrane were stained and counted under a light microscope in 5 different fields (×400). Data were expressed as the mean number of migrated cells/well in the representative experiments performed in triplicate.

Tumor samples

Specimens from patients with previously untreated OSCC were obtained at the time of resection, in accordance with an institutional review board–approved protocol. Tissue specimens from tumor (79 cases gathered from Department of Oral & Maxillofacial Surgery, Peking University, Shenzhen Hospital) and adjacent normal-appearing mucosa (79 cases) were frozen in liquid nitrogen and stored at −80°C. OSCC specimens used for immunohistochemistry were obtained from archived, paraffin-embedded tissue blocks from patients with histologically proven OSCC.

Immunohistochemical staining of hBD-3

The avidin-biotin complex method was used to detect the proteins of hBD-3 (anti-hBD-3, dilution 1:100; both from PeproTech). Formalin-fixed and paraffin-embedded tissues were deparaffinized and subsequently microwaved in sodium citrate buffer. After preincubation with hydrogen peroxide, avidin-biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA), and rabbit serum (Vector Laboratories), the primary antibody was applied at room temperature for 1 hour. After incubation with the secondary antibody, the avidin-biotin complex was added and stained with diaminobenzidine. Subsequently, the slides were rinsed in tap water and counterstained with hematoxylin. Finally, the slides were dehydrated with ethanol, rinsed with xylene, and coverslipped.

Evaluation of hBD-3 immunostaining

hBD-3 immunoreactivity was evaluated independently and in a blinded manner by 2 investigators for the 79 OSCC specimens, and evaluation was based on the following criteria: 0, no staining; 1+, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); 2+, moderate to strong granular cytoplasmic staining in 10% to 90% of the cancer cells; 3+, more than 90% of the tumor cells stained with strong intensity. Elevated expression of hBD-3 protein was defined as moderate (2+) or strong (3+) cytoplasmic granular hBD-3 immunoreactivity. The percentage agreement between the 2 independent and blinded investigators
HBD-3 expression and EGFR activation are upregulated by LPS in SCC-25 cells

In this study, the effects of LPS on hBD-3 expression and EGFR activation in oral cancer cells were investigated (Fig. 1). The results indicated that hBD-3 mRNA expression increased significantly when SCC-25 cells were treated with LPS (10 μg/mL) (Fig. 1, A), and the release of hBD-3 began to increase slowly when SCC-25 cells were treated with LPS (10 μg/mL) from 1 to 2 hours, but not significantly different compared with controls. After SCC-25 cells were treated with LPS (10 μg/mL) for 4 hours, the maximal effect of LPS on the release of hBD-3 was obtained, the release of hBD-3 began to decrease after 4 hours, and then increased and reached the second peak at 10 hours (Fig. 1, B). EGFR phosphorylation was induced when SCC-25 cells were treated with LPS (10 μg/mL) for 30 minutes. The maximal effect of LPS on EGFR phosphorylation was observed after SCC-25 cells were treated with LPS (10 μg/mL) for 1 hour, which preceded the phase of induced release of hBD-3. LPS-induced EGFR phosphorylation levels began to decrease after 1 hour, and the levels began to increase again after 6 hours, then returned to baseline at 10 hours. However, no change in EGFR mRNA expression was observed when SCC-25 cells were treated with LPS (data not shown).

HBD-3 expression is upregulated by LPS through EGFR in SCC-25 cells

In our previous experiment we demonstrated that LPS can improve hBD-3 expression and EGFR activation in OSCC cells. For this subsequent study, cetuximab, an EGFR inhibitor, was used to explore whether LPS can improve hBD-3 expression through EGFR activation. The results showed that the LPS-induced increase in hBD-3 expression is inversely correlated with an increased dose of cetuximab in SCC-25 cells and is almost blocked by cetuximab at the concentration of 1000 μg/mL (Fig. 2). These data demonstrate that the LPS-induced increase in hBD-3 expression requires EGFR activation.

HBD-3 expression and EGFR activation improved by LPS through TLR4 in SCC-25 cells

TLR4, the receptor of LPS, is expressed in SCC-25 cells (data not shown). We examined whether LPS increases EGFR activation by binding to TLR4 in the plasma membrane. SCC-25 cells were preincubated with TLR4-neutralizing Abs (TLR4 Ab, 10 μg/mL) or an isotype-matched control, Ab TLR5-neutralizing Abs (TLR5 Ab, 10 μg/mL) for 30 minutes and then treated with LPS (10 μg/mL) for 4 hours. The results reveal that LPS can increase EGFR phosphorylation (Fig. 1, coulombs D) as well as hBD-3 expression (Fig. 1, E–F). Preincubation of the cells with TLR4 Ab, but not TLR5 Ab, significantly inhibited EGFR phosphorylation and hBD-3 expression (Fig. 3 B). This evidence confirms that TLR4 plays a role in increasing EGFR activation and hBD-3 expression in response to LPS stimulation of SCC-25 cells.

Effect of hBD-3 on cancer metastasis in vitro

Transwell chemotaxis assays were performed to examine the mobilizing effect of LPS or hBD-3 on SCC-25 cells. The results indicated that SCC-25 cells exhibited significant chemotaxis responses to increased concentrations of hBD-3, the correlations with hBD-3 concentration level (Fig. 4, A and G). To confirm this, we used hBD-3 small interfering RNA (siRNA) to knockdown hBD-3 expression in SCC-25 cells and then examined chemotaxis response to LPS. Compared with SCC-25 cells transfected with control vector, hBD-3 protein expression and chemotaxis response to LPS (10 μg/mL) in SCC-25 cells transfected with siRNA-hBD-3 were inhibited significantly (Fig. 4, B–F). Thus, confirming induction of chemotactic response by signals through hBD-3.

HBD-3 overexpression in OSCC tissues and associated with lymph node metastasis

The expected RT-PCR product of hBD-3 is 182 base pairs. The mRNA expression of hBD-3 appeared in both OSCC tissues and normal oral mucosal tissues (Fig. 5, A), but the intensity of mRNA expression is different, the former is higher than the latter. Paired t test of RT-qPCR indicated that expression of hBD-3 mRNA significantly increased in tumor tissues (Fig. 5,
Fig. 1. LPS has the ability to stimulate both hBD-3 expression and EGFR activation in SCC-25 cells. A, RT-qPCR of hBD-3 using total RNA samples extracted from SCC-25 cells treated with LPS for 6 h. B, ELISA of hBD-3 using culture supernatants collected from SCC-25 cells treated with LPS for various amounts of time (0-10 h); *P < .05, compared with control at the same time. C, At each time point, the phosphorylation status of EGFR was analyzed by immunoblotting with an anti-p-Tyr992-EGFR. Total EGFR protein was evaluated by western blot using anti-EGFR. D, Quantification of phospho-protein is shown as the ratio between the phosphorylated forms and total protein levels of EGFR. Results are shown as percentage above the ratio observed at time 0 (without LPS). *P < .05, compared with time 0.
B. P = .031). Fig. 5, A, shows representative mRNA hBD-3 expression. The protein expression of hBD-3 was detected in both normal oral mucosa and OSCC tissue samples. In OSCC tissue, elevated expression of hBD-3 protein was observed, which was localized exclusively to the cytoplasm of malignant epithelium cells and in inflammatory cells, whereas the stroma was either negative or weakly positive (Fig. 5, C). In the normal oral mucosa, weak expression of hBD-3 (Fig. 5, D) was sporadically detected within the cytoplasm. Fig. 5, C and D, shows representative immunohistochemical hBD-3 staining.

The statistical correlation of the hBD-3 mRNA status with clinicopathological parameters was investigated.
Fig. 4. Dose–response of cancer cell SCC-25 chemotaxis in response to hBD-3 (A, G). Chemotaxis of cells was determined by counting cells in 5 fields under a microscope in each lower chamber (×400). *P < .05, compared with concentration 0. hBD-3 protein expression of control vector (B, ×600) or siRNA-hBD-3 (C, ×600) transfected cells was confirmed by immunofluorescence; the green in the cytoplasm is hBD3. The ability of chemotaxis of SCC-25 cells transfected with control vector or siRNA-hBD-3 vector in response to LPS (D, E, F); *P < .05, compared with control.
As shown in Table I, in $\chi^2$-analysis hBD-3 overexpression was not associated with tumor size, histologic grade, and histologic type. However, hBD-3 overexpression was positively associated with lymph node metastasis ($P < .05$). The expression of hBD-3 between in lymph node–negative and lymph node–positive tumor tissues was evaluated by using Wilcoxon rank-sum test. The data showed that hBD-3 expression was more frequently found in tumors with lymph node metastasis ($P < .05$).

**DISCUSSION**

The possible relationship between bacteria and oral cancer development has been reported previously.\(^1,6\) Innate immunity is the first line of defense against invading pathogens. hBDs are critical components of innate immunity at mucosal surfaces.\(^7\) It has been demonstrated that hBD-3 is highly expressed in oral cancer and is involved in cancer progression.\(^18\) LPS is a major integral component of the outer membrane of gram-negative bacteria. In this study, we demonstrated that in OSCC cells, hBD-3 expression can be stimulated by LPS exposure.

EGFR is one of the peptide growth factors; it plays a central role in the pathogenesis and progression of different carcinoma types, and it is overexpressed in many cancers, including oral cancer.\(^15,19-21\) Based on the expression data of growth factor receptors, therapeu tic targeting of the EGFR receptor has been attempted in various tumor patients. The agents, which target EGFR, can be classified into 2 groups: tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, and monoclonal antibodies, such as cetuximab or panitumumab. It is well established that EGFR activation can induce the expression of many cytokines, including hBD-3,\(^12\) and EGFR activation can be induced by LPS.\(^4,5\) Although LPS can induce EGFR activation,
hBD-3 expression can be regulated by EGFR.\textsuperscript{4,5,12} In this study, it was seen that EGFR activation and hBD-3 expression were both significantly increased when SCC-25 cells were stimulated by LPS, and LPS-induced hBD-3 expression was blocked by the EGFR antagonist cetuximab. The result validates the hypothesis that LPS-induced hBD-3 expression is dependent on EGFR activation in OSCC cells, and demonstrates that EGFR activation plays a key role in LPS-improved hBD-3 expression in OSCC cells.

The importance of TLRs in recognizing exogenous microbial pathogens by the innate defense system is well established,\textsuperscript{22} and it has been shown that TLRs have high expression in different cancer types, including OSCC, and is involved in cancer progression.\textsuperscript{23} In this study, a TLR4 antagonist was used to demonstrate that LPS can stimulate EGFR activation and hBD-3 expression via the interaction of LPS with TLR4 in SCC-25 cells. TLR5 neutralizing antibody was used to the isotype-matched control in the study, which confirms that LPS can upregulate hBD-3 expression through specific binding of TLR4.

The mobilizing effect of hBD-3 on SCC-25 cells was detected by transwell chemotaxis assay, and the results showed that SCC-25 cells exhibited significant chemotaxis response to hBD-3, correlating with hBD-3 concentration level. To confirm this, we used hBD-3 siRNA to knockdown hBD-3 expression in SCC-25 cells and then examined chemotaxis response in response to LPS. The results showed that the chemotaxis response to LPS of SCC-25 cells was partly blocked by siRNA target hBD-3. These findings suggested that in OSCC cells, hBD-3 expression upregulated by LPS will contribute to cancer metastasis, which will partly explain the mechanism of LPS enhanced metastasis. Previous reports demonstrated loss of human hBD-3 expression in OSCC cell lines and tissues.\textsuperscript{14,15} In this study, the results indicated that hBD-3 was commonly overexpressed in OSCC tissues. Our results are in agreement with other published reports.\textsuperscript{16-18} The different results may be because of fewer cases (20 cases) in the former than the latter (79 cases). In addition, our data strongly suggested that hBD-3 was a critical determinant to predict lymph node metastasis in OSCC tumors. It seems likely that LPS-induced signaling via the TLR4 receptor activates EGFR and increases hBD-3 expression in OSCC cells. hBD-3 expression contributes to oral cancer metastasis. However, the mechanism of SCC-25 cells exhibited chemotaxis responses to hBD-3 should be further investigated.

CONCLUSIONS

Collectively, our results indicated that overexpression of hBD-3 associated with lymphatic invasion of OSCC tissue, and hBD-3 expression upregulated by LPS via EGFR signaling pathways can enhance metastasis of OSCC cells. This novel mechanism is the first evidence that hBD-3 expression can be upregulated in OSCC cells by a component of the bacterial cell wall. These findings suggest that hBD-3 expression upregulated by LPS may play an important role in the pathophysiology of OSCC, which implies some connection between bacteria in the oral cavity and OSCC development.

REFERENCES

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