Human β-defensin 3 promotes NF-κB-mediated CCR7 expression and anti-apoptotic signals in squamous cell carcinoma of the head and neck

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The microenvironment of aerodigestive cancers contains tumor-promoting inflammatory signals often involved in innate immunity. The epithelial malignancy, squamous cell carcinoma of the head and neck (SCCHN), is characterized by secretion of inflammatory mediators that can promote tumorigenesis and lymph node metastasis. Human β-defensin (hBD) 3 is one such antimicrobial mediator of innate immunity produced by squamous epithelial cells in response to tissue damage and inflammation. Here, we hypothesized that the observed overexpression of hBD3 in SCCHN may have a tumor-promoting effect or contribute to nodal metastasis, which has previously been linked to chemokine receptor (CCR) 7 overexpression. Indeed, treatment of non-metastatic SCCHN cells with hBD3 induced surface CCR7 expression and migration toward its ligand, CCL19. The hBD3-induced CCR7 upregulation in SCCHN cells was significantly reduced by inhibition of nuclear factor (NF)-κB, an inflammatory transcription factor known to influence CCR7 expression. Moreover, hBD3 stimulation provided anti-apoptotic signals to SCCHN cells, as evidenced by tumor resistance to caspatin-induced death, which was regulated by phosphoinositide-3-kinase/Akt activation. Interestingly, the observed hBD3-mediated effects were not dependent on G-protein coupled receptors or toll-like receptors, as has been previously published, but hBD3 was internalized through endocytosis, allowing intracellular signal transduction. Our findings suggest that hBD3 represents a novel NF-κB-regulated mediator of CCR7 expression and anti-apoptotic pathways, which may be exploited by developing SCCHN tumors to enhance their survival and metastasis.

Introduction

Human β-defensins (hBDs) are small antimicrobial peptides secreted by epithelial cells in response to various signals including pro-inflammatory cytokines and microbial invasion (1). The four hBDs (hBD1–4) are highly expressed in epithelial cells and tissues that are involved in host defense, such as the cutaneous and mucosal surfaces (2). In addition to innate antimicrobial properties, hBD2 and hBD3 can contribute to adaptive immunity through their effects on the maturation of antigen-presenting cells (3,4).

Recent studies have shown that β-defensins can induce various pro-inflammatory effects on immune cells. For instance, hBD2 has been shown to induce chemotactic migration of T cells and immature dendritic cells (DCs) by binding to chemokine receptor (CCR) 6 (3). In addition, (murine) mBD2 and hBD3 can bind to toll-like receptors (TLRs) and induce antigen uptake and processing as well as differentiation of antigen-presenting cells (4–7). Moreover, our data indicate that hBD3 induces the maturation of DCs, upregulating various costimulatory markers as well as the lymph node homing receptor CCR7. These interactions enhance the production of inflammatory cytokines and promote the generation of a potent adaptive immune response (8).

Despite the advances in understanding the role of β-defensins in immunity, their function in oncogenesis of aerodigestive cancers, which are derived from hBD-producing epithelial cells (9,10), has not been well characterized. In oral squamous cancers such as squamous cell carcinoma of the head and neck (SCCHN), hBD3 is frequently overexpressed and has been implicated in the pathogenesis of these malignancies (11,12), but the mechanism(s) of such an effect is undetermined (13). hBD3 expression has been correlated with the infiltration of tumor-associated macrophages, which are known to generate an inflammatory tumor microenvironment and promote tumor aggressiveness (14).

We have previously reported that CCR7 is upregulated in metastatic SCCHN cells (15), activating anti-apoptotic and invasive pathways and contributing to caspatin resistance. However, the signals, which can induce CCR7 expression in primary non-metastatic tumors and promote tumor progression are largely unknown. Here, we examine the effects of hBD3 stimulation on non-metastatic SCCHN tumor cells. We hypothesized that hBD3 might induce CCR7 expression in these primary SCCHN tumor cells, providing migratory capability as well as pro-survival signals within the tumor microenvironment. We also investigated the role of nuclear factor (NF)-κB as an inflammatory mediator of the putative hBD3-mediated CCR7 expression and pro-apoptotic effects. Our findings suggest that hBD3 expression can be exploited by developing tumors to enhance their growth, survival and evolution into a metastatic phenotype. In particular, the hBD3-stimulated induction of NF-κB-dependent CCR7 expression in primary tumors may contribute to the predictable pattern of regional lymph node metastases commonly observed in SCCHN.

Materials and methods

Cell lines

Human SCCHN cell lines PCI-6A, PCI-15A and PCI-37A were derived from the non-metastatic primary tumor site and characterized at the University of Pittsburgh (16). All cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen Corporation, Carlsbad, CA) containing 8% (vol/vol) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 U/ml penicillin G and 100 μg/ml streptomycin and 4 mM l-glutamine (Invitrogen). The cells were also tested for mycoplasma on a regular basis to ensure that only mycoplasma-free cell lines were studied in our assays.

Antibodies and reagents

Tumor necrosis factor-α and the CCR7 ligand Macrophage Inflammatory Protein-3β (MIP3β/CCL19) were both purchased from R&D Systems (Minneapolis, MN). The hBDs, hBD2, hBD3 and hBD4, were purchased from Peptides International (Louisville, KY). The phosphoinositide-3-kinase (PI3K) inhibitor LY294002 and the Akt/PI3K inhibitor (1,6-hexamethylyldene)-chiro-inositol-2-(R)-2-O-methyl-3-0-octadecyl-sn-glycero-carbonate were obtained from Calbiochem (San Diego, CA). BAY 11-7082, wortmannin, nocodazole, cytochalasin D and dimethyl amiloride were all obtained from Sigma–Aldrich (St Louis, MO). Antibodies used were: fluorescein-conjugated anti-human CCR7 (R&D Systems), rabbit anti-Akt and anti-phospho-Akt (Ser 473) (Cell Signaling Technology, Beverly, MA). NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) and γ-tubulin (Abcam, Cambridge, MA). Annexin V apoptosis detection kit was purchased from BD Biosciences (San Jose, CA). Fluorescein isothiocyanate (FITC) labeling kit used to label hBD3 peptide was obtained from Pierce (Rockford, IL).
Cells were cultured in serum-free media for 24–48 h prior to manipulation. Briefly, cells were treated with hBD3 (1 μM, 24 h) or as indicated. Following treatment, cells were washed with sterile 1× phosphate-buffered saline (PBS) buffer (Sigma–Aldrich), collected by scraping and incubated with FITC-conjugated hCCR7 mAb (150503) (R&D Systems). Irrelevant isotype-matched FITC-labeled IgG1 antibody (BD Biosciences) was used as a control. After incubation, cells were washed twice and fixed using 2% paraformaldehyde in PBS. Fluorescence was read using Beckman Coulter Epics XL cytometer, with the isotype control set to a mean fluorescence index x-mean of 5, and analyzed using Exp2 software (Beckman Coulter, Miami, FL).

**Chemoattractant assay**

Cell migration studies were performed as described previously (15). Briefly, 30 μl aliquots of chemotractant (CCL19 or CCL20 at 500 ng/ml) were added in triplicate to the wells of a disposable 96-well chemotaxis chamber (ChemoTx Neuroprobe, Gaithersburg, MD) with an 8 μm pore size filter and 5.7 mm width/well. Cell suspensions (1 × 10^6 cells/ml) were placed on top of the filter. After 4 h incubation at 37°C, the filter was washed gently with media. To release adherent tumor cells, a 30 μl aliquot of trypsin-ethylenediaminetetraacetic acid was added to the filter (5 min, 37°C) before centrifuging the plate (1400 r.p.m., 5 min). The filter was removed and the cells in each lower well were counted under a light microscope. The results are presented as chemotactic index, defined as the fold increase in cell migration in chemotractant medium over migration in media alone.

**Small interfering RNA transfection**

Cells were grown to 50% confluence and transfected using Lipofectamine RNAiMAX (Invitrogen) with 200 nM of either p65 (RelA) or non-targeting control small interfering RNA (Ambion, Austin, TX). Transfected cells were incubated for 24–48 h before analysis.

**Immuno-blot analysis**

To prepare whole cell extracts, 50–70% confluent cells were serum starved for 16–20 h and then harvested in a buffer (10 mM Tris–HCl pH 7.6, 50 mM NaPO₄, 50 mM NaF, 1 mM NaVO₄, 1% Triton X-100 and protease inhibitors), vortexed at 4°C for at least 1 h and the supernatant containing whole cell extract was collected. To prepare nuclear extracts, cells were stimulated as appropriate, washed, collected and resuspended in cell lysis buffer (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM ethylene glycol-bis(aminohexylether)-tetraacetic acid, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitors). After allowing cells to swell on ice for 15 min, 1% NP-40 was added and cell suspension vortexed. After centrifugation, the nuclear pellet was resuspended in nuclear lysis buffer (20 mM HEPES, 400 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(aminohexylether)-tetraacetic acid, glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and Protease and Phosphatase inhibitors). Samples were vortexed at 4°C for at least 1 h. After determining protein concentration using BCA Protein Assay reagents (Biorad, Hercules, CA), 50 μl of whole cell or nuclear extract was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and immunoblotted with the relevant antibodies.

**Apoptosis detection**

Cells were pretreated with hBD3 (0.25 μM, 1 h) and then treated with apoptosis-inducing agent, cisplatin (40 μM, 4 h). To examine whether hBD3-induced survival was dependent on Akt activation, cells were pretreated with an Akt inhibitor (10 μM, 3 h) prior to hBD3 and cisplatin treatment. Cellular apoptosis was assessed by Annexin V and 7-amino-actinomycin D staining using flow cytometry (where early apoptotic cells were Annexin V positive and 7-amino-actinomycin D negative). Briefly, cells were harvested by gentle scraping and washed with 1× PBS. The cells were then stained with 5 μl Annexin V-FITC and 5 μl 7-amino-actinomycin D in 100 μl binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 1.8 mM CaCl₂; pH 7.4) for 15 min at room temperature in the dark. After staining, cells were analyzed within 1 h by flow cytometry.

**Detection of fluorescent hBD3**

To analyze internalization of fluorescently labeled hBD3 peptide, tumor cells were collected by trypsinization, washed with 1× PBS, resuspended in AIM-V medium and treated with 1–2 μM hBD3-FITC for the indicated periods of time. At the conclusion of each treatment, 0.1% azide/PBS solution was added to the cells. The cells were then washed twice with 1× PBS and fixed using 2% paraformaldehyde in PBS. Fluorescence was read using either: a Beckman Coulter Epics XL cytometer and analyzed using Expo 32 software (Beckman Coulter) or using an ImageStream 100 imaging flow cytometer (AMNIS, Seattle, WA). Alternatively, cells were grown on a cover slip and treated with 1 μM hBD3-FITC for the indicated periods of time. The cells were then washed with 0.1% azide/PBS solution and then with 1× PBS, fixed using 2% paraformaldehyde/PBS and stained with PKH26 (membrane dye) and 4’6-diamidino-2-phenylindole (nuclear dye) (Sigma–Aldrich) and then examined by fluorescence microscopy using an Olympus Fluoview 1000 Confocal Microscope at ×60 magnification.

**Statistical analysis**

Data are expressed as mean ± standard error of at least three experiments. An unpaired t-test was used to calculate whether observed differences were statistically significant. P < 0.05 was considered significant.

**Results**

hBD3 upregulates CCR7 expression on primary non-metastatic SCCHN tumor cells. We previously showed that CCR7 is upregulated in metastatic SCCHN cell lines (PCI-6B, -15B and -37B) but not in the parental non-metastatic SCCHN cell lines derived from the primary head and neck tumor site (15,17). To determine whether hBD3 could stimulate CCR7 expression in non-metastatic tumor cells, we treated the parental primary SCCHN tumor cells, PCI-6A, -15A and -37A, with hBD3 (1 μM for 24 h at 37°C) and examined CCR7 expression by flow cytometry (Figure 1A). Under these conditions, we found that CCR7 surface expression was significantly upregulated in primary SCCHN tumor cells following hBD3 stimulation (P < 0.05).

The induction of CCR7 expression showed a dose response to hBD3 concentration and was detected at hBD3 concentrations ≥0.25 μM (data not shown), and CCR7 expression reached a plateau at ~1 μM. Notably, the observed increase in CCR7 expression could not be attributed to a nonspecific increase in cellular protein synthesis as the expression of human leukocyte antigen-class I molecules did not increase under these conditions (Figure 1B). Furthermore, the use of heat-inactivated hBD3 or the use of other defensins hBD2 or hBD4 did not result in CCR7 upregulation (data not shown).

hBD3-induced CCR7 promotes migration toward CCL19

To evaluate the functional significance of hBD3-induced CCR7 expression on primary SCCHN tumor cells, we assayed the migration of hBD3-treated tumor cells to the CCR7 ligand CCL19, using in vitro transwell chemotaxis chambers. Untreated cells did not migrate toward CCL19 in the transwell assay, however, after hBD3 exposure (0.25 μM, 24 h), we observed a significant (P < 0.006) increase in CCR7 and tumor cell migration toward CCL19 (Figure 2). These data show that hBD3 stimulation of these primary, non-metastatic SCCHN tumor cells results in the upregulation of a functional CCR7 receptor. Interestingly, we observed a 2-fold increase in migratory response in both PCI-6A and -37A despite differing levels of CCR7 upregulation (Figure 1A), suggesting that even a small increase in CCR7 expression is sufficient to induce a functional response in these cells.

hBD3 stimulation of CCR7 expression is NF-κB dependent

Previous studies have suggested that CCR7 is an NF-κB target gene (18) and we have observed that NF-κB activation can regulate basal CCR7 expression in metastatic SCCHN tumor cells (19). To determine whether NF-κB activation mediated hBD3-induced CCR7 upregulation, we utilized small interfering RNA to specifically knock down NF-κB p65 expression in PCI-15A and -37A cells. Our findings show that, following p65 knockdown, the hBD3-mediated CCR7 expression was reduced (Figure 3A). Furthermore, by using the Inhibitor of κB phosphorylation inhibitor BAY 11-7082 (10–100 μM, 4 h), we observed significant inhibition of CCR7 induction after hBD3 treatment in the presence of increasing concentrations of the inhibitor. This occurred in a dose-dependent fashion (P < 0.05) (Figure 3B). The ability of these inhibitors to block NF-κB activation was demonstrated by examining their ability to block NF-κB p65 activation in tumor necrosis factor-α-induced nuclear extracts. Additionally, the
expression of human leukocyte antigen-class I was not significantly affected by NF-κB inhibition thereby ruling out nonspecific effects of these inhibitors (data not shown). Taken together, these findings strongly indicate that hBD3 upregulates CCR7 expression in an NF-κB-dependent manner.

hBD3 activates PI3K/Akt pro-survival signaling pathways

We have previously published that CCR7+ metastatic SCCHN cells can activate PI3K/Akt-mediated pro-survival and invasive pathways in response to CCL19 stimulation (20,21). The activation of the Akt signaling pathway conferred a survival advantage to tumor cells. To determine whether hBD3 stimulation could also activate the PI3K/Akt pro-survival pathway, we stimulated tumor cells with hBD3 (1 μM, 30 min) and measured the expression of phosphorylated Akt in the presence or absence of the PI3K inhibitors, wortmannin (200 nM, 2 h) (Figure 4A) or LY294002 (data not shown). These experiments showed that phosphorylated Akt expression was enhanced following hBD3 stimulation and was blocked in the presence of wortmannin or LY294002. These inhibitors blocked both basal and hBD3-induced phosphorylated Akt activation. Thus, our findings show that the activation of Akt by hBD3 is dependent on PI3K activation.

We then examined whether the hBD3-induced Akt activation enhanced the survival of tumor cells that were exposed to cisplatin, a commonly used pro-apoptotic chemotherapeutic agent for the treatment of SCCHN. PCI-6A and -37A tumor cells were pretreated with hBD3 (0.25 μM, 1 h) and then exposed to cisplatin (40 μM, 4–6 h) (Figure 4B and C). Treatment of SCCHN cells with cisplatin alone induced apoptosis in 37 and 40% of tumor cells, respectively. However, pretreatment of these SCCHN tumor cells with hBD3 led to a reduction in apoptotic cells to 7 and 3%, respectively. To investigate whether this hBD3-induced protection from cisplatin-induced apoptosis was mediated by the Akt survival pathway, we pretreated the tumor cells with an Akt inhibitor (10 μM, 2 h). In the presence of Akt inhibition, the anti-apoptotic effects of hBD3 were abrogated and cisplatin-induced apoptosis was found in 26 and 33% of SCCHN cells, respectively (P < 0.04). Notably, treatment with the Akt inhibitor alone did not significantly increase apoptosis of the tumor cells (data not shown). Thus, hBD3 stimulation appears to contribute to

Fig. 1. hBD3 stimulation activates CCR7 expression in primary tumor-derived SCCHN tumor cells. PCI-6A, -15A and -37A were stimulated with hBD3 (1 μM, 24 h) and assayed for (A) CCR7 expression or (B) human leukocyte antigen-class I molecules by flow cytometry. Results shown are representative of at least three experiments with similar results.

Fig. 2. hBD3-induced CCR7 is functional. PCI-6A and -37A tumors were stimulated with hBD3 (0.25 μM, 24 h). The cells were collected and added in triplicate to the upper chamber of a chemotaxis plate containing either medium or CCL19 (500 ng/ml) in the lower wells. After 4 h, the number of cells migrated into the lower wells was counted. Results are expressed as chemotactic index (cells migrating toward chemokine/cells migrating toward medium); (P < 0.006).
anti-apoptotic signaling in SCCHN tumor cells, and this pro-survival effect was mediated mainly through an Akt-dependent pathway.

**hBD3-mediated CCR7 upregulation is independent of G-protein coupled receptor and TLR signaling**

To investigate the receptor(s) mediating hBD3-induced migratory effects on SCCHN cells, we studied the role of G-protein coupled receptor (GPCRs) and TLRs, two classes of receptors previously associated with the binding of \( \beta \)-defensins and NF-\( \kappa \)B pathway activation (3,4). Since PCI-6A, -15A and -37A tumor cells express both of these types of receptors (22,23), we investigated whether these receptors were involved in mediating hBD3-induced CCR7 upregulation. SCCHN tumor cells were pretreated with the \( \alpha \)i inhibitor pertussis toxin, with a MyD88-peptide inhibitor, or with a Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-\( \beta \) (TRIF)-peptide inhibitor and then were stimulated with hBD3 in the continued presence of each inhibitor. Interestingly, neither GPCR nor TLR blockade appeared to inhibit hBD3 stimulation of CCR7 upregulation (data not shown). These findings suggest that the observed hBD3 upregulation of CCR7 is not mediated through GPCRs or TLRs.

**hBD3 is internalized through an endocytic mechanism**

Several reports in the literature suggest that small cationic and antimicrobial peptides possess the ability to directly penetrate cellular membranes and potentially induce their effects through binding to intracellular receptors (24–26). To evaluate whether hBD3 was capable of penetrating the cellular membrane, we examined cellular fluorescence from cells incubated at 4°C or 37°C with 1–2 \( \mu \)M fluorescein-labeled hBD3 for different periods of time (Figure 5A). The data show significantly increased hBD3 fluorescence at 37°C compared with 4°C at each time point (\( P < 0.01 \)), suggesting that there is an active mechanism facilitating the internalization of hBD3 into tumor cells. Moreover, staining using fluorescein-labeled cetuximab antibody to epidermal growth factor receptor, which displays predominantly surface binding remained the same at 4°C and 37°C (data not shown). We further used imaging flow cytometry to examine the relative fluorescence from intracellular sources compared with that from the cell surface (Figure 5B). In our analysis, the intracellular fluorescence increased steadily over 4 h, whereas the surface fluorescence remained constant. This strongly suggests that the observed increase in fluorescence at 37°C is due to the intracellular internalization and accumulation of hBD3 peptide. The internalized hBD3 was further visualized using confocal microscopy in order to determine its intracellular location. PCI-15A and -37A tumor cells were treated with hBD3-FITC and then observed by microscopy (Figure 5C). The results show detectable pockets of hBD3-FITC within the cytoplasm, which appear to congregate and accumulate in the perinuclear regions.

To determine the mechanism of the observed accumulation of intracellular hBD3, we inhibited endocytic mechanisms in the tumor cells using the microtubule polymerization inhibitor, nocodazole (10 \( \mu \)g/ml, 1 h). Interestingly, the use of nocodazole significantly blocked but did not completely abrogate intracellular uptake of hBD3-FITC (Figure 5D) into SCCHN cells (\( P < 0.05 \)). The inhibition of pinocytosis or actin polymerization using dimethyl amiloride and cytochalasin D, respectively, did not significantly reduce hBD3 uptake or signaling (data not shown). Thus, in tumors, hBD3 appears accumulate intracellularly using active endocytic mechanisms.

**Fig. 3.** hBD3-induced CCR7 upregulation is dependent on NF-\( \kappa \)B activation. PCI-6A, -15A and -37A tumors were pretreated with: (A) NF-\( \kappa \)B p65 small interfering RNA (200 nM, 24–48 h) or (B) varying concentrations of BAY 11-7082 (4 h) and then stimulated with hBD3 (1 \( \mu \)M, 24 h). CCR7 expression was analyzed by flow cytometry. The mean fluorescence index ± standard error is plotted on the histograms (\( * P < 0.05 \)). Below each figure is an immunoblot of nuclear extracts showing the expression of NF-\( \kappa \)B p65 (or control \( \gamma \)-tubulin) when cells are stimulated with tumor necrosis factor-\( \alpha \) (20 ng/ml, 30 min) in the presence or absence of the above inhibitors. All samples were run on the same gel.
Discussion

hBDs comprise a class of inflammatory molecules, whose primary role at epithelial surfaces appears to be the initiation of an early host immune response to clear invading microorganisms (27,28). They are capable of bridging innate and adaptive immunity through their ability to attract antigen-presenting cells to inflammatory sites and to provide ‘danger signals’ that facilitate the maturation of these immune cells. However, despite our understanding of the role of defensins in host–pathogen interactions and immunity, little is known about their functions within the context of an inflammatory tumor microenvironment. Particularly for squamous mucosal malignancies, which are a source of hBD secretion, even less is known about any potential pro-tumorigenic properties that may result from a dysregulation of inflammatory mediators such as defensins. In this paper, we demonstrate that hBD3 induces CCR7 expression in primary SCCHN tumor cells in an NF-κB dependent manner, providing migratory and pro-survival signals within the tumor microenvironment. Furthermore, the stimulation of tumors with hBD3 appears to confer a survival benefit to these tumors, mediated by PI3K/Akt activation. Our findings suggest that hBD3 overexpression by epithelia can be exploited by developing tumors in autocrine and paracrine fashion to enhance their growth, survival and evolution into a metastatic phenotype. In particular, the hBD3-stimulated induction of CCR7 expression in primary tumors may contribute to the predictable pattern of regional lymph node metastases commonly observed in SCCHN.

Others have begun to characterize the inflammatory effects of hBDs, which may be pro-tumorigenic. In mouse ovarian carcinoma, the expression of β-defensin 29, the murine homologue to human β-defensin 2, was found to chemoattract DCs through CCR6 and enhance tumor vascularization and growth through cooperation with vascular endothelial growth factor A (29). Human tumors overexpressing β-defensins have been found to possess increased populations of infiltrating monocytes, macrophages and DCs suggesting that the pro-angiogenic and pro-tumorigenic functions of β-defensins may play a role in the development of these tumors. Indeed, a recent study by Jin et al. (30) reported that hBD3 expression by oral tumors was associated with increased macrophage recruitment via CCR2 and increased secretion of tumor-promoting cytokines. Our studies indicate that SCCHN cell lines secrete low levels of hBD3 into the culture medium. Interestingly, stimulation with pro-inflammatory cytokines such as interferon-γ can stimulate increased secretion of hBD3 by these cell lines (Mburu,Y.K., Ferris,R.L, unpublished results). However, any direct effects of hBD3 on tumor growth and metastasis have not yet been elucidated.
Here, we tested the ability of hBD3 to directly affect the survival and migratory ability of human SCCHN tumors. We previously identified a distinct pattern of chemokine expression in SCCHN tumors whereby CCR7 was upregulated in patient-matched metastatic tumors (15). The expression of CCR7 by metastatic SCCHN tumors may contribute to the frequent lymph node metastases that are reported in this and other malignancies. However, since CCR7 expression has generally been identified on tumors that are already present at the metastatic lymph node sites, the induction signals responsible for, and significance of, CCR7 expression on primary tumor cells emigrating from the mucosal tumor site are still unclear. Our work suggests that pro-inflammatory and cellular mechanisms cooperate to induce CCR7 expression and anti-apoptotic effects on the parental non-metastatic SCCHN tumors.

We show here that hBD3 is an inflammatory signal that stimulates the expression of CCR7 in tumors. Interestingly, hBD3 treatment had no significant effects on the expression of CCR6 and CXCR4, which are also expressed by head and neck cancers. Furthermore, we report that the induction of CCR7 by hBD3 was dependent on NF-kB activation. Our data is consistent with the notion that CCR7 is an NF-kB target gene (18) since the inhibition of NF-kB activation significantly abolished the observable hBD3-induced CCR7 upregulation. The tumorigenic effects of deregulated NF-kB activation in oral squamous cell carcinoma have been well documented since this transcription factor has been shown to regulate various oncogenes, including CCR7 (31). Indeed, we have examined the CCR7 promoter and found the existence of functional NF-kB-binding sites, which are important in the regulation of CCR7 expression (Mburu, Y.K., Walker, W.H., and Ferris, R.L., unpublished results). In SCCHN, various signals are known to induce constitutive NF-kB activation (32). Our studies suggest that the exposure to hBD3 within an inflammatory tumor microenvironment results in upregulation of CCR7 expression via NF-kB-dependent mechanisms.

In previous reports, the expression of CCR7 in immune cells and tumors has been shown to provide a survival advantage to cells and is correlated with tumor aggressiveness (20,21,33). This increased survival has been linked to the activation of the PI3K/Akt signaling pathway. In this study, we found that hBD3 stimulation activates PI3K-dependent Akt signaling. This activation and phosphorylation of Akt was biologically significant as it enhanced the survival of SCCHN tumor cells in the presence of cisplatin. Moreover, since the cells were pretreated with hBD3 for only 1 h prior to cisplatin treatment, we can attribute the observed pro-survival benefit directly to hBD3 stimulation of PI3K/Akt.

We also examined the potential extracellular receptors involved in the observed hBD3 effects on primary SCCHN cells. Remarkably, even though our primary SCCHN tumor cells express various GPCRs and TLRs (22,23), the hBD3-mediated CCR7 upregulation was not
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blocked by the Gz inhibitor pertussis toxin or by TLR peptide inhibi-
tors, both of which inhibit β-defensin effects on immune cells (3,4).
Interestingly, we found that hBD3 is taken up through endocytic
mechanisms and accumulates in the perinuclear regions in tumor
cells. It is also valuable to note that although other hBDs (such as
hBD2) do not have similar effects on tumor CCR7 expression as
hBD3, it appears that they penetrate tumor cells in a similar manner
to that described for hBD3 (data not shown). Indeed, evidence in
the literature suggests that the small cationic nature of these peptides
allows them to associate with cellular membranes and translocate
through the membrane using temperature and energy-dependent
processes (34,35). Our findings show that incubation at low temperatures
or the inhibition of microtubule polymerization usingnocodazole
significantly reduced the observed internalization of hBD3 (P < 0.05).
It is notable, however, that over an extended incubation period, hBD3
overcomes nocodazole inhibition and penetrates cells. This observation,
along with the toxicity of nocodazole at higher concentrations made it
difficult to directly assess the effects of nocodazole treatment on hBD3-
induced CCR7 expression. Nevertheless, while we cannot exclude re-
ceptor-mediated internalization, our data suggest that hBD3 signaling
can also occur through direct binding to intracellular receptors/targets
as has been described for other antimicrobial peptides (25,26). Indeed,
hBD3 could be binding to yet unidentified receptors on tumors.

To our knowledge, this is the first study directly linking defensin
expression to tumor chemokine expression. Our data show that hBD3
stimulation induces pro-inflammatory, NF-κB-mediated functional
CCR7 expression in SCCHN cells derived from primary, non-metastatic
tumors. This gain in CCR7 expression may be important in facilitating
the chemotactic migration of the tumor cells toward CCL19. Furthermore,
we found that hBD3 stimulation provides post-survival signals to
primary SCCHN tumor cells through the activation of PI3K/Akt
signaling. Future investigations into the receptor(s) involved in hBD3
stimulation on tumors could provide potential therapeutic avenues for
clinical intervention.

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