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Cutting Edge: Human β Defensin 3—A Novel Antagonist of the HIV-1 Coreceptor CXCR4

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Previously, we showed that human epithelial cell-derived β-defensins (hBDs)-2 and -3 block HIV-1 replication via a direct interaction with virions and through modulation of the CXCR4 coreceptor on immunocompetent cells. In the present study, we show that hBD-3 promotes directly the internalization of CXCR4 yet does not induce calcium flux. ERK (ERK-1/2) phosphorylation, or chemotaxis. hBD-3 competes with stromal-derived factor 1 (SDF-1), the natural ligand for CXCR4, for cellular binding and blocks SDF-1-induced calcium flux, ERK-1/2 phosphorylation, and chemotaxis, without effects on other G protein-coupled receptors. The novel activity of this endogenous CXCR4 antagonist may provide a new strategy for HIV therapies or immunomodulation. Moreover, since the SDF-1/CXCR4 axis plays an important role in hemopoiesis, neurogenesis, cardiogenesis, and angiogenesis, endogenous agents such as hBD-3 or its derivatives offer a new paradigm in immunoregulatory therapeutics and provide the opportunity to enhance future drug design. The Journal of Immunology, 2006, 177: 782–786.

Recent findings are shedding new light into the importance of host defense peptides in immunity. With the discoveries of mucosal epithilium of human β-defensins (hBDs), a 4- to 5-kDa cationic, amphipathic peptides with antimicrobial and immunosurveillance properties (1–3), there is reason to propose that these innate defense peptides may function not only to protect the host against microbial pathogens at the mucosal barrier, but also to marshal adaptive host immune responses to both prevent and clear infection. Our previous studies demonstrated that HIV-1 induces hBD-2 and -3 expression in human oral epithelial cells and that these peptides block HIV-1 replication via a direct interaction with virions and through modulation of CXCR4, the coreceptor used by HIV-1 X4 isolates to infect target cells (4). The present study explored the mechanism of the interaction between hBD-3 and CXCR4. We found that this peptide could compete with stromal-derived factor 1 (SDF-1), the CXCR4 ligand, for binding to cells and also could induce internalization of CXCR4 in immunocompetent cells without promoting calcium mobilization, activation of ERK-1/2 MAPK, or chemotaxis. This activity was specific for CXCR4 since there was no inhibition of calcium mobilization as a result of RANTES, fMLP, ATP, or carbachol binding to their respective receptors.

Materials and Methods

**Cell culture**

CEM X4/R5 cells (human T cell line expressing CXCR4 and CCR5) and THP-1 cells (human monocytic leukemia cell line) were cultured and maintained as described previously (4). To induce fMLP receptor expression, THP-1 cells were differentiated in RPMI 1640 medium supplemented with 300 μM D-glucose, 1 mg/ml BSA, and 20 mM NaHEPES (pH 7.5). One million cells in 1 ml RPMI 1640 medium containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 1 mg/ml BSA, and 20 mM NaHEPES (pH 7.5). One million cells in a final volume of 300 μl were placed in each tube along with 0.1 μM 125I-labeled SDF-1 (PerkinElmer NEN) or RANTES (PerkinElmer NEN). Unlabeled SDF-1 or RANTES (1 μg/ml for each) or various concentrations of hBD-3 were added simultaneously. Only 125I-labeled SDF-1 or 125I-labeled RANTES was added to control tubes. Cells were incubated at 37°C for 30 min, or on ice for 4 h, and then washed three times with BSS. Cellular binding of 125I-labeled SDF-1 or 125I-labeled RANTES was determined by gamma counter readings. All experiments were performed in triplicate.

**Generation and isolation of recombinant hBD-3 (rhBD-3)**

rhBD-3 was produced by cloning hBD-3 cDNA into pET-30c (from J. Härdor and J. Schröder, Kiel University, Germany) (Novagen) as described previously (5). LPS presence was deemed negligible by Charles River Laboratories, i.e., <5 EU/mg rhBD-3 and representing <0.01 ng/ml in our assay system.

**Competitive binding assays using 125I labeled SDF-1/RANTES**

CEM X4/R5 cells were washed and resuspended in balanced salt solution (BSS) containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM D-glucose, 1 mg/ml BSA, and 20 mM NaHEPES (pH 7.5). One million cells in a final volume of 300 μl were placed in each tube along with 0.1 μM 125I-labeled SDF-1 or RANTES (PerkinElmer NEN). Unlabeled SDF-1 or RANTES (1 μg/ml for each) or various concentrations of hBD-3 were added simultaneously. Only 125I-labeled SDF-1 or 125I-labeled RANTES was added to control tubes. Cells were incubated at 37°C for 30 min, or on ice for 4 h, and then washed three times with BSS. Cellular binding of 125I-labeled SDF-1 or 125I-labeled RANTES was determined by gamma counter readings. All experiments were performed in triplicate.

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Fluorometric analysis of cytosolic Ca\(^{2+}\):

CEM and THP-1 cells were washed in PBS and resuspended at a concentration of \(-10^6\) cells/ml in BSS. The cell suspension was supplemented with 1 mM fura 2-AM (Molecular Probes) and incubated at 37°C for 45 min. Cells were pelleted, washed once, and resuspended in fresh BSS to a density of 10^6 cells/ml. Cytosolic Ca\(^{2+}\) levels in 1.5 ml of stirred cell suspensions were assayed fluorometrically at 37°C using equipment and calibration protocols that have been described previously (6). The cells were treated with hBD-3, SDF-1, rhBD-3 before SDF-1, carbachol, ATP, or fMLP (all from Sigma-Aldrich) that were added as 0.5- to 15-μM aliquots from concentrated stocks. Cells were then permeabilized with 50 μg/ml digitonin (Sigma-Aldrich) to facilitate calibration of fura-2 fluorescence as a function of extracellular Ca\(^{2+}\) levels.

Confluent microscopy

CEM X4/R5 cells or CD4\(^+\) PBMCs were collected, washed twice with PBS, resuspended in medium containing 0.5% FBS, with or without 20 μg/ml rhBD-3, followed by incubation at 37°C in 5% CO\(_2\) for 2 h. Cells were treated with FACS/Perm (BD Pharmingen) at room temperature, 90 min, followed by washing three times with PBS. Cells were stained with PE-labeled anti-CXCR4 mAb (clone 12G5; BD Pharmingen) at room temperature, 10 min, and then washed three times with PBS. Samples were observed using a dual scanning confocal microscope system (Zeiss LSM 510; Oberkochem) and analyzed with the Zeiss LSM Image Browser.

Western blot analysis of ERK1/2 activation

CEM X4/R5 cells or CD4\(^+\) PBMCs were resuspended at a concentration of 10^6 cells/ml in BSS. Aliquots (500 μl) were pretreated with either 10 nM SDF-1, 20 μg/ml, hBD-3 before SDF-1, carbachol (10 μM), or phorbol myristate acetate (Sigma-Aldrich) for 5 min. Reactions were terminated by transfer to an ice-bath and centrifugation to pellet the cells. Following aspiration and ice-cold PBS washing, the pelleted cells were routinely disrupted in 200 μl of lysis buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM β-glycerol phosphate, 2 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 2 mM PMSF, 0.1% aprotinin, and 10 μg/ml leupeptin. After 20 min on ice, the lysate was centrifuged at 10,000 × g for 10 min at 4°C. 150 μl of the supernatant was denatured by addition of 4× Laemmli sample buffer (7) and boiling for 5 min. The extracted proteins were separated by SDS-PAGE on 12% gels (Bio-Rad) and transferred to 0.45-μm Immobilon-P polyvinylidene difluoride membranes (Millipore). The blots were serially probed with anti-phospho-ERK1/2 (Thr\(^{202}\)/Tyr\(^{204}\)) mouse mAbs and then anti-total ERK1/2 rabbit polyclonal Abs (Cell Signaling Technology). Bound primary Abs were visualized with appropriate peroxidase-coupled secondary Abs (Santa Cruz Biotechnology) using Enhanced Luminol Chemiluminescence Reagent (Amersham Biosciences).

Chemotaxis assays

Chemotaxis assays were performed as described by Chernock et al. (8). Briefly, Jurkat cells (clone E6–1; human T cell line; ATCC TIB-152) or activated primary T lymphoblasts (from Dr. A. Levine, Department of Gastroenterology, Case Western Reserve University, Cleveland, OH; prepared as previously described (9)) were washed and resuspended in RPMI 1640 with 0.5% FBS at a concentration of 1 × 10^7 cells/ml. A 100-μl aliquot of each cell type was added into respective Transwell inserts of chemotaxis chambers (Trounwell Permeable Supports; Corning), where the lower compartment contained 600 μl of RPMI 1640 and 0.5% FBS. SDF-1 (100 ng/ml) and hBD-3 (0–40 μg/ml) were added into each lower compartment except in control chambers. After incubation at 37°C for 3 h, inserts were removed, and cells that migrated into the lower chamber were counted.

Results and Discussion

We previously reported that interaction of hBDs with the CEM lymphoid cell line resulted in decreased surface CXCR4 expression and concomitant internalization of the defensin peptides (4). Given these observations, we surmised that CXCR4 receptors were also internalized in response to hBD exposure. Herein, we show that hBD-3 treatment of CEM cells results in CXCR4 internalization as detected by confocal microscopic analysis of permeabilized cells stained with anti-CXCR4 Abs (Fig. 1B). This is particularly apparent in confocal views of cells appearing in the Z axis (arrows), where hBD-3 treated cells show dense internal staining. Untreated cells demonstrate

![FIGURE 1](image1.png)

**FIGURE 1.** Confocal microscopy analysis of hBD-3 on CXCR4 trafficking. Live CEM X4/R5 cells were permeabilized and stained with anti-CXCR4 PE Abs (A) or pre-treated with hBD-3, followed by permeabilization and staining with anti-CXCR4 PE Abs (B). Arrows show cells in the Z axis.

![FIGURE 2](image2.png)

**FIGURE 2.** Inhibition of 125I-labeled SDF-1 binding to CEM cells by hBD-3. CEM X4/R5 cells were treated simultaneously with either 125I-labeled SDF-1 (A and B), 125I-labeled RANTES (C) together with increasing concentrations of hBD-3, or unlabeled chemokine as indicated. Ligand binding to cells was assessed by gamma counter readings. Lane 1 (A and B), 125I-labeled SDF-1 alone; lane 1 (C), 125I-labeled RANTES; lane 2–6, increasing concentrations of hBD-3 at 1, 5, 10, 20, and 40 μg/ml, respectively; lane 7 (A and B), 1 μg/ml unlabeled SDF-1; lane 7 (C), 1 μg/ml unlabeled RANTES. Data are presented as mean ± SD of three independent experiments. Experiments shown in A and C were conducted at 37°C, whereas the experiment shown in B was conducted on ice.
CXCR4 surface localization (Fig. 1A). These results are consistent with our previous FACS findings showing a significant reduction in CXCR4 surface expression when unstimulated human PBMCs or CEM cells were incubated with hBD-3 (4).

To elucidate further the interaction of hBD-3 with CXCR4, we conducted a competition assay to assess the ability of hBD-3 to inhibit the binding of the CXCR4 ligand SDF-1 to CEM cells expressing both CXCR4 and CCR5. hBD-3 inhibited the binding of 125I-labeled SDF-1 (Fig. 2A) but not the binding of 125I RANTES (Fig. 2C) in a dose-dependent manner (Fig. 2A). When the experiment was conducted at 4°C to inhibit internalization of CXCR4, inhibition of 125I-SDF-1 binding was also seen (Fig. 2B), indicating that hBD-3 and SDF-1 compete for binding to common or proximate cell surface sites.

Since hBD-3 promoted internalization of CXCR4 and blocked SDF-1 binding, we anticipated that hBD-3 would also elicit calcium mobilization by activating the G protein-effector cascades regulated by agonist-occupied CXCR4. To our surprise, at concentrations ranging from 2.5 to 20 μg/ml (0.48–3.9 μM), hBD-3 did not induce a calcium mobilization response in CEM cells but rather blocked the calcium flux induced by SDF-1 in a dose-dependent manner (Fig. 3, A–C) as soon as 1 min after application (data not shown). Importantly, hBD-3 could not block the calcium mobilization induced by carbachol (Fig. 3, A–C), indicating that this defensin had no effect on the M1-muscarinic receptors also expressed in CEM cells. This result prompted us to test other ligands to assess hBD-3 specificity in inhibiting calcium signaling through other GPCRs.

**FIGURE 3.** Inhibition of SDF-1-induced calcium mobilization by hBD-3. CEM X4/R5 cells, differentiated THP-1 cells, or human T lymphocytes were loaded with fura-2 AM and assayed for changes in cytosolic Ca²⁺ as described in Materials and Methods. A–C, CEM cells were treated with the indicated concentrations of hBD-3 (or water vehicle) for 5 min before stimulation with 10 nM SDF-1; after an additional 2 min, the cells were stimulated with 10 μM carbachol. D, CEM cells were treated without (black line) or with (blue line) 20 μg/ml hBD-3 for 5 min before stimulation with 30 nM RANTES and then 10 μM carbachol. E, THP-1 cells were treated without (black line) or with (blue line) 20 μg/ml hBD-3 before stimulation with 10 nM SDF-1 and then 0.3 μM fMLP. F and G, Human T lymphocytes were treated without (black line) or with (blue line) 20 μg/ml hBD-3 before stimulation with 10 nM SDF-1. Con A was used as a positive control (G). HBD-3 did not inhibit Con A-induced calcium mobilization (G).

**FIGURE 4.** Inhibition of SDF-1-induced ERK-1/2 phosphorylation by hBD-3. Protein extracts from either CEM cells (A) or CD4⁺ T cells (B) pretreated with either SDF-1, hBD-3, hBD-3 before SDF-1, carbachol, or PMA (+ control) were separated by SDS-PAGE and probed with the anti-phospho-ERK-1/2 mAb (lower panel in A and B) or the anti-ERK-1/2 Ab (upper panel in A and B).
G protein-coupled receptors (GPCRs). We found that hBD-3 had no effect on RANTES-induced calcium signaling in CEM cells (Fig. 3D) or human PBMCs (data not shown), or on calcium signaling elicited by fMLP (Fig. 3E) or ATP (data not shown) in the THP-1 monocyte cell line. Similar to its actions in CEM cells, hBD-3 inhibited SDF-1-induced calcium mobilization in these THP-1 cells (Fig. 3E) and in peripheral blood human T lymphocytes (Fig. 3, F and G). Thus, hBD-3 can repress SDF-1-dependent calcium signaling in both lymphoid and myeloid cell lines and in primary human lymphocytes.

In addition to mobilizing calcium, signal transduction through SDF-1/CXCR4 binding coordinates activation of MAPK pathways, such as ERK (ERK-1/2) (10, 11). Therefore, to define further the antagonism of SDF-1/CXCR4 interactions by hBD-3, we assessed the ability of hBD-3 to inhibit SDF-1-induced phosphorylation of the ERK-1/2 MAPK. We found that hBD-3 did not induce ERK-1 or ERK-2 phosphorylation but abrogated SDF-1-dependent ERK1/2 activation in both CEM X4/R5 cells and in freshly isolated CD4+ PBMCs (Fig. 4).

Finally, since SDF-1/CXCR4-dependent lymphocyte locomotion is well established (12) and since our experiments up to this point indicated that hBD-3 acts antagonistically toward CXCR4, we surmised that it would block SDF-1-induced T cell chemotaxis. Our chemotaxis assays demonstrated that hBD3 did indeed reduce SDF-1-dependent chemotaxis in a dose-dependent manner in both Jurkat cells and activated human T cells (Fig. 5, A and B).

The experiments reported here demonstrate that rhBD-3 induces internalization of CXCR4 but elicits little internalization of the receptor (22). Cai et al. (21) reported recently that rSDF-1 lacking the C-terminal propeptide (residues 12 through 17 of SDF-1) is required for functional activation of CXCR4 but elicits little internalization of the receptor (22). Taken together, these data indicate that CXCR4 may conceivably assume multiple conformations that can independently regulate G protein coupling vs receptor internalization.

SDF-1/CXCR4 interactions have been implicated in a number of vital biological functions, including hemopoiesis, neurogenesis, angiogenesis, cardiogenesis, and immune cell trafficking (12, 23–25). Our findings indicate that hBD-3 may regulate some of these activities at selected sites such as those proximate to mucosal surfaces where the concentration of hBDs is high (26, 27). These findings also indicate that calcium influx and ERK1/2 phosphorylation are not necessary to induce CXCR4 internalization and thus implicate the likely importance of other as yet undescribed mechanisms in trafficking of CXCR4. This novel finding also may lead to a better understanding of how to inhibit the SDF-1/CXCR4 axis by use of endogenous agents such as hBD-3 or its derivatives and also may provide a new direction for drug design.

**FIGURE 5.** Inhibition of SDF-1-induced chemotaxis in Jurkat cells and activated human T cells. Jurkat cells or activated human T cells, in the presence or absence of 100 ng/ml SDF-1, along with differing concentrations of hBD-3 (0–40 μg/ml), were assayed for chemotaxis as described in Materials and Methods. A, Results for Jurkat cells; B, results for activated T cells. Data are presented as mean ± SD of three independent experiments.
Disclosures
The authors have no financial conflict of interest.

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