Journal of Cellular Biochemistry 104:2286–2297 (2008)

Human Beta Defensin 2 Promotes Intestinal Wound Healing In Vitro

Jan-Michel Otte,[1]* Ilka Werner,[1] Stephan Brand,[2] Ansgar M. Chromik,[3] Frank Schmitz,[1] Michael Kleine,[4] and Wolfgang E. Schmidt[1]

[1]Department of Internal Medicine I, St. Josef-Hospital, Ruhr-University, Bochum, Germany
[2]Department of Medicine II, Ludwig-Maximilians-University, Munich, Germany
[3]Department of Surgery, St. Josef-Hospital, Ruhr-University, Bochum, Germany
[4]Planton GmbH, Kiel, Germany

Abstract Limiting microbial threats, maintenance and re-establishment of the mucosal barrier are vital for intestinal homeostasis. Antimicrobial peptides have been recognized as essential defence molecules and decreased expression of these peptides has been attributed to chronic inflammation of the human intestinal mucosa. Recently, pluripotent properties, including stimulation of proliferation and migration have been suggested for a number of antimicrobial peptides. However, it is currently unknown, whether the human β-defensin 2 (hBD-2) in addition to its known antimicrobial properties has further effects on healing and protection of the intestinal epithelial barrier. Caco-2 and HT-29 cells were stimulated with 0.1–10 μg/ml hBD-2 for 6–72 h. Effects on cell viability and apoptosis were monitored and proliferation was quantified by bromo-deoxyuridine incorporation. Migration was quantified in wounding assays and characterized by immunohistochemistry. Expression of mucins was determined by quantitative PCR and slot-blot analysis. Furthermore, anti-apoptotic capacities of hBD-2 were studied. Over a broad range of concentrations and stimulation periods, hBD-2 was well tolerated by IECs and did not induce apoptosis. hBD-2 significantly increased migration but not proliferation of intestinal epithelial cells. Furthermore, hBD-2 induced cell line specific the expression of mucins 2 and 3 and ameliorated TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. In addition to its known antimicrobial properties, hBD-2 might have further protective effects on the intestinal epithelium. Results of this in vitro study suggest, that hBD-2 expression may play a dual role in vivo, i.e. in impaired intestinal barrier function observed in patients with inflammatory bowel disease. J. Cell. Biochem. 104: 2286–2297, 2008.

Key words: Crohn's disease; human beta defensin 2; inflammatory bowel disease; intestinal barrier function; wound healing

Mucosal surfaces of vertebrates such as the one in the human gastrointestinal tract are constantly exposed to environmental threats including a multitude of microbial organisms. In addition to forming a physical barrier, the intestinal epithelium has been identified to actively participate in host defence by sensing and responding to luminal and invading microorganisms [Rakoff-Nahoum et al., 2004]. Antimicrobial peptides expressed and secreted by epithelial cells represent essential effector molecules of the innate immune system [Boman, 2000]. One member of the cathelicidin family and three subfamilies of the so-called defensins, α-, β- and θ-defensins have yet been identified in vertebrates including humans [Lehrer, 2004]. Defensins have been shown to most effectively kill microbes mostly by compromising their membrane integrity [Matsuzaki et al., 1999; Shai, 1999]. Limiting invasion by pathogens as well as controlling the composition of the luminal flora are one prerequisite of intestinal homeostasis, the other being re-establishment of the barrier integrity once the epithelial layer has been disrupted. Proliferation and migration

Abbreviations used: BrdU, bromo-deoxyuridine; hBD-2, human beta-defensin 2; IEC, intestinal epithelial cell; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium), PTX, pertussistoxin.

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: OT 186/10-1.

*Correspondence to: Jan-Michel Otte, MD, Department of Medicine I, St. Josef-Hospital, Ruhr-University, Gudrunstrasse 56, 44791 Bochum, Germany.
E-mail: jan-michel.otte@rub.de

Received 21 June 2007; Accepted 17 March 2008
DOI 10.1002/jcb.21787

have been described as key mechanisms of mucosal repair, which are induced by a plenitude of mitogens via autocrine and paracrine pathways [Podolsky, 1997]. In addition to its direct antimicrobial capacities, hBD-2 has been reported to exert additional receptor mediated effects. It has been shown to promote adaptive immune responses by attracting dendritic and T cells as well as neutrophils by specifically binding to expressed CCR6 receptors [Yang et al., 1999; Niyonsaba et al., 2004]. Furthermore, hBD-2 exerted dose dependent effects on viability and proliferation of cultured human cervical cells [Markeeva et al., 2005]. Putative mitogenic and motogenic effects of this antimicrobial peptide on intestinal epithelial cells, however, have not been reported yet. Interestingly, we recently demonstrated that the CC chemokine ligand (CCL) 20 (MIP-3α, LARC, Exodus), which is – similar to hBD-2 – also a chemokine receptor (CCR) 6 ligand, increases IEC migration and proliferation [Brand et al., 2006a].

Disruption of the barrier integrity and invasion of microbes with subsequent chronic inflammation and further disturbance of the mucosal architecture are hallmarks of inflammatory bowel disease such as Crohn's disease (CD) and ulcerative colitis (UC). The sought for key pathophysiological mechanisms has identified a decreased response to microbes due to the mutated intracellular pattern recognition receptor NOD2/CARD15 with subsequent decreased expression of alpha-defensins in individuals with ileal Crohn as well as decreased gene copy numbers of hBD-2 in patients with colonic CD [Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001; Wehkamp et al., 2005a; Fellermann et al., 2006]. Of note, the decrease of Paneth cell defensins can be found in all patients with ileal CD with NOD2 mutations further aggravating this decrease. This lack of defensins has been shown to result in a lack of antibacterial killing as well as in changes of the intestinal flora [Wehkamp et al., 2005b]. It is intriguing to speculate that decreased expression of hBD-2 contributes to perpetuated intestinal inflammation not only by diminished microbial clearance but additionally by impaired mucosal healing. The current study was therefore designed to characterize potential effects of hBD-2 on intestinal epithelial cell survival and mucosal repair. Data from this study indicate, that hBD-2 is not toxic for IEC, but rather exerts anti-apoptotic properties. This peptide was furthermore identified to promote migration of IEC and to induce the expression of protective mucins.

MATERIAL AND METHODS

Reagents and Chemicals

Recombinant hBD-2 was purchased from Platon (Kiel, Germany) and diluted to final concentrations as previously described [Sahly et al., 2006].

Cell Types and Culture Conditions

Analysis was performed utilizing the human intestinal epithelial cell lines HT-29 and Caco-2, which were obtained from the American Type Culture Collection (LGC-ATCC; Wesel, Germany) and cultured in DMEM (4 g/l glucose) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany), 2 mM L-glutamine and 1.5 g/l $NaHCO_3$. Cells were maintained at 37°C in 5% CO_2.

MTT and BrdU Assays

Cell viability in hBD-2 stimulated cultures was monitored by means of a colorimetric thiazolyl blue (MTT, Roche Diagnostics, Mannheim, Germany) assay. Briefly, 10^4 cells/ml IEC were cultured until subconfluency and then exposed to 0.1–10 μg/ml hBD-2 for 6–72 h. Utilizing the same concentrations of hBD-2 and stimulation periods, proliferation of IEC was monitored utilizing the 5-bromo-2-deoxyuridine incorporation method following the manufacturer's instructions (BrdU labelling and detection kit II, Roche Diagnostics). Proliferation was quantified as the index of BrdU-positive cells.

Apoptosis Assays

A putative induction of apoptosis in IEC challenged with various doses of hBD-2 was analyzed using a commercial detection kit following the manufacturer's guidelines (Cell Death Detection Elisa plus; Roche Diagnostics). Ameliorating effects of hBD-2 on TRAIL (10 ng/ml; Roche Diagnostics) induced apoptosis of IEC were furthermore analyzed by co-incubation versus a 12 h pre-incubation of the cells with 2 μg/ml hBD-2 followed by a 6 h exposure to TRAIL.

RNA Extraction and Expression Analysis

Total RNA was isolated from hBD-2 stimulated IEC utilizing the Qiagen Rneasy Mini kit (Qiagen, Hilden, Germany) with additional DNase treatments included. The RT reaction was performed utilizing a M-MLV (Promega, Mannheim, Germany) and PCR amplification was performed with *Taq* polymerase (Perkin-Elmer, Wiesbaden, Germany) for 32 cycles at 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min. Expression of GAPDH served as internal control and amplification of samples without prior RT was used as negative control. Primers for the detection of mucins 1, 2, 3 and 5AC as well as the chemokine receptor CCR6 were chosen according to published gene sequences and are provided in Table I.

PCR Conditions for Real-Time Analysis

PCR amplification was done with Fast Start DNA Sybr Green I (Roche) and automated quantification in real-time utilizing the Light Cycler (Roche). Two microliters of the total reverse transcription volume were used for individual PCRs, which were performed in a total volume of 20 μl containing 1× PCR buffer [3 mM $MgCl_2$; 10 mM Tris–HCl (pH, 8.3); 50 mM KCl; 5% glycerol; 200 μM dATP, dCTP, and dGTP; 400 μM dUTP], 500 nM of each primer, H_2O and Fast Start DNA Sybr Green I. Thermal cycling was edited to include 10 min at 95°C, and then 45 cycles at 95°C for 15 s, 54–56°C for 5 s and 72°C for 15 s followed by a melting curve analysis. For individual primer pairs, optimal annealing temperatures were calculated and adapted under experimental conditions. All analysis comprehended independent RNA extraction and reverse transcription, which were performed in duplicate. In addition to GAPDH, which served as a positive control, two standard negative controls were included. Quantitative PCR results were calculated by the ΔΔCt cycle threshold method. For quality analysis, PCR products were subjected to electrophoresis on 1.5% agarose gels and ethidium bromide staining.

TABLE I. Sequences of Primer Pairs Utilized for Specific Amplification of the Genes Indicated

Gene	Sequence
GAPDH fwd	5′-cggagtcaacggatttggtcgtat-3′
GAPDH rev	5′-agccttctccatggtggtgaagac-3′
CCR6 fwd	5′-gcattagcatggaccg-3′
CCR6 rev	5′-gcagcttccacctgat-3′
MUC1 fwd	5′-gtgccccctagcagtaccg-3′
MUC1 rev	5′-gacgtgcccctacaagttgg-3′
MUC2 fwd	5′-actgcacattcttcagctgc-3′
MUC2 rev	5′-attcatgaggacggtcttgg-3′
MUC3 fwd	5′-cctcattgcaaacttcactc-3′
MUC3 rev	5′-agcccacattttctgtactg-3′
MUC5AC fwd	5′-ccaaggagaacctcccatat-3′
MUC5AC rev	5′-ccaagcgtcattcctgag-3′

Wounding Assays

Effects of hBD-2 on IEC migration was analyzed in wounding assays as described previously [Dignass et al., 1994]. Briefly, standard wounds were set in confluent IEC monolayers cultured under serum-free conditions. Cellular debris was removed and the cells were stimulated with hBD-2 in concentrations ranging from 0.1–10 μg/ml for 12–48 h. Migration of IEC across the wound margins was documented by video microscopy of four to five locations per wound at the times indicated.

A putative role of the CCR6 receptor in hBD-2 mediated migration of IEC was furthermore characterized by pre-incubating the cells for 1 h with a blocking anti-CCR6 antibody [Wang et al., 2004] (4 μg/10 μl/10^6 cells; R&D Systems Inc., Minneapolis, MN). Using an isotype control, the migration assay was performed as described above. Furthermore, an involvement of heterotrimeric G protein components was analyzed by adding pertussistoxin (100 ng/ml; Sigma; Munich, Germany) to the culture medium 1h prior to stimulation with hBD-2 (5 μg/ml; 24 h).

Immunohistochemistry

IEC were plated into 6-well plates containing glass coverslips at a density of 3×10^6/well. Twenty-four hours later the cultures were rendered quiescent by serum withdrawal for an additional 24 h. At various times, unstimulated and hBD-2 or TGF-β stimulated cells were fixed in 4% paraformaldehyde and washed in serum-free cell culture medium. The cells were permeabilized for 10 min with 0.2% Triton X-100 and then blocked with 5% dry milk in PBS containing 0.1% Tween 20 (TBS-T). To visualize filamentous actin, TRICT-coupled phalloidin (1:5,000; Sigma) was applied to cultures for 1 h. Following staining procedures, coverslips were washed and mounted (Vectashield antifade mounting medium; Vector Laboratories, Burlingame CA). Labeling of the cells was

analyzed by fluorescence microscopy including digital processing of the images (Zeiss Axioplan, Zeiss, Göttingen, Germany).

Western Blot Analysis

Quiescent and hBD-2 (5 μg/ml) stimulated HT-29 and Caco-2 cells were lyzed and homogenized in ice-cold buffer containing protease inhibitors. Protein contents were determined using the Bradford method. Ten micrograms of total protein were transferred onto nitrocellulose membranes (BioRad, Munich, Germany) following 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were blocked with 5% dry milk in PBS containing 0.1% Tween 20 (TBS-T) and incubated for 2 h at room temperature with a monoclonal anti-CCR6 antibody (1:1,000; Santa Cruz, Santa Cruz, CA). Following multiple washing steps in TBS-T and incubation with a secondary antibody (45 min; 1:1,500; Santa Cruz) specific bands were detected by use of the enhanced chemiluminescence Western blotting system (GE Healthcare, Munich, Germany). Blots were scanned (Epson Perfection 1640SU-Photo scanner; Epson, Meerbusch, Germany) and quantified by densitometry (BioRad).

Mucin Expression

Expression of mucins 1, 2, 3 and 5AC was quantified in unstimulated and hBD-2 stimulated IEC by real-time PCR. IEC were directly stimulated for 24–48 h with 2 μg/ml hBD-2. Expression of mucins 2 and 3 was furthermore characterized by slot-blot analysis utilizing specific antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Briefly, 10 μg protein were applied to a slot-blot apparatus and transferred to nitrocellulose membranes (Bio-Rad), air dried and blocked for 1 h in 5% dry milk in PBS containing 0.1% Tween 20 (TBS-T). Blotted proteins were detected by use of specific primary antibodies (Santa Cruz Biotechnology Inc.), visualized and quantified as described above for Western blot analysis.

Statistical Analysis

Data are expressed as mean ± SEM of at least three independent experiments. Comparison between two experimental groups was performed using the Student's t test. Mean values among more than two groups were compared by the one-way analysis of variance. A P value of less than 0.05 was considered as significant.

RESULTS

Cell viability is not Affected by hBD-2

hBD-2 is not toxic for IEC. Over a broad range of concentrations (0.1–10 μg/ml) and incubation periods (6–72 h) hBD-2 did not affect incorporation of MTT in Caco-2 and HT-29 cells. Figure 1A shows data obtained in Caco-2 cells following 24 h of stimulation with various doses of hBD-2. Comparable results were detected in HT-29 cells incubated with hBD-2 (data not shown). Furthermore, a challenge to hBD-2 for 6–72 h in concentrations ranging from 0.1–10 μg/ml did not significantly increase the level of apoptosis in Caco-2 (Fig. 1B) and HT-29 cells (data not shown).

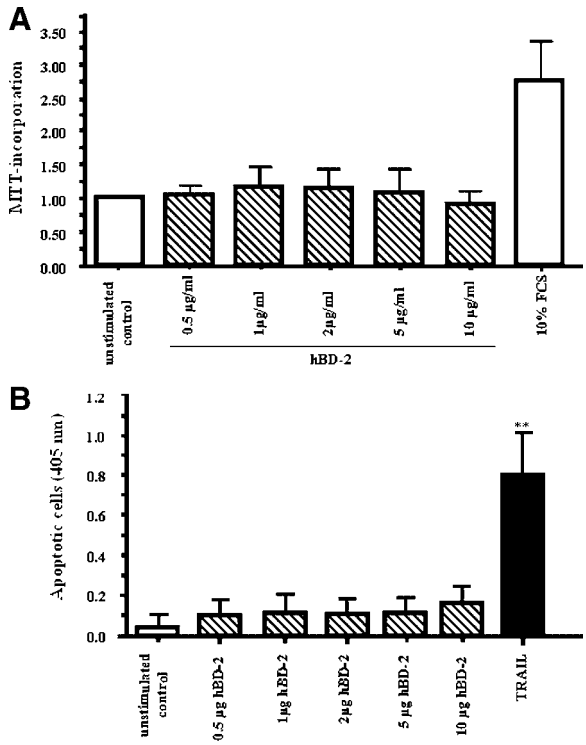

Fig. 1. Effects of hBD-2 on cell viability and apoptosis in IEC. Caco-2 cells were incubated for 24 h with hBD-2 using concentrations as indicated (cross hatched bars). Cell viability was determined by a MTT assay (**A**) with results detected in unstimulated controls set as 1 (white bar). Furthermore, apoptotic cells were identified by labeling histone-complex DNA fragments in unstimulated (white bar) and hBD-2 stimulated IEC (cross hatched bars). Incubation with TRAIL (10 ng/ml; black bar) served as a positive control (**B**). Shown are results obtained with Caco-2 cells after 24 h of stimulation with hBD-2 in the concentration indicated. Values are means ± SEM of five independent experiments. AU, arbitrary units; BrdU, 5-bromo-2-deoxy-uridine; FCS, fetal calf serum; MTT, colorimetric thiazolyl blue assay; MUC, mucin; ns, non-significant.

hBD-2 Does not Induce IEC Proliferation

Proliferation of intestinal epithelial cells Caco-2 and HT-29 as assessed by means of a BrdU incorporation assay was not significantly altered by hBD-2 (0.1–10 μg/ml) applied to cultured cells for 12–72 h. Results determined after 24–48 h of stimulation in Caco-2 cells are shown in Figure 2. Similar results were observed in HT-29 cells (data not shown).

hBD-2 Significantly Stimulates Migration of IEC

Effects of hBD-2 on cell migration as an important characteristic of intestinal wound healing was monitored in IEC following stimulation with hBD-2 in various concentrations (1–5 μg/ml) for 12–72 h. A significant increase in cells migrating across wound margins was observed in IEC monolayers after 24 h of stimulation with hBD-2 with a further increase observed up to 72 h. Maximal increase in cell migration compared to unstimulated controls was detected when cells were stimulated for 48 h with 2 μg/ml hBD-2. Figure 3 shows representative photographs and data obtained with HT-29 cells. A migratory response upon stimulation with hBD-2 was also observed in Caco-2 cells although due to morphological characteristic the effect was difficult to quantify in that cell line.

Effects of hBD-2 on IEC migration were furthermore characterized by analyzing the hBD-2 stimulated dissolution and re-organization of stress fibres. As shown in Figure 4, F-actin staining revealed a striking organization of stress fibres in quiescent IEC (Fig. 4A). Stimulation with hBD-2 for 15 min dramatically reduced both the number and the intensity of stress fibres (Fig. 4B). On later time points, the stress fibres were re-organized and showed an expression concentrated in the periphery of cells (Fig. 4C).

To test the hypothesis that the migratory response evoked by hBD-2 is mediated via CCR6 receptors, expression of this receptor was analyzed by real-time PCR (data not shown) and Western blotting. As shown in Figure 5, quiescent IEC express CCR6 protein with a further, significant increase observed in hBD-2 stimulated cells (Fig. 5A). Furthermore, following blockage of expressed CCR6 receptors by use of a specific antibody, a significant decrease in hBD-2 induced migration of IEC was observed (Fig. 5B).

In addition, a putative role of G-protein coupled receptors in hBD-2 stimulated migration was

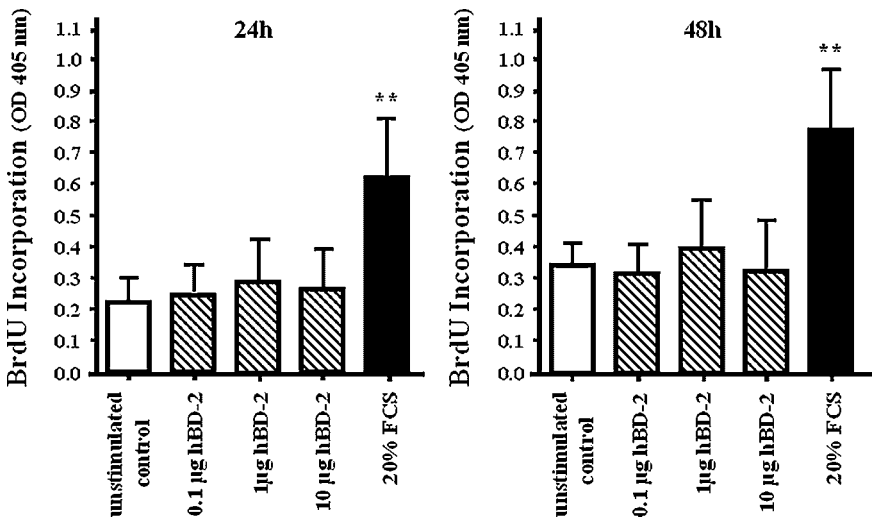

Fig. 2. Effects of hBD-2 on IEC proliferation. Proliferation in hBD-2 stimulated IEC was analyzed by a BrdU incorporation assays. IEC were incubated with hBD-2 (0.1–10 μg/ml; cross hatched bars) for 12–72 h with BrdU being added during the last hour of incubation. Shown is the percentage of the BrdU-positive Caco-2 cells (BrdU incorporation index) after 24 h and 48 h of stimulation. Serum stimulated cells served as positive control (black bars). Data are expressed as means ± SEM of four independent experiments, $^{**}P < 0.01$ (compared to unstimulated controls; white bars). Refer Figure 1 for abbreviations.

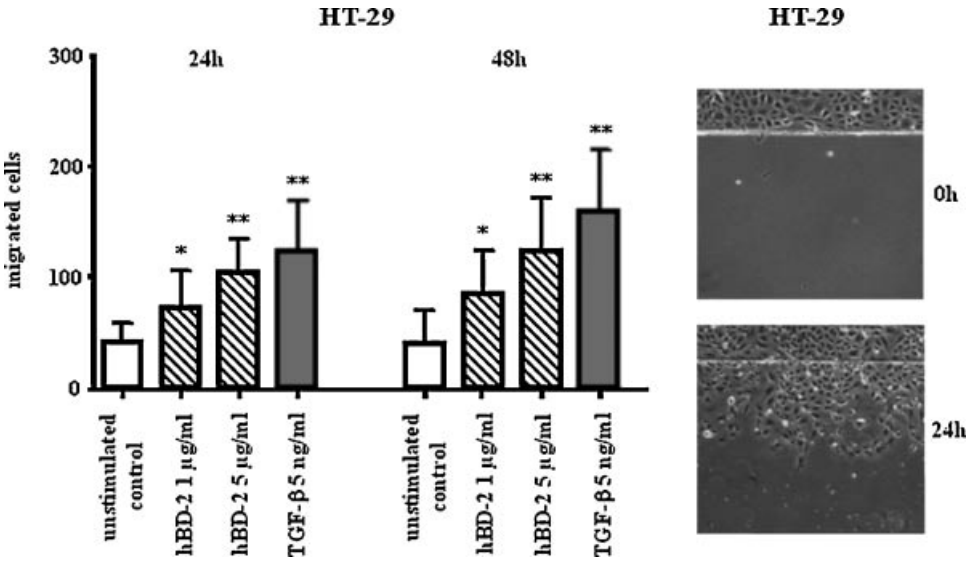

Fig. 3. Effect of hBD-2 on intestinal epithelial wound healing. Mechanically wounded HT-29 cells were incubated with different concentrations of hBD-2 (cross hatched bars) or, as a control, 5 ng/ml TGF-β (grey bars). Photomicrographs at 100× magnification were taken at 24 and 48 h after wounding, and the number of cells migrated across the wound margin were counted. Shown are representative photographs and the means ± SEM of six independent experiments (n = 15 per group); $^{**}P < 0.01$; $^{*}P < 0.05$ (compared to unstimulated controls; white bars). Refer Figure 1 for abbreviations.

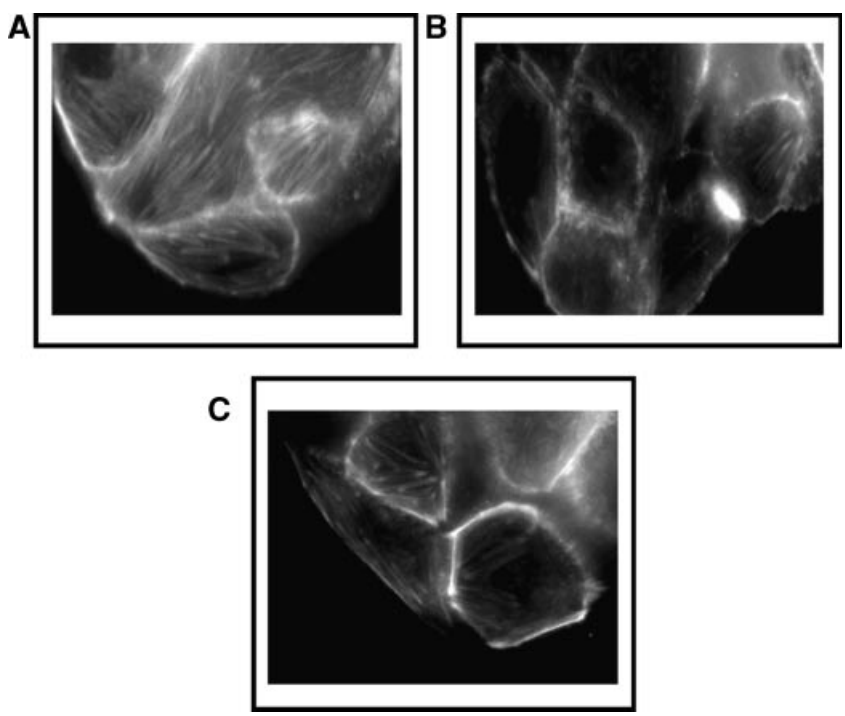

Fig. 4. Acute effect of hBD-2 on the reorganization of cytoskeletal actin in IEC. HT-29 cells remained unstimulated (**A**) or were stimulated with 5 µg/ml hBD-2 for 15 min (**B**) or 60 min (**C**). Effects on the number and intensity of stress fibres was determined by phalloidin staining. Shown are representative photographs of n = 5 independent evaluations.

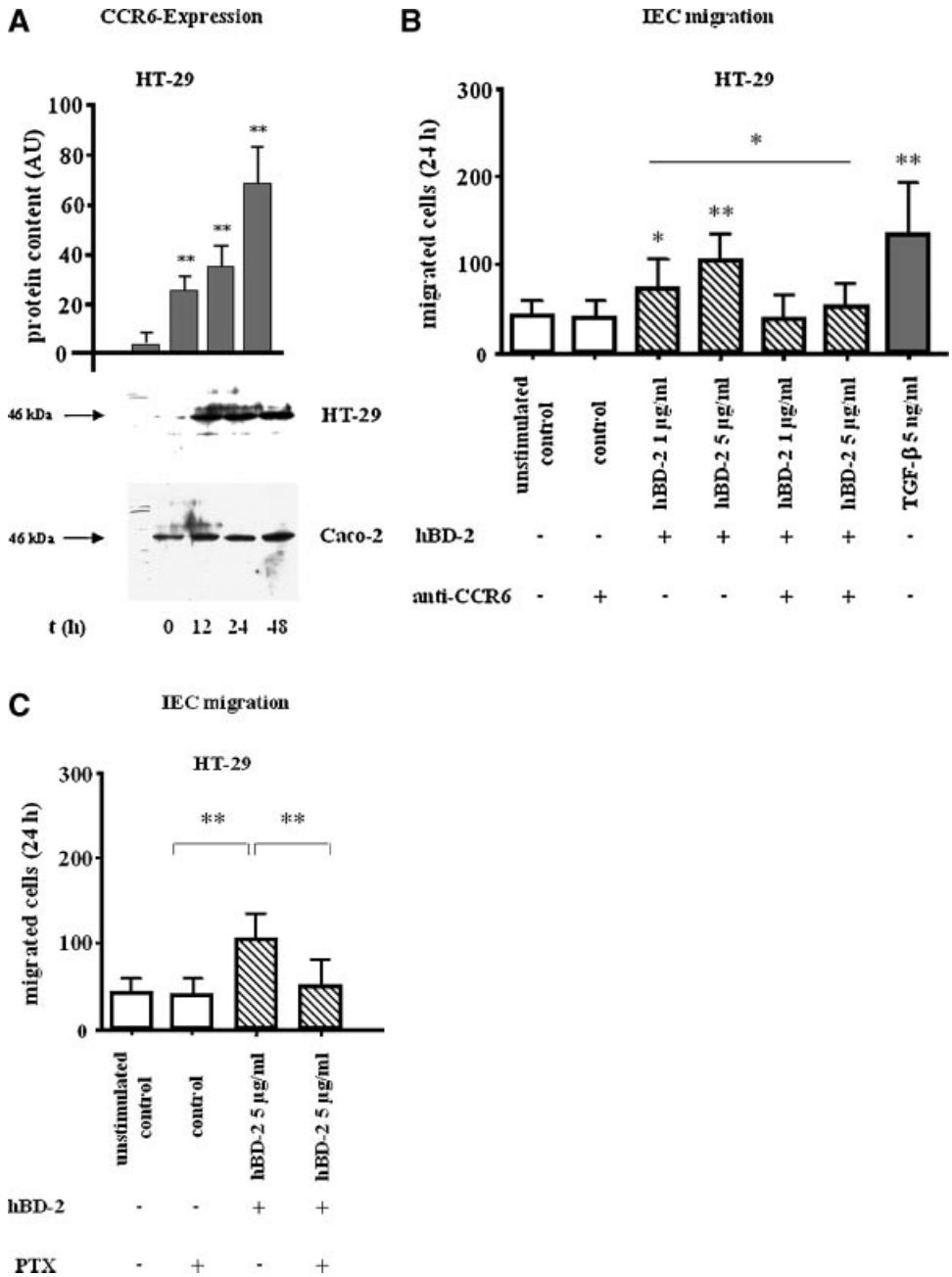

Fig. 5. Role of CCR6 in hBD-2 induced migration of IEC. Protein expression of the CCR6 receptor was analyzed in quiescent and hBD-2 (5 µg/ml; 12–48 h) stimulated IEC. Shown are the means ± SEM (**$P < 0.01$) of 6 independent experiments compared to unstimulated controls) and a representative, original Western blot (**A**). CCR6 receptors were furthermore blocked by pre-incubating IECs prior to stimulation with hBD-2 (1 and 5 µg/ml; 24 h) with an anti-CCR6 antibody. Shown are the means ± SEM (*$P < 0.05$; **$P < 0.01$) of four independent assays obtained in HT-29 cells (**B**). IEC were incubated with PTX (100 ng/ml) and stimulated with hBD-2 (5 µg/ml; 24 h). Migration was assayed as described. Shown are the results of n = 6 independent assays; **$P < 0.01$ (**C**). Refer Figure 1 for abbreviations.

analyzed by pre-incubating IEC with PTX prior to stimulation with hBD-2. As shown in Figure 5C, blockage of G-proteins significantly decreased the migratory response of IEC to hBD-2.

It was furthermore analyzed whether wounding of epithelial layers induces expression of hBD-2. Expression of this defensin was therefore analyzed in resting and wounded IEC. HBD-2 was detected at low levels in quiescent

IEC (Caco-2), with no further increase observed in wounded or migrating cells (data not shown).

Expression of Mucins 2 and 3 is Significantly Upregulated by hBD-2

Mucins (MUC) are known as essential elements of the protective barrier separating the luminal content from the epithelial cells. To test the possibility of the induction of this protective factor by a defensin, expression of MUC1, 2, 3 and 5AC was analyzed in IECs stimulated with hBD-2 (1–10 μg/ml). Maximal and significant alterations were observed following 24 h and more pronounced after 48 h of incubation with 5 μg/ml hBD-2. Whereas expression of MUC1 and 5AC was not significantly altered in HT-29 cells, expression levels for MUC2 and 3 were significantly upregulated (Fig. 6). In Caco-2 cells, hBD-2 significantly upregulated the expression of MUC2 only, whereas the other mucins were not upregulated (data not shown). In correlation to mRNA data, protein of MUC2 and 3 was upregulated following stimulation of IEC with hBD-2. Following a 24 h incubation period significantly upregulated MUC2 and 3 levels were detected in HT-29 cells by slot-blot analysis, whereas in Caco-2 cells expression of MUC2 only was upregulated. Regulation of mucin expression was dose dependent with an increase already detected following stimulation of IEC with 2 μg/ml of hBD-2 and maximum levels reached at 10 μg/ml.

Effect of hBD-2 on IEC Apoptosis

As indicated above, various concentrations of hBD-2 did not affect the rate of apoptosis in stimulated IEC whereas TRAIL, a well known pro-apoptotic substance [Begue et al., 2006], induced a significant increase in apoptotic cells in Caco-2 and HT-29 cultures when applied for 6 h (10 ng/ml). However, after a 12 h pre-incubation period with 2 μg/ml hBD-2 followed by a 6 h challenge to TRAIL (10 ng/ml), the TRAIL induced increase in IEC apoptosis was significantly diminished (Fig. 7). This effect was not observed when the IEC were co-incubated with hBD-2 and TRAIL (Fig. 7).

DISCUSSION

Defensins as endogenous peptides with broad antimicrobial activity have been recognized as key effector molecules of innate immune responses [Lehrer, 2004]. Expression of these peptides has been detected in various human tissues including the epithelial lineages of the gastrointestinal tract [Bevins et al., 1999; Ouellette, 2004; Wehkamp et al., 2005b]. In addition to constitutively expressed defensins such as hBD-1, inducible forms of this

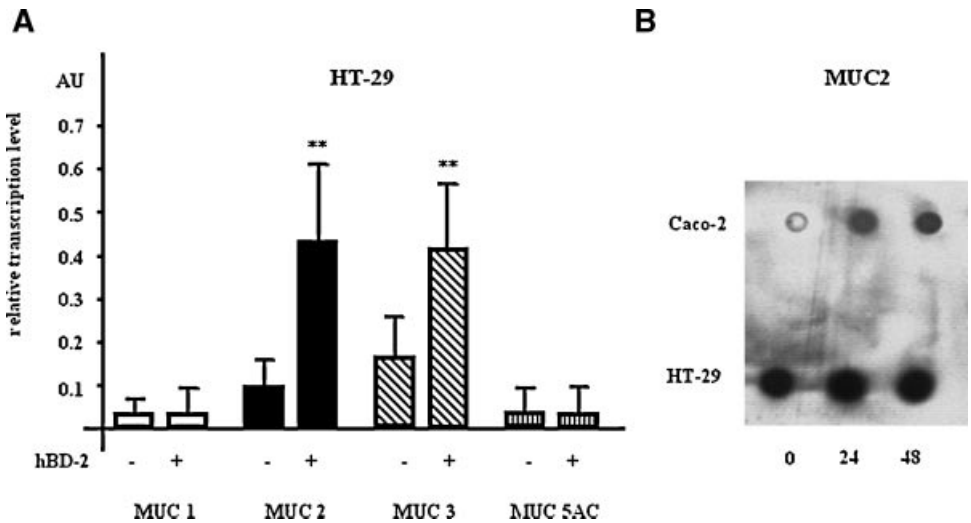

Fig. 6. hBD-2 induced mucin expression in IEC. Basal and hBD-2 induced expression of MUC1 (white bars), 2 (black bars), 3 (cross hatched bars) and 5AC (striped bars) in intestinal epithelial cells. Shown are results of HT-29 cells stimulated with 2 μg/ml hBD-2 for 24 h. Expression of mucins was quantified by real-time PCR (means ± SEM of four independent assays) Also shown are representative blots for MUC2 expression in HT-29 and Caco-2 cells as well as the densitometric evaluation of 3 independent experiments; $^{**}P < 0.01$ (compared to unstimulated controls). Refer Figure 1 for abbreviations.

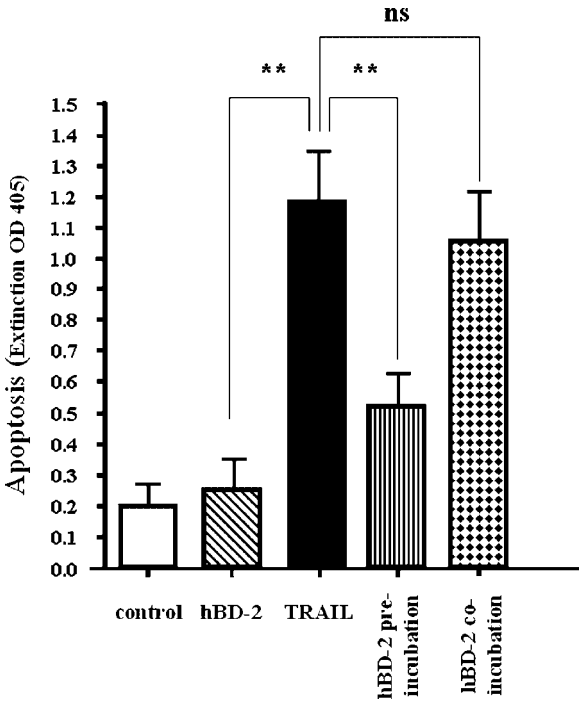

Fig. 7. Anti-apoptotic properties of hBD-2 in IEC. Putative modulating effects of hBD-2 on induced apoptosis in IEC were assayed by pre-incubating intestinal cells for 12 h with 2 μg/ml hBD-2 (striped bars) with a subsequent challenge to TRAIL (10 ng/ml) for 6 h. In addition, cells were co-incubated with hBD-2 (spotted bars) and TRAIL for 6 h. Shown are results from analysis with Caco-2 cells. Values are means ± SEM of three independent experiments; $^{**}P < 0.01$; $^{*}P < 0.05$ (compared to cells treated with 2 μg/ml hBD-2 (cross hatched bars) or 10 ng/ml TRAIL (black bars) only). The apoptotic index in unstimulated controls is represented by white bars. Refer Figure 1 for abbreviations.

antimicrobial peptide family including hBD-2 have been described [Wehkamp et al., 2002; Fahlgren et al., 2004]. hBD-2 is expressed at low levels in epithelial cells of the intestinal mucosa with a significantly upregulated expression being observed in inflammation, i.e. following microbial invasion or stimulation by pro-inflammatory cytokines [Ogushi et al., 2001; Wehkamp et al., 2002]. In addition, an induction of hBD-2 expression has been identified as a unique mechanism of action of the probiotic organism *E. coli* Nissle 1917 [Wehkamp et al., 2004]. Collectively, these data suggest an important role for hBD-2 in mucosal defence and intestinal barrier function. Furthermore, lack of hBD-2 has been attributed to diminished antimicrobial capacity thereby facilitating the attachment and invasion of microbial organisms with a potential subsequent chronification of inflammatory responses as detected in inflammatory bowel disease such as CD [Fellermann et al., 2006]. Perpetuated mucosal inflammation is associated with extensive disturbance of the mucosal architecture which might facilitate further infiltration of microbial components. Protection and re-establishment of the intestinal barrier function are therefore prerequisites for the limitation of inflammatory processes and the maintenance of intestinal homeostasis. However, putative functions of defensins, namely hBD-2, on conservation and healing of the intestinal mucosa have not been reported yet. Indeed, antimicrobial proteins are increasingly regarded as pluripotent peptides [Hiemstra, 2006]. LL-37, the only cathelicidin found in humans, has been shown to promote proliferation and migration of epithelial cells of the respiratory tract as well as migration of keratinocytes [Shaykhiev et al., 2005; Tokumaru et al., 2005].

In the current study, hBD-2 did not alter IEC viability or cell proliferation, but dose dependently induced migration of IEC, a key feature of intestinal wound healing. Exposure of IEC to hBD-2 resulted in a reorganization of stress fibres, a well-known features of migratory actions. Although the individual effects were only moderately pronounced, a pro-migratory potential of hBD-2 can be assumed as it has also been described for human mast cells and neutrophils. In those cells, the migratory response was most likely mediated by the chemokine receptor CCR6, which is also expressed on IEC as shown in this study in agreement with previous reports [Niyonsaba et al., 2002; Niyonsaba et al., 2004; Brand et al., 2006a]. In addition, we demonstrate that blockage of CCR6 receptors resulted in a significantly decreased migratory response in hBD-2 stimulated IECs without reaching baseline levels. These data suggest, that hBD-2 induced migration is at least in part mediated by CCR6 receptors. Furthermore, inhibition of the heterotrimeric G protein i/o by pertussistoxin (PTX) significantly decreased the hBD-2 elicited migratory response of IEC, suggesting the involvement of serpentine G-protein-linked transmembrane receptors such as the chemokine receptor CCR6. In agreement, an involvement of the CCR6 receptor in hBD-2 elicited migration has previously been reported [Yang et al., 1999]. However, hBD-2 induced activation of different cell types such as mast cells has been shown to involve alternative pathways

including the G protein-phospholipase C signalling pathway [Niyonsaba et al., 2002] as well as G-alpha proteins and the MAPK pathway in macrophages [Soruri et al., 2007]. Furthermore, we have previously shown that binding of the chemokine CCL20 in IECs results in activation of ERK1/2, SAPK/JNK and Akt [Brand et al., 2006a]. CCL20, although structurally unrelated to hBD-2 has been shown to share a number of similarities with this defensin, including antimicrobial and chemotactic activities, binding to the CCR6 receptor, regulation by IL-1β and TNF-α, and the coordinated regulation by IL-17 [Kao et al., 2005].

Interestingly, we recently demonstrated that IL-22 increases cell migration and wound healing of IEC. IL-22 also increases mRNA expression of hBD-2 [Brand et al., 2006b]. Given the results of the current study, these IL-22 evoked effects may be mediated via hBD-2. However, data obtained in wounded monolayers did not reveal an upregulated expression of hBD-2 in migrating cells, making an autocrine fashion of stimulation implausible. In addition, hBD-2 might have further protective features by inducing expression of mucins, which have long been regarded as the only active defence mechanism of the intestinal epithelium but are now considered as important elements of the complex barrier network in IEC. The mucus coat plays a central role in the protection against mechanical insults, luminal proteases derived from mucosal and bacterial cells and the colonization by pathogenic bacteria and their toxins. In humans, up to date 20 different mucins have been identified, which are divided into secreted and membrane-bound mucins [Liévin-Le Moal et al., 2006]. In this aspect, we analyzed the expression of secreted, gel-forming mucins MUC2, MUC5AC and prominent membrane-associated mucins MUC1 and MUC3 in quiescent and hBD-2 stimulated IEC. As shown, hBD-2 significantly induced the expression of MUC2 and 3 in IEC, suggesting that the release of hBD-2 might induced further protective mechanisms in the intestinal mucosa. Furthermore, the decreased induction of mucin expression following blockage of CCR6 receptors suggests that the observed effect of hBD-2 on mucin expression is at least mediated in part by CCR6. Of note, previous work has detected the predominant expression and secretion of MUC2 in the healthy colonic epithelium of rodents and humans as well as in the chronically inflamed intestinal mucosa [Tytgat et al., 1994, 1995, 1996]. More recently, the important role of MUC2 in intestinal barrier formation and its potential (patho-) physiological relevance in inflammatory conditions of the intestine have been addressed in greater detail. Utilizing MUC2 deficient mice, a lack of this mucin has been associated with the onset an perpetuation of colonic inflammation [Van der Sluis et al., 2006].

It has been previously shown, that during intestinal inflammation TRAIL activated the intracellular caspase cascade in IEC leading to a rapid and potent elimination of IEC ultimately resulting in epithelial lesions as observed in infectious or chronic inflammatory disorders of the intestine [Begue et al., 2006]. We therefore hypothesized that hBD-2 might strengthen the mucosal barrier by alleviating TRAIL induced IEC apoptosis, thereby contributing to the maintenance of the epithelial barrier. As indicated, pre-incubation with hBD-2 significantly decreased TRAIL induced apoptosis in IEC. Although these preliminary data suggest an additional role of hBD-2 in the complex network underlying mucosal integrity, further research is needed to clarify the mechanisms involved. Furthermore, up to date conflicting data have been reported on the role of antimicrobial peptides in cell survival [Barlow et al., 2006]. Based on the peptide used and the cellular system studied, LL-37, for example, has been shown to be toxic in pulmonary epithelial cells but to suppress apoptosis of neutrophils and leukocytes [Shaykhiev et al., 2005; Nagaoka et al., 2006]. Differences in cellular systems and concentrations applied require further studies to fully elucidate the role of antimicrobial peptides in apoptosis. In addition, since TRAIL has been associated with the induction of the pattern recognition receptor NOD2/CARD15 in intestinal epithelial cells as well as to exert pro-inflammatory properties, one might speculate that hBD-2 also ameliorates innate immune responses of the intestinal epithelium. This should however be addressed in further studies.

In summary, the in vitro results obtained in this study suggest, that hBD-2 may contribute to the stability of the intestinal barrier function by promoting intestinal epithelial cell survival, expression of further protective factors and by increasing intestinal wound healing. Lack of or diminished expression in genetically altered individuals may be associated with decreased

microbial clearance delayed mucosal healing with perpetuation of inflammatory processes by continuous translocation of microbial components through disrupted areas of the epithelial cell layer. Further clinical studies will be needed to corroborate this hypothesis.

ACKNOWLEDGMENTS

We are grateful for the excellent technical assistance of Sarah Strauss (Bochum). This work was supported by a grant from the Deutsche Forschungsgemeinschaft (OT 186/10-1 to J.-M. Otte).

REFERENCES

Barlow PG, Li Y, Wilkinson TS, Bowdish DM, Lau YE, Cosseau C, Haslett C, Simpson AJ, Hancock RE, Davidson DJ. 2006. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. J Leukoc Biol 80(3):509–520.

Begue B, Wajant H, Bambou JC, Dubuquoy L, Siegmund D, Beaulieu JF, Canioni D, Berrebi D, Brousse N, Desreumaux P, Schmitz J, Lentze MJ, Goulet O, Cerf-Bensussan N, Ruemmele FM. 2006. Implications of TNF-related apoptosis-inducing ligand in inflammatory intestinal epithelial lesions. Gastroenterology 130(7):1962–1974.

Bevins CL, Martin-Porter E, Ganz T. 1999. Defensins and innate host defence of the gastrointestinal tract. Gut 45(6):911–915.

Boman HG. 2000. Innate immunity and the normal microflora. Immunol Rev 173:5–16. Review

Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, Goke B, Dambacher J. 2006a. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. J Cell Biochem 97(4): 709–7023.

Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkuhn T, Goke B, Auernhammer CJ, Dambacher J. 2006b. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 290(4):G827–G838.

Dignass AU, Tsunekawa S, Podolsky DK. 1994. Fibroblast growth factors modulate intestinal epithelial cell growth and migration. Gastroenterology 106(5):1254–1262.

Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML. 2004. Beta-defensin-3 and -4 in intestinal epithelial cells display increased mRNA expression in ulcerative colitis. Clin Exp Immunol 137(2):379–385.

Fellermann K, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, Bevins CL, Reinisch W, Teml A, Schwab M, Lichter P, Radlwimmer B, Stange EF. 2006. A chromosome 8 gene-cluster polymorphism with low human Beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. Am J Hum Genet 79(3):439–448.

Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG. 2001. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 357(9272):1925–1928.

Hiemstra PS. 2006. Defensins and cathelicidins in inflammatory lung disease: Beyond antimicrobial activity. Biochem Soc Trans 34(Pt 2):276–278.

Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411(6837):599–603.

Kao CY, Huang F, Chen Y, Thai P, Wachi S, Kim C, Tam L, Wu R. 2005. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. J Immunol 175(10):6676–6685.

Lehrer RI. 2004. Primate defensins. Nat Rev Microbiol 2(9):727–738.

Liévin-Le Moal V, Servin AL. 2006. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: Mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev 19(2):315–337.

Markeeva N, Lysovskiy I, Zhuravel E, Soldatkina M, Lyzogubov V, Usenko V, Potapov V, Pogrebnoy P. 2005. Involvement of human beta-defensin-2 in proliferation of transformed cells of human cervix. Exp Oncol 27(4):308–313.

Matsuzaki K, Sugishita K, Miyajima K. 1999. Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of gram-negative bacteria. FEBS Lett 449 (2–3):221–224.

Nagaoka I, Tamura H, Hirata M. 2006. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2 X7. J Immunol 176(5):3044–3052.

Niyonsaba F, Iwabuchi K, Matsuda H, Ogawa H, Nagaoka I. 2002. Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway. Int Immunol 14(4):421–426.

Niyonsaba F, Ogawa H, Nagaoka I. 2004. Human beta-defensin-2 functions as a chemotactic agent for tumour necrosis factor-alpha-treated human neutrophils. Immunology 111(3):273–281.

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411(6837):603–606.

Ogushi K, Wada A, Niidome T, Mori N, Oishi K, Nagatake T, Takahashi A, Asakura H, Makino S, Hojo H, Nakahara Y, Ohsaki M, Hatakeyama T, Aoyagi H, Kurazono H, Moss J, Hirayama T. 2001. Salmonella enteritidis FliC (flagella filament protein) induces human beta-defensin-2

mRNA production by Caco-2 cells. J Biol Chem 276(32): 30521–30526.

Ouellette AJ. 2004. Defensin-mediated innate immunity in the small intestine. Best Pract Res Clin Gastroenterol 18(2):405–419.

Podolsky DK. 1997. Healing the epithelium: Solving the problem from two sides. J Gastroenterol 32(1):122–126.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118(2):229–241.

Sahly H, Schubert S, Harder J, Kleine M, Sandvang D, Ullmann U, Schroder JM, Podschun R. 2006. Activity of human beta-defensins 2 and 3 against ESBL-producing Klebsiella strains. J Antimicrob Chemother 57(3):562–565.

Shai Y. 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462(1–2): 55–70.

Shaykhiev R, Beisswenger C, Kandler K, Senske J, Puchner A, Damm T, Behr J, Bals R. 2005. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. Am J Physiol Lung Cell Mol Physiol 289(5):L842–L848.

Soruri A, Grigat J, Forssmann U, Riggert J, Zwirner J. 2007. Beta-defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved. Eur J Immunol 37(9):2474–2486.

Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H, Yang L, Higashiyama S, Yoshimura A, Sugai M, Hashimoto K. 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J Immunol 175(7):4662–4668.

Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. 1994. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 107(5):1352–1363.

Tytgat KM, Bovelander FJ, Opdam FJ, Einerhand AW, Buller HA, Dekker J. 1995. Biosynthesis of rat MUC2 in colon and its analogy with human M UC2. Biochem J 309(1):221–229.

Tytgat KM, Opdam FJ, Einerhand AW, Buller HA, Dekker J. 1996. MUC2 is the prominent colonic mucin expressed in ulcerative colitis. Gut 38(4):554–563.

Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Buller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131(1):117–129.

Wang J, Xi L, Hunt JL, Gooding W, Whiteside TL, Chen Z, Godfrey TE, Ferris RL. 2004. Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. Cancer Res 64(5):1861–1866.

Wehkamp J, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B, Duchrow M, Wohlschläger C, Feller AC, Stange EF. 2002. Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. Eur J Gastroenterol Hepatol 14(7):745–752.

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, Schroder JM, Stange EF. 2004. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: A novel effect of a probiotic bacterium. Infect Immun 72(10):5750–5758.

Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, Shen B, Schaeffeler E, Schwab M, Linzmeier R, Feathers RW, Chu H, Lima H Jr, Fellermann K, Ganz T, Stange EF, Bevins CL. 2005a. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proc Natl Acad Sci U S A 102(50):18129–18134.

Wehkamp J, Fellermann K, Herrlinger KR, Bevins CL, Stange EF. 2005b. Mechanisms of disease: Defensins in gastrointestinal diseases. Nat Clin Pract Gastroenterol Hepatol 2(9):406–415.

Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schröder JM, Wang JM, Howard OM, Oppenheim JJ. 1999. Beta-defensins: Linking innate and adaptive immunity through dendritic and T cell C CR6. Science 286(5439):525–528.