Neutrophil elastase up-regulates human β-defensin-2 expression in human bronchial epithelial cells

Siobhan Griffin, Clifford C. Taggart, Catherine M. Greene, Shane O'Neill, Noel G. McElvaney*

Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland

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Abstract **Human β-defensin-2 (HBD-2) gene expression is induced by tumour necrosis factor-α, interleukin-1β and lipopolysaccharide. The objective of this study was to investigate the effect of neutrophil elastase (NE), a major pro-inflammatory protease, on HBD-2 expression. HBD-2 gene expression was assessed by reverse transcription polymerase chain reaction in the human bronchial epithelial cell line 16HBE14o− and primary normal human bronchial epithelial (NHBE) cells. Optimal HBD-2 expression was induced with 100 nM NE. Using a HBD-2-luciferase reporter construct, luciferase activity increased significantly in 16HBE14o− cells following incubation with NE. An increase in HBD-2 protein expression was observed in primary NHBE cells after incubation with NE as assessed by laser scanning cytometry. In conclusion, NE up-regulates HBD-2 expression in bronchial epithelial cells.**

Key words: Human β-defensin-2; Neutrophil elastase; Innate immunity

1. Introduction

Neutrophil elastase (NE) is a serine protease specifically expressed by neutrophils and stored in their primary granules [1]. It is an omnivorous protease, which is capable of degrading a wide variety of proteins and has been implicated in the pathogenesis of a number of lung diseases including cystic fibrosis (CF), pneumonia and emphysema [2–4]. However, recent evidence points towards NE directly participating in the process by which neutrophils kill bacteria [5]. Comparison of wild type and NE deficient (NE−/−) mice has revealed that NE−/− mice are more susceptible than their normal littermates to sepsis and death following intraperitoneal infection with Gram-negative bacteria [6]. Further studies have revealed that NE can cleave virulence products of *Shigella*, *Salmonella* and *Yersinia* [7]. NE can also impair host defence mechanisms by cleaving immunoglobulin and complement receptors [8]. Apart from its antibacterial activity, NE has been shown to up-regulate interleukin (IL)-8 expression by epithelial cells via a MyD88/IRAK/TRAF-6 pathway [9]. Due to the large NE burden in the CF lung, NE activation of IL-8 expression may lead to further neutrophil recruitment to the lung thus setting up a cycle of inflammation.

Not all factors secreted by the respiratory tract are pro-inflammatory. A number of proteins expressed by the respiratory tract play a part in host defence including the small cationic β-defensins. Four β-defensin proteins have been isolated, human β-defensin 1, 2, 3 and 4 (HBD-1, -2, -3 and -4), and many more β-defensin genes have been identified following a genomewide search [10]. HBD-1–4 are expressed by epithelial cells either constitutively or up-regulated in response to bacterial or pro-inflammatory stimuli [11].

In this report we demonstrate that NE up-regulates HBD-2 expression in human bronchial epithelial (HBE) cells. This result indicates that apart from having a direct bactericidal effect, NE can also up-regulate antimicrobial peptides such as HBD-2, which also have microbicidal activity. This activity of NE may be particularly important in the setting of acute infection in which NE participates in bacterial clearance, both directly and indirectly.

2. Materials and methods

2.1. Culture and stimulation of airway epithelial cells

Primary airway normal human bronchial epithelial (NHBE) cells (Clonetics Bio Whittaker, Wokingham, UK) were cultured in BEGM® BulletKit® medium supplied by the manufacturer. Before stimulation cells were seeded on a six-well plate (Greiner, Solingen, Germany) at 1×10^6 cells/well and allowed to adhere. Cells were then treated with NE (Elastin Products, Owensville, MO, USA) at doses of 0, 50 and 100 nM and cultured for 24 h before harvesting.

Lab-Tek chamber slides (Nalgene Nunc, Roskilde, Denmark) were seeded with NHBE cells at 1×10^5 cells per well. Cells were treated with 10 nM NE. Stimulation was blocked with recombinant 50 nM α1-antitrypsin (α1AT, Sigma-Ireland, Tallaght, Dublin, Ireland).

HBE cells 16HBE14o− (a gift from Dr Gruenert, University of Vermont, VT, USA) were cultured in minimum essential medium (Gibco BRL, Karlsruhe, Germany) containing 10% foetal calf serum (FCS). Before stimulation 1×10^6 cells/well were cultured in a six well plate for 24 h in FCS-free medium and were incubated with NE at 0–100 nM. Cells were cultured for 24 h before harvesting.

2.2. Laser scanning cytometry (LSC)

NHBE cells were washed in phosphate-buffered saline (PBS) and blocked with 2% bovine serum albumin and slides prepared as above were incubated at room temperature for 15 min. A 1:100 dilution of goat anti-HBD-2 antibody (a gift from Dr Paul McCray, University

*Corresponding author. Fax: (353)-1-8093808.
E-mail address: gmcelvaney@rcsi.ie (N.G. McElvaney).

Abbreviations: HBD-2, human β-defensin-2; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NE, neutrophil elastase; NHBE, normal human bronchial epithelial; HBE, human bronchial epithelial; CF, cystic fibrosis; LSC, laser scanning cytometry; α1AT, α1-antitrypsin

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of Iowa, Iowa City, IA, USA) or 1:1000 dilution of 1 mg/ml stock of isotype goat IgG was added and incubated at 4°C in the dark for 30 min. Slides were washed three times in PBS and probed with 1:10 dilution of anti-goat IgG fluorescein isothiocyanate (FITC, Dako, Glostrup, Denmark) at 4°C in the dark for 30 min. The washing step was repeated and cells were permeabilised in a 1:1 ratio of permeabilisation solution (Dako) and 0.2 μg/ml solution of propidium iodide (PI, Molecular Probes, Leiden, The Netherlands). Slides were washed in PBS and HBD-2 expression was quantified on a CompuCyte laser scanning cytometer (CompuCyte, Cambridge, MA, USA). FITC and PI cellular fluorescence of at least 5×10^3 cells were measured. The threshold contour was set on red fluorescence to detect all PI-stained nuclei. Artificially contoured debris was gated out based on contour size. Aggregated cells were gated out using an algorithm in the LSC software that finds and marks multiple cells. Individual HBD-2-expressing cells were identified and quantified using CompuCyte software on the basis of integrated green fluorescence reflecting binding of anti-goat FITC antibody.

2.3. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

After treatment cells were harvested in Tri reagent (Sigma-Ireland) and RNA was extracted as detailed in the manufacturer's protocol. RNA (1 μg) was reverse-transcribed at 42°C using the first strand cDNA synthesis kit for RT-PCR (Roche, Lewes, UK) with 1 mM deoxynucleotide mix, 1.6 μg oligo-p[dT]15 primer and 0.8 μl AMV reverse transcriptase in a 20 μl volume as described in the manufacturer's protocol. 2 μl of each cDNA was amplified with 1.25 U Taq DNA polymerase, 1×PCR buffer and 10 mM dNTPs (Promega, Southampton, UK) in a 50 μl volume containing 100 pmol each of the following primers: 5′-GGT ATA GGC GAT CCT GTT ACC TGC-3′ (sense) and 5′-TCA TGG CTT TTT GCA GCA TTT TGT TC-3′ (antisense) for HBD-2; 5′-AAC TCT GGT AAA GTG GAT-3′ (sense) and 5′-TAC TCA GCG CCA CCA GCA TCG-3′ (antisense) for GAPDH. After a hot start the amplification profile for HBD-2 was 32 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C. RT-PCR amplification of HBD-2 and GAPDH generated products of 174 bp and 211 bp respectively.

2.4. Densitometric analysis of PCR results

PCR products were resolved on a 1% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma). The gels were analysed by densitometry and compared in a semiquantitative manner using ImageMaster® TotalLab Software (Amersham Pharmacia, Amersham, UK). The ratio of PCR fragment intensities of HBD-2 relative to GAPDH was determined. All expression values were verified by at least two independent RT-PCRs.

2.5. Transfection and reporter gene studies

To construct the HBD-2 luciferase reporter plasmid, the human HBD-2 promoter was PCR-amplified using Vent Polymerase (New England Biolabs, Herts., UK) from genomic DNA with 5′-GGCTCGAGGCTCAGACATCAGCACCCAAA-3′ (forward) and 5′-AAAAGCTTGAGTCTGGGGAGGACATCAA-3′ (reverse), and was cloned on a *Xho*I-*Hin*dIII fragment (underlined) into the pGL3-PV plasmid (Promega). HBE cells were seeded at 5×10^4 on coated 24-well plates 24 h before transfection. Transfections were performed with TransFast Transfection Reagent (Promega) in a 1:1 ratio according to the manufacturer's instructions, using 1 μg of HBD-2 luciferase plasmid. Uniform transfection efficiencies were achieved by initially optimising transfection conditions using a constitutive luciferase expression vector, pGL3-control (Promega). Transfections were incubated for 1 h at 37°C. Cells were then supplemented with additional growth medium (1 ml/well) for 48 h at 37°C before being left untreated or stimulated with NE as indicated. Cells were lysed with Reporter Lysis Buffer (1×) (Promega) (300 μl/well), protein concentrations were determined, and reporter gene activity was quantified by luminometry (Wallac Victor^2, 1420 multilabel counter, Turku, Finland) using the Promega luciferase assay system according to the manufacturer's instructions.

2.6. Statistical analysis

Data were analysed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± S.E.M. and were compared by Mann–Whitney one-tailed test. Differences were considered significant when the P value was ≤ 0.05.

3. Results

3.1. Up-regulation of HBD-2 mRNA by NE

Inducibility of antimicrobial factors such as β-defensins by bacterial products and/or inflammatory mediators is a key feature of the immune response. To evaluate the effect of NE on HBD-2 mRNA levels by RT-PCR, (1×10^6) 16HBE14o− cells and NHBE primary cells were incubated for 24 h with 0, 50 or 100 nM NE. HBD-2 mRNA expression increased significantly in both 16HBE14o− cell line and NHBE primary cells after treatment with 50 ($P\leq0.05$) and 100 ($P\leq0.05$) nM NE (Fig. 1A,B).

3.2. HBD-2 surface protein expression in NHBE primary cells

LSC analysis using a HBD-2 primary antibody and an anti-goat FITC-labelled secondary antibody was carried out on NHBE primary cells. HBD-2 surface protein expression increased after treatment with 30 μg lipopolysaccharide (LPS) for 24 h (Fig. 2A). 10 nM NE treatment of cells for 24 h

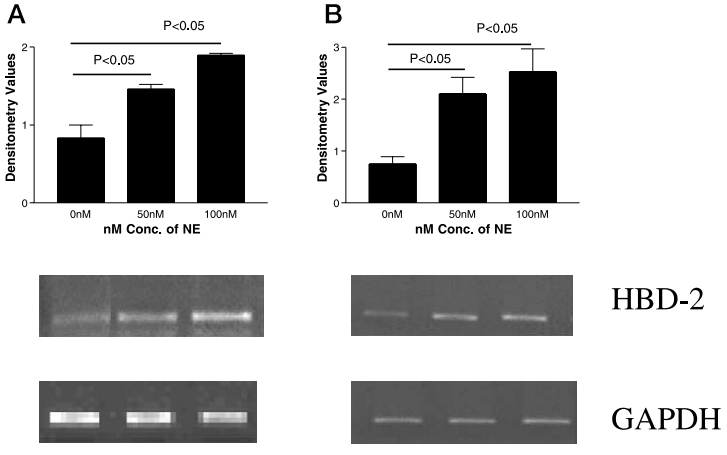

Fig. 1. HBD-2 mRNA expression is up-regulated by NE. Analysis of gene expression of HBD-2 in cultured 16HBE14o− cell line (A) and primary NHBE cells (B) using RT-PCR. 1×10^6 cells seeded on a six-well plate were treated with 0, 50, or 100 nM NE for 24 h and revealed HBD-2- (174 bp) and GAPDH- (211 bp) specific amplification products.

significantly elevated protein expression compared with control cells ($P \leq 0.05$) (Fig. 2B). Pre-treatment with 50 nM α1AT decreased HBD-2 protein expression to that of control or untreated cells (Fig. 2C,D).

3.3. *Effect of nM concentrations of active NE on HBD-2 promoter activity*

To investigate the regulation of HBD-2 mRNA expression in 16HBE14o− cells by NE, the HBD-2 promoter was amplified by PCR and subcloned into a luciferase reporter plasmid pGL3-Basic. 16HBE14o− cells were transfected with the construct and stimulated with NE. Treatment of cells with 20 and 50 nM NE for 4 h increased the relative luciferase activity significantly ($P \leq 0.05$) (Fig. 3).

4. Discussion

This report is the first identifying NE as a stimulus for the up-regulation of HBD-2 mRNA and surface protein expression.

β-Defensins are antimicrobial peptides that protect the epithelial mucosa from bacterial infection and colonisation. The first human β-defensin, HBD-1, was isolated as a trace peptide from human haemofiltrates, its mRNA is predominantly expressed in epithelia of the urogenital tract [12–14]. HBD-2 and HBD-3 are highly expressed in inflamed skin but all three β-defensins are also expressed in epithelia of the trachea and lung [15–17]. Gene expression of HBD-1 has been reported to be constitutive whereas HBD-2 gene expression is inducible by various pro-inflammatory agents such as tumour necrosis factor-α, IL-1β, LPS, bacteria and yeasts, stimuli commonly found in lung infection and inflammation [10,11,18].

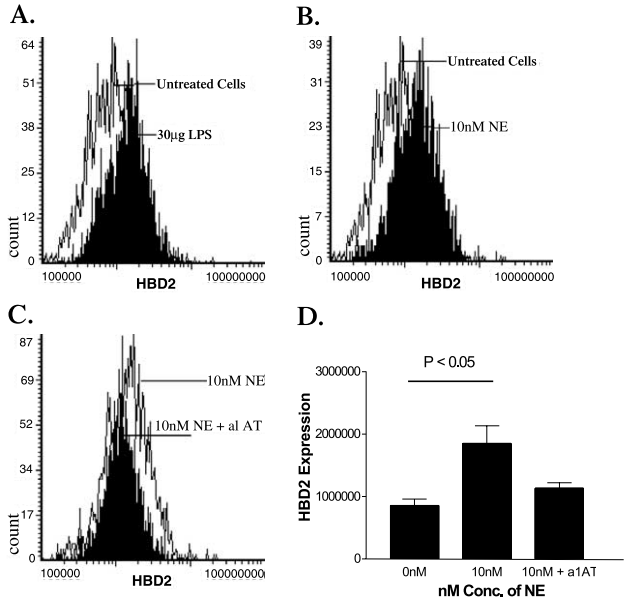

Fig. 2. Representative histograms of HBD-2 surface protein expression. Lab-Tek chamber slides were seeded with NHBE cells at 1×10^5 cells/well. A: Histogram showing HBD-2 expression in untreated cells (clear) and after treatment with 30 μg LPS (black) for 24 h. B: Histogram representing HBD-2 expression in untreated cells (clear) and after treatment with 10 nM NE (black) for 24 h. C: Histogram shows HBD-2 expression returns to that of unstimulated cells (clear) after inhibition with α1AT (black). D: Bar graph representing the median peak values for each treatment in C.

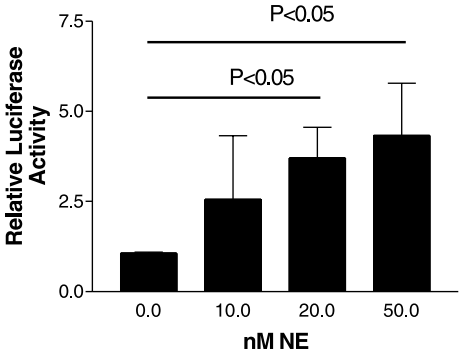

Fig. 3. Effect of NE on induction of HBD-2 promoter activity of pGL3 luciferase construct in 16HBE14o− cells. Transfected cells were incubated for 4 h with the indicated doses of NE before assay of luciferase activity.

The inducible nature of HBD-2 suggests it may play an important role in innate immune defence. It acts as a host defence molecule whose production is induced in response to infection thus maintaining a pathogen-free environment. HBD-2 is expressed in epithelia of the inner and outer surfaces of the human body such as the skin, the respiratory tract and the gastrointestinal tract. HBD-2 is also expressed in diseased tissue such as psoriatic skin, the CF lung and the gastric mucosal tissue in response to *Helicobacter pylori* infection [16,19,20]. We have previously shown that NE up-regulates IL-8 via NF-κB; therefore, due to the presence of NF-κB-like consensus binding sequences on the 5′ flanking region of the HBD-2 gene, we construed that HDB2 expression may also up-regulated by NE. This was shown for the first time in this study.

NE is a serine protease expressed at the promyelocytic stage of neutrophil development and stored in azurophilic granules in mature neutrophils. Apart from having an enormous elastolytic potential, recent studies have demonstrated that NE has important antibacterial properties. Survival of NE−/− mice is decreased following infection with Gram-negative bacteria [6]. In addition, neutrophil proteases, not oxidants, are primarily responsible for neutrophil activity against staphylococcal and fungal infections [7,21]. It has been shown that the bacterial cell wall protein, OmpA, is a prime target for NE cleavage and degradation although other proteins in Gram-negative bacteria may also be targeted by NE [22]. In this regard production of NE may be beneficial in the normal lung during acute infection, facilitating clearance of bacteria from the respiratory tract [23]. In addition, in this study we have shown the release of NE onto the epithelial surface of the respiratory tract may lead to increased expression of HBD-2, which will also participate in bacterial killing.

In conclusion, although the deleterious effects of NE have previously been described, such as impairment of mucociliary clearance, increased mucin production [24] and cleavage of immunoglobulin and complement receptors, NE is clearly beneficial in host defence by directly killing bacteria and, as shown in this study, by up-regulating the expression of the antimicrobial peptide HBD-2.

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