FEBS 27285

FEBS Letters 544 (2003) 129–132

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Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4

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Received 25 February 2003; revised 14 April 2003; accepted 14 April 2003

First published online 12 May 2003

Edited by Beat Imhof

Abstract **Cystic fibrosis is characterised in the lungs by high levels of neutrophil elastase (NE). NE induces interleukin-8 (IL-8) expression via an IL-1 receptor-associated kinase signalling pathway. Here, we show that these events involve the cell surface membrane bound toll-like receptor 4 (TLR4). We demonstrate that human embryonic kidney (HEK)293 cells transfected with a TLR4 cDNA (HEK-TLR4) express TLR4 mRNA and protein and induce IL-8 promoter activity in response to NE. Treatment of both HEK-TLR4 and human bronchial epithelial cells with NE decreases TLR4 protein expression. Furthermore, a TLR4 neutralising antibody abrogates NE-induced IL-8 production, and induces tolerance to a secondary lipopolysaccharide stimulus. These data implicate TLR4 in NE induced IL-8 expression in bronchial epithelium.**
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Key words: Toll-like receptor 4; Cystic fibrosis; Neutrophil elastase

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive inherited disorder, characterised by mutations in the gene encoding the CF transmembrane conductance regulator protein – CFTR [1,2]. It is one of the most common lethal hereditary disorders among caucasians of European descent [3]. The clinical characteristics include bacterial colonisation in the lung, production of thick mucous and recurrent infection, leading to morbidity and mortality in 95% of patients [4]. Inflammation in the CF lung is dominated by neutrophils and their products, including neutrophil elastase (NE), an omnivorous protease capable of degrading components of the lung matrix. The interaction between neutrophils and lung cytokines largely determines the nature and persistence of the inflammatory response in CF [5].

We have previously elucidated the intracellular signalling pathways involved in NE induction of interleukin-8 (IL-8) gene expression in human bronchial epithelial (HBE) cells [6]. These data demonstrate that HBEs express the IL-8 gene and produce IL-8 protein, and that these responses are up-regulated by NE. We further demonstrated the involvement of the signal transducing molecules IL-1 receptor-associated kinase (IRAK), MyD88 and TRAF6, in NE-induced NFκB activation and IL-8 expression. This pathway has been reported to transduce signals for the IL-1 receptor (IL-1R)/toll-like receptor (TLR) superfamily, a recently defined and expanding group of receptors that participate in immune and inflammatory responses [7]. TLRs compose a large family with 10 members (TLR1–10) [8–12], which play an important role in the recognition of microbial components, with subsequent activation of innate immunity leading to development of adaptive immune responses [13]. The prototypical TLR is TLR4, which is the recognised receptor for lipopolysaccharide (LPS) [14,15].

Neutrophil accumulation on the airway epithelial surface is an essential component of normal host defence against infection. When exaggerated it can cause progressive damage to the bronchial epithelium. In CF, this damage is mediated significantly by NE [16,17]. We have previously shown that IL-8, a potent chemoattractant and activator of neutrophils, is released by bronchial epithelial cells in response to NE [6]. In this study, we show that this IL-8 up-regulation by NE occurs in part through the cell surface membrane bound TLR4.

2. Materials and methods

2.1. Cell culture

The human embryonic kidney cell line, HEK293, (ECACC-85120602) was obtained from the European Collection of Cell Cultures. Cells were cultured at 37°C in Eagle's minimal essential medium (EMEM, Biowhittaker) supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 1% NEAA (Gibco-BRL). 16HBE14o$^-$ (HBE) cells, an SV-40-transformed HBE cell line [18] was obtained as a gift from D. Gruenert (University of Vermont). The cells were cultured as above without 1% NEAA.

2.2. Transfection and reporter gene studies

Construction of the IL-8 luciferase reporter plasmid was previously described [6]. HEK cells were seeded at 5×10^5 on six-well plates 24 h before transfection. Transfections were performed with TransFast (Promega) using 200 ng of IL-8 luciferase plasmid and 200 ng of either pcDNA3 or a TLR4 expression plasmid (a gift from Marta Muzio [19]). All cells were transfected with a β-galactosidase control plasmid for normalising transfection efficiencies. Cells were lysed with reporter lysis buffer (Promega), protein concentrations were determined [20], and IL-8 reporter gene activity was quantified by luminometry (Wallac Victor^2, 1420 multilabel counter). Reporter gene expression is expressed as light units (L.U.)/μg total protein.

2.3. RNA isolation and analysis

Total RNA was isolated from HEK cells using TRI reagent (Sigma). 1 μg total RNA was reverse transcribed into cDNA with an oligo(dT)$_{15}$ primer, using first strand cDNA synthesis kit (Roche). TLR4- or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-spe-

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Abbreviations: CF, cystic fibrosis; ELISA, enzyme-linked immunosorbent assay; HBE, human bronchial epithelia(l); HEK, human embryonic kidney; IRAK, interleukin-1 receptor-associated kinase; NE, neutrophil elastase; TLR, toll-like receptor

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doi:10.1016/S0014-5793(03)00482-4

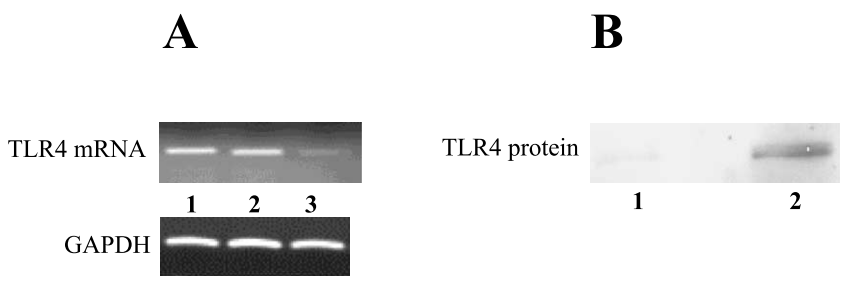

Fig. 1. HEK293 cell line transfected with human TLR4 cDNA expresses TLR4 mRNA and TLR4 protein. HEK293 cells (5×10^5/ml) were transfected with 200 ng of pcDNA3 or pcDNA-TLR4. A: Total RNA was extracted, 1 ug was reverse transcribed into cDNA and used in PCRs. Lane 1, PCR positive control (pcDNA3-TLR4); lane 2, HEK-TLR4; lane 3, HEK-pcDNA3 ($n=3$). B: Total protein was extracted and 10 ug was used in Western blots. Lane 1, HEK293; lane 2, HEK-TLR4 ($n=3$).

cific polymerase chain reactions (PCRs) were performed followed by resolution of the 481-bp TLR4 products and the 211-bp GAPDH products on a 1.5% TBE agarose gel containing 0.5 μg/ml ethidium bromide (Sigma).

2.4. Western blotting

HBE or HEK293 cells (1×10^6/ml) were exposed to NE (50 nM) for 20 min and total protein extracts were prepared as described [20]. Protein extracts (10 μg) were electrophoresed on a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding was blocked and immunoreactive proteins were detected by specific antibodies (TLR4 from Serotec; IRAK from Transduction Laboratories), alkaline phosphatase-conjugated anti-mouse IgG (Promega) and CDP-Star chemiluminescent substrate solution (Tropix).

2.5. Laser scanning cytometry (LSC)

To quantify TLR4 cell surface expression on HBE cells, 2×10^4 cells were seeded in eight-well chamber slides in serum-free medium for 24 h prior to exposure to NE (50 nM, 20 min). Cells were Fc-blocked with 1 μg/ml goat IgG then labelled with mouse IgG_{2A} anti-human TLR4 and an fluorescein isothiocyanate (FITC)-labelled anti-mouse $F(ab)_2$ (Dako). Isotype control samples were also prepared. Cells were counterstained with propidium iodide (Molecular Probes) and LSC (Compucyte, Cambridge, MA, USA) was used to quantify cell surface TLR4 expression. FITC and PI cellular fluorescence of at least 5×10^3 cells was measured by separate photomultipliers at 530 ± 20 nm and >610 nm, respectively. Individual TLR4-expressing cells were identified and quantified using CompuCyte software on the basis of integrated green fluorescence.

2.6. IL-8 protein production

HBE cells were seeded at 1×10^5 on 24-well plates 24 h before stimulation. Cells were left untreated or stimulated with 50 nM NE for 4 h, or 20 μg/ml LPS (Sigma) for 20 h. Prior to NE treatment, some cells were incubated for 1 h with an isotype control antibody (R&D Systems Inc., Minneapolis, MN, USA) or a mouse anti-TLR4 neutralising antibody (Serotec) and IL-8 protein concentrations in the cell supernatants were determined by enzyme-linked immunosorbent assays (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). Values are expressed as pg/ml IL-8.

2.7. Statistical analyses

Data were analysed with GraphPad Prism 2.0 software package (GraphPad Software, San Diego, CA, USA). Results are expressed as means ± S.E.M. and were compared by Mann–Whitney test. Differences were considered significant when the P value was ≤0.05.

3. Results

3.1. HEK-TLR4 cells express TLR4 mRNA and protein and IL-8 is up-regulated following stimulation with NE

HEK293 cells transiently transfected with a human TLR4 cDNA (HEK-TLR4) expressed both TLR4 mRNA and TLR4 protein (Fig. 1A and B). HEK-TLR4 cells cotransfected with an IL-8 luciferase reporter gene expressed higher basal levels of luciferase compared to mock-transfected cells. Upon exposure to NE, HEK-TLR4 cells showed a two-fold increase in luciferase expression compared to HEK-TLR4 cells alone ($P<0.0001$). This is compared to no increase between HEK cells treated with NE or left untreated (Fig. 2). HEK-TLR4 cells exposed to NE also showed decreased TLR4 by Western blot analysis (Fig. 3A), with simultaneous degradation of IRAK (Fig. 3B).

3.2. NE decreases TLR4 surface expression on HBE cells

In subsequent experiments using the human cell line 16HBE14o$^-$ (HBE), NE was shown by LSC to decrease cell surface expression of TLR4 (Fig. 4A), with simultaneous IRAK degradation shown by Western blotting (Fig. 4, inset). HBE cells exposed to NE also showed decreased TLR4 by Western blot analysis (Fig. 4B).

3.3. A TLR4 neutralising antibody inhibits NE-induced IL-8 production from HBE cells

HBE cells were next analysed for IL-8 protein production (Fig. 5). Cells treated with NE expressed two-fold higher levels of IL-8 than control cells (129 ± 12 vs. 70 ± 9 pg/ml IL-8). Pre-treatment with a TLR4 neutralising antibody prior to exposure to NE significantly inhibited NE-induced IL-8 production (78 ± 7 pg/ml IL-8) ($P=0.0178$). An isotype antibody had no effect (not shown).

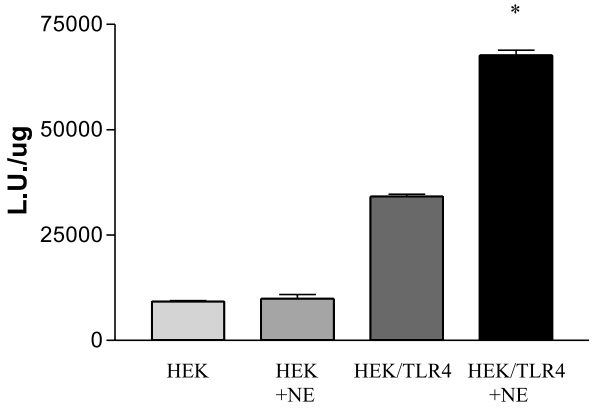

Fig. 2. NE up-regulates IL-8 via TLR4. HEK293 and HEK-TLR4 cells (5×10^5/ml) were left untreated or stimulated with NE (50 nM, 4 h). Levels of luciferase in cell extracts were measured and values are expressed as L.U./μg protein. (*$P<0.0001$). Assays were performed in duplicate ($n=3$).

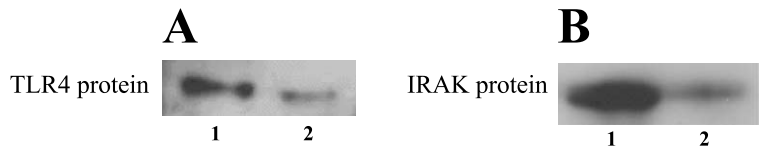

Fig. 3. NE decreases TLR4 expression. HEK-TLR4 cells (5×10^5/ml) were left untreated or stimulated with NE (50 nM, 20 min). Total protein was extracted and 10 ug was used in Western blots to detect TLR4 (A) or IRAK (B). Lane 1, HEK-TLR4; lane 2, HEK-TLR4 plus 50 nM NE/20 min ($n=5$).

3.4. NE induces tolerance to LPS in HBE cells

We next examined whether NE could induce tolerance to a secondary LPS stimulus in HBE cells (Fig. 6). Both NE and LPS increased IL-8 protein production from HBE cells at 4 and 20 h, respectively (120 ± 10, 216 ± 10 and 1364 ± 20 pg/ml IL-8 for control, NE and LPS, respectively) ($^*P=0.05$). Pretreatment for 4 h with NE, followed by removal of the NE and stimulation with LPS for a further 20 h blunted the LPS response (440 ± 30 pg/ml IL-8) ($\#P=0.05$), indicating that NE can induce LPS tolerance.

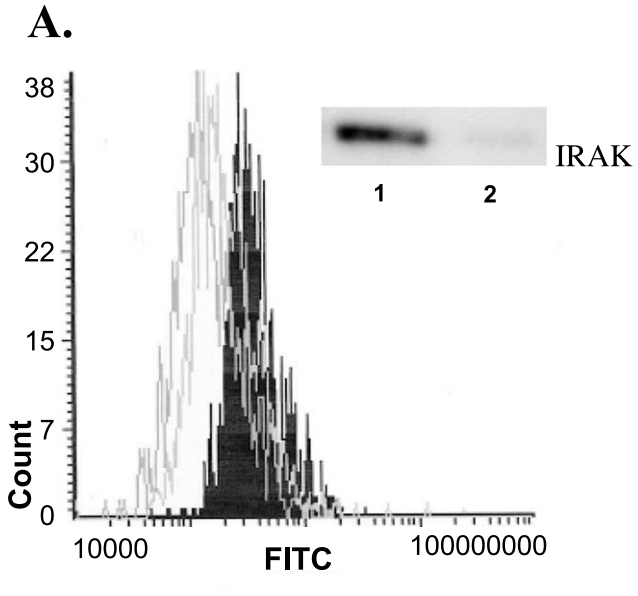

Fig. 4. NE decreases TLR4 expression in 16HBE14o$^-$ cells. HBE cells (2×10^4/ml) were left untreated or stimulated with NE (50 nM, 20 min). A: Black clear, isotype control; black solid, untreated cells; grey, 50 nM NE/20 min. Inset: IRAK Western blot: lane 1, untreated cells; lane 2, 50 nM NE/20 min ($n=5$). B: HBE cells (5×10^5/ml) were left untreated or stimulated with NE (50 nM, 20 min). Total protein was extracted and 10 ug was used in Western blots to detect TLR4. Lane 1, untreated; lane 2, 50 nM NE/20 min ($n=3$).

4. Discussion

The intracellular mechanism by which NE up-regulates IL-8 in bronchial epithelial cells is mediated in part by MyD88/IRAK/TRAF6 resulting in activation of NFκB [6]. These signal transducers have been implicated in pathways activated by the TLR/IL-1R family of proteins [7,8]. In this study, we show that NE acts to up-regulate IL-8 through TLR4, via the IRAK pathway.

Neutrophil-dominated inflammation is a major component of CF airway disease. Much of the lung damage mediated by neutrophils in CF is through the action of NE [16,21]. Attempts to inhibit NE activity in vivo have been hampered by the enormous NE burden [22].

IL-8 is an important proinflammatory chemokine in CF. Clinical studies have demonstrated high levels of active IL-8 in the airways and sputum of CF patients at virtually all stages of disease [5,23,24]. In this regard, identification of the signalling pathways and cellular events involved in NE-induced inflammation is of critical importance in that inhibition at these levels may diminish the inflammatory response more effectively than standard anti-protease therapies.

We have previously elucidated part of the intracellular signalling pathways involved in NE induction of IL-8 gene expression in BECs [6] by demonstrating the involvement of the signal transducers IRAK, MyD88, and TRAF-6. Here we show that TLR4 is involved in NE-induced IL-8 protein production in HBE cells and HEK cells expressing a TLR4 cDNA.

The HEK-TLR4 cells showed increased basal level of IL-8 luciferase activity compared to parental cells. Transfection of the cDNA for the functional TLR4 receptor led to expression of TLR4 mRNA and protein (Fig. 1), this overexpression can lead to activation of downstream effectors. It has previously been shown that this effect can be inhibited by the LPS antagonist E5531 [25]. There was no effect on IRAK-1 in HEK-

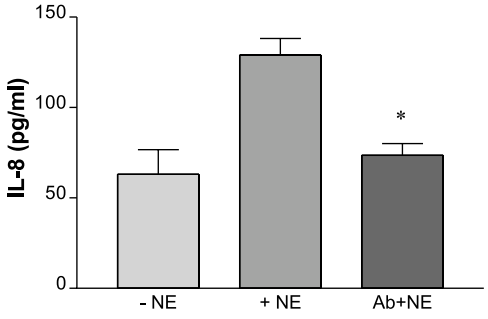

Fig. 5. TLR4 neutralising antibody blocks NE-induced up-regulation of IL-8. HBE cells (1×10^6/ml) were left untreated or were stimulated with NE (50 nM, 4 h). Addition of TLR4 antibody (Ab) to HBE cells attenuated the effects of NE exposure ($^*P=0.0178$). Assays were performed in duplicate ($n=3$).

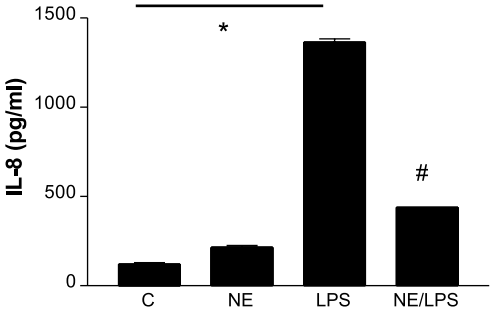

Fig. 6. NE induces LPS tolerance in HBE cells. HBE cells (1×10^5/ml) were left untreated or were stimulated with NE (50 nM, 4 h), LPS (20 μg/ml, 24 h) or NE for 4 h followed by LPS for a further 20 h. IL-8 protein production was measured in cell supernatants by ELISA (*$P=0.05$ for NE and LPS vs. control, #$P=0.05$ for NE/LPS vs. LPS). Assays were performed in duplicate ($n=3$).

TLR4 cells. We found that the cells responded normally to NE stimulation by degrading IRAK (Fig. 3B). However, it is possible that IRAK-2 or IRAK-4 may be responsible for transducing the 'basal' signal.

LPS is ubiquitous in the CF lung and TLR4 is recognised as the receptor responsible for transducing the LPS signal. This response is enhanced by the presence of MD-2 [26]. In this study we show that NE can transduce a signal through TLR4 in the absence of MD-2, since HEK293 cells have been shown not to express MD-2. The mechanism by which NE initiates inflammation via TLR4 is associated with decreased TLR4 surface expression. This may be similar to the mechanism of LPS-induced TLR4 internalisation [27] or alternatively could be a result of cleavage by NE.

The observed NE-induced decrease in TLR4 expression on HBE cells (Fig. 4) suggested that NE may be inducing tolerance in HBE cells. This is the phenomenon by which activated cells become recalcitrant to secondary stimulation and has been studied in detail for LPS and other TLR ligands. Using NE as a primary stimulus and LPS as a secondary stimulus, we found that the NE could blunt the LPS-induced IL-8 response compared to stimulation with LPS alone. This provides further evidence that NE is exerting its effects via TLR4.

Acknowledgements: This research was funded by The Higher Education Authority and the Cystic Fibrosis Association of Ireland.

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