Effect of nuclear factor-κB inhibition on interleukin-1β-stimulated matrix metalloproteinase-3 production by gingival fibroblasts from a patient with aggressive periodontitis

KEY WORDS aggressive periodontitis, IL-1β, MMP-3, NBD peptide, NF-κB

Background: Aggressive periodontitis (AgP) is a group of diseases characterised by rapid, severe periodontal destruction. Matrix metalloproteinase-3 (MMP-3) degrades the extracellular matrix and is associated with periodontitis. Interleukin-1β (IL-1β) upregulates MMP-3 in human gingival fibroblasts, activating genes via transcription factors (i.e. nuclear factor κB [NF-κB]). Cytoplasmic NF-κB is bound to inhibitors of κB (IκB), which are phosphorylated and degraded after cellular stimulation by IL-1β, allowing nuclear translocation of NF-κB subunits (e.g. p65). This involves activation of IκB kinase (IKK), requiring a regulatory subunit [NF-κB essential modifier (NEMO)]. The objective of this study was to investigate NF-κB involvement in IL-1β upregulation of MMP-3 in AgP gingival fibroblasts by inhibiting NF-κB activation with NEMO binding domain (NBD) peptide, blocking NEMO-IKK binding.

Study design: Constitutive and IL-1β-stimulated MMP-3 was measured by enzyme-linked immunosorbent assay (ELISA). For effects on NF-κB activation, cells were preincubated with 50 to 200μM NBD or control peptide (CP) for 6 to 48h, stimulated with IL-1β, and nuclear p65 levels were measured. For effects on MMP-3, cells were preincubated with 50μM NBD or CP for 24 or 48h, stimulated with IL-1β, and MMP-3 was measured.

Results: There were time-dependent increases in constitutive MMP-3 production, and IL-1β significantly increased MMP-3 at each time point. NBD peptide specifically decreased IL-1β-stimulated nuclear p65 by approximately 55%, and decreased IL-1β-stimulated MMP-3 production by a maximum of 26%.

Conclusions: IL-1β stimulation of MMP-3 may be partially regulated by NF-κB. Blocking MMP-3 gene expression by inhibitors such as NBD peptide may be an important approach for treatment of AgP.

Introduction

Aggressive periodontitis (AgP) is a group of diseases characterised by rapid, severe periodontal destruction. Fibroblasts and other cells in periodontal tissues produce proteolytic enzymes called matrix metalloproteinases (MMPs), which contribute to tissue destruction in periodontitis. Together, MMPs can degrade all components of the periodontal extracellular matrix (ECM), and are the main degradative pathway for ECM breakdown in periodontal tissues1. MMP-3 (stromelysin 1) degrades many ECM
structural molecules. Work in this laboratory suggested that human AgP gingival fibroblasts may contribute to periodontal tissue destruction via increased production of MMP-3. In other studies, MMP-3 in gingiva has been associated with periodontitis, and it may be a key enzyme causing tissue destruction in inflammatory diseases such as arthritis.

Several cytokines also participate in the pathogenesis of periodontitis. Interleukin-1β (IL-1β) expression is highly correlated with the severity of periodontitis and other inflammatory diseases. IL-1β upregulates MMP-3 in human gingival fibroblasts, and work in this laboratory showed that it also upregulates MMP-3 in human temperomandibular joint (TMJ) synovial fibroblasts. IL-1β causes rapid expression of genes that encode proinflammatory proteins, via transcription factors such as nuclear factor-κB (NF-κB). IL-1β binding to its cell surface receptor initiates a signalling cascade leading to NF-κB activation.

NF-κB is typically a homodimer or heterodimer, composed of p65 and p50 subunits, but it can also exist as a diverse variety of protein complexes. In resting cells, inactive NF-κB is located in the cytoplasm attached to inhibitory proteins (inhibitors of κB, IκB). IL-1β activates the IκB kinase (IKK) complex, which in turn phosphorylates IκB proteins. Phospho-IκB then dissociates from NF-κB and undergoes proteosomal degradation, allowing free NF-κB to enter the nucleus. IKKs require the regulatory subunit NF-κB essential modifier (NEMO) for activation. The NEMO-binding domain (NBD) of NEMO associates with IKKs. NBD peptide is a cell-permeable peptide that blocks NEMO-IKK association complex, thus inhibiting NF-κB activation.

Activator protein 1 (AP-1) is considered the dominant transcription factor for MMP-3 because there are AP-1 sites in the promoter region of this gene. There may also be AP-1-independent mechanisms of MMP-3 induction, including involvement of NF-κB, in rabbit and human cells including dermal fibroblasts, vascular smooth muscle cells, and chondrocytes. The role of NF-κB in the regulation of MMP-3 expression in human gingival fibroblasts is unknown. The present study investigated the involvement of NF-κB in IL-1β upregulation of MMP-3 in human AgP gingival fibroblasts, through the use of NBD peptide to inhibit NF-κB activation. Understanding the regulation of MMP-3 and other factors involved in periodontal destruction may lead to new generations of anti-inflammatory drugs that are more effective and have fewer side effects than drugs currently available.

### Study design

#### Human gingival fibroblasts

A human gingival fibroblast cell line derived from a patient with AgP was used in this study. This patient was an African American female, aged 16, who presented to the University of Tennessee College of Dentistry Periodontology Clinic with >80% generalised alveolar bone loss and generalised erythematous, oedematous gingival tissue. The cell line was established from gingival explants using standard techniques and identified in culture by cellular morphology and production of type I collagen. The cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) newborn calf serum (Gibco) and 100μg/ml gentamicin (Sigma Chemical Company; St Louis, MO, USA) (complete medium), at 37ºC in a humidified atmosphere of 5% CO₂ in air. Cells between passages 5 and 14 were used in the experiments described below.

#### NF-κB inhibitors

NBD peptide (Imgenex; San Diego, CA, USA) is a cell-permeable peptide that blocks the association of NEMO with the IKK complex, thereby inhibiting IKK activity, and ultimately selectively inhibits NF-κB activation. The amino acid sequence of NDB peptide is DROIKWFQNRMKWDDTDWSWLTE (the underlined portion of sequence represents the IKK/NEMO binding sequence). A control peptide (CP) (Imgenex) is also supplied with the NBD peptide, which has a sequence identical to the NBD peptide, except that it lacks the IKK/NEMO binding sequence. Stock solutions of both peptides were prepared in dimethyl sulphoxide (DMSO; Sigma) at a strength of 100x and diluted 1:100 in serum-free DMEM containing 100μg/ml gentamicin (DMEM-gent).

In addition, NS-398 (Cayman Chemical Company; Ann Arbor, MI, USA), a selective cyclooxygenen-
ase 2 (COX-2) inhibitor that also inhibits IL-1ß-stimulated NF-κB activation, was used in this study. NS-398 is a sulphonamide class drug (N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulphonamide; MW 314.36) that is soluble in DMSO. Stock solutions of NS-398 were prepared in DMSO at a strength of 100x and diluted 1:100 in DMEM-gent before addition to cells. Concentrations of DMSO ≤1% are not toxic to AgP gingival fibroblasts.

### Determination of constitutive and IL-1ß-stimulated production of MMP-3

Production of MMP-3 was measured in AgP fibroblast-conditioned media using an enzyme-linked immunosorbent assay (ELISA) kit (Amersham Life Science; Arlington Heights, IL, USA). This assay measured the total amount of MMP-3 (pro-enzyme, latent enzyme, and inhibitor/MMP complex). To obtain conditioned media, the fibroblasts were seeded in 6-well plates (Costar; Corning, NY, USA) in complete medium and cultured overnight at 37°C. The medium was removed, the wells washed once with phosphate-buffered saline (PBS), and DMEM-gent ± human recombinant IL-1ß (1x10⁻⁹ mol/l or 1x10⁻¹⁰ mol/l) (R&D Systems; Minneapolis, MN, USA) was added. The cells were incubated at 37°C, and aliquots of the cell supernatants were removed at days 1, 3, and 6 and stored at -80°C until assayed. Results were expressed as μg MMP-3/mg protein (measured in the supernatants using a modification of the method of Lowry et al).

### Determination of the effect of NBD peptide or NS-398 on IL-1ß-stimulated MMP-3 production

The effect of inhibition of NF-κB activation, using NBD peptide or NS-398, on IL-1ß-stimulated production of MMP-3 was determined using the MMP-3 ELISA described above. Fibroblasts were seeded at 3x10⁵ cells/well in 6-well plates in complete medium and cultured overnight at 37°C. The medium was removed and the wells washed once with PBS. To determine the effect of the peptides, DMEM-gent containing NBD peptide (50μmol/l) or CP (50μmol/l) was added and the cells were cultured for 24 or 48h. Control medium was DMEM-gent/DMSO. After this pre-incubation period, IL-1ß (1x10⁻¹⁰ mol/l) was added and the cells were further incubated at 37°C. Aliquots of the cell supernatants were removed daily for 6 days and stored at -80°C until assayed. To determine the effect of NS-398 (10nmol/l or 100nmol/l), the drug was added concurrently with IL-1ß (1x10⁻¹⁰ mol/l), conditions that inhibited IL-1ß-stimulated prostaglandin E₂ (PGE₂) production and activation of NF-κB. Control medium was DMEM-gent/DMSO. The cells were incubated at 37°C for 6 days, at which time aliquots were removed and stored at -80°C.

The ELISA was performed as described above. The results were calculated as μg MMP-3/mg protein in the supernatants, then converted to percent control (amount of MMP-3 in non-stimulated cells, set as 100%). Specific decreases in IL-1ß-stimulated MMP-3 caused by NBD peptide were calculated as the difference between the inhibition caused by NBD peptide and any non-specific inhibition caused by the CP.
Statistical analysis

All experiments were performed with duplicate or triplicate samples and were repeated at least twice. The data were expressed as mean ± standard deviation and were analysed using a one-way analysis of variance (ANOVA) and Scheffe’s F procedure for post hoc comparisons, using StatView® software (SAS Institute, Cary, NC, USA).

Results

Constitutive and IL-1β-stimulated production of MMP-3

Before determining the effects of NF-κB inhibition, both constitutive and IL-1β-stimulated production of MMP-3 by AgP fibroblasts was determined (Fig 1). Constitutive MMP-3 production increased over a time period from 1 to 6 days from 0.19 μg/mg protein at day 1, to 0.48 μg/mg protein at day 3 (P < 0.0001 vs. day 1), to 1.15 μg/mg protein at day 6 (P < 0.0001 vs. day 1). Constitutive MMP-3 production at day 6 was also greater than that at day 3 (P < 0.0001). When stimulated with IL-1β, the amount of MMP-3 produced by fibroblasts was also increased over the 6-day time period. At day 3, both 1x10^-10 mol/l and 1x10^-9 mol/l IL-1β concentrations significantly increased the amount of MMP-3 produced, to 0.79 μg/mg protein and 0.84 μg/mg protein respectively (P = 0.02 vs. day 1), and at day 6, to 3.23 and 2.7 μg/mg protein respectively (P < 0.0001 vs. day 1). Day 6 MMP-3 levels for both IL-1β concentrations were significantly elevated compared with those at day 3 (P < 0.0001). Significant differences between control and IL-1β-stimulated MMP levels were seen at day 1 (P = 0.0005 for 1x10^-10 mol/l IL-1β only), day 3 (P < 0.0001) and at day 6 (P < 0.001).

Effect of NBD peptide on IL-1β-stimulated nuclear NF-κB p65 levels

The specific effects of NBD peptide on nuclear levels of NF-κB p65 were determined 45 min after the addition of IL-1β, when maximum IL-1β-stimulated nuclear p65 levels occur in these cells (Table 1). There were specific decreases of 30 to 40% in nuclear p65 levels when the fibroblasts were exposed to 100 μmol/l and 200 μmol/l NBD for 6 and 24 h. However, these concentrations of both the NBD peptide and CP caused significant cellular toxicity, manifested in morphological changes and cell death, as noted by microscopic examination (not shown), and most likely this data did not represent true, specific inhibition of nuclear p65 levels. Therefore, the concentration of the peptides was decreased to a level that did not cause cellular toxicity (50 μmol/l), as...
observed microscopically, and the fibroblasts were incubated with the peptides for 24 or 48h before stimulation with IL-1β. A maximum decrease of 57% in nuclear NF-κB p65 levels was seen under these conditions (Table 1); non-specific inhibition caused by the control peptide averaged 15%.

**Effect of NBD peptide on IL-1β-stimulated MMP-3 production**

As pre-incubation with 50μmol/l NBD peptide for 24 or 48h resulted in >50% specific inhibition of IL-1β-stimulated nuclear p65 levels (with little toxicity and non-specific inhibition by the CP), these conditions were used to determine its effect on IL-1β-stimulated MMP-3 production. Figure 2 shows that NBD peptide specifically decreased IL-1β-stimulated MMP-3 by 19% (P=0.01) and 26% (P=0.007) at days 4 and 5, respectively, when added 24h before the addition of IL-1β. When the fibroblasts were pre-incubated with NBD peptide for 48h before IL-1β-stimulation, specific decreases in MMP-3 production in general were low and did not reach statistical significance.

**Effect of NS-398 on IL-1β-stimulated MMP-3 production**

NS-398, a selective COX-2 inhibitor that also inhibits IL-1β-stimulated NF-κB activation, was evaluated for its ability to regulate MMP-3 production by AgP fibroblasts (Fig 3). IL-1β increased MMP-3 production by approximately 7-fold over control (P=0.0004). In contrast to the inhibitory effects of NBD peptide, concurrent addition of NS-398 and IL-1β further increased IL-1β-stimulated MMP-3 production, to 8.5 to 9 fold over control (P<0.0001).

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>50 μmol/l NBD</th>
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1x10^6 fibroblasts were pre-incubated with the indicated concentrations of NBD or control peptides for 6, 24 or 48h, then exposed to IL-1β (1x10^-10 mol/l) for 45min. Nuclear fractions were isolated and NF-κB p65 levels were measured using an ELISA-based assay. Results are expressed as % specific decrease in nuclear p65 levels (A450/mg protein in the nuclear pellet) compared with cells incubated with IL-1β alone. ND = not determined.
Discussion

The present study demonstrates a role for NF-kB, previously shown to be activated by IL-1ß, in the regulation of IL-1ß-stimulated MMP-3 production. NBD and control peptide concentrations of 100 to 200μmol/l apparently caused cell death, and under these conditions, decreases in nuclear p65 may have been due to this toxicity. At 50μmol/l, there was little apparent cytotoxicity, and nuclear p65 was inhibited by approximately 55%. This specific, although partial, inhibition demonstrated indirectly that NBD peptide entered the cells. To facilitate its entry into cells, NBD peptide is conjugated to a small sequence from the antennapedia homeodomain of Drosophila, and although not 100% efficient, it is more competent than other conjugates. There may also be an equilibrium of antennapedia-conjugated peptides between the intra- and extracellular domains of gingival fibroblasts. Therefore, the intracellular concentration of NBD peptide might not have been high enough to fully inhibit NF-kB activation, dependent upon factors such as the dissociation constant of NBD peptide/NEMO interaction. Alternatively, the peptide may have undergone partial proteolysis, or there may be some regulatory defect in these cells that results in only partial inhibition of NF-kB activation by NBD peptide.

IL-1ß stimulation of MMP-3 in AgP fibroblasts is consistent with reports that in AgP patients, elevated IL-1ß increases transcription of genes for gingival MMP-3 and MMP-8. MMP-3 levels are much higher in gingival crevicular fluid (GCF) of gingivitis and periodontitis sites compared with disease-free sites. Scaling and root planing significantly decreases these MMP-3 levels. Because of the potential importance of MMP-3 in periodontal destruction, therapeutic means of decreasing it might be beneficial, particularly in patients with AgP or refractory cases of chronic periodontitis.

In gingival fibroblasts, IL-1ß activates NF-kB and the transcription factor AP-1, which is necessary, but not sufficient, for transcription and upregulation of the MMP3 gene. NF-kB is required for transcription of the MMP3 gene in several cell types. NF-kB and AP-1 have binding sites in the promoter region of MMP3 (GenBank® accession no. U43511), and in human articular chondrocytes, IL-1ß upregulated MMP-3 production via NF-kB activation. Borg-haei et al. found that NF-kB binds to a repressor in the MMP3 promoter in human gingival fibroblasts, as opposed to being an activator for MMP3. However, NF-kB may still activate MMP3 transcription through another related site, or indirectly by activating other transcription factor(s), while still acting as a repressor at the MMP3 promoter site. NF-kB may also regulate genes, including MMP3, via interactions with other transcription factors, such as AP-1, that regulate MMP3 expression.

Pre-incubation with 50μmol/l NBD peptide for 24h before exposure to IL-1ß significantly decreased IL-1ß-stimulated MMP-3 production. NBD peptide
decreased MMP-3 by approximately 20 to 25%, while inhibiting NF-κB activation by approximately 55%. The relatively small, yet significant, decrease in MMP-3 may be related to partial inhibition of NF-κB and to the fact that AP-1 regulates MMP3 transcription in addition to NF-κB. This is the first demonstration that NF-κB inhibition results in inhibition of IL-1β-stimulated MMP-3 production in human gingival fibroblasts. Others showed that in human neutrophils, inhibition of NF-κB and AP-1 with an antibiotic resulted in partial inhibition of MMP-9.

Bondeson et al. showed that in human synovial cells, blocking NF-κB by overexpression of its inhibitor IκBα decreased MMP-3 production, which might also have been due to indirect effects of cytokine (i.e. IL-1β) inhibition. With 48-hours preincubation, NBD peptide had no significant effect on IL-1β-stimulated MMP-3 production, possibly because of decreased activity of the peptide over time, due to its degradation. These data suggest that NF-κB has a role in MMP-3 production by human gingival fibroblasts, whether it is direct or indirect.

In contrast to the inhibitory effect of NBD peptide, NS-398 increased IL-1β-stimulated MMP-3 production. This COX-2 inhibitor downregulates PGE2 production by fibroblasts stimulated with IL-1β. PGE2 can increase cAMP, downregulating MMP-3 through a protein kinase A-dependent pathway. Therefore, by inhibiting PGE2, NS-398 caused a net increase in MMP-3 production. However, PGE2 can also increase intracellular Ca2+, which interacts with protein kinase C, a major stimulator of MMP-3, to upregulate MMP-3. Studies show that in normal human gingival fibroblasts, PGE2 downregulates or upregulates IL-1β-stimulated MMP-3 via activation of different PGE2 receptors. Owing to these multiple effects, NS-398 may not be suitable for investigating NF-κB regulation of MMP-3.

With the advent of medications that modify host response to periodontal infection, it is important to understand key steps in the inflammatory cascade associated with periodontitis. COX-2 inhibitors, which block NF-κB activation, have been evaluated for reducing periodontal inflammation, but some COX-2 inhibitors are linked to cardiac and renal failure. In rabbits, the COX inhibitor indomethacin reduced intra-articular PGE2 levels and decreased clinical joint swelling, but loss of articular cartilage proteoglycans (MMP-3 substrates) was accelerated. Indomethacin may block the intracellular pathway that downregulates MMP3 transcription, consistent with failure of non-steroidal anti-inflammatory drugs (NSAIDs) to halt tissue loss in patients with rheumatoid arthritis. This may limit their use in reducing inflammation in other chronic inflammatory diseases such as periodontitis.

The NBD of NEMO is an extremely specific target for drugs designed to inhibit NF-κB activation. Activated NF-κB is found in 75% of chronic periodontitis gingival tissue samples compared with 5% of healthy gingival samples, suggesting that NBD peptide could be used to treat periodontitis. In mice, NBD peptide inhibited osteoclastogenesis and bone erosion in arthritic joints, but in humans, the use of such peptides has been hampered by their instability and low bioavailability.

When using in vitro cellular systems, it is important to consider heterogeneous cellular responses caused by species-, tissue- and individual-specific cellular phenotypic differences. Cell selection during establishment of cell lines and changes in cellular phenotype while in culture must be considered when interpreting results of in vitro studies. Future studies using additional gingival fibroblast cell lines from different individuals will add to understanding the response of these cells to NF-κB inhibition. However, the results of the present study support the use of novel NF-κB inhibitors, such as NBD peptide, as an important approach for treating not only AgP, but also TMJ disease, arthritis and other chronic inflammatory diseases.

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References


