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

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Background: Aggressive periodontitis (AgP) causes rapid periodontal destruction. Matrix metalloproteinase-3 (MMP-3) degrades the extracellular matrix and is associated with periodontitis. Interleukin-1ß (IL-1ß) 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-1ß, 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-1ß 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-1ß-stimulated MMP-3 was measured by enzyme-linked immunosorbent assay (ELISA). For effects on NF-κB activation, cells were preincubated with 50 to 200 µM NBD or control peptide (CP) for 6 to 48 h, stimulated with IL-1ß, and nuclear p65 levels were measured. For effects on MMP-3, cells were preincubated with 50 µM NBD or CP for 24 or 48 h, stimulated with IL-1ß, and MMP-3 was measured.
Results: There were time-dependent increases in constitutive MMP-3 production, and IL-1ß significantly increased MMP-3 at each time point. NBD peptide specifically decreased IL-1ß-stimulated nuclear p65 by approximately 55%, and decreased IL-1ß-stimulated MMP-3 production by a maximum of 26%.
Conclusions: IL-1ß 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 tissues[1]. 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[2]. 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[3,4].

Several cytokines also participate in the pathogenesis of periodontitis[5-7]. Interleukin-1ß (IL-1ß) expression is highly correlated with the severity of periodontitis and other inflammatory diseases[8-15]. IL-1ß upregulates MMP-3 in human gingival fibroblasts[16], and work in this laboratory showed that it also upregulates MMP-3 in human temperomandibular joint (TMJ) synovial fibroblasts[17,18]. IL-1ß causes rapid expression of genes that encode proinflammatory proteins, via transcription factors such as nuclear factor-κB (NF-κB)[9,19-26]. IL-1ß 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[16,27]. In resting cells, inactive NF-κB is located in the cytoplasm attached to inhibitory proteins (inhibitors of κB, IκB). IL-1ß 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[28-31]. IKKs require the regulatory subunit NF-κB essential modifier (NEMO) for activation[32]. 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[33].

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[34,35]. 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[36-44]. 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-1ß 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[45]. The cell line was established from gingival explants using standard techniques and identified in culture by cellular morphology and production of type I collagen[46-49]. 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 37°C in a humidified atmosphere of 5% CO_2 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 DRQIKIWFQNRRMKWDDTALDWSWLQTE (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 cyclooxygen-

ase 2 (COX-2) inhibitor that also inhibits IL-1ß-stimulated NF-κB activation[50], was used in this study. NS-398 is a sulphonamide class drug (N-[2-(cyclohexyloxyl)-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[51].

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 inibitor/MMP complex). To obtain conditioned media, the fibroblasts were seeded in 6-well plates (Costar, Corning; 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[52,53]).

Determination of the effect of NBD peptide on IL-1ß-stimulated nuclear p65 levels

Fibroblasts were seeded in 25 cm^2 flask (Corning) and incubated overnight at 37°C. The medium was removed, the flasks washed once with PBS, and DMEM-gent containing NBD or control peptides (50 to 200 µmol/l) was added and incubated for 6 to 48 h. Control medium was DMEM-gent containing 1% DMSO (DMEM-gent/DMSO). After incubation with the peptides, IL-1ß ($1x10^{-10}$ mol/l) was added and the cells were incubated for an additional 45 min, when maximum IL-1ß-stimulated nuclear p65 levels occur in these cells[50]. The cells were then harvested by scraping, nuclear extracts were prepared using the reagents of an assay kit (Nuclear Extract Kit, Active Motif; North America, Carlsbad, CA, USA) and stored at -80°C until assayed. The nuclear extracts were assayed for p65 using the TransAm™ NF-κB p65 Transcription Factor Assay Kit (Active Motif). Results were calculated as A_{450}/mg protein in the nuclear pellet[52,53]. These values were then converted to per cent control (amount of nuclear p65 in non-stimulated cells, set as 100%). Specific decreases in IL-1ß-stimulated nuclear p65 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.

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 $3x10^5$ 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 (50 µmol/l) or CP (50 µmol/l) was added and the cells were cultured for 24 or 48 h. 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 (10 nmol/l or 100 nmol/l), the drug was added concurrently with IL-1ß ($1x10^{-10}$ mol/l), conditions that inhibited IL-1ß-stimulated prostaglandin E_2 (PGE_2) production and activation of NF-κB[50,50]. 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[52,53], then converted to per cent 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-1ß-stimulated production of MMP-3

Before determining the effects of NF-κB inhibition, both constitutive and IL-1ß-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-1ß, 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-1ß 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-1ß concentrations were significantly elevated compared with those at day 3 ($P<0.0001$). Significant differences between control and IL-1ß-stimulated MMP levels were seen at day 1 ($P=0.0005$ for $1x10^{-10}$ mol/l IL-1ß only), day 3 ($P<0.0001$) and at day 6 ($P<0.001$).

Effect of NBD peptide on IL-1ß-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-1ß, when maximum IL-1ß-stimulated nuclear p65 levels occur in these cells[50] (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

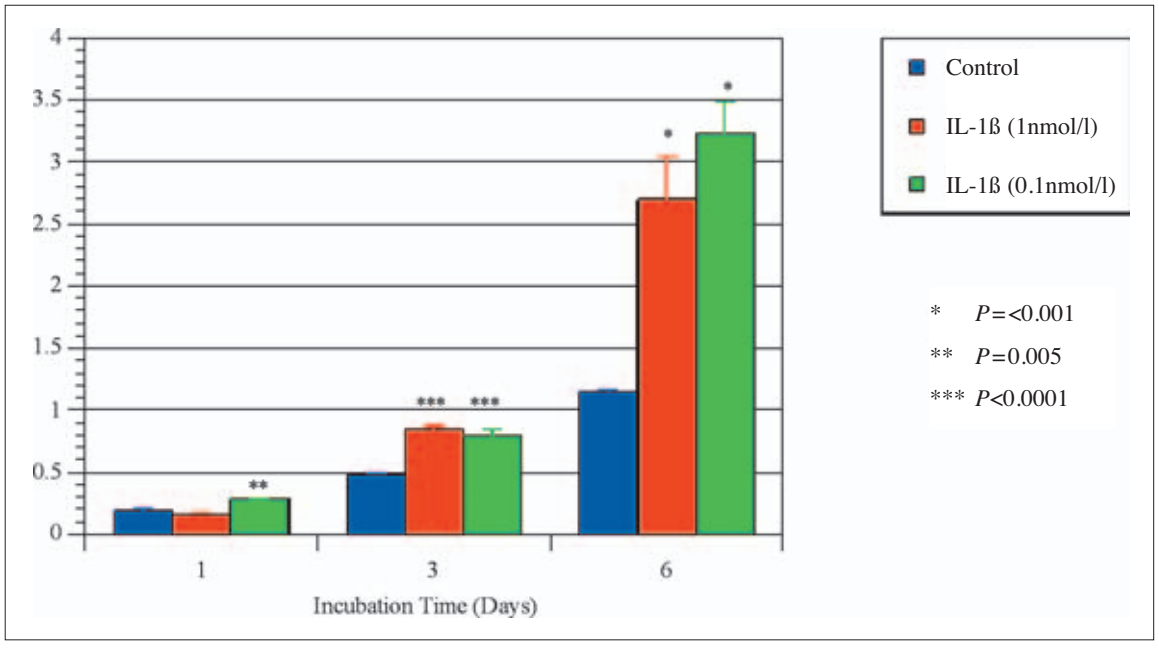

Fig 1 Constitutive and IL-1ß-stimulated MMP-3 production by AgP gingival fibroblasts. Fibroblasts were incubated in DMEM-gent ± human recombinant IL-1ß ($1x10^{-9}$ mol/l or $1x10^{-10}$ mol/l) for 1, 3 or 6 days. Total MMP-3 was measured using ELISA. Results of multiple experiments each with triplicate determinations were reported as µg MMP-3/mg protein in the cell monolayer.

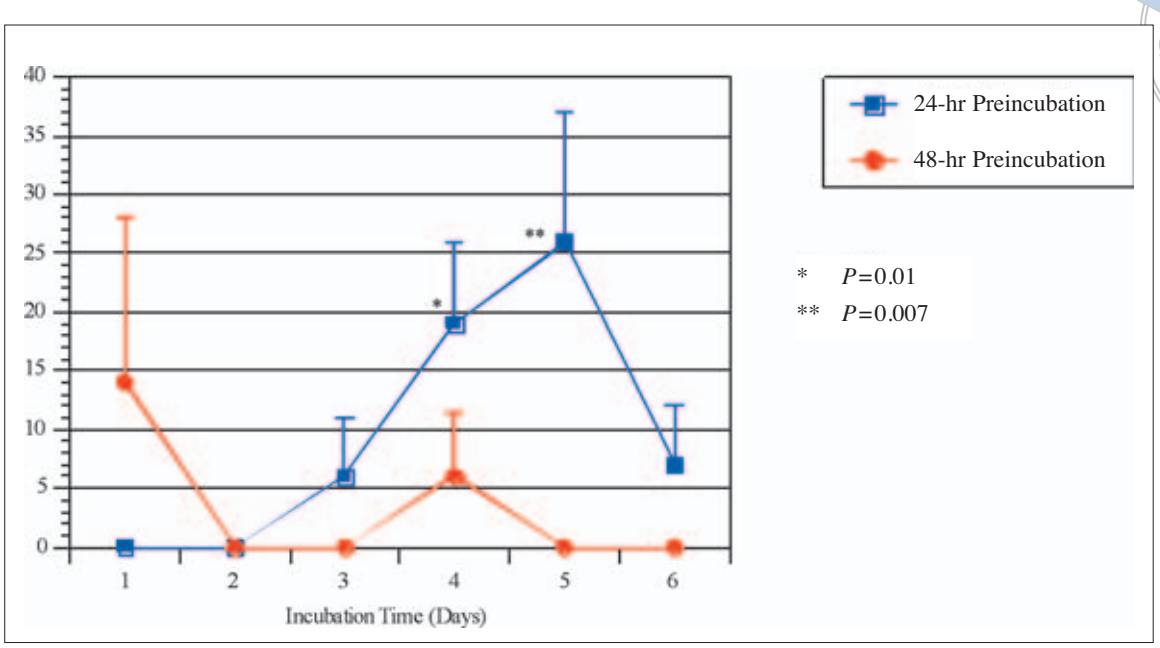

Fig 2 Effect of NBD peptide on IL-1ß-stimulated MMP-3 production by AgP gingival fibroblasts. Fibroblasts were incubated in DMEM-gent containing NBD peptide or control peptide (50 µmol/l) for 24 or 48 h. Control medium was DMEM-gent/DMSO. Human recombinant IL-1ß ($1x10^{-10}$ mol/l) was added and aliquots were removed daily for 6 days and stored at -80°C until assayed. Total MMP-3 was measured using ELISA. The results were calculated as µg MMP-3/mg protein in the conditioned media, and converted to per cent 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.

observed microscopically, and the fibroblasts were incubated with the peptides for 24 or 48 h before stimulation with IL-1ß. 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-1ß-stimulated MMP-3 production

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

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

NS-398, a selective COX-2 inhibitor that also inhibits IL-1ß-stimulated NF-κB activation, was evaluated for its ability to regulate MMP-3 production by AgP fibroblasts (Fig 3). IL-1ß 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-1ß further increased IL-1ß-stimulated MMP-3 production, to 8.5 to 9 fold over control ($P<0.0001$).

Exposure time	50 µmol/l NBD	100 µmol/l NBD	200 µmol/l NBD
6 hr	ND	30	ND
24 hr	55	43	40
48 hr	57	ND	ND

Table 1 Specific decreases in IL-1ß-stimulated nuclear NF-κB p65 levels caused by NBD peptide.

$1x10^6$ fibroblasts were pre-incubated with the indicated concentrations of NBD or control peptides for 6, 24 or 48 h, then exposed to IL-1ß ($1x10^{-10}$ mol/l) for 45 min. 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 (A_{450}/mg protein in the nuclear pellet) compared with cells incubated with IL-1ß alone. ND = not determined.

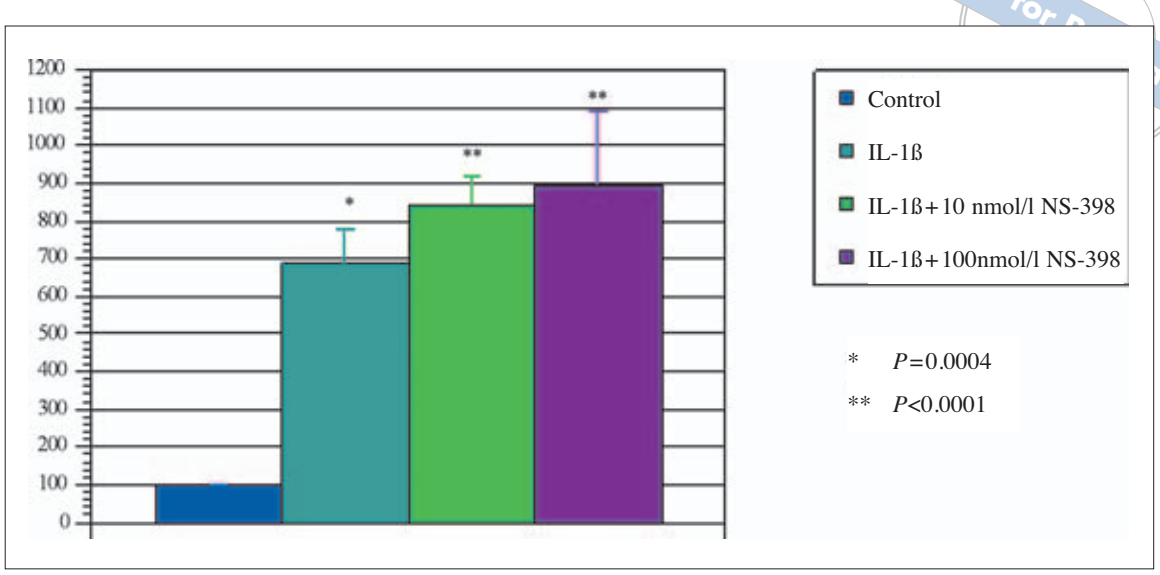

Fig 3 Effect of NS-398 on IL-1ß-stimulated MMP-3 production by AgP gingival fibroblasts. Fibroblasts were cultured for 6 days in DMEM-gent containing NS-398 (10 nmol/l or 100 nmol/l) and human recombinant IL-1ß ($1x10^{-10}$ mol/l). Control medium was DMEM-gent/DMSO. Total MMP-3 was measured using ELISA. The results were calculated as µg MMP-3/mg protein in the conditioned media, then converted to per cent control (amount of MMP-3 in non-stimulated cells, set as 100%).

■ Discussion

The present study demonstrates a role for NF-κB, previously shown to be activated by IL-1ß, in the regulation of IL-1ß-stimulated MMP-3 production[54]. NBD and control peptide concentrations of 100 to 200 µmol/l apparently caused cell death, and under these conditions, decreases in nuclear p65 may have been due to this toxicity. At 50 µmol/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*[55], and although not 100% efficient, it is more competent than other conjugates[56,57]. There may also be an equilibrium of antennapedia-conjugated peptides between the intra- and extracellular domains of gingival fibroblasts[58]. Therefore, the intracellular concentration of NBD peptide might not have been high enough to fully inhibit NF-κB 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-κB 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[59,60]. MMP-3 levels are much higher in gingival crevicular fluid (GCF) of gingivitis and periodontitis sites compared with disease-free sites[60]. Scaling and root planing significantly decreases these MMP-3 levels[61-63]. 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-κB and the transcription factor AP-1, which is necessary, but not sufficient, for transcription and upregulation of the *MMP3* gene[64,65]. NF-κB is required for transcription of the *MMP3* gene in several cell types[38]. NF-κB 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-κB activation[36,37,66]. Borghaei et al[67] found that NF-κB binds to a repressor in the *MMP3* promoter in human gingival fibroblasts, as opposed to being an activator for *MMP3*. However, NF-κB 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-κB may also regulate genes, including *MMP3*, via interactions with other transcription factors, such as AP-1, that regulate *MMP3* expression[37,68].

Pre-incubation with 50 µmol/l NBD peptide for 24 h 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-1ß-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[69]. Bondeson et al[37] 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-1ß) inhibition. With 48-hours preincubation, NBD peptide had no significant effect on IL-1ß-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-1ß-stimulated MMP-3 production. This COX-2 inhibitor downregulates PGE_2 production by fibroblasts stimulated with IL-1ß[50,51]. PGE_2 can increase cAMP, downregulating MMP-3 through a protein kinase A-dependent pathway[70]. Therefore, by inhibiting PGE_2, NS-398 caused a net increase in MMP-3 production. However, PGE_2 can also increase intracellular Ca^{2+}, which interacts with protein kinase C, a major stimulator of MMP-3, to upregulate MMP-3[71,72]. Studies show that in normal human gingival fibroblasts, PGE_2 downregulates or upregulates IL-1ß-stimulated MMP-3 via activation of different PGE_2 receptors[73]. 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[74-77]. In rabbits, the COX inhibitor indomethacin reduced intra-articular PGE_2 levels and decreased clinical joint swelling, but loss of articular cartilage proteoglycans (MMP-3 substrates) was accelerated[78]. 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[71].

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[79]. 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[80-82].

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

1. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal disease. J Periodontol 1993;64:474-484.
2. Edwards C, Tipton D, Vazquez R, Dabbous M. MMP and TIMP production by early onset periodontitis fibroblasts. J Dent Res 2000;79(Special Issue):229;Abstract 688.
3. Seguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinase and tissue inhibitor of metalloproteinases in human gingival tissue? J Periodontol 2001;72:1398-1406.
4. Alpagot T, Bell C, Lundergan W, Chambers DW, Rudin R. Longitudinal evaluation of GCF MMP-3 and TIMP-1 levels as prognostic factors for progression of periodontitis. J Clin Periodontol 2001;28:353-359.
5. Page R. The role of inflammatory mediators in the pathogenesis of periodontal disease. J Periodont Res 1999;26:230-242.
6. Kjeldsen M, Holmstrup M, Bendtzen K. Marginal periodontitis and cytokines: a review of literature. J Periodontol 1993;64:1013-1022.
7. Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. J Periodont Res 1993;28:500-510.
8. Arias-Negrete S, Keller K, Chadee K. Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. Biochem Biophys Res Commun 2000;208:582-589.
9. Monks, BG, Martell BA, Buras JA, Fenton MJ. An upstream protein interacts with a distinct protein that binds to the cap site of the human interleukin 1 beta gene. Mol Immunol 1994;31:139-151.
10. Feldmann M, Maini RN. The role of cytokines in the pathogenesis of rheumatoid arthritis. Rheumatology 1999;38:3-7.
11. Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. J Clin Periodontol 2003;30:1046-1052.
12. Giannopoulou C, Cappuyns I, Mombelli A. Effect of smoking on gingival crevicular fluid cytokine profile during experimental gingivitis. J Clin Periodontol 2003;30:996-1002.
13. Kornman KS, Crane A, Wang HY, di Giovine FS. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol1997;24:72-77.
14. Cullinan MP, Westerman B, Hamlet SM, Palmer JE. A longitudinal study of interleukin-1 gene polymorphisms and periodontal disease in a general adult population. J Clin Periodontol 2001;28:1137-1144.
15. Thomson WM, Edwards SJ, Dobson-Le DP, Tompkins GR. IL-1 genotype and adult periodontitis among young New Zealanders. J Dent Res 2001;80:1700-1703.
16. Domeij H, Yucel-Lindberg T, Modeer T. Signal pathways involved in the production of MMP-1 and MMP-3 in human gingival fibroblasts. Eur J Oral Sci 2002;110:302-306.
17. Baber M, Tipton D, Dabbous M. MMP and TIMP production by human TMJ synovial fibroblasts. J Dent Res 2002;81(Special Issue A):A-384;Abstract 3090.
18. Lyle B, Tipton D, Dabbous M. Immunolocalization of MMPs and TIMPs in human TMJ synovial tissue. J Dent Res 2003;82(Special Issue A):Abstract 1116.
19. Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. J Clin Periodontol 2003;30:1046-1052.
20. Giannopoulou C, Kamma JJ, Mombelli A. Effect of inflammation, smoking, and stress on gingival crevicular fluid cytokine levels. J Clin Periodontol 2003;30:145-153.
21. Wu YF, Tan C, Zhang JY, Meng S, Guo YH. Interleukin-1beta and IL-1 receptor antagonist levels in gingival crevicular fluid and their relationship to clinical indices of periodontitis. Sichuan Da Xue Xue Bao Yi Xue Ban 2004;35:683-686.
22. Goutoudi P, Diza E, Arvanitidou M. Effect of periodontal therapy on crevicular fluid interleukin-1beta and interleukin-10 levels in chronic periodontitis. J Dent 2004;32:511-520.
23. Cullinan MP, Westerman B, Hamlet SM, Palmer JE. A longitudinal study of interleukin-1 gene polymorphisms and periodontal disease in a general adult population. J Clin Periodontol 2001;28:1137-1144.
24. Thomson WM, Edwards SL, Dobson-Le DP, Tompkins GR. IL-1 genotype and adult periodontitis among young New Zealanders. J Dent Res 2001;80:1700-1703.
25. Bankers-Fullright JL, Kalli KR, McKean DJ. Interleukin-1 signal transduction. Life Sci 1996;59:61-83.
26. Chen F, Castranova V, Shi X, Demers LM. New insight into the role of nuclear factor-κB, a ubiquitous transcription factor in the initiation of disease. Clin Chem 1999;45:7-17.
27. Baldwin AS. The NF-kappa B and I kappa B proteins: new discoveries and insights. Ann Rev Immunol 1996;14:649-683.
28. Palombella VJ, Conner EM, Fuseler JW, Destree A, Davis JM, Laroux FS et al. Role of the proteasome and NF-kappa B in streptococcal cell wall-induced polyarthritis. Proc Natl Acad Sci USA 1998;95:15671-15676.
29. Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev 1995;9:1586-1597.
30. Scherer D, Brockman JA, Chen Z, Maniatis T, Ballard DW. Signal-induced degradation of IκBα requites site-specific ubiquination. Proc Natl Acad Sci USA 1995;92:11259-11263.
31. DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, Karin M. Mapping of inducible IkB phosphorylation sites that signal its ubiquination and degradation. Mol Cell Biol 1996;16:1295-1304.
32. Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F et al. Complementation cloning of NEMO, a component of the Ikappa B kinase complex essential for NF-kappa B activation. Cell 1998;93:1231-1240.
33. May MJ, D'Acquisto F, Madge LA, Glöckner J, Pober JS, Ghosh S. Selective inhibition of NF-κB activation by a peptide that blocks the interaction of NEMO and the IκB kinase complex. Science 2000;289:1550-1554.
34. Fini ME, Cook JR, Mohan R, Brinckerhoff CE. In: Parks WC, Mecham RP (eds). Matrix Metalloproteinases. San Diego: Academic Press 1998;299-356.
35. Quinones S, Buttice G, Kurkinen M. Promoter elements in the transcriptional activation of the human stromelysin-1 gene by the inflammatory cytokine interleukin 1. Biochem J 1994:302:471-477.
36. Borghei RC, Rawlings PL, Mochan E. Interleukin-4 suppression of interleukin-1-induced transcription of collagenase (MMP-1) and stromelysis-1 (MMP-3) in human synovial fibroblasts. Arth Rheum 1998;41:1398-1406.
37. Bondeson J, Foxwell B, Brennan F, Feldman M. Defining therapeutic targets by using adenovirus: blocking NF-κB inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares ant-inflammatory mediators. Proc Natl Acad Sci USA 1999;96:5668-5673.
38. Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-κB reduces matrix metalloproteinase-1, -3, and -9 production by vascular smooth muscle cells. Cardiovasc Res 2001;50:556-565.

39. Bond M, Baker AH, Newby AC. Nuclear factor kappa B activity is essential for MMP-1 and -3 upregulation in rabbit dermal fibroblasts. Biochem Biophys Res Commun 1999;264:561-567.
40. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1 stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol 2002;21:251-262.
41. Kim HA, Cho M-L, Choi H, Yoon CS, Jhun JY, Oh HJ, Kim HY. The catabolic pathway mediated by toll-like receptors in human osteoarthritic chondrocytes. Arth Rheum 2006;54: 2152-2163.
42. Hiramitsu T, Yasuda T, Ito H, Shimizu M, Julovi SM, Kakinuma T et al. Intercellular adhesion molecule-1 mediates the inhibitory effects of hyaluronan on interleukin-1,-induced matrix metalloproteinase production in rheumatoid synovial fibroblasts via down-regulation of NF-κB and p38. Rheumatology 2006;45:824-832.
43. Asano K, Sakai M, Matsuda T, Tanaka H, Fujii K, Hisamitsu T. Suppression of matrix metalloproteinase production from synovial fibroblasts by meloxicam in vitro. J Pharm Pharmacol 2006;58:359-366.
44. Sakai T, Kambe F, Mitsuyama H. Tumor necrosis factor alpha induces expression of genes for matrix degradation in human chondrocyte-like HCS-2/8 cells through activation of NF-kappa B: abrogation of the tumor necrosis factor alpha effect by proteasome inhibition. J Bone Min Res 2001;16:1272-1280.
45. Tipton DA, Pabst MJ, Thomas EL, Babu JP, Stein SH, Fry H. Association of high levels of Prevotella intermedia with aggressive periodontitis in a 16-year-old female. PERIO 2006;3:115-122.
46. Tipton DA, Pabst MJ, Dabbous MKh. Interleukin 1ß and tumor necrosis factor · independent monocyte stimulation of fibroblast collagenase activity. J Cell Biochem 1990;44:253 264.
47. Freshney RI. Characterization. In: Culture of Animal Cells. A Manual of Basic Technique. Hoboken: Wiley-Liss 2005;247-280.
48. Freshney RI. Culture of specific cell types. In: Culture of Animal Cells. A Manual of Basic Technique. Hoboken: Wiley-Liss 2005;375-420.
49. Gabbiani G, Rungger-Brändle E. The fibroblast. In: Glynn L (ed). Handbook of Inflammation. Tissue Repair and Regeneration. Vol. 3. Amsterdam: Elsevier/North Holland Biomedical Press 1981;1-50.
50. Tipton DA, Gay D, DeCoster V. Effect of a COX-2 inhibitor on IL-1ß-stimulated activation of the transcription factor NF-κB in human gingival fibroblasts. J Periodontol 2007;78:542-549.
51. Tipton, DA, Flynn JC, Stein SH, Dabbous MKH. Cyclooxygenase-2 inhibitors decrease interleukin-1ß-stimulated prostaglandin E_2 and IL-6 production by human gingival fibroblasts. J Periodontol 2003;74:1754-1763.
52. Lowry OH, Rosebrough NJ, Farr L, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265 275.
53. Oyama VI, Eagle H. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc Soc Exp Biol Med 1956;91:305-307.
54. Nakao S, Ogata Y, Shimizu-Sasaki E. Activation of NF-κB is necessary for IL-1ß-induced cycloxygenase-2 (COX-2) expression in human gingival fibroblasts. Mol Cell Biochem 2000;209:113-118.
55. Morris MC, Depollier J, Mery J, Heitz F, Divita G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 2001;19:1173-1176.
56. Fischer R, Waizenegger T, Kohler K, Brock R. A quantitative validation of fluorophore-labelled cell-permeable peptide conjugates: flurorphore and cargo dependence of import. Biochim Biophys Acta 2002;564:365-374.
57. Terrone D, Sang SL, Roudaia L, Silvius JR. Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. Biochemistry 2003;42:13787-13799.
58. Jarajapu YP, Baltunis J, Knot HJ, Sullivan SM. Biological evaluation of penetration domain and killing domain peptides. J Gene Med 2005;7:908-917.
59. Liu RK, Cao CF, Meng HX, Gao Y. Polymorphonuclear neutrophils and their mediators in gingival tissues from generalized aggressive periodontitis. J Periodontol 2001;72:545-553.
60. Haerian A, Adonogianaki E, Mooney J, Docherty JP, Kinane DF. Gingival crevicular stromelysin, collagenase and tissue inhibitor of metalloproteinases levels in healthy and diseased sites. J Clin Periodontol 1995;22:505-509.
61. Haerian A, Adonogianaki E, Mooney J, Manos A, Kinane DF. Effects of treatment on gingival crevicular collagenase, stromelysin and tissue inhibitor of metalloproteinases and their ability to predict response to treatment. J Clin Periodontol 1996;23:83-91.
62. Tuter G, Kurtis B, Serdar M, Yücel A, Ayhan E, Karaduman B, Ozcan G. Effects of phase I periodontal treatment on gingival crevicular fluid levels of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1. J Clin Periodontol 2005;32:1011-1015.
63. Pourtaghi N, Radvar M, Mooney J, Kinane DF. The effect of subgingival antimicrobial therapy on the levels of stromelysin and tissue inhibitor of metalloproteinases in gingival crevicular fluid. J Periodontol 1996;67:866-870.
64. Hamid QA, Reddy PJ, Tewari M, Uematsu S, Tuncay OC, Tewari DS. Regulation of IL-1-induced collagenase gene expression by activator protein-1 (c-Fos/c-Jun). Cytokine 2000;12:1609-1619.
65. Sirum-Connolly K, Brinckerhoff CE. Interleukin-1 or phorbol induction of the stromelysin promoter requires an element that cooperates with AP-1. Nucleic Acids Res 1991;19:335-341.
66. Schulze-Tanzil G, Mobasheri A, Sendzik J, John T, Shakebaei M. Effects of curcumin (diferuloylmethane) on nuclear factor kappa B signaling in interleukin-1beta-stimulated chondrocytes. Ann NY Acad Sci 2004;1030:578-586.
67. Borghaei RC, Rawlings PL, Javadi M, Woloshin J. NF-κB binds to a polymorphic repressor element in the MMP-3 promoter. Biochem Biophys Res Commun 2004;316:182-188.
68. Stein B, Baldwin AS Jr, Ballard DW, Greene WC, Angel P, Herrlich P. NF-κB potentiates transcription for promoters that lack a consensus NF-κB binding site through interaction with AP-1. EMBO J 1993;12:3879-3891.
69. Kanai K-I, Asano K, Hisamitsu T, Suxaki H. Supression of matrix metalloproteinase-9 production from neutrophils by a macrolide antibiotic roxithromycin, in vitro. Med Inflamm 2004;13:313-319.
70. Kerr LD, Olashaw NE, Matrisian LM. Transforming growth facto b1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. J Biol Chem 1988;263:16999-17005.
71. Case JP, Lafyatis R, Kumkumian GK, Remmers EF, Wilder RL. IL-1 regulation of transin/stromelysin transcription in rheumatoid synovial fibroblasts appear to involve two antagonistic transduction pathways, an inhibitory prostaglandin-dependent pathway mediated by cAMP, and a stimulatory protein kinase-C dependent pathway. J Immunol 1990;145:3755-3761.
72. Rodland KD, Lenormand P, Muldoon LL, Magun BE. Regulation of transin/stomelysin and VL30 gene expression by intracellular calcium. J Invest Dermatol 1992;98:12S-16S.

73. Ruwanpura SMPM, Noguchi K, Ishikawa I. Prostaglandin E2 regulates interleukin-1beta-induced matrix metalloproteinase-3 production in human gingival fibroblasts. J Dent Res 2004;83:260-265.
74. Paquette DW, Williams RC. Modulation of host inflammatory mediators as a treatment strategy for periodontal diseases. Periodontol 2000 2000;24:239-252.
75. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 2001;15:2057-2072.
76. Kammerl MB, Debler J, Riegger GA, Kramer BK. COX-2 inhibitors and risk of heart failure. Lancet 2004;364: 1486-1487.
77. Ahmad SR, Kortepeter C, Brinker A, Chen M, Beitz J. Renal failure associated with the use of celecoxib and rofecoxib. Drug Saf 2002;25:537-544.
78. Pettipher ER, Henderson B, Edwards JCW, Higgs GA. Effect of indomethacin on swelling, lymphocyte influx, and cartilage proteoglycan depletion in experimental arthritis. Ann Rheum Dis 1989;48:623-627.
79. Ambili R, Santhi WS, Janam P, Nandakumar K, Pillai M. Expression of activated transcription factor nuclear factor-κB in periodontally diseased tissue. J Periodontol 2005;76:1148-1153.
80. Dia S, Hirayama T, Abbas S, Abu-Amer Y. The IκB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. J Biol Chem 2004;279:37219-37222.
81. Agou F, Courtois G, Chiaravalli J, Baleux F, Coïc YM, Traincard F et al. Inhibition of NF-κB activation by peptides targeting NF-κB essential modulator (NEMO) oligomerization. J Biol Chem 2004;279:54248-54257.
82. Latham PW. Therapeutic peptides revisited. Nat Biotechnol 1999;17:755-757.