

ORIGINAL ARTICLE

# The Effect of Phenytoin on the Matrix Metalloprotease-3 Production in HGFs

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## SYNOPSIS

Special care must be taken in the orthodontic treatment of patients who are medicating with phenytoin, which has a side effect of gingival overgrowth. However, the mechanism of this side effect is not yet known in detail.

Gingival overgrowth is termed hatred by inflammation, and it has been reported that when inflammation occurs during phenytoin treatment the movement of the teeth is delayed. Therefore, in the present study, it was decided to investigate the mechanisms of phenytoin by focusing on matrix metalloproteinases (MMPs) that are expressed as a result of inflammation.

In this study, we demonstrated that the production of MMP-3 in TNF- $\alpha$ -stimulated Human gingival fibroblasts (HGFs) is suppressed by phenytoin. In addition, phenytoin suppressed the phosphorylation of NF $\kappa$ B in TNF- $\alpha$  stimulated HGFs. These results suggested that phenytoin stimulation regulated the production of MMP-3 in inflammatory HGFs, and that phenytoin stimulation involves NF $\kappa$ B signaling.

**Key words:** Phenytoin, Matrix metalloproteinase-3, Human gingival fibroblast

## INTRODUCTION

Drug-induced gingival overgrowth is a disfiguring side effect of anti-covulsants, calcineurin inhibitors, and calcium channel blocking agents. Phenytoin is an antiepileptic drug that acts on sodium channels in mammal cells<sup>1</sup>. Clinically detectable gingival overgrowth occurs as the principal side-effect of phenytoin therapy in approximately half of the treated patients<sup>2</sup>. Although the number of patients being treated with this drug is increasing, not all patients on phenytoin medication develop gingival overgrowth. Chronic

periodontitis exacerbates the diagnosis of gingival overgrowth associated with phenytoin<sup>3</sup>. Gingival overgrowth presents major problems for the maintenance of oral hygiene. Increased swelling and disfiguration of gingiva also elevate the risk of infections and inflammatory complications in patients. Additional ramifications of gingival overgrowth occur difficult orthodontic treatment<sup>4</sup>. Orthodontic treatment could contribute to the pathogenesis of this side effect.

Tumor necrosis factor (TNF)- $\alpha$  is an inflammatory cytokine that induces cel-

lular proliferation<sup>5</sup>. TNF- $\alpha$  also inhibits collagen synthesis<sup>6</sup> and increases matrix metalloproteinases (MMP) synthesis by fibroblasts<sup>7</sup>. In contrast, phenytoin decreases matrix MMP synthesis in human gingival fibroblasts<sup>8</sup>. The effect of phenytoin is specific for the reduction of total protein and collagen synthesis and phenytoin does not influence the cell proliferation of gingival fibroblasts in vitro<sup>9</sup>. Dental plaque is the most important factor for the enhancement of the deterioration that is caused by phenytoin-induced gingival overgrowth<sup>10</sup>.

Transcription factor nuclear factor kappa-B (NF- $\kappa$ B) is a key player in controlling both immune and inflammatory responses in periodontally diseased tissues<sup>11,12</sup>. In unstimulated cells, NF- $\kappa$ B is bound to its inhibitor protein, inhibitor kappa B (I $\kappa$ B), and, in this form, it is inactive in the cytoplasm. Various agents such as proinflammatory cytokines, bacterial and viral agents, lipopolysaccharides, and reactive oxygen species can activate NF- $\kappa$ B and translocate it into the nucleus. TNF- $\alpha$  is one of the most important factors for inducing the NF- $\kappa$ B signaling cascade. In the nucleus, NF- $\kappa$ B binds to its specific DNA regulatory sites, called  $\kappa$ B sites, and induces transcription of the mRNA of several MMPs<sup>13, 14</sup>. As NF- $\kappa$ B closely influences the upregulation of these mediators, it is possible that it may play a role in the complex mechanism of phenytoin activity.

It is likely that human gingival fibroblasts exposed to TNF- $\alpha$  would show greater susceptibility to phenytoin than non-exposed fibroblasts. However, a synergistic effect of phenytoin and TNF- $\alpha$  on gingival overgrowth has rarely been examined in vitro. We therefore examined the effect of phenytoin on the production of the collagenases by human gingival fibroblasts exposed to TNF- $\alpha$ . A further aim of this study was to investigate the activation of NF- $\kappa$ B and

its possible relationship to these cellular mediators in gingival fibroblasts with phenytoin-induced gingival overgrowth.

## MATERIALS AND METHODS

### 1. Cell culture

Human gingival fibroblasts (HGFs) were grown from explants obtained from the healthy marginal gingiva of healthy donors. Primary cultures were grown in minimum essential medium alpha modification ( $\alpha$ -MEM; Wako Pure Chemical Industries, Japan) containing 10% fetal bovine serum (FBS; Equitech-Bio, Inc., CA), penicillin G sodium 100 units/mL, streptomycin 100  $\mu$ g / mL and L-glutamine 292  $\mu$ g / mL (Invitrogen Co., CA, USA) in an atmosphere of 5% CO<sub>2</sub>-95% air at 37 °C. The first subcultures were obtained 20 to 30 days later, were maintained in an atmosphere of 5% CO<sub>2</sub>-95% air at 37 °C, and were routinely subcultured after the use of trypsin-EDTA (0.05% trypsin, 0.53 mmol / L EDTA-4NA, Invitrogen Co.) for cell release. Experiments with HGFs were performed between passage 3 and 10. This study was approved by the Ethical Review Board of Osaka Dental University. (Approval No. 110762)

### 2. Antibodies and Reagents

Human TNF- $\alpha$  was obtained from Miltenyi Biotec Inc. (USA) and phenytoin (5, 5-Diphenylhydantoin sodium salt) was obtained from Sigma-Aldrich Inc. (MO). Human TNF- $\alpha$  and phenytoin were dissolved in deionized sterile-filtered water. Primary antibodies used for western blotting were: anti-MMP-3 (Daiichi Fine Chemical Co., Ltd., Japan) and the anti-phosphospecific NF- $\kappa$ B antibody, p-NF $\kappa$ B p65 (Ser536) (Rabbit mAb 93H1; Cell Signaling Technology Japan, K.K., Japan). p-NF $\kappa$ B p65 (Ser536) antibodies were dissolved in deionized sterile-filtered water. Second antibodies used for western blotting

were: anti-NF $\kappa$ B p65 (D14E12)(Rabbit mAb; Cell Signaling Technology Japan, K.K., Japan) and anti- $\beta$ -actin (BioLegend, CA, USA). The cell proliferation reagent WST-1 (Roche Diagnostics, Basel, Switzerland) was used to assess cell proliferation. Anti-Collagen Type 1-FITC (Millipore Corp, Bedford, MA, USA) was used for Flow Cytometric analysis.

### **3. Western Blot analysis**

HGFs were incubated in serum-free  $\alpha$ -MEM containing phenytoin (0, 20 or 50  $\mu$ M) for 24 h. Conditioned media were collected, centrifuged to remove debris, and concentrated up to 10-fold in Amicon Centriprep concentrators (Millipore Corp.) so that proteins could be visualized by western blotting. Total cell lysates were prepared by dissolving cells in sodium dodecyl sulfate (SDS)-sample buffer. For some studies, HGFs were preincubated with 0% FBS  $\alpha$ -MEM with phenytoin (20 or 50  $\mu$ M) and TNF- $\alpha$  (2 ng/mL) for 24 h at 37 °C. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% SDS polyacrylamide gels under reducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated for 1 h with anti-MMP-3 antibodies in PBS containing 0.05% Tween 20 and 5% BSA. Peroxidase-conjugated secondary antibody was used at a 1:1,000 dilution, and immunoreactive bands were visualized using a substrate. Signals of each membrane were analyzed using a Chemi Doc MP system (Bio-Rad, Hercules, CA, USA).

### **4. Flow Cytometric Analysis**

HGFs were stained with anti-Collagen Type 1 FITC-conjugated monoclonal antibodies for 30 min at 4 °C and were then washed with FACS buffer. Stained cells were analyzed by flow cytometry

using a FACS Verse with the BD FACSuite software (BD Biosciences, San Jose, CA, USA).

### **5. Gelatin zymography**

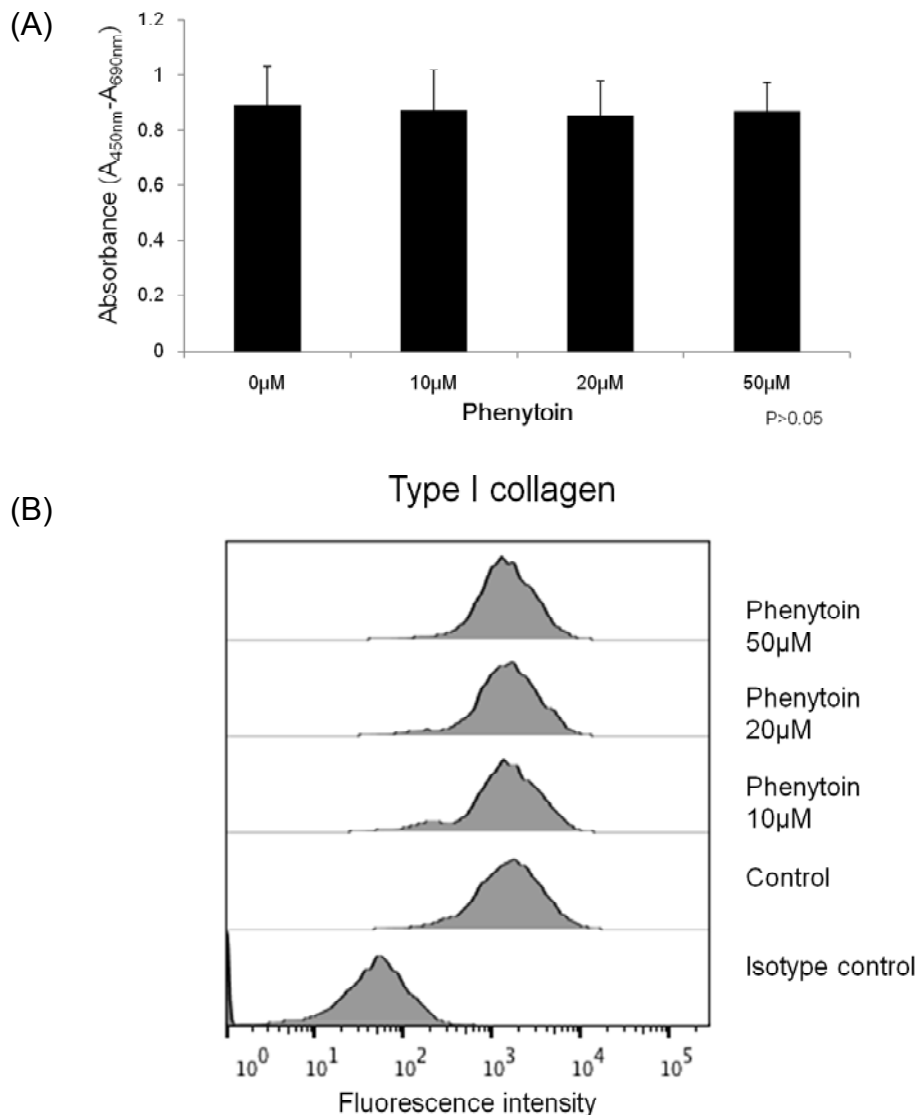
HGFs were placed in a 24-well plate ( $5.0 \times 10^5$  cells/well), and incubated in  $\alpha$ -MEM (10% FBS) for 24 h. Each well was washed with PBS and the cells were then incubated with  $\alpha$ -MEM (serum-free) containing 20 or 50  $\mu$ M phenytoin for 24 h. The culture solution in each well was centrifuged at 10,000 rpm for 10 min, following which sample buffer (0.0645 M Tris buffer (pH 6.8), 2% SDS, 10% glycerol and bromophenol blue (BPB)) was added to 10  $\mu$ L of the supernatant for electrophoresis. Each sample (15  $\mu$ L) was applied to a lane of a 10% SDS-polyacrylamide gel electrophoresis (PAGE) that included 1 mg/mL of gelatin, and electrophoresis was performed with a constant voltage of 200 mV for 60 min in concentration and isolation gels, respectively. After electrophoresis, the gel was washed at room temperature in 2.5% Triton X-100 (Katayama Kagaku Kogyo, Osaka, Japan) for 20 min. The 2.5% Triton X-100 buffer was then exchanged and the gel was washed again at room temperature for 20 min before soaking in 10 mL of activation buffer (200 ml H<sub>2</sub>O plus 10 mL zymosan (in 200 mM Tris-HCl (pH 7.5) containing 100 mM CaCl<sub>2</sub>, 20 mM ZnCl<sub>2</sub> and 2.5 mL Triton X-100) at 37 °C for 16 h. The gel was then washed in 2.5% Triton X-100 for 5 min, soaked in Coomassie blue solution for 3 h, destained using destain buffer (methanol:acetic acid:water, 50:10:40), and scanned (EPSON GT-9600; Seiko-Epson, Tokyo, Japan) for detection of MMP-2 activity.

### **Proliferation experiment**

HGFs were seeded onto a 96-well plate at a density of  $1.0 \times 10^5$  cells/well and were then incubated for up to 24 h in

$\alpha$ -MEM (plus 10% FBS) containing phenytoin (0, 10, 20 or 50  $\mu$ M). The cell proliferation reagent WST-1 (Roche Diagnostics) was used to assess cell proliferation by measurement of the absorbance at 450/650 nm using a Spectra Max M 5 (Molecular Devices,

Sunnyvale, CA, USA). The data were statistically analyzed using one-way analysis of variance with the Statcel software (Excel add-in program, OMS Publishing Inc, Japan).



**Figure 1** Effect of phenytoin on the proliferation and the production of type I collagen in HGFs.

(A) HGF growth rate. Human gingival fibroblasts were cultured in 96-well culture plates. After overnight phenytoin stimulation (0, 10, 20 or 50  $\mu$ M), the growth rate was evaluated using the WST-1 assay. Three independent experiments were performed in triplicate. There was no significant difference between the proliferation of phenytoin treated and untreated cells.

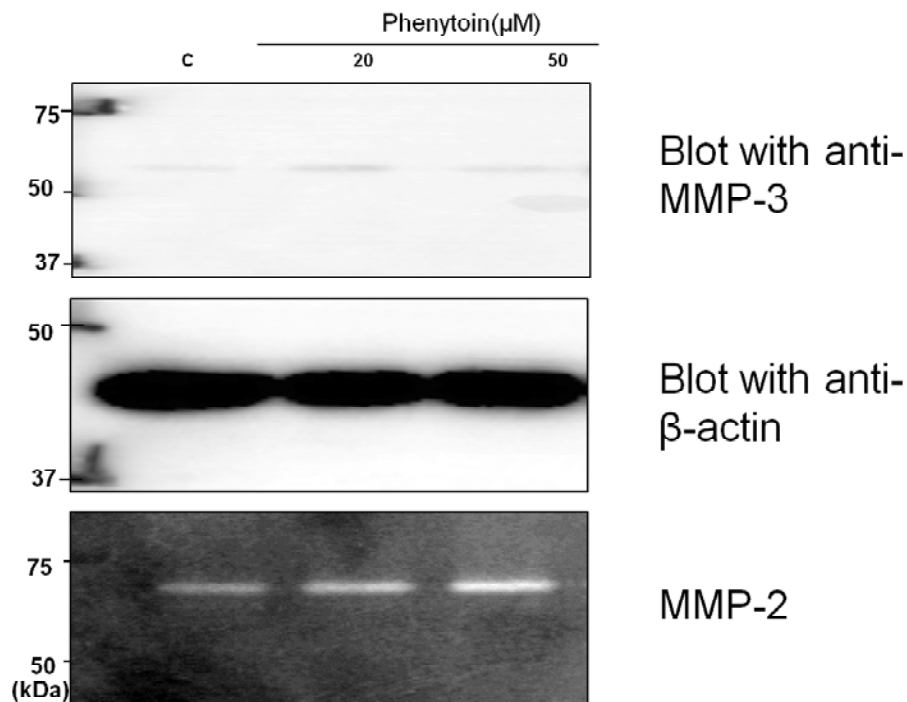
(B) Type-1 collagen production. Human gingival fibroblasts were stimulated overnight with phenytoin (0, 10, 20 or 50  $\mu$ M) and were then stained with an anti-type 1 collagen antibody and subjected to flow cytometric analysis. The control sample was an isotype control antibody. Both control cells without phenytoin stimulus and phenytoin-treated cells were stained with the type 1 collagen antibody. There was no significant difference between collagen production of control and phenytoin-treated cells.

## RESULTS

### 1. Effect of phenytoin on the proliferation and the production of MMPs by HGFs.

To investigate the effect of phenytoin on HGFs proliferation, HGFs were incubated in a 96-well plate ( $1.0 \times 10^5$  cells/well) and 0, 10, 20 or 50  $\mu\text{M}$  of phenytoin were added after 24 h and the plates were incubated overnight. Phenytoin did not affect the proliferation of the HGFs (Figure 1A). We then tested the effect of phenytoin on the production of type I collagen by the HGFs using flow cytometry. Unstimulated HGFs spontaneously produced type I collagen, and this production of type I collagen was not altered by HGF stimulation with 10, 20 or 50  $\mu\text{M}$  phenytoin (Figure 1B).

We then evaluated the production of MMP-2 and MMP-3 by HGFs in the presence of phenytoin. HGFs were incubated in the absence or presence of 10, 20 or 50  $\mu\text{M}$  phenytoin, as described in the Materials and Methods, and MMP-3 production was analyzed by western blotting of the concentrated culture medium. Importantly, MMP-3 generation was not observed when the cells were cultured in the presence of phenytoin (Figure 2 upper gel). Samples of the concentrated conditioned medium of the same HGFs were also separated by gelatin zymography to visualize MMP-2 (Figure 2, bottom gel). Unstimulated HGFs spontaneously produced the latent form of MMP-2. However, neither the total amount of MMP-2



**Figure 2** Effect of phenytoin on the production of MMPs by HGFs.

HGFs were seeded in a 24-well plate ( $5.0 \times 10^5$  cells/well), incubated in  $\alpha$ -MEM (10% FBS) for 24 h, washed with PBS, incubated with phenytoin (0, 20 or 50  $\mu\text{M}$ ) at 37  $^{\circ}\text{C}$  for 24 h and analyzed as follows. (1) The concentrated conditioned medium was separated by SDS-PAGE and analyzed by western blotting using an anti-MMP-3 antibody. (2) Cells of the same sample were lysed by adding 100  $\mu\text{l}$  lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% tert-octylphenoxy polyethanol, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/mL leupeptin, and 20 mg/mL aprotinin). The lysates were analyzed by western blotting to assess the expression of  $\beta$ -actin. For (1) and (2) antibody binding was visualized using the Super Signal West Pico chemiluminescent substrate. Molecular markers (kDa) are shown in the left column. (Top gel).

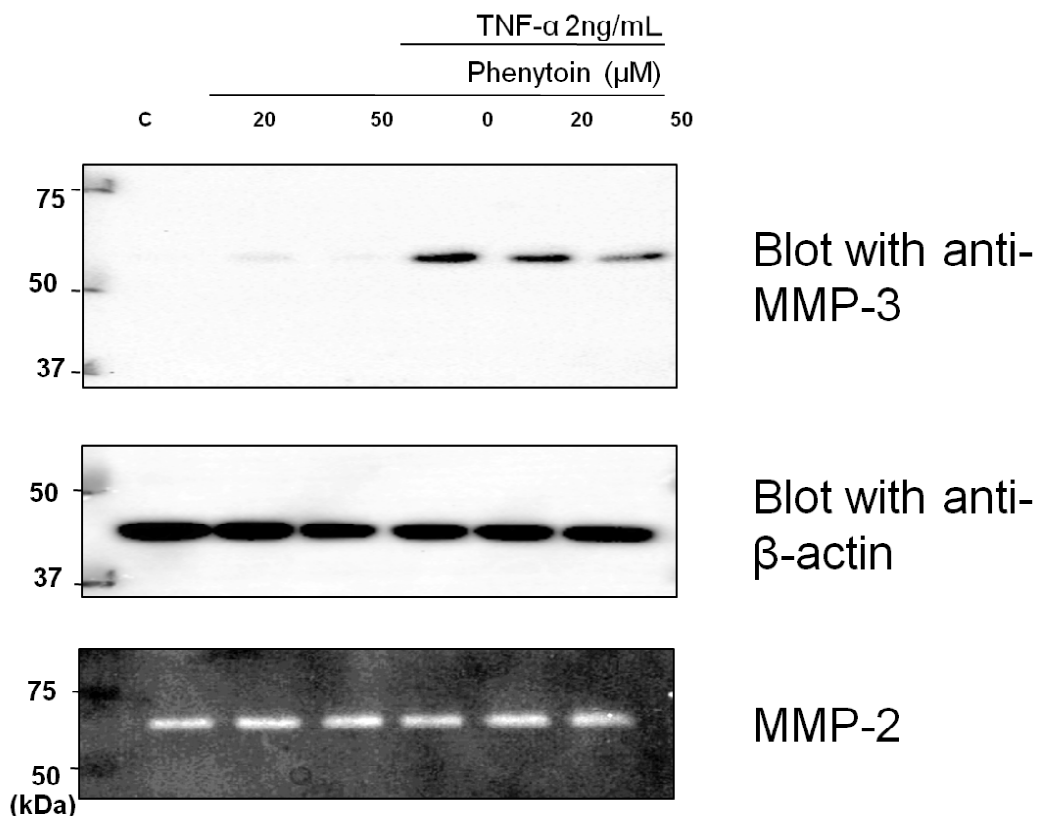
(3) Samples of the same concentrated conditioned medium were analyzed by gelatin-zymography to assess the expression of MMP-2 as described in the Materials and Methods (Lower gel).

produced nor the activation status of this enzyme was altered by stimulation with phenytoin. These results suggested that phenytoin did not affect the production of MMPs by HGFs.

## 2. Phenytoin suppressed the production of MMP-3 by TNF- $\alpha$ activated-HGFs.

It has been reported that the production of MMP-3 from fibroblasts is enhanced in the presence of TNF- $\alpha$ <sup>7</sup>. The production of MMP-3 from TNF- $\alpha$  activated-HGFs was therefore evaluated in the presence of phenytoin, by western blotting of the concentrated HGF culture

medium of treated cells. When HGFs were cultured in the presence of TNF- $\alpha$  (2 ng/ml) for 24 h, the generation of MMP-3 was enhanced compared to the non-treated control cells, whereas the level of  $\beta$ -actin, which was used as a loading control, remained unchanged (Figure 3). Phenytoin addition markedly suppressed the production of MMP-3 by TNF- $\alpha$ -stimulated HGFs in a dose dependent manner, whereas the total amount of  $\beta$ -actin was not altered by phenytoin treatment (Figure 3). Phenytoin did not affect the production of collagen by TNF- $\alpha$ -stimulated HGFs (data not shown).



**Figure 3** Phenytoin suppressed the production of MMP-3 by TNF- $\alpha$  activated-HGFs. HGFs were seeded in a 24-well plate ( $5.0 \times 10^5$  cells/well), and were then incubated with phenytoin (0, 20, or 50  $\mu$ M) and TNF- $\alpha$  (2 ng/mL) for 24 h at 37 °C. The concentrated conditioned medium was separated by SDS-PAGE and analyzed by western blotting using an anti-MMP-3 antibody. The lysates were analyzed by western blotting to assess the expression of  $\beta$ -actin. Samples of the same concentrated medium were also analyzed by gelatin-zymography to assess the expression of MMP-2 as described in the Materials and Methods.

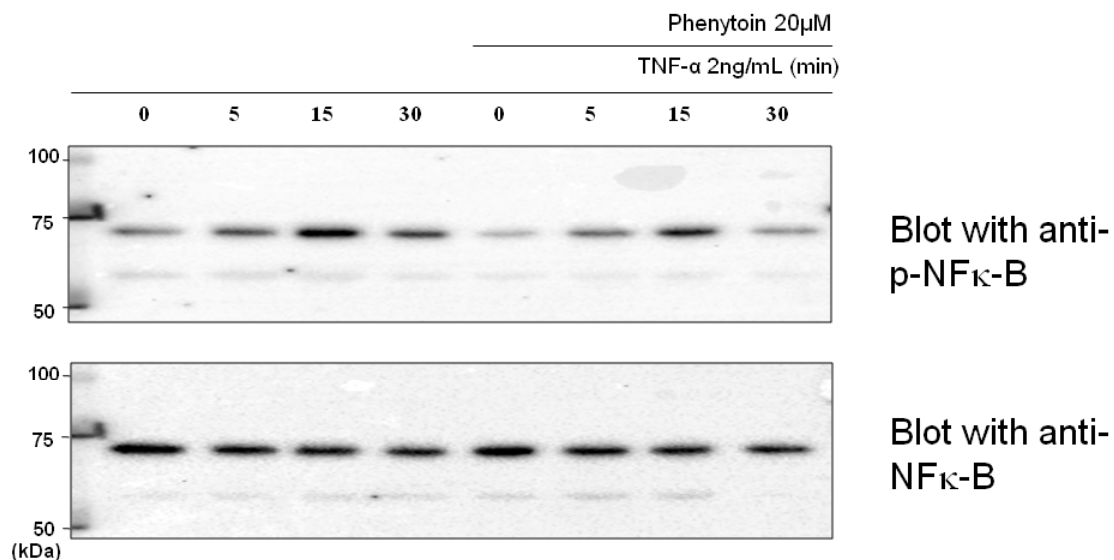
### 3. Phenytoin affects the NF-κB -dependent signaling pathway

Phosphorylation of NF-κB leads to increased promoter activation potential<sup>15</sup>. NF-κB modulates transcriptional activation of the human stromelysin (also known as MMP-3) promoter<sup>16, 17</sup>. Therefore, the present study used western blotting to investigate whether TNF-α-induced phosphorylation of NF-κB is mediated by phenytoin. TNF-α enhanced the phosphorylation of NF-κB in HGFs in a time dependent manner. However, the TNF-α-induced phosphorylation of NF-κB was decreased in the presence, compared to the absence of phenytoin treatment (Figure 4).

### DISCUSSION

For patients on phenytoin medication, the side effects can be so severe that they stop administering their medication during orthodontic treatment. Orthodontists will encounter increasing numbers of patients who have received phenytoin treatment as the drug becomes more widely used. Therefore, the mechanism of phenytoin action in inflammation is considered to be of importance for orthodontic treatment and should be studied, particularly since understanding the mechanism of phenytoin action could lead to shorter orthodontic treatment times.

The present study analyzed the ef-



**Figure 4** Phenytoin suppressed production of phosphorylated NFκB in TNF-α stimulated-HGFs. HGF cells ( $5.0 \times 10^5$  cells/well) were cultured in 24-well plates and were stimulated with phenytoin for 24 h. The cells were then further stimulated with TNF-α (2 ng/mL) for 0, 5, 10, 15 or 30 minutes. Cell lysates were separated by SDS-PAGE, and blots were probed with anti-phospho-NFκB antibodies. Signals were visualized using the Immobilon Western chemiluminescent HRP substrate. The same membrane was then stripped and reprobed with anti-oxidant NFκB antibodies.

fect of phenytoin on the regulation of the degradation of connective tissue proteins by proteases such as MMPs. We demonstrated that phenytoin inhibited the production of MMP-3 in the conditioned media of TNF- $\alpha$ -stimulated HGFs. However, phenytoin treatment did not affect the production or activation of MMP-2 by HGFs under our experimental conditions. MMP-3 has a broad substrate specificity for ECM proteins<sup>18</sup> and induces angiogenesis and fibroblast wound healing<sup>19</sup>. No significant difference was previously reported in the expression of collagen between phenytoin treated and untreated fibroblasts<sup>20</sup>. Alteration of the cytokine balance has been suggested to exert a greater influence on gingival overgrowth compared to a direct effect of a drug on the regulation of extracellular matrix metabolism or on the proliferation of gingival overgrowth<sup>21</sup>. Overgrowth is worsened in cases of chronic gingival inflammation<sup>22</sup>. TNF- $\alpha$  is a major inflammatory cytokine that is found in chronically inflamed gingival tissue<sup>23</sup>, and it has been reported to mediate gingival overgrowth in periodontal tissue<sup>24</sup>. Our data showed that the production of MMP-3, but not of MMP-2, in TNF- $\alpha$ -stimulated HGFs is inhibited by phenytoin. Since MMP-3 activates other collagenase, these data suggested that phenytoin might enhance collagen accumulation in gingival fibroblasts exposed to low levels of TNF- $\alpha$ . Elevated levels of TNF- $\alpha$  could contribute to the pathogenesis of this side effect of phenytoin.

Activation of NF- $\kappa$ B is known to be involved in MMP-3 expression<sup>25</sup>. A previous study demonstrated that activated NF- $\kappa$ B levels are elevated in chronic inflamed gingival tissues and in gingival overgrowth<sup>26</sup>. We demonstrated that NF- $\kappa$ B was involved in MMP-3 expression in TNF- $\alpha$ -stimulated HGFs and that phenytoin inhibited the activation of

NF- $\kappa$ B and production of MMP-3. Our data suggested that phenytoin-related gingival overgrowth involves fine scale regulation of transcription factor NF- $\kappa$ B, growth factors, and inflammatory cytokines by phenytoin.

This study may provide a basis for future therapeutic strategies in NF- $\kappa$ B inhibition for prevention of phenytoin-related side effects in gingival connective tissues and in orthodontic therapy.

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