Stimulation of Cells Derived from Nifedipine-induced Gingival Overgrowth with *Porphyromonas gingivalis* , Lipopolysaccharide, and Interleukin-1β

H.-K. Lu, H.-P. Chou, C.-L. Li, M.-Y. Wang and L.-F. Wang

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H.-K. Lu[1,2], H.-P. Chou[2], C.-L. Li[2], M.-Y. Wang[3], and L.-F. Wang[4*]

[1]Periodontal Clinic of the Dental Department, Taipei Medical University Hospital, Taipei, Taiwan; [2]College of Oral Medicine, Taipei Medical University, Taipei, Taiwan; [3]School of Dentistry, College of Medicine, National Taiwan University, Taipei, Taiwan; and [4]Department of Biochemistry, College of Medicine, Taipei Medical University, 250, Wu-shing Street, Taipei, Taiwan; *corresponding author, wanglf@tmu.edu.tw

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ABSTRACT

The purpose of this study was to clarify the main contributory factor of nifedipine-induced gingival overgrowth either by *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS) or interleukin-1beta (IL-1β). Human gingival fibroblasts from healthy tissues and nifedipine-induced gingival overgrowth tissues were stimulated with nifedipine, IL-1β, *Escherichia coli* lipopolysaccharide (*Ec*-LPS), and *Pg*-LPS, and the gene expressions were analyzed by RT-PCR. Analysis of the data showed no strong evidence of a synergistic effect of nifedipine and *Pg*-LPS on IL-6, connective tissue growth factor (CTGF), and type 1 collagen gene expression of either healthy cells or nifedipine-induced gingival overgrowth cells. Among the three stimulants—IL-1β, *Pg*-LPS, and *Ec*-LPS—androgen receptor and IL-6 gene expressions in both the healthy and nifedipine-induced gingival overgrowth groups were strongly up-regulated by the presence of IL-1β only. Furthermore, the responses to IL-1β in the nifedipine-induced gingival overgrowth group were stronger than those of the healthy group. It can be concluded that IL-1β is an important mediator responsible for the higher IL-6 and androgen receptor expression of nifedipine-induced gingival overgrowth cells.

KEY WORDS: nifedipine-induced gingival overgrowth, androgen receptor, IL-6, *Porphyromonas gingivalis*, IL-1β.

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A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

INTRODUCTION

Numerous studies have demonstrated that gingival overgrowth may be associated with androgen and cytokine expressions in tissues (Southren *et al.*, 1978; Ojanotko *et al.*, 1980; Ojanotko-Harri *et al.*, 1992). In a comprehensive review and analysis of nifedipine-induced gingival overgrowth, a combination of deficient intracellular folic acid uptake, an increase in 5-α-dihydrotestosterone receptors, and suppression of both Th1 cytokine production and T-cell proliferation were postulated as the "three models" for drug-induced gingival hyperplasia (Harel-Raviv *et al.*, 1995). Our previous *in vivo* study confirmed that the overexpression of androgen-receptor-positive cells present in nifedipine-induced gingival overgrowth tissues seems to be one of the major differences in the pathogenesis of nifedipine-induced gingival overgrowth (Huang *et al.*, 2003). It also showed that the cytokine profile of T-cells in nifedipine-induced gingival overgrowth tissue was preferentially toward Th1 activity.

The aim of the present study was to clarify the main contributory factor of nifedipine-induced gingival overgrowth, either by stimulation with *Pg*-LPS or the pro-inflammatory cytokine, IL-1β, *in vitro* over a predetermined time interval, to investigate the expressions of IL-6 and androgen receptor by nifedipine-induced gingival overgrowth cells.

MATERIALS & METHODS

Participant Selection

Four male nifedipine-induced gingival overgrowth responders (age range, 48-65 yrs) and four periodontally healthy individuals without smoking habits were included in this study. Informed consent was received from each participant, under a protocol approved by the Ethics Committee of the Taipei Medical University Hospital.

Diagnostic criteria for the nifedipine-induced gingival overgrowth group included individuals who had taken nifedipine for 6~36 mos, and whose gingival overgrowth was classified as grade III. Their plaque control scores ranged from 70 to 100%. Gingival bleeding scores ranged from 55 to 92%. Persons were selected for tissue sampling 6 wks after scaling and root planing. The probing depth of the sampled area was still ≥ 5 mm deep after scaling and root planing.

Healthy gingival tissue was harvested from donors who were undergoing implant surgery or selective oral surgery. Diagnostic criteria for healthy individuals included having received no antibiotic therapy for any reason in the preceding 3 mos, lacking any systemic condition that might contribute to periodontal conditions, and no regular consumption of any non-steroidal anti-inflammatory drugs. Clinically, the healthy tissue appeared firm and pink, and had no erythematous changes.

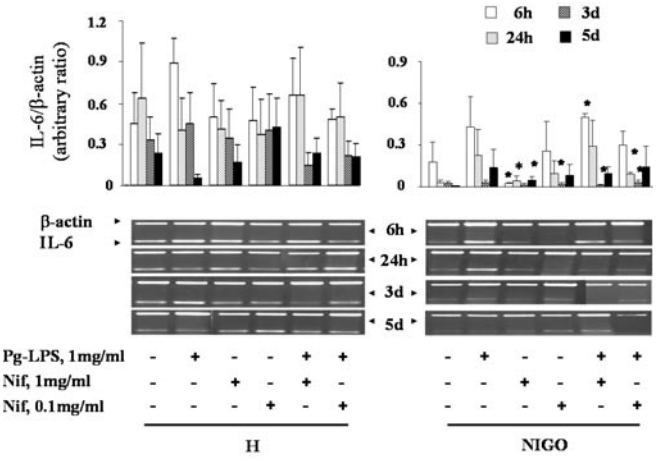

Figure 1. mRNA expression of IL-6 in gingival fibroblasts of healthy (n = 3) and nifedipine-induced gingival overgrowth individuals (n = 3) cultured with *Pg*-LPS and/or nifedipine. The expression of mRNA encoding IL-6 was examined in gingival fibroblasts cultured with *Pg*-LPS and/or nifedipine for the indicated time periods. Data are presented as means ± standard deviation of triplicate experiments. Note that there is no evidence to show the synergistic effect of nifedipine upon *Pg*-LPS in stimulating both healthy and nifedipine-induced gingival overgrowth cells for the expression of the IL-6 gene.

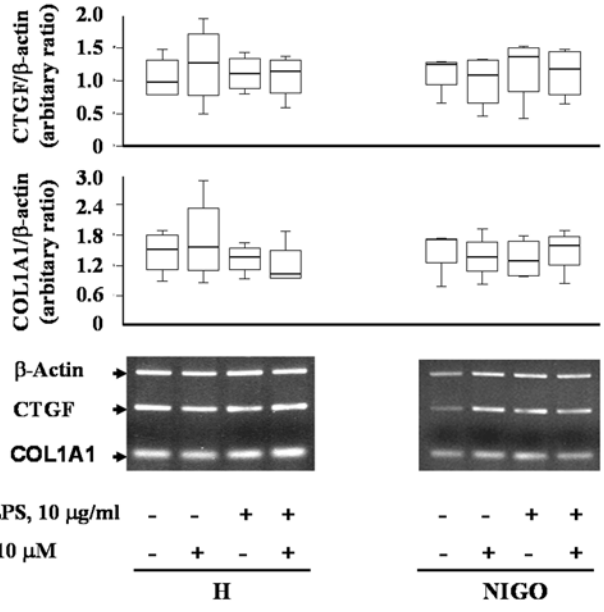

Figure 2. Effects of *Pg*-LPS and nifedipine on gene expression of CTGF and COL1A1 in gingival fibroblasts from healthy (n = 4) and nifedipine-induced gingival overgrowth groups (n = 4). Each box represents the values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of β-actin expression. $*p < 0.05$, statistically significantly different compared with the Kruskal-Wallis test. There was no significant difference between healthy and nifedipine-induced gingival overgrowth responses for all stimulants. Furthermore, gene expression was not changed when stimulated either by *Pg*-LPS and nifedipine alone, or by both ($p > 0.05$).

Fibroblast Cell Culture

Gingival fibroblasts obtained from nifedipine-induced gingival overgrowth and healthy tissues were cultured in DMEM medium supplemented with sodium pyruvate, L-glutamine, streptomycin, penicillin, and 10% fetal calf serum (Gibco, Grand Island, NY, USA). Cells between the fourth and eighth passages were used in this investigation (see APPENDIX for preliminary culture conditioning).

Reverse-transcription Polymerase Chain-reaction (RT-PCR)

Since there was reason to expect that nifedipine may possibly be the major stimulant for androgen receptor and IL-6 expression (Johnson *et al.*, 2003), the co-administration of *Pg*-LPS and nifedipine was also studied for investigation of their synergistic effect on IL-6, connective tissue growth factor (CTGF), and type 1 collagen (COL1A1) gene expression. Total RNA was isolated from gingival fibroblasts with the RNeasy mini kit (QIAGEN, Valencia, CA, USA) and treated with *Pg*-LPS (1 μg/mL) and/or nifedipine (0.1 or 1.0 μg/mL). The mRNAs coding for IL-6, CTGF, COL1A1, and β-actin were reverse-transcribed and amplified with sequence-specific primer pairs. The resultant products were separated on 2.0% agarose gels, and the results were expressed relative to β-actin. (Forward and reverse primers are shown in the APPENDIX.) All of the primers of the target genes in this study were designed to span introns. Triplicate experiments were performed, and all assays were repeated 3 times to confirm their reproducibility. PCR products were analyzed by electrophoresis on agarose gels, stained with ethidium bromide, and photographed. The band densities of the gels were determined with the use of an image analysis system (EagleEye II, Stratagene, La Jolla, CA, USA). The signal intensities of the PCR products were divided by that of β-actin for each sample.

Enzyme Immunoassay for the Detection of IL-6

Amounts of IL-6 secreted into the culture medium were determined by means of a commercially available ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Statistical Analysis

The investigation of the synergistic effects of *Pg*-LPS and nifedipine on IL-6 mRNA expression was analyzed by the Student's *t* test, and the *p* value was set at 0.05 for significance in intra-group comparison. After estimation by the Shapiro-Wilk test for the verification of normality, CTGF and COL1A1 gene expression for intra-group comparisons and ELISA/PCR data for IL-6 and androgen receptors for healthy and nifedipine-induced gingival overgrowth groups' comparison were analyzed by the Kruskal-Wallis test and the Mann-Whitney U test, respectively, with significance set at $p < 0.05$.

RESULTS

Analysis of the data showed that less IL-6 expression was detected in nifedipine-induced gingival overgrowth after stimulation with nifedipine (0, 0.1, 1 μg/mL) and/or *Pg*-LPS at the indicated times ($*p < 0.05$) (Fig. 1). There was no synergistic effect of *Pg*-LPS and nifedipine upon either healthy or nifedipine-induced gingival overgrowth cells, in comparison with control data, with either single or co-stimulants used at different times.

Furthermore, neither *Pg*-LPS- nor nifedipine-stimulated gene expression of CTGF and COL1A1 in healthy and nifedipine-induced gingival overgrowth groups showed any significant difference when both stimulants were used alone or

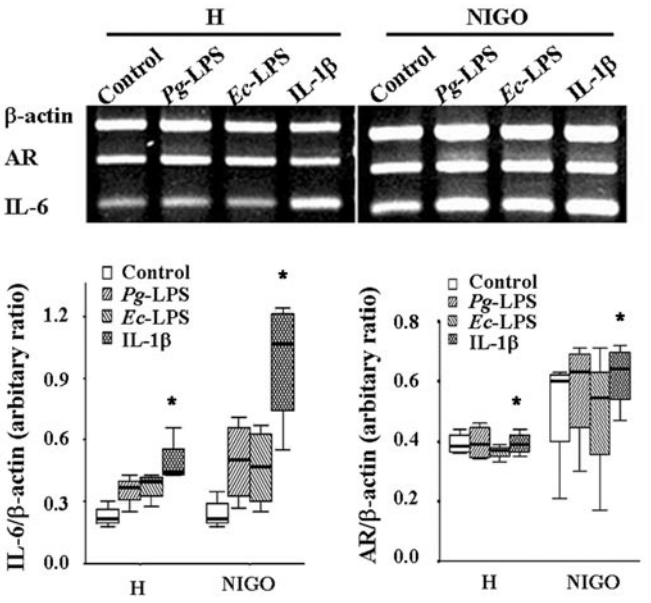

Figure 3. Gene expression of IL-6 and androgen receptor in gingival fibroblasts cultured with *Pg*-LPS, *Ec*-LPS, and IL-1β. The expressions of mRNAs encoding androgen receptor and IL-6 were examined in gingival fibroblasts cultured with *Pg*-LPS, *Ec*-LPS, and IL-β for 48 hrs. Each box represents the values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of β-actin expression. $*p < 0.05$, statistically significantly different compared with the healthy group. Among these 3 stimulants, only IL-1β increased the expression of androgen receptor and IL-6 genes significantly for nifedipine-induced gingival overgrowth cells as compared with healthy cells.

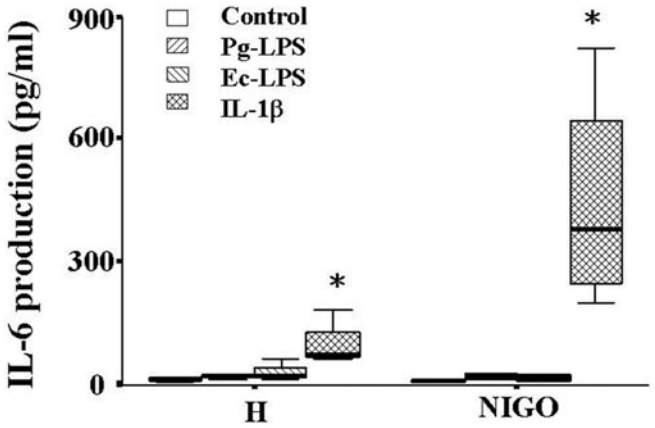

Figure 4. Effects of *Pg*-LPS, *Ec*-LPS, and IL-1β on IL-6 secretion by gingival fibroblasts from the healthy and nifedipine-induced gingival overgrowth groups, determined by ELISA. Each box represents the values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of β-actin expression. Data represent triplicate assays from 4 separate specimens. $*p < 0.05$, statistically significantly different compared with the healthy group.

simultaneously (Fig. 2 and APPENDIX).

Gene expression of androgen receptor and IL-6 was not significantly different between healthy and nifedipine-induced gingival overgrowth fibroblasts stimulated with *Ec*-LPS or *Pg*-LPS (Fig. 3). However, both androgen receptor and IL-6 mRNA levels significantly increased in the nifedipine-induced gingival overgrowth group compared with the healthy group after IL-1β stimulation ($*p < 0.05$).

Among the 3 stimulants, only IL-1β induced a prominent increase in IL-6 secretion in both healthy and nifedipine-induced gingival overgrowth fibroblasts (Fig. 4). The response of the nifedipine-induced gingival overgrowth group was much higher than that of healthy cells for IL-6 production ($*p < 0.05$). Neither *Pg*-LPS nor *Ec*-LPS showed any significant effects on both groups.

DISCUSSION

By analyzing the potential of *Pg*-LPS and IL-1β to stimulate gingival fibroblasts, we found that IL-1β appears to be the major factor responsible for the significantly increased gene and protein expressions of IL-6 and the androgen receptor in nifedipine-induced gingival overgrowth responder cells. Quantitative histomorphometric and immunohistomorphometric analyses postulated that CTGF might contribute to fibrosis in phenytoin- and nifedipine-induced gingival overgrowth tissues (Uzel *et al.* 2001). Results of our experiments showed that neither nifedipine nor *Pg*-LPS was the major stimulant inducing significant expression of androgen receptor, IL-6, CTGF, and collagen mRNA in nifedipine-induced gingival overgrowth cells.

Pg-LPS are implicated in the initiation and development of periodontal diseases, but the mechanisms are still unknown. Wang *et al.* (1998) showed that the inhibition of *Pg* LPS-induced IL-6 production by anti-CD14 antibody is dose-dependent. However, our experiment demonstrated that the differences in gene expression of androgen receptor and IL-6 between the healthy and nifedipine-induced gingival overgrowth groups stimulated by *Pg*-LPS were not appreciable, even in comparison with their control groups. It has been shown that the production of cytokines induced by *Pg*-LPS was negligible when compared with that of *Ec*-LPS (Hirschfeld *et al.*, 2001), although the chemical and biological properties of *Pg*-LPS and its active center, lipid A, are identical to those of LPS from *Bacteroides fragilis*, and *Pg* lipid A has a structure distinctly different from that of enterobacterial lipid A. The lack of a 4-O-phosphoryl group in the lipid A backbone may be the cause of the very low toxic activities of *Pg*-LPS (Johne *et al.*, 1988; Weintraub *et al.*, 1989; Gangloff *et al.*, 1999). LPS purified from enteric bacteria, such as *E. coli,* are supposedly extremely potent inducers of pro-inflammatory cytokine synthesis (Cavaillon and Haeffner-Cavaillon, 1990; Dinarello, 1990). However, the immunologic potency of *Pg*-LPS and *Ec*-LPS on the nifedipine-induced gingival overgrowth responder cells was less than that of IL-1β in the present study. Furthermore, only IL-1β induced the expression of androgen receptor and IL-6 in nifedipine-induced gingival overgrowth and healthy fibroblasts.

Several hormones and growth factors can regulate the expression of androgen receptor. An increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) by the calcium ionophore effectively reduces androgen receptor expression in the human prostate cancer cell line and platelets (Gong *et al.*, 1995; Cabeza *et al.*, 2004). Meanwhile, the $[Ca^{2+}]_i$ of rat mesangial cells and cultured bovine adrenal chromaffin cells might be decreased by IL-1β (Ikeda *et al.*, 1991; Morita *et al.*,

2004). Interestingly, our previous *in vivo* study showed that nifedipine administration in susceptible gingiva of certain patients increased androgen receptor expression two-fold higher than that in control gingival fibroblasts (Huang *et al.*, 2003). In the present *in vitro* study, the induction of androgen receptor gene expression by IL-1β was significantly higher in nifedipine-induced gingival overgrowth group than in the healthy group. We infer that nifedipine reduces $[Ca^{2+}]_i$ as a result of a blockade of L-type Ca^{2+} channels in nifedipine-induced gingival overgrowth responder cells, and that IL-1β may enhance the inhibition of Ca^{2+} influx from an independent non-genomic pathway through P/O-type Ca^{2+} channels, and synergistically increase the expression of androgen receptors on these cells (Chen *et al.*, 1988; Baumgarten *et al.*, 1992). The stimulation of gingival tissue by the co-administration of nifedipine and IL-1β seems to be the other possible issue in the pathogenesis of nifedipine-induced gingival overgrowth and will be the major focus of our further investigations.

Androgen exerts a marked anabolic effect in both non-drug-induced and drug-induced gingival overgrowth. Free androgen may passively diffuse into cells, *e.g.*, gingival fibroblasts, and be preferentially retained in target cells through the formation of a high-affinity complex with the steroid-specific receptor, causing an increase in collagen synthesis and/or a decrease in collagenase activity (Sooriyamoorthy and Gower, 1989; Soory and Gower, 1990). Meanwhile, androgen administration was found to inhibit the production of IL-4, IL-5, and IFN-γ by activated murine T-cells without affecting their capacity to produce IL-2 (Araneo *et al.*, 1991). In our previous study, strong Th2-type cytokine (IL-4, IL-10, and IL-13) expression was found in inflammatory cells of severe periodontal lesions. Stronger expressions of androgen receptor, IL-2, and IFN-γ were found in the nifedipine-induced gingival overgrowth tissues (Huang *et al.*, 2003). In the present study, the unique hyper-responsiveness of androgen receptor mRNA levels in response to IL-1β in nifedipine-induced gingival overgrowth cells may explain the link between the stimulatory effect of pro-inflammatory cytokine, elevated levels of adrenal androgen, and increased repair potential in nifedipine-induced gingival overgrowth fibroblasts. It was also shown that IL-1α, a potent IL-1β analogue, exerted a synergistic effect with nifedipine on cell proliferation and DNA synthesis in cultured human gingival fibroblasts (Sato *et al.*, 2005). We propose that this pro-inflammatory cytokine plays a major role in modulating soft-tissue enlargement in nifedipine-induced gingival overgrowth.

IL-6 is a multifunctional cytokine produced mainly by lymphocytes, monocytes, and fibroblasts. Many studies have shown that IL-6 levels are dramatically elevated in inflammatory periodontal lesions, and this cytokine is also thought to be a useful indicator or a diagnostic marker for periodontitis (Reinhardt *et al.*, 1993; Takahashi *et al.*, 1994; Mogi *et al.*, 1999). In a study of the identification of the osteoprotegerin/receptor activator of the nuclear factor-kappa B ligand (RANKL) system in gingival crevicular fluid and tissue of persons with chronic periodontitis, analysis of our data showed evidence of the positive correlations among RANKL, IL-6/oncostatin M in gingival crevicular fluid, and disease severity (Lu *et al.*, 2006). The RANKL produced by osteoblasts can act in either a paracrine manner, to activate osteoclast activity directly, or in an autocrine manner, to stimulate osteoblasts to produce further RANKL, which directly activates osteoclasts (Udagawa *et al.*, 1995). Since IL-6-type cytokines promote osteoclast formation both *in vitro* and *in vivo* (Black *et al.*, 1991; Jilka *et al.*, 1992; Girasole *et al.*, 1994; Suda *et al.*, 1996), we postulate that modulation of Th2-type cytokines by IL-6 levels may play a role in the immunocytologic response to periodontitis and the promotion of osteoclastogenesis (Lin *et al.*, 2005). The dual role of IL-6 in regulating $CD4^+$ T-cells to become Th1/Th2 effector cells had been proposed in 2002 (Diehl and Rincon, 2002). IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization through two independent molecular mechanisms. IL-6 activates transcription mediated by the nuclear factor of activated T-cells (NFAT), leading to production of IL-4 by native CD4+ T-cells and their differentiation into effector Th2 cells. While the induction of Th2 differentiation by IL-6 is dependent upon endogenous IL-4, the inhibition of Th1 differentiation by IL-6 is IL-4- and NFAT-independent (Diehl and Rincon, 2002). The activation of inflammatory responses in persons with nifedipine-induced gingival overgrowth by bacterial complexes may elicit a cascade of cytokine production, including IL-1β and IL-6, and terminating in more severe bone destruction. Theoretically, this is contradictory to our findings, which showed that stimulation with nifedipine or *Pg*-LPS resulted in less IL-6 expression detected in nifedipine-induced overgrowth cells. Severe bone breakdown was rarely seen in our study participants, nor did the cytokine profile of T-cells in nifedipine-induced gingival overgrowth tissue indicate a preferential trend toward Th2 activity (Huang *et al.*, 2003).

It can be concluded that IL-1β is the major mediator responsible for IL-6 and androgen receptor gene expression. Based on the results of the current study, it is conceivable that, in the prevention of nifedipine-induced gingival overgrowth, traditional periodontal treatment together with anti-inflammatory drugs or anti-IL-1β agents may be a possible therapeutic strategy.

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