EFFECT OF NICOTINE ON PERIODONTAL LIGAMENT DERIVED CELLS CULTURED UNDER MECHANICAL STRESS

ABSTRACT

Background and Aim: The purpose of this study was to investigate the effects of nicotine on periodontal ligament (PDL) cells cultured under mechanical stress.

Materials And Methods: Periodontal ligament-derived cells obtained from rat incisors were cultured with nicotine (20 ng/ml, NI), under centrifugal force (2000 rpm/20 min, MS) as experimental group (NI+MS). The control groups were treated with NI alone (NI), under MS without NI (MS), and without any treatment (CONT). An immunofluorescence study using an anti-receptor activator of nuclear factor-kappa B ligand (RANKL) primary antibody was also performed. The mRNA levels of RANKL, osteoprotegerin (OPG), interleukin-6 (IL-6), and vascular endothelial growth factor (VEGF) were analyzed using quantitative real-time polymerase chain reaction at day 1 of culture.

Results: RANKL positive cells and RANKL mRNA expression in the NI+MS group, NI group, and MS group were significantly higher than that of the CONT group. Expression of RANKL mRNA of the NI+MS group was higher than that of the CONT group and NI group. Expression of OPG mRNA in the MS group was significantly higher than that of other 3 groups. Expression of IL-6 and VEGF mRNA in the NI+MS group, NI group and MS group were significantly higher than that of the CONT group.

Conclusion: Nicotine suppressed expression of OPG and accelerated osteoclastogenesis in PDL-derived cells under centrifugal force.

Keywords: mechanical stress, nicotine, periodontal ligament cells
MEKANİK STRESS ALTINDA KÜLTÜRE EDİLEN PERIODONTAL LİGAMENT KAYNAKLı HÜCRELERDE NİKOTİNİN ETKİLERİ

ÖZ

Amaç: Bu çalışmanın amacı mekanik stress altında kültüre edilen periodontal ligament hücrelerinde (PDL) nikotinin etkilerini araştırmaktır.

Gereç ve yöntemler: Fare kesici dişlerinden elde edilen periodontal kaynaklı hücreler, deney grubu olarak (NI+MS) nikotin ile (20 ng/ml, NI) santrifüjel kuvvet altında (2000 rpm/20 dk, MS) kültüre edilmişlerdir. Kontrol grupları, sadece NI (NI), MS altında NI içermeyen (MS), ve herhangi bir işlem uygulanmamış (CONT) olarak ayrılmıştır. Nüklear factor-kappa B ligand (RANKL) primer antikorunun antireseptör aktivatörü kullanılarak immünoflorasan bir çalışma yapılmıştır. RANKL, osteoprotegerin (OPG), interlökin-6 (IL-6), ve vasküler endotelyal büyüme faktörü (VEGF) mRNA seviyeleri kuantitatif real-time polimeraz zincir reaksiyonu kullanılarak 1 günlük kültürde analiz edilmiştir.

Bulgular: RANKL pozitif hücreler ve NI+MS, NI ve MS gruplarındaki mRNA salınımı kontrol grubundan belirgin bir şekilde daha yüksek bulunmuştur. NI+MS grubundaki RANKL mRNA salınımı CONT ve NI gruplarından daha yüksek bulunmuştur. MS grubundaki OPG mRNA salınımı diğer üç gruptan belirgin bir şekilde yüksektir. NI+MS, NI ve MS gruplardaki VEGF ve IL-6 mRNA salınımı CONT grubundan belirgin bir şekilde yüksektir.

Sonuçlar: Nikotin, santrifüjel kuvvet altındaki PDL-kaynaklı hücrelerdeki OPG salınımı baskılamış ve osteoklastogenezizi hızlandırmıştır.

Anahtar Kelimeler: Mekanik stres, nikotin, periodontal ligament hücreleri
INTRODUCTION

Periodontal ligament (PDL) plays an important role in bone remodeling during orthodontic tooth movement. There are many reports on the effects of nicotine or mechanical stress on periodontal tissue.\cite{1,2} It is widely accepted that nicotine acts on cellular and tissue metabolism. Few studies, however, have investigated the action of the nicotine on orthodontic tooth movement. In a study investigating how nicotine influenced speed of orthodontic tooth movement, Sodagar et al.\cite{5} observed a dose-dependent accelerating effect. Kirschneck et al.\cite{6} reported that application of orthodontic force in vivo led to a significant increase in nicotine-induced periodontal bone loss. Meanwhile, Shintcovsk et al.\cite{7} reported that nicotine affected bone remodeling during orthodontic treatment and that delaying the collagen maturation process in the developing bone matrix resulted in slower tooth movement. Thus, the evidence on the effects of nicotine on tooth movement is conflicting.

Receptor activator of nuclear factor-kappa B ligand (RANKL), which is normally expressed by mesenchymal cells, including those found in PDL, is a key factor in osteoclast differentiation and activation.\cite{3} This protein binds to RANK, which is present in pre-osteoclast membrane, promoting an increase in nuclear factors responsible for eliciting differentiation into osteoclasts. On the other hand, osteoprotegerin (OPG) acts as a decoy receptor for RANKL, inhibiting osteoclastogenesis by preventing RANKL-RANK interactions. Interleukin-6 (IL-6), a pro-inflammatory cytokine, regulates immune responses at sites of inflammation. It also exhibits autocrine/paracrine activity, stimulating osteoclast formation and the bone resorption activity of preformed osteoclasts.\cite{8} Vascular endothelial growth factor (VEGF), the primary mediator of angiogenesis, serves various biological functions. It increases vascular permeability and is also involved in bone resorption.\cite{9,10}

The aim of the present study was to investigate the effects of nicotine on PDL cells cultured under mechanical stress in terms of expression of RANKL, OPG, IL-6, and VEGF.

MATERIALS AND METHODS

PDL cell culture

The study protocol complied with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College (Approval number: 272801). Periodontal tissues were obtained from the upper incisors of 4-week-old, male, Sprague-Dawley rats weighing 100 g each (Sankyo Labo Service, Tokyo, Japan) according to the method of Inoue et al.\cite{11} The lingual root surface was placed on a dish for culture of PDL-derived cells. The derived cells were then incubated at 37°C in a humidified atmosphere comprising 95% air and 5% CO₂ for primary culture using α-minimal essential medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) supplemented with 0.4% gentamycin (Sigma) and 0.1% fungizone (Gibco). Periodontal ligament-derived cells obtained at the fifth passage were used for all experiments.

Mechanical stress

The PDL-derived cells were centrifuged using a desk centrifuge (Himac CT6D, Hitachi Ltd., Tokyo, Japan) for 20 min at 2000 rpm (11.7 kPa, 573 g, 119 g/cm²). The following equation was used to determine how much force should be used: \[ P = \frac{m \times r \times r \times \pi^2}{A \times 9.8 \times 900} \], where \( P \) = kg pressure per cm² cells, \( m \) = mass of medium (0.001 kg), \( r \) = radius (0.09 m), \( rpm \) = revolution/min (2000), and \( A \) = area of contact between medium and cells (3.38 cm²).\cite{12}

This amount of centrifugal force is nearly equivalent to that produced by clinical orthodontics.\cite{13}

Nicotine concentration

Nicotine (Wako, Tokyo, Japan) was used at 20 ng/ml based on reported plasma levels in smokers.\cite{2,14} The concentration of nicotine to be used was determined in preliminary experiments.

Experimental design

The PDL-derived cells in the experimental group were exposed to both nicotine and mechanical stress (NI+MS). The other cells were divided into 3 groups: one in which the PDL-derived cells were exposed to neither mechanical stress nor nicotine (control group, CONT); one in which they were exposed to nicotine only (nicotine group, NI); and one in which they were exposed to mechanical stress only (mechanical stress group, MS).

The cells were seeded at a density of 5.0 x 10³ cells per cm² in 12-well dishes (BD Falcon, Franklin Lakes, NJ, USA). Two days later, the medium was changed and the MS and NI + MS groups exposed to centrifugal force (Figure 1).

Immunofluorescence Staining

At day 1 (24 hr) after commencement of culture, the cells were washed twice with PBS, fixed in 10% formalin for 30 min at room temperature, and permeabilized in 1% Triton X-100 (IBI Scientific, Peosta, IA, USA) for 5 min. The cells
were then washed twice with PBS. Non-specific binding was blocked with 1% BSA (BSA; Roche Applied Science, Indianapolis, IN, USA). The primary antibody used was anti-RANKL goat polyclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the secondary antibody used was rabbit anti-goat IgG conjugated to Alexa Fluor 555 (1:100, Invitrogen). Alexa Fluor phalloidin (1:100, Invitrogen) was used for staining of filamentous actin (F-actin) and DAPI (4',6-diamino-2-phenylindole; 1:100, Invitrogen) for identification of nuclei. Immunofluorescence was observed using a LSM5 Duo confocal laser scanning microscope (Carl Zeiss Microimaging, Oberkochen, Germany) and ZEN 2012 imaging software (Carl Zeiss Microimaging). Shooting conditions were as follows: total magnification, 63 x; channels/filters, 405/420-480, 488/505-550, and 543/548-687.

**RT-PCR analysis**

At day 1 (24 hr) after commencement of culture, total RNA from the cells in each group was isolated with an RNeasy Kit (Qiagen, Tokyo, Japan) according to the protocol provided by the manufacturer. The quantity of isolated RNA in each tube was measured by absorbance using Nanodrop (Nanodrop ND-1000; Thermo Fisher Scientific, Wilmington DE, USA). Reverse-transcription to cDNA was performed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan). RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the target genes: RANKL (Rn00589289_m1), OPG (Rn00563499_m1), IL-6 (Rn01410330_m1), and VEGF (Rn01511601_m1). The TaqMan Endogenous Control (Applied Biosystems) for the target gene GAPDH (Rn01775763_g1) was used as a control. RT-PCR was performed using the 7500 fast real-time PCR system (Applied Biosystems).

Gene expression was quantified using the TaqMan Gene Expression Assay system. Assays were performed in 20-μl single-plex reactions containing TaqMan Fast Universal PCR Master Mix, TaqMan Gene Expression Assays, distilled water, and cDNA according to the manufacturer's protocol (Applied Biosystems). Reaction conditions consisted of a primary denaturation at 95°C for 20 sec and then 50 cycles of 95°C for 3 sec and 62°C for 30 sec. Relative mRNA expression levels were determined after normalizing the cycle threshold values for each gene with the internal control (GAPDH). Quantitative RT-PCR analyses were reproduced 4 times.

**Statistical analysis**

All data except for those pertaining to immunofluorescence staining were tested for normal distribution and homogeneity of variance (Bartlett test) using the software application Statcel 3 (The Publisher OMS Ltd., Saitama, Japan). Data were analyzed statistically using a one-way ANOVA and Sheffe's test. A p value of < 0.05/0.01 was considered to be statistically significant.

**RESULTS**

**Immunofluorescence observations**

The PDL-derived cells were spindle-shaped and had many processes. No significant differences were observed in their morphology in any group (Figure 2).

**Expression of RANKL mRNA**

Expression of RANKL mRNA in the treated groups was significantly higher than that in the CONT group (p< 0.05/0.01) (Figure 3) (Table 1). There was no significant difference between the MS group and the NI+MS group, but expression of RANKL mRNA in the MS and NI+MS groups was significantly higher than that in the NI group (p< 0.01).
Expression of OPG mRNA

Expression of OPG mRNA in the MS group was significantly higher than that in the CONT group (p< 0.01). Expression of OPG mRNA in the MS group was also significantly higher than that in the NI and NI+MS groups (p< 0.05/0.01) (Figure 4). No significant difference was observed between the NI and NI+MS groups.

Expression of IL-6 mRNA

Expression of IL-6 mRNA in the NI, MS, and NI+MS groups was significantly higher than that in the CONT group (p< 0.05/0.01) (Figure 5). There was no significant difference in the treated groups.

Expression of VEGF mRNA

Expression of VEGF mRNA in the NI, MS, and NI+MS groups was significantly higher than that in the CONT group (p< 0.05) (Figure 6). There was no significant difference in the treated groups.

DISCUSSION

One earlier study found that plasma nicotine concentrations in smokers generally ranged from 10 to 50 ng/ml (0.06 to 0.31 μM), with trough concentrations typically at 5 to 37 ng/ml (0.03 to 0.23 μM). Furthermore, in one in vitro study, vacuolization of PDL fibroblasts was observed soon after exposure to nicotine at a concentration of 1 μg/ml. Based on these earlier findings, the nicotine concentration to be used in the present study was set at 20 ng/ml to mimic nicotine plasma levels in smokers. A number of studies have demonstrated that various types of mechanical stress, including compressive, tensile, and centrifugal forces, stimulate expression of inflammatory-, osteogenesis- and osteoclastogenesis-related proteins in PDL cells. It has been suggested that this is due to the integrin-FAK complex acting as a mechanoreceptor. Application of strong compression forces, however, such as

Table 1. Expression of mRNA in each group at day 1 (mean + standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>RANKL</th>
<th>OPG</th>
<th>IL-6</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>NI</td>
<td>1.92 ± 0.16</td>
<td>0.92 ± 0.07</td>
<td>2.73 ± 0.32</td>
<td>2.37 ± 0.10</td>
</tr>
<tr>
<td>MS</td>
<td>2.80 ± 0.36</td>
<td>1.49 ± 0.07</td>
<td>3.81 ± 0.38</td>
<td>2.51 ± 0.24</td>
</tr>
<tr>
<td>NI+MS</td>
<td>2.91 ± 0.59</td>
<td>1.18 ± 0.24</td>
<td>3.65 ± 0.87</td>
<td>2.79 ± 0.38</td>
</tr>
</tbody>
</table>
Effect of nicotine on PDL cells with mechanical stress

by using a glass cylinder, for example, may damage or even kill the cell, making such investigations difficult in terms of managing culture conditions. It was possible to avoid this problem in the present study by application of the appropriate amount of centrifugal force and concentration of nicotine.

Compression has been demonstrated to promote secretion of osteoclastogenesis-stimulating cytokines, such as macrophage-colony stimulating factor, TNF-α, and RANKL. Centrifugal force induces up-regulation of OPG expression through extracellular signal-regulated kinase-mediated signaling.

Nicotine induces expression of cyclooxygenase-2 via the α7 nicotine acetylcholine receptor (nAChR)/NF-κB pathway in human PDL fibroblasts. A predominant subunit of nAChRs, α7 nAChR is also a potent target of nicotine binding receptors. Cyclooxygenase-2 induces prostaglandin E2, which acts via an autocrine mechanism, resulting in up-regulation of RANKL expression in PDL cells. Kirshneck et al. reported that RANKL expression could be increased by applying nicotine and compressive forces to human PDL fibroblasts. In contrast, OPG expression is decreased by applying nicotine and compressive forces to human PDL fibroblasts.

In the present study, RANKL expression was significantly higher in the NI, MS, and NI+MS groups than that in the CONT group. However, RANKL expression was higher in the MS and NI+MS groups than that in the NI group, and there was no significant difference between the MS and NI+MS groups. Moreover, OPG expression was significantly higher in the MS group than in the CONT, NI, or EXP groups, but there was no significant difference between the latter three. This suggests that nicotine has no direct effect on OPG expression, but suppresses expression of OPG in PDL-derived cells exposed to centrifugal force. Expression of RANKL was significantly higher and OPG significantly lower in the NI+MS group. Taken together, the patterns of expression of RANKL and OPG observed here suggest that nicotine accelerates osteoclastogenesis.

A number of studies have reported up-regulation of IL-6 by nicotine or mechanical stress. One such study found that compressive force significantly increased expression of IL-6 mRNA and that subsequent increase in release of IL-6 occurred in a time-dependent manner. Many studies have demonstrated that TNF-α, IL-1, and IL-6 accelerate RANKL expression and promote osteoclastogenesis.

Expression of IL-6 mRNA in the present study was higher in the NI, MS, and NI+MS groups than that in the CONT group. However, no significant difference was observed among the NI, MS, and NI+MS groups, suggesting that IL-6

![Figure 4. Expression of OPG mRNA in PDL-derived cells in MS group was significantly higher than that in other 3 groups. Mean values ± SD. *p< 0.05, **p< 0.01](image)

![Figure 5. Expression of IL-6 mRNA in PDL-derived cells in NI, MS, and NI+MS groups was significantly higher than that in CONT group. Mean values ± SD. *p< 0.05, **p< 0.01](image)

![Figure 6. Expression of VEGF mRNA in PDL-derived cells in NI, MS, and NI+MS groups was significantly higher than that in CONT group. Bars indicate mean values ± SD. *p< 0.05](image)
produced by PDL-derived cells in response to either nicotine or mechanical stress stimulates production of RANKL. Expression of VEGF is up-regulated by mechanical stress in periodontal tissue. Nakai et al. reported that mechanical stress up-regulated RANKL expression via a VEGF autocrine pathway in osteoblastic MC3T3-Ea cells. Few studies, however, have investigated the effects of nicotine on expression of VEGF in PDL-derived cells. One study found that nicotine significantly increased endothelial cell VEGF mRNA levels and that nAChR was present in endothelial cells. These findings suggest that nicotine also induces expression of VEGF in PDL-derived cells. In the present study, expression of VEGF mRNA in PDL-derived cells was significantly higher in the NI, MS, and NI + MS groups than that in the CONT group. However, no significant difference was observed among those three groups. This suggests that VEGF produced by PDL-derived cells with exposure to either nicotine or mechanical stress also stimulates production of RANKL.

CONCLUSION
Application of mechanical stress and/or nicotine enhanced expression of RANKL in cultured PDL-derived cells, stimulating production of both IL-6 and VEGF. Moreover, nicotine suppressed expression of OPG in these cells when exposed to centrifugal force, accelerating osteoclastogenesis.

REFERENCES


