A Novel Single Nucleotide Polymorphism of the Interleukin-8 Promoter: Its Transcriptional Regulation and Analysis of the Mutation in Periodontal Disease in the Japanese Population

Yasuhiro Imamura¹, Yoshihisa Fujigaki², Kouichi Higaki³, Nobuo Yoshinari³ and Pao-Li Wang⁴

¹ Department of Pharmacology, Matsumoto Dental University, Nagano, Japan
² Department of Oral Preventive Dentistry, Matsumoto Dental University, Nagano, Japan
³ Department of Periodontology, Matsumoto Dental University, Nagano, Japan
⁴ Department of Dental Education Innovation, Osaka Dental University, Osaka, Japan

(Received for publication, July 15, 2012)

Abstract: Chronic periodontitis is a major dental disease associated with continuous inflammation and the mediation of cytokines and chemokines such as Interleukin (IL)-8. Periodontitis processes may also be associated with quantitative changes in IL-8 gene expression. We examined the transcriptional activation and mediation of a novel single nucleotide polymorphism (SNP) at position -845 (from the transcription initiation site) of the IL8 promoter. Moreover, we investigated whether the frequencies of this SNP were associated with susceptibility to chronic periodontitis among Japanese. The fragments of cloned IL8 promoter (-866 to +30) containing the novel SNP (-845T and -845C) were ligated to the luciferase gene. A luciferase assay was then conducted using these reporter plasmids in SCCTM cells. The presence and molecular weight of the -845T-specific binding proteins were analyzed by gel mobility shift and UV-crosslinking competition assays, respectively. SNP analysis was performed with DNA from 56 control subjects and 52 patients by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and disease associations were analyzed by a Chi-square test. These results indicate that transcriptional activity of the IL8 promoter containing -845T was approximately 9-fold higher than when the promoter contained -845C. -845T-specific binding proteins (approximately 55 and 60 kDa) were present in SCCTM cells. Genotype frequencies (T/T and T/C) between the control and patient groups were not significantly different (P = 0.586). These results suggest that transcription of the IL8 gene, particularly mediation of -845T/C, appears to be strictly regulated. This SNP was not associated with chronic periodontitis in the Japanese population.

Keywords: Interleukin-8, Periodontitis, SNPs, Transcriptional regulation

Introduction

Periodontitis is a major and widespread dental disease that is associated with environmental factors, such as infections of gram-negative bacteria. Excessive interaction between host defenses and microorganisms leads to inflammation of gingival tissues and severe pocketing, resulting in alveolar bone loss. Given that periodontitis is associated with systemic diseases such as cardiovascular disease and diabetes, the treatment of periodontal disease is considered both essential and important for human health.

The incidence of periodontitis also appears to be associated with genetic factors, such as genetic variability. Indeed, recent studies have shown that single nucleotide polymorphisms (SNPs) in genes such as interleukin (IL)-2, IL-6, IL-10, matrix metalloprotease 1, and tumor necrosis factor receptor (TNFR) 2 are associated with periodontal disease. We also previously showed that SNP-frequencies of the IL-13 gene are associated with periodontitis in the Japanese population, but not for those of the IL-1A, IL-1B, toll-like receptor (TLR) 4, TLR2, and histatin genes. However, because periodontitis is a complex and multifaceted disease, there are likely to be more candidate genes associated with the condition.

IL-8, a chemokine that is responsible for inducing chemotaxis (migration of cells to the site of inflammation), is expressed and secreted by cells such as lymphocytes, monocytes, and fibroblasts. Moreover, in an inflamed region, IL-8 attracts and activates polymorphonuclear leukocytes, inducing their...
transendothelial migration and granule enzyme release\textsuperscript{17,18}. The IL8 gene, which consists of four exons, is located on chromosome 4q13-q21\textsuperscript{19}. Transcription of the IL8 gene has been examined using a variety of gene mutation and deletion analyses, which have shown that a sequence spanning nucleotides -1 to -133 within the 5'-flanking region of the gene is both essential and sufficient for its transcriptional regulation\textsuperscript{20,21}. Interestingly, numerous SNPs have been found in the IL8 gene, some of which are known to be associated with susceptibility to diseases such as asthma and oral lichen planus\textsuperscript{22,23}. A recent study showed that the SNP at position -251 of the IL8 promoter was associated with chronic periodontitis in Brazilian subjects\textsuperscript{24}. Increased expression of the IL8 gene has also been observed in affected periodontal tissues\textsuperscript{25}. Thus, given the ability of SNPs to affect the transcription and the production of IL-8 through the IL8 promoter, SNP analysis could be an important means of assessing susceptibility of a population to periodontitis.

In this study, we identified a novel SNP (T/C) at position -845 (from the transcriptional initiation site) of the IL8 promoter. Based on observations of the transcriptional activity of the promoter containing -845T, we confirmed the presence and molecular weight of the -845T-specific binding proteins. We also clarified the relationship between SNP frequencies and periodontitis in the Japanese population.

**Materials and Methods**

**Cell culture**

SCCTM (human gingival carcinoma) cells were cultured in Dulbecco’s Modified Eagle Medium (Sigma-Aldrich Co., St. Louis, MO, USA) with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a 5% CO\textsubscript{2} and 95% air humidified incubator.

**Plasmid constructions**

All polymerase chain reaction (PCR) experiments were carried out using 1.75 U of Expand High Fidelity polymerase (Roche Diagnostics, Tokyo, Japan), genomic DNA (50 ng), and 100 pmol each of the following primers: for phIL-8(-845T)-Luc, primers hIL-8-4 (5'-GGGTACCGAA\ TACTG\ TTCCTA\ TCTG\ GAAT\ GTGC-3') and hIL-8D (5'-ATGCTAGTTAACAG\ AGTGAA\ GGGGC\ ACATGTCTTC-3') (amplified fragment, 336 bp), and hIL-8-1 (5'-CTGTTAACTAGCA\ TTAGAAAAACAAA\ TC-3') and hIL-8A (5'-GGCTCGAGGC\ GTGTCTCTG\ AAAGTTTG\ TGC\ CTATTATGGAG-3') (amplified fragment, 589 bp). The 336 bp fragment was digested with Kpn\textsubscript{I} and Hinc\textsubscript{II} and the 589 bp fragment was digested with Hinc\textsubscript{II} and Xho\textsubscript{I}. The digested fragments were then inserted into the Kpn\textsubscript{I} and Xho\textsubscript{I} sites of pUC-Luc. For phIL-8(-845C)-Luc, primers hIL-8-5 (5'-GGGTACCGAATACTGTTCCTATCTG\ GAACGTGC-3') and hIL-8D were used. The amplified fragment was digested with Kpn\textsubscript{I} and Hinc\textsubscript{II}. This fragment and the Hinc\textsubscript{II}-Xho\textsubscript{I} fragment described above were then inserted into the Kpn\textsubscript{I} and Xho\textsubscript{I} sites of pUC-Luc.

**Transfection, luciferase assay, gel mobility shift assay and UV-crosslinking assay**

Transfection, the luciferase assay, the gel mobility shift assay, and the UV-crosslinking assay have all been described previously.

### Table 1

<table>
<thead>
<tr>
<th>Diagnostic criteria</th>
<th>BOP*</th>
<th>PD** (mm)</th>
<th>AL*** (mm)</th>
<th>BL *** (%)</th>
<th>n</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>&lt;4</td>
<td>0</td>
<td>-</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>Periodontitis (total)</td>
<td>+</td>
<td>≥4</td>
<td>2-9</td>
<td>38 ± 0.18</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>Periodontitis (moderate)</td>
<td>+</td>
<td>4-6</td>
<td>2-8</td>
<td>35 ± 0.18</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>Periodontitis (severe)</td>
<td>+</td>
<td>≥7</td>
<td>2-9</td>
<td>55 ± 0.11</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

*BOP, bleeding on probing; **PD, probing depth; ***AL, attachment loss; ****BL, bone loss.

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n = 56)</th>
<th>Total (n = 52)</th>
<th>Moderate (n = 44)</th>
<th>Severe (n = 8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-845T/T</td>
<td>36 (64.3)</td>
<td>36 (69.2)</td>
<td>31 (70.5)</td>
<td>5 (62.5)</td>
<td>0.586</td>
</tr>
<tr>
<td>-845T/C</td>
<td>20 (35.7)</td>
<td>16 (30.8)</td>
<td>13 (29.5)</td>
<td>3 (37.5)</td>
<td></td>
</tr>
<tr>
<td>-845C/C</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>
by Imamura et al.\textsuperscript{27}. Figure 1B shows a representative example of experiments performed in triplicate with essentially identical results. Gel mobility shift and UV-crosslinking assays were performed with double-stranded oligonucleotides hIL-8-845(T) (5'-CTGTTCCTATCTGGAATGTGCTGTTCTCTT-3', for the probe and competitor) and hIL-8-845(C) (5'-CTGTTCCTATCTGGAACGTGCTGTTCTCTT-3', for the competitor). Nuclear extracts derived from SCCTM cells were
Prepared as described elsewhere.25

Subjects
Subjects included 108 unrelated Japanese individuals, of which 56 (age range, 26 to 55 years; mean ± SD, 40.7 ± 8.5) were controls (i.e., healthy volunteers) and 52 (age range, 27 to 56 years; mean ± SD, 42.6 ± 7.9) were periodontal patients who visited the Periodontic Department at Matsumoto Dental University Hospital. All subjects gave informed consent prior to entering the study, which was approved by the Ethics Committee of Matsumoto Dental University (No. 0021). Conditions and diagnostic criteria for all subjects have been described by Komazaki et al.15. The baseline clinical parameters of the subject population are presented in Table 1.

Extraction of DNA from lingual mucosal cells
Lingual mucosal cells from subjects were collected with a toothbrush. Genomic DNA was extracted from the cells as described elsewhere.15

Genotyping
The sequence of the IL8 gene was obtained from GenBank (Accession number: M28130). The IL8 gene at position -845 was amplified by PCR using a Gene Amp® PCR System 9700 (Life Technologies, Inc., Carlsbad, CA, USA) using the specific primers hIL-8(-845)Fw (5′-CAACCTTTCCA TTGATAACACCTCCCTCCC-3′) and hIL-8(-845)Rv (5′-T A G G A G G C T T C A T A G A G G A A G A G AGAACTGTAC-3′). Thermal cycle conditions included an initial denaturation at 96°C for 2 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1.5 min, extension at 72°C for 1 min, and a final extension step at 74°C for 7 min. The PCR products were digested with 1 unit of Tat I (Fermentas International Inc., ON, Canada) at 65°C for 2 h (detected bands: T allele, 167 bp; C allele, 127 and 40 bp). All of the digested and undigested PCR products were separated by electrophoresis on a 5% polyacrylamide gel (19:1) and stained with ethidium bromide.

Statistical analysis
All variables were subjected to logistic regression analyses that corrected for potential confounding parameters such as age, gender, and smoking status. The association of a gene with periodontal disease susceptibility was assessed by Chi-square analysis to compare genotype frequencies for controls and cases. Statistical significance was set at the 95% level and a two-sided P-value was calculated.

Results
Transcriptional regulation mediating a novel SNP at position -845 of the IL8 promoter

We cloned and sequenced the IL8 promoter from subjects to examine transcriptional regulation of the IL8 gene. The results indicated the existence of a novel SNP in the upstream of the IL8 gene at position -845, the alleles for which were T (-845T) and C (-845C). We then examined the transcriptional regulation mediating this SNP in the IL8 gene. The region from -866 to +30 of the IL8 promoter containing either -845T or -845C was linked to the luciferase reporter gene (Fig. 1A, named pIL-8(-845T)-Luc and pIL-8 (-845C)-Luc). These reporter plasmids were transfected into SCCTM cells and luciferase assay was performed. As can be seen in Fig. 1B, the transcriptional activity of the IL8 (-845T) promoter in SCCTM cells was approximately 9-fold higher than that of the IL8 (+845C) promoter. This finding suggests that the process of transcriptional activation mediating the T allele at position -845 of the IL8 gene cooperates in some way with the region downstream of the allele.

Presence of nuclear factors bound to the region containing the SNP at position -845 of the IL8 promoter

In order to examine for the presence of nuclear factors bound specifically to, and dependent upon, the -845 site, a gel mobility shift competition assay was carried out. A double-stranded and labeled IL8(-845T) probe was mixed with nuclear extracts from SCCTM cells in the presence or absence of competitors (Fig. 2A, non-labeled IL8 (-845T) and IL8 (-845C) oligonucleotides), and the mixtures were then electrophoresed on a native polyacrylamide gel. As shown in Fig. 2B, at least one band was observed in the sample to which no competitors were added. While the band disappeared completely when competitor IL8(-845T) was added, the band was not completely outcompeted in the presence of competitor IL8(-845C). These results indicate that specific nuclear factor(s) are bound to the region containing T allele at position -845 of the IL8 promoter.

Molecular weight of the proteins bound to the region containing the SNP at position -845 of the IL8 promoter

Next, we measured the molecular weight of the protein(s) bound to the region containing -845T of the IL8 promoter by a UV-crosslinking competition assay using the IL8 (-845T) probe with SCCTM nuclear extracts. As shown in Fig. 2C, proteins of approximately 55 and 60 kDa recognized the IL8 (-845T) region in the absence of competitors. However, these proteins were unable to bind to the IL8 (-845T) region after the addition of IL8(-845T) competitor in a dose-dependent manner. Indeed, in the presence of IL8 (-845C) competitor, these proteins remained bound to the IL8 (-845T) region. Taken together, these results suggest that the approximately 55 and 60 kDa proteins bind specifically to the region containing the T allele at position -845 of the IL8 promoter, suggesting that the site is an essential nucleotide for binding of these proteins.
The SNP at position -845 of the *IL8* promoter affected the transcriptional activation of the *IL8* gene as well as the DNA binding ability of proteins measuring approximately 55 and 60 kDa. We therefore investigated whether the frequencies of the SNP were associated with periodontitis susceptibility in the Japanese population. SNP analysis was performed by a PCR-RFLP method using DNA from subjects (control: 56, periodontitis patients: 52, Table 2) and primers specific for the *IL8* gene. These results showed that the -845T/T and -845T/C genotypes were prevalent in 64.3% and 35.7% of the control subjects and 69.2% and 30.8% of the patients, respectively. In contrast, the -845C/C genotype was not observed in either the control or patient groups.

### Discussion

We examined the *IL8* promoter region and found a novel SNP (T/C) in the *IL8* promoter at position -845 (from the transcriptional initiation site). Transcription of the *IL8* promoter that mediated -845T was higher than that mediating -845C. Interestingly, -845T-specific binding proteins with molecular weights of approximately 55 and 60 kDa were present in SCCTM cells. In addition, we found that the genotype (T/T and T/C) frequencies of this SNP in the control and patient groups were not associated with susceptibility to periodontitis in the Japanese population.

In general, transcriptional regulation of genes involves a variety of complex interactions between nuclear factors and cis-acting elements found in the promoter region. Expression of the *IL8* gene may also be precisely and strictly regulated by transcription factors. Indeed, the binding sequences of the transcription factors nuclear factor (NF)-κB, CAAT/enhancer-binding protein (C/EBP), and activating protein (AP)-1, all of which are activated by mitogen-activated protein kinases (MAPK: extracellular-regulated protein kinase (ERK), JUN-N-terminal protein kinase (JNK), and p38), exist in the upstream region (from -1 to -133) of the *IL8* gene. Genetic polymorphisms consisting of SNPs in the *IL8* promoter may also affect transcriptional regulation. It has been reported that the A allele at position -251 in the *IL8* promoter upregulates IL-8 levels in the leucocytes of healthy blood donors. This SNP (-251T/A) was associated with chronic periodontitis and other diseases like bronchiolitis, oral squamous cell carcinoma, and gastric cancer. Our findings showed that the transcriptional activity of the *IL8* gene mediating -845T was 9-fold higher than that mediating -845C in SCCTM cells (Fig. 1B), which produce IL-8 under ordinary culture conditions (data not shown). Furthermore, it is possible that the -845T-specific nuclear binding proteins (approximately 55 and 60 kDa) observed in SCCTM cells (Fig. 2A, B) may have been heat shock factor (HSF) 1 (57.3 kDa) and HSF 2 (60.3 kDa) based on searches for consensus sequences of transcription factors. HSFs regulate the activation of heat shock gene transcription, and HSFs 1 and 2 are ubiquitous. We tested this hypothesis using a gel mobility supershift assay with anti-HSFs 1 and 2 antibodies and nuclear extracts from SCCTM cells. However, despite similarities in molecular weights of the obtained proteins and HSFs, the obtained proteins were not HSFs 1 and 2 (data not shown). It is thus possible that the -845T-specific binding proteins interact with factors that are bound to the *IL8* promoter at a site downstream of the -845T site, and in so doing, affect the transcription of the *IL8* gene.

Previous studies have reported that IL-8 gene expression and protein levels are increased in cells of periodontal tissues and other diseases like bronchiolitis, oral squamous cell carcinoma, and gastric cancer. We are grateful to the individuals who graciously to be the
subjects of this investigation. This research was supported by a special fellowship grant from Matsumoto Dental University.

References

25. Dongari-Bagtzoglou AI and Ebersole JL. Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis. J Periodontol 69: 899-910, 1998


36. Dongari-Bagtzoglou AI and Ebersole JL. Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis. J Periodontol 69: 899-910, 1998


