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ORIGINAL ARTICLE

Lipopolysaccharide extracted from *Porphyromonas gingivalis* induces DNA hypermethylation of runt-related transcription factor 2 in human periodontal fibroblasts

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Osteoblastic differentiation;
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Background/Purpose: Epigenetic alterations such as DNA methylation and histone acetylation are described as changes in the pattern of gene expression not involving the DNA sequence. Lipopolysaccharide (LPS) derived from *Porphyromonas gingivalis* has been shown to inhibit osteoblastic cell differentiation. We examined whether DNA hypermethylation was involved in the inhibitory effect of LPS on osteoblastic differentiation of fibroblasts derived from human periodontal ligament (HPDL).
Methods: The HPDL cells were incubated with LPS derived from *P. gingivalis* at a concentration of 10 μg/ml for 24 h. The cells were treated with DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5Aza). Untreated cells were used as a control. Cell viability was determined using cell proliferation reagent. DNA methyltransferase (DNMT1) and runt-related transcription factor 2 (RUNX2) mRNAs were evaluated by quantitative polymerase chain reaction (RT-PCR). Analysis of RUNX2 DNA methylation was performed using quantitative methylation-specific PCR.

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Results: The expression level of RUNX2 was significantly lower in the cells stimulated with LPS than the controls. The presence of 5Aza increased the expression of RUNX2 in cells stimulated with LPS. The expression levels of DNMT1 mRNA in the cells stimulated with LPS were significantly higher than in the control. The presence of 5Aza completely abolished the upregulated expression of DNMT1 in cells stimulated with LPS. The methylation of DNA at 0.1 kb and −1.9 kb in the cells stimulated with LPS was significantly higher than the control.
Conclusion: The results indicate that DNA hypermethylation may be involved in the inhibitory effect of LPS on osteoblastic differentiation in HPDL.

Introduction

Epigenetics is described as changes in the pattern of gene expression not involving the DNA sequence. Epigenetic events act through chemical modification of DNA and by selectively activating or inactivating genes to determine their expression during development.[1] DNA methylation and histone deacetylation are two major mechanisms of epigenetic alteration in human cells. DNA methylation is characterized by the addition of a methyl group to cytosines within CpG regions. Histone deacetylation is a mechanism that involves the removal of the acetyl group leading to alteration of the charge and packing of DNA around histones.[2] These epigenetic modifications are often observed in malignant and premalignant lesions.[3] It has recently been shown that epigenetic modifications may cause other common diseases such as diabetes, metabolic diseases, allergies, autoimmune diseases, and neurodegenerative diseases.[4] Epigenetic alterations have also recently been implicated in periodontal disease.[5] The expression profiles of several cytokines may be epigenetically modified.[6] Preliminary data suggest that expression of periodontal disease-related cytokines such as IL-6 may be altered by an epigenetic modification.[7] Methylation of the collagen-a1 gene has been observed in the periodontal ligament during the aging process.[8] Some local risk factors for periodontal disease such as tobacco smoke, alcohol and infectious agents can cause epigenetic modification.[9,10]

Epigenetic modification can be caused by certain types of bacteria and lipopolysaccharide (LPS).[11] For example, oral pathogens including *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were recently shown to cause epigenetic modifications.[12] Epigenetic modifications such as DNA hypermethylation often induce downregulation of transcriptional levels.[13] LPS derived from *P gingivalis* has been demonstrated to inhibit osteoblastic differentiation of osteoprogenitor cells derived from fatal rat calvaria.[14] Runt-related transcription factor 2 (RUNX2) is a key transcription factor associated with osteoblast differentiation, and the inhibition of osteoblastic cell differentiation is accompanied by downregulated expression of RUNX2.[15] We hypothesized that the downregulated expression of RUNX2 is involved in DNA hypermethylation. Fibroblasts derived from periodontal tissues have the potential for osteoblastic differentiation. We examined whether LPS derived from *P gingivalis* caused downregulated expression of RUNX2 in fibroblasts derived from periodontal ligaments, and whether DNA hypermethylation is involved in the downregulated expression.

Methods

Cell culture

Human periodontal ligament (HPDL) cells from normal human periodontal ligament tissue were purchased from Lonza Walkersville (Basel, Switzerland) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

HPDL cells were spread onto 60 mm tissue culture plates at a density of 4.0×10^5 cells/ml. After overnight incubation, the cells were cultured with *P gingivalis* LPS (LPS: InvivoGen, San Diego, CA, USA) at a concentration of 10 μg/ml, or with 5-Aza-2'-deoxycytidine (5Aza: Sigma), an inhibitor of DNA methyltransferase, at concentrations of 1μM, 10 μM, and 100 μM. Untreated cells were used as controls.

In vitro cytotoxicity assays

Cell viability was determined using cell proliferation reagent WST-1 (Roch Diagnostics, Mannheim, Germany). HPDL cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium and cultured overnight. The cells were treated with different concentrations of LPS or 5Aza. After incubation for 12 and 24 h, 10 μl of WST-1 was added to each well and cultured for 1 hour. The absorbance at 450 nm was determined using an Infinite F200 microplate reader (Tecan, Männedorf, Switzerland).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from HPDL cells using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. Total RNA was reversely transcribed into cDNA using an Omniscript RT Kit (Qiagen). Nucleotide contents were measured on a Nanodrop ND-1000 spectral photometer (Nanodrop Technologies, Wilmington, DE, USA). The cDNA levels were measured using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster

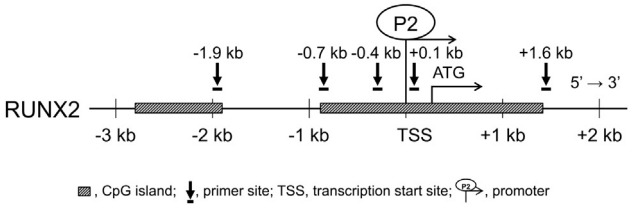

Figure 1. Schematic diagram of the coverage of the CpG islands and the retro-element distributions in the 5'-end regions of RUNX2.

City, CA, USA). Amplifications were obtained using on demand TaqMan probes: DNMT1 (Hs00154749_m1), RUNX2 (Hs00231692_m1), and GAPDH (Hs99999905_m1; Applied Biosystems). For the PCR (25 μl), 1.0 μl of cDNA was mixed with 12.5 μl of 2×TaqMan Universal PCR master mix (Applied Biosystems) and TaqMan probes 1.25 μl. The PCR conditions included initial incubation at 50°C for 2 minutes, denaturing at 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 1 minutes. The relative expression of each mRNA was calculated as the Ct (the value obtained by subtracting the Ct value of the GAPDH mRNA from the Ct value of the target mRNA) using the ΔΔCt method.[16] Specifically, the amount of target mRNA relative to GAPDH mRNA is expressed as $2^{-(\Delta Ct)}$. Data are expressed as the ratio of target mRNA to GAPDH mRNA.

Analysis of DNA methylation

Genomic DNA was extracted from HPDL cells using the DNeasy Blood & Tissue Kit (Qiagen). The DNA samples (Control, LPS, 5Aza, LPS+5Aza) were treated with sodium bisulfite using the EpiTect Plus Bisulfite Kits (Qiagen). DNA methylation of RUNX2 was analyzed using SYBR green-based quantitative methylation-specific PCR (qMSP). Two sets of PCR primers were designed using the Methyl Primer Express Software version 1.0 (Applied Biosystems): one for unmethylated and one for methylated DNA sequences. The methylated and unmethylated primers were designed between the promoters and retro-elements shown in Fig. 1 and Tables 1 and 2. Some of the primers were designed as described in a previous study.[17] In the PCR (25 μl), 1.0 μl of bisulfite-treated DNA template was mixed with 12.5 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems) and a pair of primers in a final concentration of 400 nM. The PCR conditions included initial incubation at 50°C for 2 minutes, denaturing at 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 58°C for 1 minute. After PCR amplification, a dissociation curve was generated to confirm the size of the PCR product. The percentage of RUNX2 methylation in a sample was estimated using the following formula:

Methylated $RUNX2\ (\%) = M/M + U \times 100 = 1/1 + U/M \times 100 = 1/1 + 2^{-\Delta Ct} \times 100$, where M is the copy number of methylated RUNX2, U is the copy number of unmethylated RUNX2, and $\Delta Ct = Ct_U - Ct_M$.[18]

Statistical analysis

Statistical analysis was performed on a database using the IBM SPSS Statistics for Windows (version 19; SPSS Inc, Chicago, IL). Results were compared by the Mann-Whitney U test and Kruskal-Wallis test with $p < 0.05$ accepted as statistically significant.

Results

The cytotoxity of the inhibitor for DNA methylation, 5Aza, was estimated by cell counts at 12 and 24 hours after the 5Aza was added to the cell culture systems, since 5Aza may affect cell viability. No significant differences in the cell numbers were observed between the controls and the 5Aza samples up to 100 μM. No cytotoxic effect of 5Aza on the cells was observed even in the presence of 10 μM LPS (Fig. 2).

We examined whether LPS caused downregulation of RUNX2 mRNA in HPDL cells. The expression levels were evaluated by quantitative RT-PCR. The expression level of RUNX2 was significantly lower in the cells stimulated with LPS than the controls. The DNA methyltransferase inhibitor, 5Aza, increased the expression of RUNX2 in the cells stimulated with LPS, although the increased expression level was significantly lower than the control. No significant difference in the expression level was observed between the 5Aza samples and the control groups (Fig. 3). The results suggest that DNA hypermethylation is, at least in part, involved in the decreased expression of RUNX2 in the cells stimulated with LPS. We examined whether LPS induces DNA methylation by regulating DNA methyltransferase. The expression level of DNMT1 mRNA in the cells stimulated with LPS was significantly higher than the controls. The presence of 5Aza completely abolished the upregulated expression of DNMT1 in the cells stimulated with LPS (Fig. 4). In order to confirm the percentage of methylation in RUNX2 DNA, quantitative RT-PCR was carried out after bisulphate modification of RUNX2 DNA.

Table 1 Summary of CpG sites

Gene symbol	Gene description and RefSeq ID	Gene size (bp)	CpG island size (bp)	CpG amplicon (bp)		Amplicon sites
				Unmethylation (U)	Methylation (M)	
RUNX2	NM_004348 (P2 promoter)	128,905	2226	−1947	−1947	−1.9 kb
				−852	−852	−0.7 kb
				−450	−450	−0.4 kb
				+21	+21	+0.1 kb
				+1573	+1573	+1.6 kb

Table 2 Primer sequences used in methylation

Amplicon sites	Primer	Forward	Reverse	Amplicon Size (bp)	References
−1.9 kb	U	GGATTTTTTGGTTTTTGTGGGT	CTCTAACTAAATCAATCATTACA	127	(17)
	M	GATTTTTCGGTTTTTGCGGGC	CTCTAACTAAATCGATCATTACG	126	
−0.7 kb	U	GGTTTTGGAAATTGTATATGGTGT	AAACAACAAATCTCAAACCTACA	96	(17)
	M	TTTCGGAAATTGTATACGGCGC	AACAACGAATCTCGAACCTACG	93	
−0.4 kb	U	TTTCGGAAATTGTATACGGC	TCGTAAACAACGAATCTCGA	113	This study
	M	AGGTTTTGGAAATTGTATATGGT	TCATAAACAACAAATCTCAAAAC	113	
+0.1 kb	U	GGGTGTTAGTGTATTTAGGATGT	ACACAAATCTTTACCAAAATCTT	146	This study
	M	CGTTAGCGTATTTAGGACGC	CGCAAATCTTTACCGAAATC	146	
+1.6 kb	U	GTTTGAGGGTGGGTGGTAGTTGT	ACTACCCCAAAAAATCTAAATCA	127	(17)
	M	GTTTGAGGGCGGGTGGTAGTCGC	ACTACCCCGAAAAATCTAAATCG	127	

Since two CpG islands exist at the promoter areas of +1573 to −852 and −1947 to −3061 up to approximately −3000, we estimated the percentage of DNA methylation of these areas using qMSP. The methylation of DNA at 0.1 kb (40%) and −1.9 kb (20%) in the cells stimulated with LPS was significantly higher than the control (Fig. 5).

Discussion

The present study first demonstrated that *P gingivalis* LPS caused DNA hypermethylation accompanied by downregulated expression of RUNX2 in fibroblasts derived from the periodontal ligament. Both LPS and lipids extract from *P gingivalis* inhibited osteoblastic differentiation, and the inhibition was probably associated with downregulated expression of RUNX2.[14,15] The mechanism of downregulated expression of RUNX2 is, however, not clear. In the present study, the presence of 5Aza completely abolished the upregulated expression of DNMT1 in the cells stimulated with LPS. The RUNX2 mRNA level did not, however, revert to the control level in the presence of 5Aza. DNA hypermethylation may not be fully, but at least in part, involved in the downregulated expression of RUNX2.

The LPS extracted from periodontal pathogens negatively affects many kinds of cells including epithelial cells, fibroblasts, osteoblasts, and lymphocytes in the periodontal tissues.[19] These cell responses induce the development and progression of periodontal destruction mainly via cytokine production.[20] Although epigenetic modifications are important factors in the regulation of cell responses, little information is available about the involvement of epigenetic modifications in the development and progression of periodontal destruction.[21] Our results may support the theory that epigenetic modifications are involved in the progression of periodontal disease.

Recent studies have speculated about the role of epigenetics in the development and progression of periodontal diseases.[5] Epigenetic mechanisms have been evaluated in some cytokine genes related to the progression of periodontal diseases such as IL-2, IFN-γ, IL-10, IL-6, and TNF-α.[22–26] The epigenome can be modified by risk factors for periodontal disease including environment, nutrition, tobacco smoke and alcohol, and periodontal pathogens may also affect placental development.[26–28] *P gingivalis* and *F nucleatum* have been shown to alter the expression of epigenetic markers in gingival epithelial cells.[12] Both *P gingivalis* and *F nucleatum* significantly decreased expression of DNMT1, indicating that these bacteria may induce

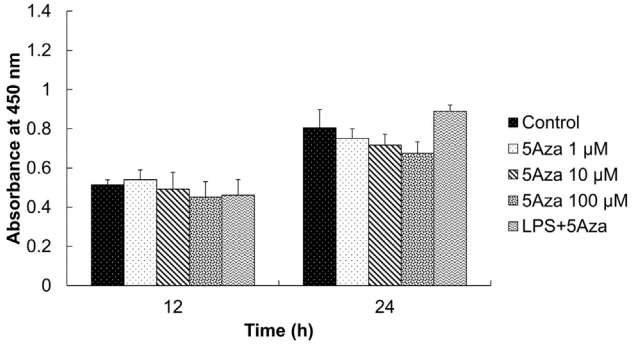

Figure 2. The cytotoxic effect of 5Aza on HPDL cells. The cytotoxity of 5Aza was estimated by cell counts at 12 and 24 hours after the 5Aza was added to the cell culture system. No significant differences in cell numbers were observed between the controls and the 5Aza samples up to 100 μM at 12 and 24 hours. No cytotoxic effects of 5Aza were observed on HPDL cells, even in the presence of 10 μg/ml LPS.

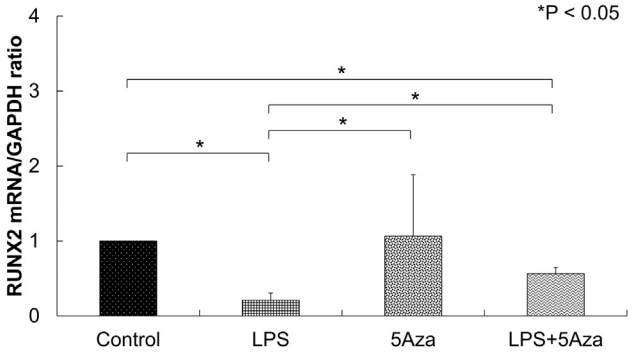

Figure 3. The expression of mRNA in HPDL cells in the presence of LPS and/or 5Aza. The expression levels of RUNX2 were evaluated by quantitative RT-PCR. The expression of RUNX2 was significantly lower in cells stimulated with 10 μg/ml LPS than the control (*$p < 0.05$). The presence of 100 μM 5Aza increased the expression of RUNX2 in cells stimulated with 10 μg/ml LPS.

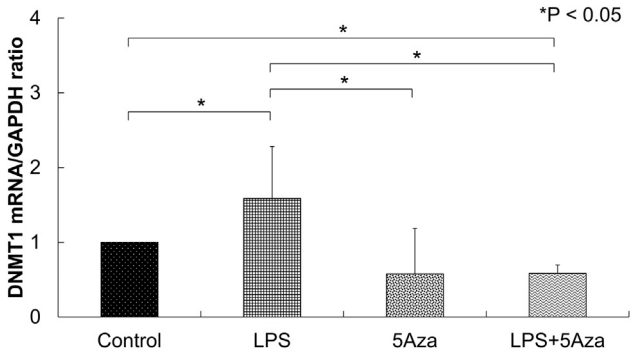

Figure 4. The expression of DNMT1 mRNA in HPDL cells in the presence of LPS and/or 5Aza. The expression levels of DNMT1 were evaluated by quantitative RT-PCR. The expression of DNMT1 mRNA in cells stimulated with 10 μg/ml LPS was significantly higher than the control (*$p < 0.05$). The presence of 100 μM 5Aza completely abolished the upregulated expression of DNMT1 in cells stimulated with 10 μg/ml LPS.

hypomethylation of the genes in gingival epithelial cells. In contrast to these findings, our results showed that LPS extract from *P gingivalis* significantly increased expression of DNMT1 and induced hypermethylation of RUNX2 in HPDL cells. The expression profile of DNMT1 by stimulated with *P gingivalis* may depend on the type of cells. The gingival epithelial cells play an important role in protective barrier against bacterial infection, whereas HPDL cells do not.[29] These functional differences between cells may be involved in the different expression profile of DNMT1. *F nucleatum* and another oral bacteria, *Campylobacter rectus*, induced hypermethylation of MALT1 and Igf2 genes.[28,30] LPS extracted from *Escherichia coli* induced hypermethylation of Toll-like receptor 4 genes in large intestinal epithelial cells.[31] Taken together, these findings suggest that different bacteria and their extracts may cause different methylation changes in genes in different types of cells.

Some studies have shown hypomethylation of RUNX2 during osteogenic and chondrogenic differentiation.

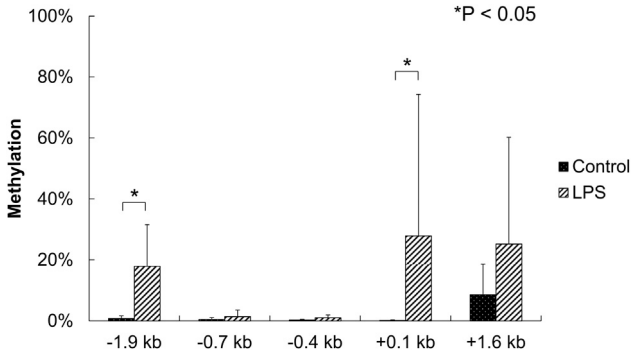

Figure 5. The percentage of DNA methylation of CpG islands areas as measured by qMSP. The percentage of metylation in RUNX2 was estimated by quantitative RT-PCR after bisulphate modification of RUNX2 DNA. The methylation of DNA at 0.1 kb and −1.9 kb in cells stimulated with 10 μg/ml LPS was significantly higher than the control (*$p < 0.05$).

Previous studies have found hypermethylation of RUNX2 at −868 to −509; however, hypermethylation of RUNX2 is poorly documented so far.[32] We found that the hypermethylation site was limited to the area near the P2 promoter, at 0.1 kb ansd −1.9 kb but not at −0.7 kb. Further studies are needed to clarify areas of frequent hypermethylation of the RUNX2 gene.[33]

In conclusion, we first demonstrated that LPS extracted from *P gingivalis* induced DNA hypermethylation of RUNX2 in fibroblasts derived from periodontal ligaments. LPS causes periodontal destruction and impedes periodontal regeneration.[34] The decreased expression of RUNX2 in HPDL cells may directly impede periodontal regeneration. DNA hypermethylation is chemically reversible, and may therefore be a therapeutic target for negatively regulating gene expression through DNA hypermethylation. There is growing acceptance of the concept of epigenetic therapy, and several agents that alter methylation patterns are currently being tested in clinical trials for cancer therapy.[6,35] The findings of this study may contribute to the development of new therapies for periodontal disease involving epigenetic modification. The 5Aza, an inhibitor for DNA methyltransferase, may be one of candidates for the epigenetic therapy.[36] Recently, microRNAs have found to regulate DNMT-associated DNA methylation in specific genes.[37] The microRNAs may be more gene-specific epigenetic therapy. Further investigations need to establish the therapy.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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