ORIGINAL ARTICLE

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Comprehensive gene expression analysis in human periodontal ligaments of the mandibular third molars performing vertical movement and the maxillary second premolars with occlusal contact

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Structured Abstract

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Objectives – The periodontal ligament (PDL) is thought to be an important tissue in vertical movement during tooth eruption, but the precise molecular mechanism is not known. Thereto, comprehensive gene expression was analyzed in human PDL of mandibular third molars performing vertical movement and maxillary second premolars with occlusal contact.

Design – The expression profile of 9,243 genes in the PDL of one subject was compared between vertically moving third molars and second premolars with occlusal contact by DNA microarray.

Results – The expression of 27 genes showed more than a 10-fold difference between third molars and second premolars. The expression of CALB1 (encoding calbindin 1), CYP26A1 (encoding cytochrome P450, family 26, subfamily A, polypeptide 1), SPOCK3 (encoding testican-3), CCK (encoding cholecystokinin) and SCRG1 (encoding scrapie responsive protein 1) was more than 30-fold higher in PDLs of the third molars than the second premolars. CALB1 is reported to increase at the pressure side of PDL during experimental orthodontic tooth movement in rats. Interestingly, in this study, CALB1 expression showed the largest difference. In contrast, CRCT1 (encoding cysteine-rich C-terminal 1), SPRP3 (encoding small proline-rich protein 3), IL8 (encoding interleukin 8) and MMP12 (encoding matrix metalloproteinase 12) showed more than 100-fold higher expression in PDLs of the second premolars than the third molars.

Conclusion – The present comprehensive gene expression in PDLs provides new insights into the molecular mechanism during the vertical tooth movement.

Key words: DNA microarray; gene expression; periodontal ligament; tooth eruption; vertical tooth movement

Introduction

Tooth eruption is a complicated process that can be divided into two simultaneous and synchronous phases, the formation of eruption pathway and the vertical tooth movement in and through the alveolar bone to

the oral cavity (1). The formation of the eruption pathway requires the recruitment and generation of numerous osteoclasts and active alveolar bone resorption (2). It is known that over half of the alveolar bone surface overlaying the tooth is occupied by osteoclasts during tooth eruption. Colony stimulating factor (CSF) 1 (3), receptor activator of NFκ-B ligand (RANKL) (4, 5), epidermal growth factor (EGF) (6) and monocyte chemotactic protein (MCP) 1 (7) are expressed from the periodontal ligament (PDL) and the dental follicle to recruit and generate osteoclasts in the eruption pathway. Furthermore, our and other studies have proposed the importance of parathyroid hormone-related protein (PTHrP) on the eruption pathway formation (8–10). A high level of PTHrP is produced from the enamel epithelium of teeth and the type 1 PTH/PTHrP receptor is expressed in the cells of the dental follicle, PDL and alveolar bone. PTHrP activates RANKL expression of cells in these tissues and creates a local environment highly inductive for osteoclast recruitment and formation.

Regarding the vertical tooth movement during tooth eruption, classical theories have been suggested to be relevant to this event (11). First is the root formation, but rootless mutant mice lacking nuclear factor I transcription-replication protein (NFI-C/CTF) showed tooth eruption as did wild-type mice, suggesting that the root formation is not essential for tooth eruption (12); Secondly is the bone formation at the base of tooth. Thirdly is the contractile property of PDL fibroblasts providing the force for the vertical traction of teeth (11). This is the accepted theory for tooth eruption at least in rodent incisors. In order to clarify the molecular mechanism in the human vertical tooth movement, the expression profile of human PDLs in the vertically moving mandibular third molars and the maxillary second premolars with occlusal contact was examined by DNA microarray in this study.

Materials and methods

Tissue preparation and RNA extraction

Periodontal ligaments were obtained from a 19-year-old healthy female during the course of orthodontic treatment. She had no congenital anomaly or endocrine problem. Panoramic radiographs showed that the maxillary left and right second premolars had occlusal contact with the mandibular teeth (Fig. 1). The position of these teeth did not change in radiographs between the age of 15 years and 5 months and 18 years and 8 months. The mandibular left and right third molars were moving vertically in and through the alveolar bone during this period (Fig. 1). There were no apparent symptoms of periodontal disease. Extracted maxillary left and right second premolars and mandibular left and right third molars were washed with phosphate-buffer saline. To minimize contamination of gingiva and the root forming odontoblasts, only PDLs attached to the mid-third of the root were isolated with a scrape and used in the experiment. Total RNA was isolated by using Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) from PDLs of the maxillary second premolars and the mandibular third molars, according to the manufacturer's instructions. The protocol for the experiment was reviewed and approved by the Ethics Committee of Tokyo Medical and Dental University. The patient provided informed written consent before extraction.

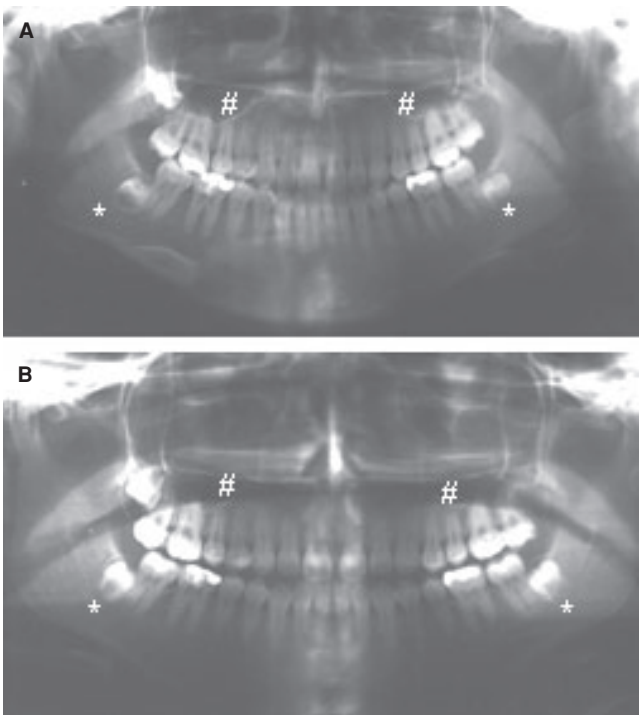

Fig. 1. Panoramic radiographs of a Japanese female whose periodontal ligaments were used. Radiographs at 15 years and 5 months of age (A) and 18 years and 8 months (B). Note that the mandibular third molars (*) were moving vertically, but the vertical movement of maxillary second premolars (#) had terminated and contacted to the mandibular teeth.

Target preparation and DNA microarray

Target cRNA preparation and DNA microarray were carried out as previously reported (13). In brief, 5 μg of total RNA were reverse transcribed using T7-(dT)24 oligonucleotide primer and Superscript^{TM} II RNase H$^-$ reverse transcriptase (Invitrogen). The second-strand cDNA was synthesized using DNA polymerase I (Amersham Bioscience, Piscataway, NJ, USA) and purified using a QIAquick purification kit (Qiagen, Valencia, CA, USA). The cDNA was resuspended and incubated in IVT reaction mix, containing nuclease-free water, 10x reaction buffer, ATP, GTP, CTP, UTP, Biotin 11-CTP, Biotin 11-UTP and T7 RNA polymerase. The cRNA target was purified using an RNeasy kit (Qiagen). Ten micrograms of fragmented target cRNA was hybridized with each UniSet Human I Expression Bioarray chip (Amersham Biosciences) for 18 h at 37°C with shaking. After hybridization, each slide was washed in TNT buffer (0.1 M Tris-HCL, pH7.6, 0.15 M NaCl, 0.05% Tween-20) at 42°C for 60 min. The signal was developed using a streptavidin-Cy5 (Amersham Biosciences) for 30 min at room temperature. The proceeded slides were scanned using as Axon GenePix Scanner (Axon, Union City, CA, USA) with the laser set to 635 nm. Slides were scanned using GenePix Pro software (Axon) and images for each slide were analyzed using the CodeLink TM expression analysis software (Amersham Biosciences). A total of 9,243 genes were examined in this study.

Reverse transcription polymerase chain reaction analysis

For the temple of reverse transcription polymerase chain reaction (RT-PCR), the second-strand cDNA purified in the course of the target preparation for DNA microarray from PDLs of the mandibular third molars and the maxillary second premolars were used. The primers and the annealing temperatures for ACTB (encoding human β-actin), CALB1 (encoding human calbindin 1, 28 kDa), CRABP1 (encoding human cellular retinoic acid binding protein 1), CY26A1 (encoding human cytochrome P450, family 26, subfamily A, polypeptide 1), FBN2 (encoding human fibrillin 2), MMP12 (encoding human matrix metallopeptidase 12) and KLK5, -7, -13 (encoding human kallikrein-related peptidase 5, 7 and 13) are summarized in Table 1. Amplification was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Tokyo, Japan) with 20–30 cycles of denaturation at 94°C for 1 min, annealing at temperatures shown in Table 1 for 1 min and extension at 72°C for 1 min. PCR products were resolved on a 2% (w/v) agarose gel and stained with ethidium bromide. All gene expressions were normalized to the human house keeping gene, ACTB.

Results

In this study, the expression profile of PDLs from the vertically moving third molars and the second premolars with occlusal contact were examined by DNA microarray and RT-PCR. Table 2 lists 15 genes whose expression were found to be over 10-fold higher in PDLs of the third molars than the second premolars. In the 9243 genes, CALB1 showed the largest difference (×406). CYP26A1, SPOCK3 (encoding human testican 3), CCK (encoding human cholecystokinin) and SCRG1 (encoding human scrapie responsive protein 1) also showed more than 30-fold difference.

Table 3 lists 12 genes, which expression was found to be over 10-fold higher in PDLs of the second premolars with occlusal contact than the vertically moving third molars. CRCT1 (encoding human cysteine-rich C-terminal 1), SPRR3 (encoding human small proline-rich protein 3), IL8 (encoding human interleukin-8), MMP12, KRT6A (encoding human keratin 6A), KLK7 and 13, and CLDN4 (encoding human claudin 4) showed more than 50-fold difference. Among them, CRCT1 showed the largest difference (×347).

To verify the difference of expression, the expression of eight genes on the array chip together with the house keeping gene ACBT were examined by RT-PCR (Fig. 2). The expression of CALB1, CRABP1, CYP26A and FBN2 was seen in PDLs from the moving third molars but not from the second premolars with occlusal contact. For MMP12 and KLK5, 7 and 13, the expression was clearly seen in PDLs of the second premolars. The expression for MMP12 and KLK7 was quite low and that for KLK5 and 13 was not seen in PDLs of the third molars.

Discussion

The present samples of PDLs were isolated from maxillary second premolars and mandibular third molars of

Table 1. **Primers, product sizes, annealing temperatures and PCR cycles of reverse transcription polymerase chain reaction**

Gene	Oligonucleotide sequence	Product size	Annealing temperature	PCR cycles
ACTB	5′-ATGAGGATCCTCACCGAGCGCGGCTACAGC-3′	331	60	20
	5′-ACACCACTGTGTTGGCGTACAGGTCTTTGC-3′			
CALB1	5′-AGCCGAGTATACAGACCTAATG-3′	352	51	30
	5′-ATCCCCAGCACAGAGAATAAG-3′			
CRABP1	5′-CGGCACCTGGAAGATGCGCA-3′	371	65	30
	5′-CCACGTCATCGGCGCCAAACTTG-3′			
CYP26A1	5′-TCCTCGCACAAGCAGCGAAAGAAGGTG-3′	572	60	30
	5′-ATGTGGGTAGAGTCCTAGGTAAGT-3′			
FBN2	5′-TTCGCCCGGCAGCAAACTCAGC-3′	470	61	30
	5′-CCCAAGCCGCCCCGACAGC-3′			
KLK5	5′-GGATGCTTACCCGAGACAGA-3′	343	58	30
	5′-GCTGGAGAGATGAACATTCT-3′			
KLK7	5′-GAATGAGTACACCGTGCACC-3′	359	65	30
	5′-TGCCAGCGCACAGCATGGAA-3′			
KLK13	5′-CCAACATCCAACTTCGCTCA-3′	503	58	30
	5′-TTCAGGGATGCAACATCTGG-3′			
MMP12	5′-TTCCCCTGAACAGCTCTACAAGCCTGGAAA-3′	517	55	30
	5′-GATCCAGGTCCAAAAGCATGGGCTAGGATT-3′			

Table 2. **Summary of genes and encoding molecules showing markedly higher expression in periodontal ligaments of vertically moving mandibular third molars than of maxillary second premolars with occlusal contact**

Gene	Encoding molecule	Accession no.	Relative fold
CALB1	Human calbindin 1, 28 kDa	NM_004929	406
CYP26A1	Human cytochrome P450, family 26, subfamily A, polypeptide 1	NM_000783	73
SPOCK3	Human testican 3	NM_016950	43
CCK	Human cholecystokinin	NM_000729	38
SCRG1	Human scrapie responsive protein 1	NM_007281	31
SCGN	Human secretagogin, EF-hand calcium binding protein	NM_006998	28
FBN2	Human fibrillin 2	NM_001999	14
CRABP1	Human cellular retinoic acid binding protein 1	NM_004378	14
CLDN10	Human claudin 10	NM_006984	13
CABYR	Human fibrousheathin 2	NM_012189	12
TF	Human transferrin	NM_001063	11
RYR2	Human ryanodine receptor 2	NM_001035	11
CDH12	Human N-cadherin 2	NM_004061	11
FABP7	Human fatty acid binding protein 7	NM_001446	10
SCIN	Human scinderin	NM_033128	10

Table 3. **Summary of genes and encoding molecules showing markedly higher expression in periodontal ligaments of the maxillary second premolars with occlusal contact than the vertically moving mandibular third molars**

Gene	Encoding molecule	Accession no	Relative fold
CRCT1	Human cysteine-rich C-terminal 1	NM_019060	347
SPRR3	Human small proline-rich protein 3	NM_005416	150
IL8	Human interleukin 8	NM_000584	136
MMP12	Human matrix metallopeptidase 12	NM_002426	114
KRT6A	Human keratin 6A	NM_005554	56
KLK13	Human kallikrein-related peptidase 13	NM_015596	55
KLK7	Human kallikrein-related peptidase 7	NM_005046	52
CLDN4	Human claudin 4	NM_001305	51
NMU	Human neuromedin U	NM_006681	26
KLK5	Human kallikrein-related peptidase 5	NM_012427	23
DEFB4	Human defensin, beta 4	NM_004942	17
VNN1	Human varin 1	NM_004666	10

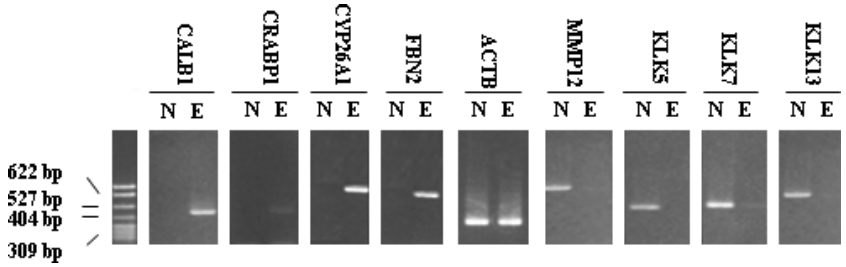

Fig. 2. Reverse transcription polymerase chain reaction analysis of the examined genes in periodontal ligaments (PDLs) of the vertically moving mandibular third molars (E) and the maxillary second premolars with occlusal contact (N). A molecular marker is shown in the left lane. Genes highly expressed in PDLs of mandibular third molars are shown on the left sides of the house keeping gene, ACTB (encoding human β-actin). Genes highly expressed in PDLs of the maxillary second premolars are shown on the right sides of ACTB.

one individual. There are many differences between these two types of teeth, e.g. shape of crown and root, number of cusps and roots, position in the jaw bone, and timing of eruption and formation. It is not fair to say that PDLs of these two types of teeth were comparable. However, only genes that showed dramatic differences in expression were selected and highlighted in Tables 2 and 3. Thus, the present findings would help clarifying the differential gene expression in PDLs during vertical tooth movement.

The expression of CALB1 showed the largest up-regulation among all the examined genes (Table 2). This gene is an intracellular soluble vitamin D-dependent Ca binding protein and a member of troponin C superfamily (14). Two classes of calbindins have been reported; 28 kDa protein (calbindin 1) and 8–10 kDa protein (calbindin 2). A study of immunohistochemistry has shown that the number of calbindin 1-positive cells increased in the PDL 12 h following the onset of experimental tooth movement in rats (15). Interestingly, this increase was only seen in the pressure side but not in the tension side. The functional significance of the increased level of calbindin 1 in PDLs of vertically moving third molars or orthodontically moved teeth remains unclear. Calbindin 1 is known to buffer an increase of the intracellular calcium level in the hippocampus at the ischemic damage (16). Further study is required to examine the relationship between the expression of CALB1 and the intracellular Ca level of PDL-cells during vertical movement and orthodontic tooth movement.

The force bringing about the vertical tooth eruption has been thought to be related to local events so far,

e.g. alveolar bone remodeling, contractile force of fibroblasts, root formation, etc., but not to the systemic events (11). It is interesting that there was a dramatic increase in the expression of CRABP1 and CYP26A1 in PDLs of the vertically moving teeth. Retinoids, derived from dietary vitamin A, are systemic signaling molecules that regulate pattering of limb buds and neural tubes, and RARs and RXRs serve as those receptors (17). An all-*trans* retinoic acid, the active metabolite of vitamin A, is categorized to polar metabolites by CYP26A1 (18). This is supported by the evidence that null mutant of Cyp26a1 gene in mice had lethal morphogenetic phenotypes mimicking those generated by excess retinoic acid administration (19). CRABP1 and 2 are known to regulate intracellular retinoic acid concentration, transport and metabolism (20). Taking these together, the concentration of retinoids is likely to be strictly regulated during the vertical tooth movement. To clarify the role of retinoids in this process, further studies are essential to monitor the concentration of active and inactive metabolites around vertically moving teeth.

The expression of CRCT1 showed the largest up-regulation in the PDLs of the premolars with occlusal contact. CRCT1 was discovered by the subtractive hybridization method using YAC probes of 1q21 region and human keratinocyte cDNA library (21). This gene is still uncharacterized but belongs to a human epidermal differentiation complex. This complex is thought to be a potential candidate involving skin diseases and epidermal differentiation. Selective expression only in the second premolars with occlusal contact would provide new insight into the unknown functions of this gene.

The reason why KLK genes (KLK5, 7 and 13) had higher expression level in PDLs of the second premolars with occlusal contact is unclear. The PDLs provided by the 19-year-old female did not have any apparent symptom of periodontal disease. Thus, it is unlikely that elevated level of kallikreins is related to the inflammation status of PDLs.

It has to be noted that the vertically moving third molars were in the stage when active alveolar bone resorption required for the generation for eruption pathway was almost completed or at the final stage (Fig. 1). Genes involved in the bone resorption are likely to be expressed prior to the onset of the generation for eruption pathway (1, 22). Thus, the gene expression profile of PDLs in the moving third molars should be reflected not by the alveolar bone resorption but by the pressure and/or tension in the PDLs during the vertical movement.

During the process of tooth eruption, the tooth root elongates. The moving third molars showed root elongation as shown in the panoramic radiographs between two stages (Fig. 1). Tissues in the root tips include root forming odontoblasts and the immature dental follicle cells (11). In this study, tissues were only isolated from the middle portion of the root where the root formation was not going on. Thus, there was only a minimum inclusion of odontoblasts and dental follicle cells in the isolated PDLs. However, it would be difficult to exclude completely the involvement of these cells in the present sample.

In summary, the gene expression of a total 9,243 genes was examined in PDLs of vertically moving third molars and second premolars with occlusal contact. Five genes, CALB1, CRCT1, SPRR3, IL8 and MMP12, showed over a 100-fold difference between these two samples. It is also suggested that the concentration of retinoids may be strictly regulated during the process of the vertical tooth movement.

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