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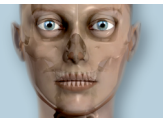

ORIGINAL ARTICLE

Tooth eruption: altered gene expression in the dental follicle of patients with cleidocranial dysplasia

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Structured Abstract

Objectives – The dental follicle plays an important role in tooth eruption by providing key regulators of osteogenesis and bone resorption. Patients with cleidocranial dysplasia (CCD) exhibit delayed tooth eruption in combination with increased bone density in the maxilla and mandible, suggesting disturbances in bone remodeling. The aim of this study was to determine the expression of genes relevant for tooth eruption and bone remodeling in the dental follicles of patients with CCD and normal subjects.

Material and Methods – Thirteen dental follicles were isolated from five unrelated patients with CCD, and fourteen dental follicles were obtained from 10 healthy individuals. All teeth were in the intraosseous phase of eruption. The expression of RANK, RANKL, OPG, and CSF-1 was determined by quantitative RT-PCR.

Results – In patients with CCD, the mRNA levels of RANK, OPG, and CSF-1 were significantly elevated compared with the control group. Accordingly, the ratios of RANKL/OPG and RANKL/RANK mRNAs were significantly decreased in patients with CCD.

Conclusion – The observed alterations in the expression and ratios of the aforementioned factors in the dental follicle of CCD individuals suggest a disturbed paracrine signaling for bone remodeling that could be responsible for the impaired tooth eruption seen in these patients.

Key words: bone remodeling; cleidocranial dysplasia; rank ligand; receptor activator of nuclear factor-kappa B; tooth eruption

Introduction

Tooth eruption is a timely programmed and locally regulated developmental process that requires both bone resorption and osteogenesis (1). The dental follicle, a loose connective tissue interposed between the alveolar bone and the tooth, is the orchestrator of tooth transposition through the bone (2), providing important regulators of bone resorption and osteogenesis.

Bone remodeling is regulated by paracrine interactions of a set of molecules, which include the following: 1) the colony-stimulating factor-1 (CSF-1); 2) the receptor activator of nuclear factor κB (RANK); 3) the ligand for receptor activator of nuclear factor κB (RANKL); and 4) osteoprotegerin (OPG), a decoy receptor for RANKL. CSF-1 is a chemokine required for growth and differentiation of preosteoclasts and for the up-regulation of RANK (3). The membrane-bound receptor RANK on osteoclast precursors promotes osteoclastogenesis upon binding its ligand RANKL (4). The latter is found in osteoblasts and stromal cells in two forms, either as a soluble or as an intramembrane molecule (5). The actions of RANKL are inhibited by OPG that halts bone resorption (6, 7).

Cleidocranial dysplasia (CCD) is a skeletal disorder of autosomal dominant inheritance. Clinical features include increased bone density of jaw bones and multiple supernumerary, late erupting teeth of the permanent dentition (8). CCD has been causatively linked to RUNX2 haploinsufficiency (9, 10), and numerous mutations in RUNX2 gene have been identified in most patients with CCD (11). Studies in a calvarial cell line have shown that RUNX2 can induce RANKL and suppress OPG gene expression and that overexpression of soluble RANKL in homozygous Runx2/Cbfa1 knockout mice increases the number and size of osteoclasts (12). The presence of binding sites for RUNX2 in murine RANKL gene indicates that RUNX2 is involved in osteoclastogenesis via activating RANKL expression and RANK-RANKL signaling (13). In cleidocranial dysplasia because of RUNX2 mutations, the induction of RANKL expression appears deranged, leading to disturbed osteoclastogenesis.

The molecular events of tooth eruption in humans are largely unknown. Evidence from studies in rodents (1) shows that an influx of mononuclear cells into the dental follicle that release CSF-1 is the initiating event for the development of the osteoclasts, needed to resorb the alveolar bone during tooth eruption. CSF-1 is required to recruit osteoclast precursors and it has been shown to up-regulate RANK in these cells. During the initial burst of osteoclastogenesis in the rat 1st molar, the expression of OPG is down-regulated in the dental follicle (14). The follicle is also a source of RANKL, at least in rodents (15). RANKL promotes osteoclastogenesis and its presence appears indispensable for tooth eruption, since in RANKL-null mice the teeth do not erupt (16).

In patients with CCD, delayed eruption is witnessed in the permanent teeth, even in regions without any anatomical obstacle, whereas the primary dentition erupts physiologically. RUNX2 haploinsufficiency in these subjects may lead to abnormal alveolar bone remodeling, because of osteoblast defects that compromise osteoblast–osteoclast interaction. The causative contribution of RUNX2 in this event may increase with age, thus affecting the permanent but not primary dentition. Evidence from Runx2 heterozygous knockout mice supports an increased impact of this factor in cancellous bone formation with advanced age (17). In addition to disturbed signals acting within the alveolar bone during tooth eruption in CCD individuals, deranged signals originating in the dental follicle may contribute to delayed eruption. Indeed, this tissue hosts important eruption regulators, as it has been shown in rodents (1). Although further research is required to shed light on the molecular pathogenesis of CCD, this condition provides a valuable model for studies of the mechanisms of tooth eruption and alveolar bone remodeling.

In this study, we hypothesized that disturbed molecular signals from the dental follicle during tooth eruption may contribute to impaired osteoclastogenesis and bone remodeling suspected in CCD. We have thus examined the expression of RANK, RANKL, OPG, and CSF-1 in dental follicles of patients with CCD, compared with healthy controls.

Material and methods

Participants and sample collection

Five not related patients with CCD, who contacted the Orthodontic Clinic of Athens University seeking therapy, were included in the study. Patients' average age was 18.4 ± 5.1 years. The diagnosis of cleidocranial dysplasia was based on clinical and radiographic examination (18).

Patients presented typical clinical findings such as the ability to bring their shoulders together owing to hypoplasia/aplasia of the clavicles, brachydactyly, and short stature, hypoplasia of the maxilla, depressed nasal bridge, and hypertelorism. The radiographic examination revealed multiple supernumerary teeth. Ten healthy persons, with clear medical history, aged 19.4 ± 3.1 years comprised the control group (Table 1). This project has been approved by the Ethical Committee of the Dental School of Athens University (protocol number 122/19.05.2009). Written informed consent was obtained from all participants prior to enrollment in the study.

The dental follicles were isolated during surgical removal or exposure of teeth for dental and/or orthodontic reasons. All operations were performed in the Clinic of Oral and Maxillofacial Surgery of the Dental School of Athens University by the same oral surgeon (DK). Thirteen dental follicle specimens were obtained from the patients with CCD. Fourteen follicles were removed from the control group, selected from routine third molar extraction cases. All teeth were in the intraosseous phase of eruption, meaning that they were entirely covered by bone (Fig. 1). The follicle specimens were similarly distributed between the maxilla and the mandible in both CCD individuals and controls. Experimental and control groups were also matched for age and sex (Table 1). Immediately after their removal, dental follicles were grounded in liquid N_2. By the end of the operation, they were stored at −80°C until use.

Table 1. **Sample characteristics**

	Subjects	Sex	DF Specimens	Age
Patients	P1	M	2	17.0
	P2	M	4	25.1
	P3	F	2	10.9
	P4	F	2	19.9
	P5	F	3	19.2
	Total	*n* = 5	*n* = 13	18.4 ± 5.1*
Controls	C1	M	3	15.2
	C2	M	1	15.3
	C3	M	1	22.5
	C4	M	1	
	C5	F	1	15.7
	C6	F	1	18.2
	C7	F	1	20.3
	C8	F	2	20.1
	C9	F	1	21.5
	C10	F	2	22.6
	Total	*n* = 10	*n* = 14	19.4 ± 3.1*

Data are provided for individual patients with CCD (P1–P5) and controls (C1–C10) regarding sex (M, male; F, female), number (*n*) of isolated dental follicle (DF) specimens and age (years. months). Average age per group is expressed in mean ± SD.
*Ages did not differ between CCD and control groups, $p = 0.62$, *Mann–Whitney* test.

Quantitative real-time PCR (qRT-PCR) analysis

The dental follicles were homogenized on ice with ULTRA – TURRAX® T25, high-performance disperser (Janke & Kunkel, IKA®- Labotechnik, Staufen, Germany). Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and treated with DNase I (Ambion, Carlsbad, CA, USA) to remove any DNA contamination. Spectrophotometric quantification of RNA was performed with BioSpec-nano (Shimadzu Biotech, Manchester, UK). RNA concentration was determined using the optical density (O.D.) reading at 260 nm and RNA purity by the ratio of $O.D._{\cdot 260}/O.D._{\cdot 280}$ that was always

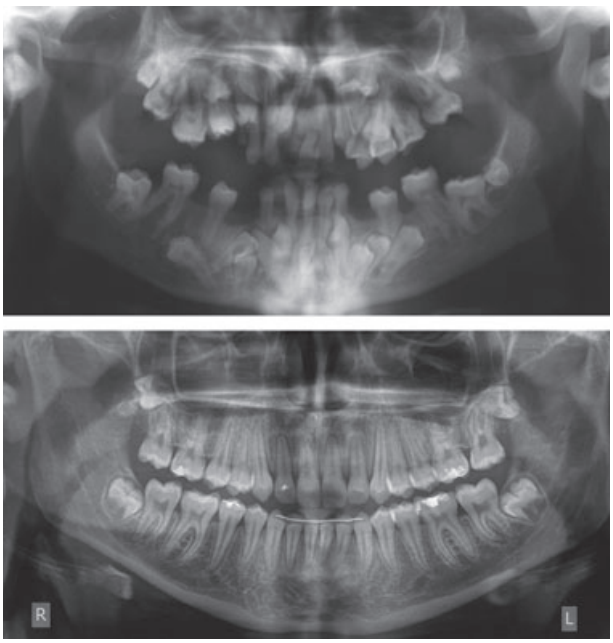

Fig. 1. Representative panoramic X-ray of a patient with CCD (A), showing multiple supernumerary teeth, and a control subject (B) with four erupting third molars.

>1.7. RNA (1 μg) was reverse transcribed using the iScript kit (Biorad, Hercules, CA, USA) for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C to generate cDNA. The primer sequences for RANK, RANKL, OPG, CSF-1, and β-ACTIN (normalization gene) were selected using the Primer 3 program. They were checked for dimers' formation with the Oligo 4 program and for specificity through BLAST network service of the National Center for Biotechnology Information (Bethesda, MD, USA). Detailed information on the primers is provided in Table 2. The PCR was carried out in a Light Cycler 1.5 apparatus (Roche, Indianapolis, IN, USA), within a 10 μl total volume consisting of 1 μl LightCycler FastStart DNA Master SYBR Green I (Roche, Indianpolis, IN, USA), 0.5 μl of each primer, 2.5 μl cDNA sample, and 5.5 μl sterile H_2O. A threshold cycle (C_t) value for each sample was generated through the Light Cycler software and was normalized by the respective β-ACTIN value. The amplification was carried out twice for each specimen, and the mean C_t value was used for the analysis. In cases of more than one specimen analyzed from the same subject, the mean C_t value of all specimens derived from the same subject was used in the analysis of the results. The comparative C_t method was used to calculate the relative gene expression by the formula $2^{(-\Delta\Delta Ct)}$ for gene expression patterns and the formula $2^{(-\Delta Ct)}$ for ratios of gene expression (19). Amplified products were checked by electrophoresis in 3% agarose gel and visualized with ethidium bromide staining.

Statistical analysis

All calculations were performed using SPSS Version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± SE gene expression values of each group. Mann–Whitney test was used to assess any age difference between CCD and control subjects and to evaluate the differences between the control and experimental group in gene expression of RANK, RANKL, OPG, and CSF-1 and gene expression ratios RANKL/RANK and RANKL/OPG. The critical value of significance was determined at $p \leq 0.05$.

Results

The mean age of the patients with CCD did not differ significantly from that of the control group (Table 1). Using a quantitative RT-PCR method, we detected the expression of genes under study in dental follicle specimens obtained from all subjects. The expression level of genes in CCD individuals was determined relative to the

Table 2. **Characteristics of the primers**

Gene	Primer sequence	Location (exon)	Product Size (bp)	Anneal. temp. (C°)	$MgCl_2$ (mM)
RANK	F: 5′ GCTTTCCCAGTGTGTGTTCA 3′ R: 5′ AAAGTCCCACCACATGTTCC 3′	10	101	54	2.5
RANKL	F: 5′ GATGAAAGGAGGAAGCACCA 3′ R: 5′ TTGGAGACCTCGATGCTGAT 3′	5	119	54	3.5
OPG	F: 5′ GGCAACACAGCTCACAAGAA 3′ R: 5′ AGGTGAGGTTAGCATGTCCAAT 3′	4 & 5	149	54	3.0
CSF-1	F: 5′ GAGATTCCCGTACCCCAAG 3′ R: 5′ GATGCAGGGAGTGGAGAAGA 3′	6	118	56	1.0
β-ACTIN	F: 5′ AGGATGCAGAAGGAGATCACTG 3′ R: 5′ GGGTGTAACGCAACTAAGTCATAG 3′	5 & 6	224	67	6.0

Data are provided for the sequence of each primer set (F = Forward, R = Reverse), the location (exon) in the gene, the size of each product (in base pairs), the annealing temperature, and the final $MgCl_2$ concentration in each reaction.

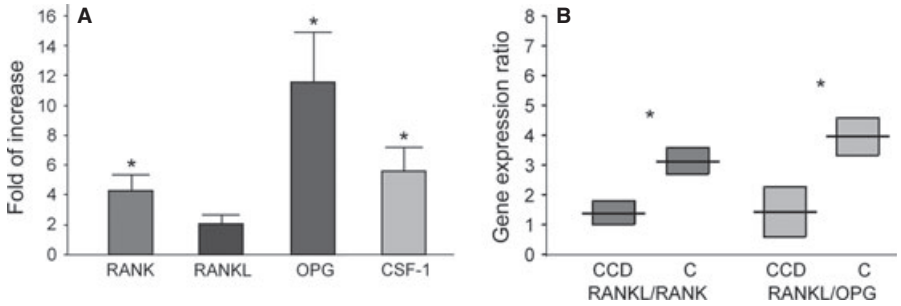

Fig. 2. (A) Gene expression of RANK, RANKL, OPG, and CSF-1 in the dental follicles of teeth in the intraosseous phase of eruption obtained from patients with CCD. The data are presented as fold increase of mRNA levels in patients relative to the control subjects. (B) Ratios for RANKL /RANK and RANKL/OPG gene expression (in the form of $2^{-\Delta Ct}$ values), in the dental follicles of CCD and Control (C) subjects. Values are presented as mean ± SE. *Statistically significant difference between CCD and Controls ($p \leq 0.05$).

controls. The ACTIN-normalized expression values per group and gene are illustrated in Fig. 2. Analysis with Mann–Whitney test detected significant alterations in gene expression in CCD individuals' dental follicles compared with controls (Fig. 2A). In patients with CCD, the mRNA levels of RANK, OPG, and CSF-1 were significantly higher than in the control group ($p \leq 0.05$). The increase in gene expression was 4.2 times for RANK, 11.7 times for OPG, and 5.6 times for CSF-1, respectively, in dental follicles of patients with CCD, compared with the controls. The difference observed in the expression of RANKL between CCD individuals and controls did not reach significance ($p \geq 0.5$) (Fig. 2A).

Statistically significant decreases were also witnessed in the mean ratios of RANKL/RANK and RANKL/OPG in patients with CCD compared with the controls ($p \leq 0.05$) (Fig. 2B) because of the observed overexpression of RANK and OPG genes.

Discussion

To our knowledge, this is the first study to analyze the expression of RANK, RANKL, OPG, and CSF-1 genes in dental follicles of human origin, derived from healthy individuals or subjects with disturbed tooth eruption. The present results revealed important differences in gene expression of key regulators of bone remodeling between patients with CCD and controls in the dental follicles of teeth at the intraosseous phase of eruption. The observed alterations in CCD individuals relative to normal subjects imply impaired osteoclastogenesis' signals and possibly alveolar bone remodeling. These findings support our hypothesis for disturbed eruption signaling originating from the dental follicle of subjects with CCD.

Animal studies have shown that RANK is required for osteoclast formation, because RANK knockout mice have osteoclast precursors but lack mature osteoclasts (20). Accordingly, the increased expression of RANK detected in subjects with CCD may represent a more efficient stimulus for osteoclastogenesis. However, in our study, the level of RANK's ligand (RANKL) was not raised proportionally in CCD individuals' compared with the controls, and as a result, the RANKL/RANK ratio was significantly decreased. Collectively, these findings are indicative of a disturbance in dental follicle signals required for osteoclastogenesis in patients with CCD.

In support to our assumption for impaired osteoclastogenesis, OPG expression in CCD individuals was considerably increased. OPG is a decoy receptor, blocking RANKL's actions in osteoclast formation. It has been proposed that a timely regulated reduction of OPG expression by CSF-1 in the rodent follicle is required for osteoclast formation during tooth eruption (14). A decrease in OPG thus creates a favorable ratio of RANKL/OPG, which is considered essential for osteoclastogenesis (1). In our study, OPG was overexpressed in CCD individuals, and consequently, RANKL/OPG was decreased against the favorable ratio detected in normal follicles. This situation could also lead to impaired osteoclastogenesis and eruption arrest. In a recent study, cultured periodontal ligament cells, from two

patients with CCD showed reduced capacity to induce differentiation of active osteoclasts because of an unfavorable RANKL/OPG mRNA ratio (21).

CSF-1 functions as a chemoattractant for monocytes (22); it promotes the up-regulation of RANK gene expression in osteoclast precursors (23) and is also required for osteoclast differentiation (24). Our results showing higher gene expression of both CSF-1 and RANK in the dental follicles of CCD individuals are in accordance with the previous *ex vivo* data, showing the expected sequel between these two factors during the initial phase of osteoclast differentiation, though at a higher level than the controls. At certain time points during eruption of the 1st molar in rodents, OPG levels in the respective dental follicle have been reported to be either down-regulated or basal as a result of the action of elevated CSF-1 (14). In our study, OPG expression in CCD individuals' dental follicles was up-regulated, along with higher CSF-1 expression, suggesting a possible disruption of CSF-1 signaling in the regulation of OPG levels. Although the majority of data about the molecular basis of tooth eruption is based on rodent molars, extrapolation of rodent data to human should be cautious concerning tooth eruption given the differences between diverse species (e.g., rodents have only one dentition). Our present data for CSF-1, RANK, and OPG indicate the existence of similar mechanisms that are disturbed in CCD, leading to impaired osteoclastogenesis.

RUNX2 haploinsufficiency may orchestrate the observed alterations in the dental follicles of subjects with CCD. Yoda et al. (25), using heterozygous RUNX2/Cbfa1 knockout mice to model CCD, reported suppressed maturation of osteoclasts in the alveolar bone during the critical timing of bone resorption. These findings support that the disturbed osteoclastogenesis is a putative cellular mechanism for the impaired tooth eruption of the permanent dentition in patients with CCD.

Furthermore, OPG was strongly expressed in a RUNX2 −/− calvaria-derived cell line, whereas RANKL showed minimal expression. Co-culture of these cells with normal bone marrow cells suppressed osteoclast differentiation of the latter. Adenoviral introduction of RUNX2 into above-mentioned deficient cells induced RANKL expression, suppressed OPG and restored osteoclast differentiation (12). Our results from human follicles are in accordance with the previous data from a rodent cell line. In the presence of RUNX2 haploinsufficiency, we detected over expression of OPG without significant increase in RANKL, in the dental follicle of patients with CCD. These changes may lead osteoclasts of the alveolar bone to differentiation arrest.

One limitation of this study is the small number of the patients with CCD. CCD is a rare condition, and therefore, only five subjects were analyzed which, however, were not relatives. Another limitation is that the dental follicles of the control group did not match exactly the same tooth in the CCDs, which is stemming from the use of human samples in research that do not permit the extraction of a tooth only for experimental reasons. The control dental follicles were collected solely from unerupted third molars that were in the intraosseous phase of eruption. In our opinion, this control group has allowed us to safely compare similar situations concerning the eruption process. Moreover, this is a comparative and not a causative study. Thus, the results cannot prove the actual cause of altered tooth eruption in CCD individuals, but still provide some insight in the completely unexplored field of tooth eruption in humans. Further studies are required to elucidate the inter-relationship between these factors and other potential osteo-inductive genes during tooth eruption in humans.

Conclusions

Overall, this study shows alterations in the expression of genes encoding regulators of osteoclastogenesis and bone remodeling in the dental follicles of human subjects suffering from cleidocranial dysplasia. This may be causatively linked to the deficient tooth eruption of these patients, highlighting the importance of a precisely regulated signaling for normal tooth eruption in humans.

Clinical Relevance

Cleidocranial dysplasia is an autosomal dominant condition characterized by supernumerary and late erupting teeth of the permanent dentition. Here, we demonstrate altered expression of certain genes, encoding regulators of osteoclastogenesis and bone remodeling in the dental follicles of individuals with cleidocranial dysplasia. The results of this work will help to elucidate some of the biological mechanisms involved in the intraosseous phase of eruption and their dysfunction in cleidocranial dysplasia. It is quite likely that this disorder arises from alterations in gene expression resulting in disturbed osteoclastogenesis or/and osteogenesis. Translation of our results in clinical practice could support the efforts for ultimate therapeutic approaches that may involve local delivery of certain gene products or specific inhibitors to promote tooth eruption.

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