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Requirement of alveolar bone formation for eruption of rat molars

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Abstract

Tooth eruption is a localized event that requires a dental follicle (DF) to regulate the resorption of alveolar bone to form an eruption pathway. During the intra-osseous phase of eruption, the tooth moves through this pathway. The mechanism or motive force that propels the tooth through this pathway is controversial but many studies have shown that alveolar bone growth at the base of the crypt occurs during eruption. To determine if this bone growth (osteogenesis) was causal, experiments were designed in which the expression of an osteogenic gene in the DF, bone morphogenetic protein-6 (BMP6), was inhibited by injection of the 1st mandibular molar of the rat with an siRNA targeted against BMP6. The injection was followed by electroporation to promote uptake of the siRNA. In 45 first molars injected, eruption either was delayed or completely inhibited (7 molars). In the impacted molars, an eruption pathway formed but bone growth at the base of the crypt was greatly reduced as compared to the erupted first molar controls. These studies show that alveolar bone growth at the base of the crypt is required for tooth eruption and that BMP6 may be an essential gene for promoting this growth.

Keywords

tooth eruption; alveolar bone formation; BMP6; siRNA; electroporation

It has been well established that teeth of limited eruption require the presence of a dental follicle (DF) which, in turn, regulates the osteoclastogenesis needed for formation of an eruption pathway in the alveolar bone (1, 2). Specifically, teeth do not erupt when the DF is surgically removed (3) and teeth do not erupt in osteopetrotic rats and mice devoid of osteoclasts (4–6). Reducing osteoclast activity by injection of a bisphosphonate such as pamidronate also delays tooth eruption (7). The molecular regulation by the DF for the osteoclastogenesis and subsequent bone resorption to form an eruption pathway has been detailed (1, 2, 8).

With the above in mind, what is required during the intra-osseous phase of eruption such that the tooth can move out of its alveolar bony socket? Work in our laboratory and others have suggested that alveolar bone growth (osteogenesis) at the base of the crypt lifts the tooth out of its bony socket; i.e., this bone growth is the motive force of the intra-osseous phase of eruption. For example, using scanning electron microscopy (SEM) we have shown that during the intra-osseous phase of eruption of the rat first mandibular molar, alveolar

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bone growth in the base of the crypt begins at day 3 postnatally and proceeds rapidly from day 9 onward until the 1st molar erupts (pierces the gingiva) at day 18 postnatally (9).

The requirement for the presence of the DF for this alveolar bone growth in eruption was first demonstrated in the dog by Marks and Cahill (10). They demonstrated that removal of the basal one-half of the dental follicle of the 4th mandibular premolar of the dog prior to the onset of eruption inhibited alveolar bone formation at the base of the crypt and resulted in no tooth eruption, despite the fact that an eruption pathway formed. With this in mind, we examined the expression of an osteogenic gene, bone morphogenetic protein-2 (BMP2), and determined that beginning at day 3 its gene expression was greater in the basal one-half of the DF than in the coronal one-half (11). In preliminary studies, we also have discovered that at least 7 osteogenic genes are expressed more in the basal one-half of the DF than in the coronal from days 3 through 11. In particular, bone morphogenetic protein-6 (BMP6) is dramatically increased in the basal one-half at day 11.

To determine if inhibition of gene expression of an osteogenic gene heavily expressed in the basal one-half of the DF might inhibit alveolar bone growth and subsequent tooth eruption, experiments were designed in which short interfering RNAs (siRNAs) targeted against BMP6 were injected into the DF followed by electroporation to enhance uptake of the siRNAs into the DF cells. The siRNAs are short, double-stranded molecules (20–25 nucleotides in length) that act to degrade the mRNA of a specific gene and thus, inhibit the effect of that gene. The effect of this inhibition of expression of BMP6 on tooth eruption and alveolar bone formation at the base of the crypt was then determined.

MATERIALS AND METHODS

In vivo expression of BMP6 in the dental follicle

To determine the chronological gene expression of BMP6 in the dental follicles (DFs), rat pups were sacrificed at postnatal days 1, 3, 5, 7, 9 and 11, and the DFs were surgically isolated from the 1st mandibular molars. Total RNA was extracted from the DFs and treated with DNase I. RNA concentration was measured with a spectrophotometer and total RNA of 3 µg was reverse transcribed into cDNA. Real-time PCR was conducted with BMP6 gene specific primers (forward: CTTACAG GAGCATCAGCAC AGA, reverse: GTCACCACCCACAGA TTGCTA) to determine the C_T values. Relative gene expression (RGE) was calculated with the Delta C_T method.

Laser capture microdissection (LCM) was used to collect the coronal and basal halves of the DFs from postnatal day 11 rat pups using procedures described in our previous publication (11). In brief, frozen sections were cut, fixed, and stained prior to regions of the DF being excised from the section using a PALM Laser Capture Microdissection system (P.A.L.M. Microlaser Technologies, Bernried, Germany). RNA was extracted using Arcturus PicoPure RNA isolation kit (MDS Analytical Technologies, Sunnyvale, CA, USA). RNA was quantitated with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). About 100 ng of RNA was reverse transcribed into cDNA and subsequent real-time PCR was conducted to determine RGE (basal vs. coronal). All of these experiments were repeated 3 times and all procedures involving animals were approved by the IACUC of Louisiana State University.

BMP6 siRNA for gene knockdown

The siRNAs for targeting BMP6 were purchased from Dharmacon (Boulder, CO, USA). The control siRNA was self-designed (sequence: UAAGGCUAUGAAGAGAUAC) and synthesized by Integrated DNA Technologies (city, state, USA).

Dental follicle cells were isolated and cultured using methods previously described (12). To determine the knockdown efficiency of BMP6 siRNA, dental follicle cells were transfected with BMP6 siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, lipofectamine was mixed with OPTI-MEM medium and siRNA, and then incubated at room temperature for 15 to 20 min for formation of the transfection complex. Next, the mixture was added into each well with the final concentration of siRNA at 50nM. Cells were collected to determine the knockdown efficiency after transfection.

Gene knockdown efficiency by BMP6 siRNA was determined by RT-PCR 48 h after transfection. To do that, total RNA from transfected cells was extracted and purified by Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA of 2 µg of each sample was reverse-transcribed into cDNA. The cDNA was used as template for conventional PCR to determine gene expression with gene specific primers; i.e., BMP6: forward CGACACCACAAG GAGT TCAA and reverse ACCTCGC TCACCTTGAAGAA; beta-actin: forward, GAGAGGGAAATC GTGCGTGAC and reverse, CATCTGCTGGAAG GTGGACA.

To assess the knockdown effect of BMP6 siRNA on BMP6 protein levels, cells were collected at 24 to 72 h after transfection, and total protein was extracted. The protein was quantitated with Pierce BCA (Thermo Scientific), and samples of 7.5 µg total protein each were electrophoresed on 10% SDS page gels. Next, the proteins on each gel were transferred to a PVDF membrane for hybridization with mouse anti-BMP6 or anti-β-actin antibodies at 4°C overnight. After washing 3× with PBS-T buffer, the membrane was hybridized with secondary antibody conjugated to HRP. After washing with PBS-T buffer, the membrane was developed using a chemiluminescence kit (Thermo Scientific) and the BMP6 and β-actin signals were captured with a ChemiDoc instrument (BioRad, Hercules, CA, USA).

***In vivo* injection and electroporation**

For *in vivo* transfection, siRNAs were first dissolved in 1X siRNA buffer and then diluted to 10 µM with nuclease free water plus saline. The rat pups then were anesthetized with Isoflurane prior to injection. Next, 20 µl siRNA solution was injected into the first mandibular molars of the rat pups at postnatal day 5 and day 7 or at day 7 and day 11 using a 31-G syringe with a stopper attached to the needle to control depth of injection of 1.8 mm, the optimal depth to reach the DF (13). For controls, first molars of pups either were injected with siRNA buffer plus saline or with scrambled siRNA. Immediately after injection, the given mandibles were clamped with forceps electrodes and the electroporation was carried out with four pulses at 120 V for 20 ms per pulse in 1 s intervals using a BTX ECM850 Electro Square Porator (Harvard Apparatus, Holliston, MA, USA) (13). The pups were evaluated for the eruption of the first mandibular molar twice daily from day 16 until the day that the molars erupted. Animals with impacted molars and comparable controls were sacrificed at various times after the controls had erupted, and the mandibles were surgically collected.

Histological analysis

The collected mandibles were fixed with neutral buffered formalin and then decalcified with Cal-EX (Fisher Scientific, Waltham, MA, USA) for 12 d before being embedded in paraffin. Tissues were cut longitudinally into 5 µm thick sections. The sections were mounted on glass slides and stained either with Masson's Trichrome or H&E before being examined by light microscopy. The thickness of the bone in the basal part of the crypt was measured with Olympus Qcolour-3 software (Olympus America Center, Valley, PA, USA) to compare the bone formation between the impacted molars and the erupted molars. In brief, for a given

age (day 20, 27 or 30), mandibles were longitudinally sectioned until the distal and mesial roots appeared. Then, the amount of alveolar bone under the roots was measured in 10 serial sections for both the impacted 1st molar and its erupted counterpart.

Statistical analysis

Three litters of rat pups were used to determine the RGE of BMP6 in different postnatal days. The experiments were conducted in a completely randomized block design with litters as blocks. Analysis of variance (ANOVA) and least significant difference (LSD) were used to compare RGE. Means were separated by LSD at $P \leq 0.05$ and a student T test was conducted to compare the RGE of BMP6 in the basal DF versus the coronal DF.

RESULTS

Chronological expression of BMP6 in the DF was analyzed using real-time RT-PCR (Fig. 1). A continuous increase of BMP6 expression was seen from postnatal days 1 to 11. On the average, its expression at day 11 was increased more than 6-fold compared to day 1 (Fig. 1). Further study of its expression in the coronal and basal one-half of the DF was conducted by coupling LCM and real-time RT-PCR. Real-time RT-PCR showed that BMP6 gene expression in the basal one-half of the DF was expressed some 8 fold greater than in the coronal half of the DF at day 11, a time of rapid alveolar bone growth in the base of the crypt. The difference was statistically significant ($P=0.022$). Conventional RT-PCR demonstrated that siRNA targeted against BMP6 inhibited the gene expression of BMP6 in the DF cells whereas the scrambled control did not (Fig. 2A). Western blotting showed that the knockdown effect on BVMP6 protein levels lasted for at least 72 h after transfection with the maximum knockdown seen at 48 h after transfection (Fig. 2B).

The effect of in vivo injection and electroporation of the siRNA targeted against BMP6 into the region of the DF in the first mandibular molar of the rat was examined in 45 molars. In 7 molars (15.5%), eruption was inhibited such that the tooth was impacted. Of the other 38, eruption was delayed by 0.8 d, a number that was statistically significant at $P \leq 0.01$ using a Student t-test. For all of the 7 first mandibular molars that were impacted, the eruption pathway formed (e.g., see Fig. 3) but the tooth did not erupt. Histological analyses of the impacted teeth showed a reduction in the amount of alveolar bone in the crypt at the base of the impacted first molar, be it in comparison to the adjacent second molar that erupted (Fig. 4A–C) or in comparison with the erupted first molar control on the contra-lateral side (Fig. 5A–D). In many instances, the alveolar bone in the bony crypt at the base of the erupted molar also appeared more compact than the comparable alveolar bone of the impacted molar (e.g., see Figure 5B). Measurements of a minimum of 10 sections of 3 different impacted 1st molars and their counterpart erupted 1st molars showed that at day 20 the depth of the alveolar bone at the base of the impacted molar was 0.44 ± 0.05 mm, whereas for the erupted 1st molar it was 1.22 ± 0.02 mm. For day 27, the impacted was 0.89 ± 0.11 mm, and the erupted was 1.34 ± 0.03 mm. For day 30, the impacted was 0.45 ± 0.05 mm, and the erupted was 1.56 ± 0.03 mm.

DISCUSSION

That alveolar bone formation at the base of the crypt might be involved in tooth eruption was suggested by the studies of CAHILL (14), in which he used transmandibular wires to temporarily impact the unerupted 4th premandibular molar of the dog. An eruption pathway formed above the tooth and when the wires were removed, the tooth erupted rapidly accompanied by a rapid growth of alveolar bone under the tooth at the base of the socket. Although not proof that this bone growth was causal, it was a strong correlation between bone growth and the intra-osseous phase of eruption. As mentioned in the Introduction, such

bone growth also is seen in eruption of the 1st mandibular molar of the rat (9). Moreover, the regulation of such alveolar bone growth is likely regulated by the basal one-half of the DF based on surgical studies in the dog in which either the basal or coronal one-half of the DF was removed (10) and by gene expression studies showing that an osteogenic gene such as BMP-2 is expressed more in the basal one-half of the DF than in the coronal half (11).

This study demonstrates that local injection of an siRNA targeted against BMP6 can inhibit alveolar bone formation and subsequent tooth eruption despite the fact that an eruption pathway is formed. That alveolar bone formation is reduced in the impacted teeth is seen in Figure 4 in which less alveolar bone is present at the base of the crypt of the impacted 1st mandibular molar as compared to the amount of bone under the erupted 2nd mandibular molar. This is particularly dramatic given that the 2nd molar erupts later (day 25) than the 1st molar (day 18) and thus the 2nd molar crypt started alveolar bone formation later than the first molar. Comparing the alveolar bone under the impacted first molar as compared to its companion erupted 1st molar also shows less bone under the impacted molar as compared to the erupted control (Fig. 5).

The reason that not every 1st molar is impacted by injection of siRNA targeted against BMP6 is because the small size of the tooth and surrounding DF makes it impossible to precisely hit the target (DF of 1st molar) every time. As we demonstrated in a previous study, in which we injected a plasmid with a lacZ reporter gene followed by electroporation and subsequent histology and X-Gal staining, success in transfecting the dental follicle ranged from about 43% to 86% for a given litter transfected (13). However, in that study many of the DFs transfected did not show staining in the basal one-half and thus, in this study, the numbers of DFs that received siRNA in the basal one-half likely is much less than 43%. Even with that, 7 out of 45 rats injected with siRNA against BMP 6 had the 1st molar impacted (15.5%). Precise injection of siRNA to the DF is important to achieve the effect on tooth eruption. We attempted an injection regimen at days 5 and 7 in one litter, but precise injection of rat pups at postnatal day 5 was difficult because of their smaller size. Considering that more rapid bone growth at the base of the tooth crypt begins on postnatal day 9 (9), the injection regimen of days 7 and 11 was more appropriate.

The impaction is not likely due to damage of the DF by electroporation because the controls also were electroporated. Furthermore, the eruption pathway formed normally, suggesting that no substantial damage to the DF by electroporation occurs. To eliminate the possibility of the off- target effect of the BMP6 siRNA, we screened the expression of other osteogenesis genes, including several BMPs, in the BMP6 siRNA transfected cell population containing dental follicle cells and dental follicle stem cells. The results indicate that the BMP6 siRNA is quite specific, and no significant off-target effect appears (data not shown). Thus, BMP6 appears to be a key molecule in eruption that is needed for promoting osteogenesis of the alveolar bone at the base of the tooth. BMP6 is a member of the TGF- β super-family and it has long been known to induce osteoprogenitor cells to differentiate into osteoblasts (15). It is present in a specific zone of the growth plate (16) and if its expression is inhibited such as in BMP6 null mice, growth plate function is impaired (17). Of particular interest is that BMP6 expression is up-regulated in human PDL under cyclic tensile strain (18). Given that the PDL is derived from the DF, the role of BMP6 in osteogenesis is consistent. In future studies, we will determine if other osteogenic genes that are expressed more in the basal one-half of the DF affect osteogenesis and eruption.

It should be noted that one early report in the literature indicated that *BMP6* knockout mice are viable, and the only skeletal defect seen was delayed ossification of the sternum (19). This is likely due to functional redundancy, common among the BMPs, in which one gene has substituted for another. In that vein, the genes for *BMP6*, *BMP5* and *BMP7* are 80 –90%

homologous in sequence (20, 21). Although mice null for each of these genes are viable (22), the double knockouts of *BMP5/BMP7* are not viable, suggesting that BMP5 and BMP7 can compensate for each other at early stages of embryonic development (23). The double knockouts of *BMP6/BMP5* display numerous skeletal defects (19). Moreover, in the developing mouse teeth, there is an extensive overlap of the BMPs in many of the tissues, suggesting a functional redundancy of the genes to insure that early development occurs (24).

Regarding the above, it is critical to note that our experiments inhibiting expression of *BMP6* are occurring postnatally (days 7 and 11), not during embryogenesis. Thus, it is likely that the redundant gene(s) that might replace the function of BMP6 in the DF have been permanently turned off and there would be no signal to upregulate them. Tooth eruption is a genetically programmed localized event directed by the DF (1) and inhibiting a gene in the program at the time it is to be expressed postnatally likely does not allow time for any reprogramming to occur. Regardless of the degree to which BMP6 regulates the alveolar bone growth, it is evident in this study that alveolar bone growth has been inhibited at the base of the crypt in the impacted molars after injection of siRNA targeted against BMP6. As a consequence, when this alveolar bone formation at the base of the crypt is inhibited, tooth eruption does not occur. Another requirement for eruption, a tooth eruption pathway, does form, indicating that the inhibition of eruption is indeed the reduced osteogenesis and not reduced osteoclastogenesis.

Finally, this osteogenesis needed for eruption is compatible with many other studies of eruption. For example, the PDL is not involved in pulling the tooth during the intra-osseous phase of eruption because it is not organized and attached to the alveolar bone until the tooth has erupted (9). Moreover, in the dog, an inert metal replica of a tooth can be substituted for the tooth and the replica will erupt (25). This latter study also eliminated vascular pressure changes in the pulp of the tooth as a motive force because there is no pulp. It also eliminated root growth as a force of eruption, which confirmed Gowgiel's findings that rootless teeth can erupt (26). Thus, we conclude that the motive force of tooth eruption during the intra-osseous phase appears to be alveolar bone formation at the base of the crypt and that this osteogenesis at the base of the crypt is required for tooth eruption.

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Figure 1.

In vivo expression of BMP6 in the dental follicles (DF) of the first mandibular molars of postnatal rats as determined by real-time RT-PCR. Chronologically increased expression of BMP6 was seen in the postnatal days examined. Different letters above the bars indicate that the means were significantly different at $P \leq 0.05$ in ANOVA and LSD tests. Bars that contain the same letter are not significantly different; e.g., Day 11 (A) and Day 9 (AB) are not significantly different.

Figure 2.

Gene knockdown efficiency of siRNA against BMP6 (BMP6-siRNA) was determined by conventional RT-PCR and Western blotting. (A) RT-PCR indicated that BMP6 gene expression in BMP6-siRNA transfected cells was reduced to an almost undetectable level at 48 h after transfection, whereas no notable reduction of BMP6 expression was seen in the cells transfected with scrambled siRNA as compared to the untreated controls. In addition, siRNA transfection appeared to have no effect on expression of the β -actin gene. (B) Western blotting analysis showed that the maximum knockdown effect on BMP6 protein was at 48 h after transfection, but the knockdown effect lasts at least 72 h after transfection.

Figure 3.

Transfection of DF with siRNA BMP6 resulted in tooth impaction. (A) Mandible showing the impacted and erupted 1st molars at day 30. Note that the eruption pathway still formed above the impacted molar. (B, C) Histology sections also show the eruption pathway above the impacted molar (B) and that the amount of alveolar bone at the base of the impacted molar was much less (B) as compared to the base of the erupted molar (C).

Figure 4.

(A) Comparison of the alveolar bone between the impacted 1st molar and the adjacent erupted 2nd molar at day 30 showing that the impacted 1st molar had less bone at its base than did the base of the erupted 2nd molar. (B & C) High power magnifications of the same area illustrates the differences in the quantity of the alveolar bone (AB) at the base of these molars; i.e., less bone under the 1st molar.

Figure 5.

Comparison of the impacted and erupted 1st molars at different postnatal days. A significant reduction of the amount of alveolar bone (AB) was seen at the base of the impacted 1st molars from pups of postnatal days 20 and 30 (A, C) vs. the large amount of bone seen at the base of the erupted 1st molars of the same age (B, D). Note that the alveolar bone (AB) at the base of the erupted molars appeared to be more compact than the bone in the comparable part of the impacted molars (A vs. B; C vs. D).