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AAO Foundation Final Report Form
(a/o 1/3/2018)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Please prepare a report that addresses the following:

Type of Award: Research Aid Award

Name(s) of Principal Investigator(s): Ginny Ching-Yun, Hsu

Institution: New York University

Title of Project:Differential regulation of PTH, PTHrP and abaloparatide on cementoblasts Collagen 1 expression.

Period of AAOF Support:07-01-17 to 06-30-18

Amount of Funding: 5000

Summary/Abstract

Parathyroid hormone plays an important role in maintains mineral homeostasis, in part by regulating calcium and phosphate absorption/reabsorption. Previously, clinical trial has shown PTH , PTHrP and abaloparatide can have different anabolic effect in osteoporosis patient , but the underlying mechanism remains unknown. Previously, we have shown that PTH, PTHrP and abaloparatide can differenctial regulate CREB and MAPK signaling pathway in osteoblast. Immortalized murine cementoblasts (OCCM.30), similar to osteoblasts and known to express collagen 1, were treated with intermittent PTH (1-34), PTHrP and abaloparatide 6 hours every 24 hours for 3 days. Western blot revealed that after intermittent treatment for 3 days, PTH showed an increase in collagen 1 right after treatment. In contrast, abaloparatide showed a latent effect in Collagen 1 expression. Immunofluorescence confirmed the same result from the western blot. This study reveal the differential anabolic effects of intermittent PTH, PTHrP and abaloparatide on cementoblasts which purpose abaloparatide may be a potential therapeutic approach for achieving improved cementogenesis.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR
2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be included.

The manuscript is in progress to be published. The manuscript is attached.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?

   The proposed research projects, resulting in one manuscript and one oral presentation. In addition, the data becoming the preliminary data in the Dr. Hsu's NIH R03 grant application.

2. Were the results published?
   a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
   b. Was AAOF support acknowledged?

      We acknowledged AAOF for the support in the manuscript.

   c. If not, are there plans to publish?
      Yes, the the menuscript is in progress to be published. The manuscript is attached.

3. Have the results of this proposal been presented?
   a. If so, list titles, author or co-authors of these presentation/s, year and locations

      Hsu C, Ricarte F, Partridge NC **Differential Signaling of PTH (1-34) from PTHrP (1-36) and Abaloparatide in Cementoblast**……Columbia University Center for Craniofacial Regeneration2018/5/28

   b. Was AAOF support acknowledged? Yes
   c.
4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

My long term goal is to become an independent clinician scientist. The has provide the opportunity and research training experience for Dr. Hsu to become an independent scientist at a dental school in an academic institution. The goals of the Research Aid Award is to support the research training and career development during orthodontic residency training in the laboratory. AAOF funding will prepare Dr. Hsu's development plan and the academic enhancement activities to be an independent, NIH-funded academic orthodontist who will contribute to the fields of craniofacial development and orthodontics.

Differential effects of PTH, PTHrP and abaloparatide on cementoblasts collagen 1 expression

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Abstract

Parathyroid hormone plays an important role in maintains mineral homeostasis, in part by regulating calcium and phosphate. Previously, clinical trial has shown PTH, PTHrP and abaloparatide can have different anabolic effects in osteoporosis patient, but the underlying mechanism remains unknown. We have shown that PTH, PTHrP and abaloparatide can differentially regulate CREB and MAPK signaling pathways in osteoblasts. Murine cementoblasts (OCCM.30), known to express collagen I, were treated with intermittent PTH (1-34), PTHrP and abaloparatide 6 hours every 24 hours for 3 days. Western blot revealed that after intermittent treatment for 3 days, PTH showed an increase in collagen I right after treatment. In contrast, abaloparatide showed a latent effect in increasing collagen 1 expression of 18 hours after treatment. Immunofluorescence confirmed the same result from the western blot. This study reveal the differential anabolic effects of intermittent PTH, PTHrP and abaloparatide on cementoblasts which purpose abaloparatide may be a potential

therapeutic approach for achieving improve cementogenesis.

Introduction

External root resorption is one of the most undesirable side effects in orthodontic treatment and is a pathological phenomenon that occurs when orthodontic force is generated along the root surface causing compressive stresses and leading to localized necrosis and hyalinization which is a condition in which normal tissue deteriorates into a homogeneous, translucent material; causing permanent root shortening. About 80% of patients undergoing orthodontic treatment develop some degree of apical external root resorption (1). Three percent of patients undergoing orthodontic treatment have severe root resorption (shortening by more than one-quarter of the root length) (2) which presents as root shortening in the maxillary central incisors. There are reports that show root resorption in adult patients is more obvious than in young patients (3). During orthodontic treatment, if root resorption is detected, treatment usually has to pause or be terminated because currently there is no treatment to prevent or reduce root resorption. Based on the different response of individuals to orthodontic forces, it is difficult for clinicians to predict the side effects other than by years of experience. Thus, having the ability to prevent and treat patients who have a tendency of root resorption and to establish good cementum health is critical.

Cementum, a specialized calcified outer layer of the root of a tooth, is the part of the periodontium that attaches the teeth to the alveolar bone by anchoring the periodontal ligament (4). Cementum is secreted by cementoblasts within the root of the tooth. If cementum is compromised by trauma or infection, it will affect periodontal conditions and cause root shortening, resorption of alveolar bone and root cavities. Cementum plays an important role in connecting the periodontal ligament (PDL) and tooth surface. Infected cementum from bacterial endotoxins and/or lacking appropriate formation due to metabolic disorders results in an inability of PDL and will compromise the periodontal condition

and healing. The organic matrix of cementum consists predominantly of collagens. In bovine cementum type I collagen accounts for more than 90% of the organic matrix and type III collagen approximately 5%. In human cementum type I collagen appears to be the only collagen type. The collagens form cross-striated fibrils in cementum and induce biological mineralization as a scaffold for the mineral crystals during mineralization and maintain the structural integrity of cementum after mineralization. (5) In the present study, we investigated the collagen I changes in cementoblasts (OCCM-30) after intermittent treatment of PTH, PTHrP and abaloparatide. These results in this study shown that PTH and its analog abaloparatide have different effects on cementogenesis in different fashion which provide the opportunity for evaluating the potential role of intermittent PTH and abaloparatide in the cementum regeneration therapies by increasing collagen 1. **Methods**

Cell culture and reagents

The immortalized mouse cementoblast cell line OCCM-30 was used in this study Briefly, OCCM-30 cells were cultured in Dulbeco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 1% penicillin G and streptomycin (Gibco, Grand Island, NY, USA). OCCM-30 cells were incubated in a humidified chamber (5%CO2/95% air) at 37 0C. Monolayer cells at 80% confluence were trypsinized and harvested for further study. PTH (1–34), PTHrP (1-36) and abaloparatide (Bachem, Torrance, CA, USA) was dissolved in 0.1% acetic acid according to the manufacturer's protocol. For intermittent PTH incubation, OCCM-30 cells were exposed to $1*10^{-8}$ PTH, PTHrP and abaloparatide for the first 6 h in each 24 h treatment session, while the vehicle culture medium of the control group also contained the same concentration of acetic acid. Then cells were all cultured in fresh medium during the remainder of the session. After 3 cycles of treatment, cells were collected and examined.

qPCR

Total RNA was extracted from OCCM-30 cells using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA as a template TaqMan® Reverse Transcription Reagents (Life Technologies, Inc.). SYBR® Green Master Mix was used for quantitative real-time RT-PCR using a real-time thermocycler machine (Realplex) manufacturer's instructions (Eppendorf, Hauppauge, NY). mRNA expression was calculated using a formula reported previously. The primer pairs were listed in Table 1.

Western blot analysis

Cells were rinsed with PBS and collected by centrifugation at 2000 rpm and 4°C for 5 min. Cell lysis was obtained by incubating in lysis containing RIPA and complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Equivalent amounts of protein (20μg) were subjected to SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat dried milk in TBST for 1 h and incubated with specific antibodies at 4 °C overnight. Proteins were detected using HRP-conjugated secondary antibody (goat-anti-rabbit) and visualized using an enhanced chemiluminescence kit (Millipore, Bedford, MA, USA). Results were captured and quantitated by ChemiDoc XRS+ software (Bio-Rad, Hercules, CA) after normalization to β-actin. For immunoblotting anti-COL1 antibody (Abcam, Cambridge, UK) were used at 1:2000 dilution

Alizarin red S staining and quantitative calcium assay

The Alizarin Red S assay was used to determine the degree of mineralization in intermittent PTH, PTHrP, abaloparatide and vehicle cultures. The mineralization solution contained ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM) (Sigma, St. Louis, MO, USA) and 2% fetal bovine serum (FBS) in DMEM. $1*10^{-8}$ PTH, PTHrP and abaloparatide was dissolved in 0.1% acetic acid in the mineralization solution, while the vehicle mineralization solution contained 0.1% acetic acid without

or PTH, PTHrP or abaloparatide. OCCM-30 cells were seeded in 6-well plates at a density of 1 *10^5 cells per well. They were exposed to PTH or vehicle mineralization solution for 3 cycles. Then cells were cultured in mineralization solution without acetic acid or PTH PTHrP or abaloparatide for 7 days. After 10 days of culture, OCCM-30 cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min for subsequent Alizarin Red S staining (2% (w/v) Alizarin red S (Sigma-Aldrich, St. Louis, MO, USA) solution; pH 4.2), which was conducted (2 ml/each well) for 5 min at room temperature. Following staining, the cells were rinsed with deionized water. For quantification, alizarin red stain was recovered in 10% acetic acid, heated to 85°C for 10 min and iced, and read in duplicate on a plate reader at 405 nm. All experiments were performed twice in triplicate.

Immunofluorescent staining

Cells were plated in BD Falcon™ chamber cell culture slides (Bedford, MA) and cultured as for TRAP and resorption assays in osteoclast medium for 9 days. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized in 0.1% Triton X-100 for 10 min. After blocking using Invitrogen BlockAid blocking solution (Carlsbad, CA) for 1 h, the cells were then incubated with the specific primary rabbit antibodies for type I collagen overnight at 4 °C. Abcam Alexa Fluor 594 goat-anti-rabbit IgG H + L (red) was used as secondary antibody for 1 h. All antibodies were purchased from Abcam (Cambridge, MA). DAPI was used to stain the cell nuclei (blue).

Statistical analysis

Data were presented as the mean ± standard error (SE). Statistical analyses were performed using ANOVA of factorial design (for factorial designed experiments) or one-way ANOVA (for three or more groups), or independent samples t test (between two groups). Values of $p < 0.05$ were defined as statistical significance. All statistical analysis was determined by using SPSS 13.0 (SPSS).

Result

Abaloparatide inhibits OCCM30 cells collagen 1 mRNA and protein expression.

We first investigated the expression of collagen 1 (COL1) mRNA, the majority of the organic part of cementum. After intermittent treatment, COL1 mRNA levels were significantly decrease in abaloparatide group treatment (* $p < 0.05$). (Figure 1).

OCCM30 cells increase collagen 1 protein level after withdrawing abaloparatide treatment for 18 hours

Next, we investigate the protein level of COL1 after intermittent treatment. Western blot analysis showed that after intermittent treatment, PTH significant increased and then followed by decrease of the expression of COL1 proteins(* $p < 0.05$). Interestingly, abaloparatide showed significant decrease(* $p < 0.05$) after treatment and followed by increase of COL1 after 18 hours after withdrawing the treatment. These results indicated that PTH and abaloparatide have differential effect on COL1 protein levels. Immunofluorescence staining of Col1 shows the same result as western blot. Collagen I expression increase after 3 cycles of intermittent treatment for PTH group. Collagen I showed decrease expression in abaloparatide group and increase after change to normal media for 18 hours.

Abaloparatide promotes cementoblast mineralization in OCCM-30 cells hours after intermittent treatment.

It was reported that PTH protected against periodontitis associated bone loss through the regulation of osteoblast activity (6). We examined the mineralization capacity of cementoblasts after intermittent PTH, PTHrP and abaloparatide treatment by Alizarin red S staining. 7 days after 3 cycles intermittent treatment, it was shown that abaloparatide enhanced the area of mineralized nodules compared to the control group. Abaloparatide showed the most effect (1.8 fold) of mineralization followed by PTHrP and 2.5 folds then PTH groups. These results suggested that intermittent abaloparatide could have a

better promotive role in cementoblast differentiation than PTH.

Discussion

Abaloparatide is the analog of PTHrP, has been used treating osteoporosis patient which has shown to have superior effect the PTH. PTH has long been used as an anabolic drug for osteoporosis. Our study revealed that intermittent abaloparatide has latent effect of increasing COL1 protein expression and have greater effect on promoting mineralization then PTH.

Type 1 collagen is the predominant type of protein that forms the extracellular matrix of bone. It determines the bending and compressive biomechanical properties of cortical bone, and is independent of bone mineral density. Collagens also play vital roles during organ development, wound healing and tissue repair through interacting with growth factors and cytokines. The fast changing in collagen expression on cementoblast might be because PTH stimulates the expression of matrix metalloproteinase-13 (MMP-13) and that MMP-13 plays a key role in bone remodeling, endochondral bone formation, Report has shown that PTH-induced down-regulation of miR-532-5p resulted in the stimulation of MMP-13 expression in rat osteoblasts (7).

Our study provides a way that abaloparatide can be a potent method to regulate mineral activities in cementoblasts. Taken together, we propose that intermittent abaloparatide has the potential to assist the prevention of tooth root resorption. However, several issues remain to be resolved before the application of intermittent abaloparatide to clinical root resorption treatment and the actions of abaloparatide in this complicated network have not yet been well established.

Conclusions

This study identified the potential of intermittent abaloparatide to promote cementogenesis. It was shown that intermittent abaloparatide treatment can enhanced the mineralization capacity of cementoblasts than PTH. Our data also indicated that intermittent abaloparatide improved the

expression of collagen I. Taken together, these findings suggest that intermittent abaloparatide can be therapeutically exploited to improve prognosis of tooth root resorption.

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