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PTHrP 1-141 and 1-86 Increase *In Vitro* Bone Formation

Blake Eason Hildreth III, D.V.M., M.S.,* Jillian L. Werbeck, B.S.,† Nandu K. Thudi, B.V.Sc., Ph.D.,† Xiyun Deng, Ph.D.,† Thomas J. Rosol, D.V.M., Ph.D.,† and Ramiro E. Toribio, D.V.M., M.S., Ph.D.*,[1]

**Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio; and †Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio*

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Background. **Parathyroid hormone-related protein (PTHrP) has anabolic effects in bone, which has led to the clinical use of N-terminal fragments of PTHrP and PTH. Since 10% to 20% of fractures demonstrate healing complications and osteoporosis continues to be a debilitating disease, the development of bone-forming agents is of utmost importance. Due to evidence that regions of PTHrP other than the N-terminus may have bone-forming effects, this study was designed to compare the effects of full-length PTHrP 1-141 to N-terminal PTHrP 1-86 on *in vitro* bone formation.**

Materials and Methods. **MC3T3-E1 pre-osteoblasts were treated once every 6 d for 36 d with 5, 25, and 50 pM of PTHrP 1-141 or 1-86 for 1 or 24 h. Cells were also treated after blocking the N-terminus, the nuclear localization sequence (NLS), and the C-terminus of PTHrP, individually and in combination. Area of mineralization, alkaline phosphatase (ALP), and osteocalcin (OCN) were measured.**

Results. **PTHrP 1-141 and 1-86 increased mineralization after 24-h treatments, but not 1-h. PTHrP 1-141 was more potent than 1-86. Treatment with PTHrP 1-141 for 24-h, but not 1-86, resulted in a concentration-dependent increase in ALP, with no effect after 1-h. Exposure to both peptides for 1- or 24-h induced a concentration-dependent increase in OCN, with 24-h exceeding 1-h. Antibody blocking revealed that the NLS and C-terminus are anabolic.**

Conclusions. **Both PTHrP 1-141 and 1-86 increased *in vitro* bone formation; however, PTHrP 1-141 was more effective. The NLS and C-terminus have anabolic effects distinct from the N-terminus. This demonstrates the advantage of PTHrP 1-141 as a skeletal anabolic agent.**

Key Words: **PTHrP 1-141; PTHrP 1-86; C-terminus; NLS; MC3T3-E1; mineralization; LCC15-MB.**

INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was first discovered as the principal endocrine factor in the development of humoral hypercalcemia of malignancy; however, it serves a vital role in many normal physiologic processes, including fetal bone development, cellular growth and differentiation, and mesenchymal-epithelial interactions [1–3]. The primary effects of PTHrP are attributed to its N-terminus, where it is equipotent with N-terminal PTH at the common PTH/PTHrP receptor (PTH1R) [4]. However, in contrast to PTH, PTHrP is not normally present in circulation, indicating that PTHrP acts primarily in an autocrine, paracrine, and intracrine role, rather than in an endocrine manner [5]. PTHrP is a prohormone capable of undergoing post-translational modification into several discrete peptides, each believed to act through its own specific receptor to elicit a spectrum of distinct, independent physiologic responses. In addition to the N-terminus, there is evidence that PTHrP has three other functional domains: (1) a mid-region, important for transplacental calcium transport, (2) a nuclear localization sequence (NLS), involved in nuclear transport with inhibition of apoptosis and promotion of cellular proliferation and differentiation, and (3) a C-terminus, historically associated with inhibiting osteoclastic bone resorption [6–9]. While the specific peptides and receptors of PTH are well-defined, less is known regarding the secretory fragments of PTHrP or their respective receptors.

[1] To whom correspondence and reprint requests should be addressed at Department of Veterinary Clinical Sciences, The Ohio State University, 601 Vernon L. Tharp Street, Columbus, OH 43210-1089. E-mail: toribio.1@osu.edu.

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Since the discovery of the anabolic effects of ancestrally-related PTH in bone, PTHrP has also received attention as a potential skeletal therapeutic agent [10]. The bone-forming effects of both peptides depend on the dose and pattern of administration, with the greatest increase in skeletal accretion generated by brief daily exposure, where at least initially, *de novo* bone formation is preferentially increased by actions on cells of the osteoblast lineage [10]. Due to the current limitations of cost and patient compliance associated with daily administration of PTH, alternative treatment regimens are being investigated, of which once-weekly administration has also proven effective [11]. In addition, recent studies have indicated that the other functional domains of PTHrP may have anabolic effects in bone distinct from, or that contribute to, those elicited by the N-terminus [12, 13]. This is of particular interest since it has been suggested that there is no difference in the biological effects of full-length PTH and PTHrP in osteoblasts compared with their equipotent N-terminal fragments [4, 14]. However, the use of full-length PTHrP 1-141 as a skeletal anabolic agent may be advantageous since (1) cells of the osteoblast lineage not only express PTH1R but may secrete PTHrP, establishing the importance of PTHrP in the commitment of osteoprogenitor cells, skeletal patterning, and the maintenance of skeletal integrity, (2) PTHrP may bind alternative receptors for regions of the peptide other than the N-terminus in addition to being the natural ligand for PTH1R, and (3) PTHrP may be both more stable and anabolic than PTH 1-34 or 1-84 and PTHrP 1-36 or 1-86 [15–18]. Despite these potential advantages, few studies have investigated the anabolic effects of full-length PTHrP 1-141 due to its lack of commercial availability and expense.

Since approximately 10% to 20% of fractures demonstrate healing complications and osteoporosis continues to be a debilitating disease, the development of locally-delivered or systemic bone-forming agents is of utmost importance [19]. Therefore, due to the potential advantages of full-length PTHrP 1-141 as a skeletal anabolic agent, the purpose of this study was to compare the effects of full-length PTHrP 1-141 and N-terminal PTHrP 1-86 on *in vitro* bone formation. Here we show that both PTHrP 1-141 and 1-86 increased *in vitro* bone formation. However, PTHrP 1-141 was more potent due to the presence of the NLS and C-terminus, which possess complimentary anabolic effects to the N-terminal domain of PTHrP.

MATERIALS AND METHODS

Transfection of the LCC15-MB Cell Line

The HARA epithelial cell line, which is derived from a human lung squamous cell carcinoma, served as a source of PTHrP 1-141 mRNA (a generous gift from Dr. H. Iguchi, National Kyushu Cancer Center, Fukuoka, Japan) [20]. Total RNA was extracted and full-length (–36 ~ +141) cDNA amplified by RT-PCR. Primers were 5′-CTA TAG GCT AGC GAG ACG ATG CAG CGG AGA-3′ (forward) and 5′-CTA TAG CTC GAG TCA ATG CCT CCG TGA ATC-3′ (reverse), with underlined sequences representing NheI and XhoI restriction sites. Sequences were cloned into a pcDNA3.1 (+) expression vector (Invitrogen Corp., Carlsbad, CA). Both the recombinant plasmid and the empty vector were stably transfected into human LCC15-MB cells [21], which secrete negligible PTHrP 1-141, to generate the LCC15-PTHrP 1-141 and LCC15-vector cell lines, respectively. Stable transfectants were selected with 1 mg/mL G418 sulfate for 3 weeks and maintained in Dulbecco's modified Eagle's media (DMEM)/F-12, 10% fetal bovine serum (FBS), and 400 μg/mL G418 sulfate at 37°C and 5% CO_2 (Invitrogen). The presence of both expression vectors was confirmed by real-time PCR, sequencing, and Western blot analysis.

Conditioned Media Preparation and PTHrP 1-141 Quantification

The LCC15-PTHrP 1-141 and LCC15-vector cell lines were plated in T-75 flasks (BD Falcon; BD Biosciences, Bedford, MA) and maintained in DMEM/F-12, 10% FBS, 250 U/mL penicillin, 250 μg/mL streptomycin, and 2 mM L-glutamine (standard growth media) at 37°C and 5% CO_2. At confluence, media was aspirated, cells were washed with phosphate-buffered saline (PBS, Invitrogen), and serum-free media applied. After 48 h, conditioned media (CM) was aspirated, centrifuged at 290 *g* for 6 min at 4°C to remove cellular debris, supernatant divided into aliquots, and LCC15-PTHrP 1-141 CM (now referred to as PTHrP 1-141) and LCC15-vector CM (now referred to as vector) frozen at –80°C. The concentrations of PTHrP 1-141 in 5 batches of each CM were measured with a two-site immunoradiometric assay (IRMA) (DSL-8100; DSL, Inc., Webster, TX). The IRMA uses antibodies specific for the N-terminus (amino acids 1–40) and mid-region (amino acids 57–80) of PTHrP. The lowest PTHrP concentration detectable by the assay was 0.30 pM.

Cell Viability Assays and Conditioned Media Protein Quantification

To determine if transfection with PTHrP 1-141 resulted in differences between CM other than PTHrP 1-141, we compared the viability of LCC15-PTHrP 1-141 and LCC15-vector cells and protein concentrations in their respective CM. Both cell lines were plated in triplicate at an initial density of 2500 cells per well in a total volume of 100 μL DMEM/F-12, 250 U/mL penicillin, 250 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen) in a 96-well microplate and maintained at 37°C and 5% CO_2. After 24, 48, and 72 h, 15 μL of MTT dye solution was applied and incubated for 4 h (CellTiter 96 non-radioactive cell proliferation assay; Promega Corp., Madison, WI). The reaction was terminated by incubation with 100 μL of stop solution for 1 h and the absorbance of formazan measured at 570 nm in a plate reader (SOFTmax PRO ver. 3.1; Molecular Devices Corp., Sunnyvale, CA). Protein concentrations in five batches of PTHrP 1-141 and vector were measured using a colorimetric assay (Bio-Rad protein assay; Bio-Rad Laboratories, Inc., Hercules, CA). In a 96-well microplate, 200 μL of dye reagent and 1 μL of CM were combined and the absorbance measured at 595 nm.

Western Blot Analysis

LCC15-PTHrP 1-141 and LCC15-vector cell lines were plated in a 6-well plate (Costar, Corning Inc., Corning, NY) and grown until confluent. Prior to harvest, media was removed, and cells were treated with either DMEM/F-12 standard growth media or standard growth media containing 1 μL/mL of a Golgi blocker to block PTHrP secretion (Golgi Plug, BD Biosciences, Franklin Lakes, NJ). Cells were treated for 5 h at 37°C, washed once with PBS and lysed in SDS lysis buffer (62.5 mM