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Development of PTH Eluting Microspheres for the Treatment of Hypoparathyroidism

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Background. **Parathyroid hormone (PTH) replacement has been demonstrated to be superior to conventional treatment with calcium supplementation and vitamin D analogs for the treatment of hypoparathyroidism. In this investigation we evaluated the feasibility of using PTH microsphere encapsulation as a potential delivery system for PTH.**

Materials and methods. **Using the spontaneous emulsion technique, PTH microspheres were created by encapsulating PTH (1–34) in a copolymer of polyglycolic and polylactic acid (PLGA). Additional microspheres were constructed by coencapsulating calmodulin with PTH (1–34) in the PLGA microspheres. Microsphere production was confirmed using electron microscopy. PTH release was measured *in vitro* using an enzyme-linked immunosorbent assay. The bioactivity of PTH released from the microspheres was confirmed *in vivo* using a hypoparathyroid rat model by measuring serum calcium concentrations before and 3 h after subcutaneous injection of PTH microspheres.**

Results. **PTH microsphere and PTH/calmodulin microspheres could be created using the spontaneous emulsion technique. Physiologically significant PTH release was measured *in vitro* for 20 days. PTH release was calcium sensitive and exhibited negative feedback. This effect was augmented by coencapsulation with calmodulin. PTH released from the microspheres caused a significant rise in serum calcium levels from an average of 6.35 (6.19–6.48 mg/dL) to 8.55 mg/dL (8.22–8.73). PTH released from the PTH/calmodulin microspheres resulted in an increase in serum calcium from a mean of 6.8 (6.7–6.9 mg/dL) to 8.1 mg/dL (7.8–8.2).**

Conclusions. **The PLGA microspheres can be used to provide calcium sensitive controlled release of biologically active PTH and offer a potential mean of providing biomimetic hormone replacement therapy.**

Key Words: **parathyroid hormone (PTH); microspheres; drug delivery; negative feedback.**

INTRODUCTION

Hypoparathyroidism is a serious metabolic disorder. The most common cause of hypoparathyroidism is iatrogenic injury to the gland during thyroid or parathyroid surgery. Estimates of transient and permanent hypoparathyroidism after surgery range between 6.9 to 46% and 0.4 to 33%, respectively [1–5]. Unlike other hormone insufficiency states, the conventional treatment of hypoparathyroidism does not involve hormone replacement. Rather, conventional treatment for hypoparathyroidism involves the use of calcium supplementation and vitamin D analogs. parathyroid hormone (PTH) replacement therapy has been demonstrated to be superior to conventional therapy for the treatment of hypoparathyroidism [6, 7]. Unfortunately, daily or twice daily subcutaneous injections of PTH are required for effective treatment [7, 8].

The purpose of this study was to explore the use of controlled release technology to deliver PTH for the purpose of maintaining calcium homeostasis without the need for daily subcutaneous injections. Controlled release technology enables the delivery of a biologically active agent at specific times with defined concentrations by encapsulating the biologically active agent within a biodegradable polymer in the form of a microsphere. In euparathyroid individuals PTH release occurs in response to serum calcium levels via a negative feedback mechanism; as serum calcium decreases, PTH release increases and *vice versa*. Using controlled

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release microsphere technology, we developed a rudimentary, calcium-sensitive, negative feedback PTH delivery system by co-encapsulating PTH with calmodulin in poly(lactic-co-glycolic) acid (PLGA) microspheres and characterized its function both *in vitro* and *in vivo*.

MATERIALS AND METHODS

To explore the use of controlled release technology to deliver PTH, PTH (1-34) was incorporated into PLGA microspheres that were studied *in vitro* and *in vivo*. *In vitro* studies were designed to demonstrate release of PTH from these particles, while *in vivo* studies were designed to show a physiological effect thereby confirming the bioactivity of the PTH released from the microspheres.

PTH Microsphere Production and Characterization

PTH Fluorescent Labeling

The PTH was fluorescently labeled to enable detection and quantification of PTH released from the microspheres. PTH (1-34) was conjugated with fluorescein isothiocyanate (FITC) by reacting 1 mg of PTH (2.47×10^{-7} mol) with 50 times molar excess (1.23×10^{-5} mol) of FITC for 1 h at 25°C in 300 μL of sodium borate and 200 μL of phosphate-buffered saline (PBS). Conjugation of the FITC-NHS to PTH was confirmed by size exclusion high performance liquid chromatography.

Microsphere Fabrication

The spontaneous emulsion technique was used to make two batches of PLGA microspheres. One batch contained PTH conjugated to FITC and calmodulin (PTH/Cal) and another batch contained PTH conjugated with only FITC (FITC-PTH). One milliliter of dichloromethane and 4 mL of trifluoroethanol were used to dissolve 200 mg of solid PLGA (50/50). To make (PTH/cal) microspheres 250 μg Calmodulin (in 100 μL PBS) and 25 ug FITC-PTH (in 100 μL solution) was added to the polymer solution, giving a 10:1 by weight CaM:PTH ratio. To make FITC-PTH microspheres 25 μg of FITC-conjugated PTH was added in 100-μL solution. The protein solutions were added together with the surfactant, then immediately pipetted into the polymer solution. The mixture was then added slowly to 100 mL of chilled 5% (in water) poly-(vinyl alcohol) solution in a 400 mL beaker and stirred for 4 h at medium speed [5, 6] on a stir plate at 25°C. After stirring, the solution was spun down in a centrifuge at 4500 rpm for 5 min and the supernatant was removed and discarded. The microspheres were then resuspended in water and centrifuged again; this wash process was repeated a total of three times. After the final wash, the microspheres were resuspended in minimum amount of water, frozen at −70°C for 30 min, and lyophilized for 2 days.

Microsphere Characterization

To confirm microsphere production, the lyophilized PTH microspheres were imaged using scanning electron microscopy. The microspheres were fixed on an aluminum stub using two-sided carbon tape and then coated with a gold/palladium mixture (60:40) using a sputter coater. The samples were imaged via electron microscopy with the acquired imaged analyzed using NIH image (available from zippy.nimh.nih.gov) to determine microsphere diameter. Average particle diameter and size distributions were calculated.

In Vitro Studies of PTH Release From Microspheres

In Vitro *Release of PTH From PTH Microspheres in PBS Without Calcium*

Ten milligrams of the PTH-loaded microspheres were suspended in 1 mL of PBS. The eppendorf tubes were incubated at 37°C on a rotary shaker. At various times points the tubes were removed, centrifuged, and the supernatant was removed and stored at −80°C for later determination of protein content. After removal of the supernatant a fresh 1 mL aliquot of additional PBS was added to the microspheres for further incubation at 37°C. After collecting the samples, the concentration of PTH (1-34) in the release medium was measured in triplicate using a fluorimeter to detect the presence of FITC. Release studies were carried out in triplicate.

In Vitro *Release Profile of PTH/Cal and FITC-PTH Microspheres in PBS With Variable Calcium Concentrations*

To determine the release profile of PTH, 5 mg of PTH/Cal and FITC-PTH microspheres were suspended in two buffer solutions created using 20 mM Tris-HCl and either 1 mM $CaCl_2$ or 2 mM $CaCl_2$. One set was suspended in a solution of 1 mM Ca^{2+}, and the other in a 2 mM Ca^{2+} solution. One milliliter of buffer was added to each tube. The tubes were placed in a Lab-Line Environ Shaker at 37°C. Measurements of PTH release were taken by centrifuging the eppendorf tubes and removing the supernatant to measure FITC presence with the fluorimeter. The microspheres were resuspended in buffer and placed back in the shaker. Measurements were taken at 2, 4, 6, 21, 24, 48, and 72 h. At 21 h, the buffer for each set was switched to the other calcium concentration to determine if the release profile followed the change in calcium concentration. All release studies were carried out in triplicate.

In Vivo Characterization of PTH Microspheres

Hypoparathyroid Animal Model

All animal studies were approved by the Institutional Animal Care and Use Committee at Yale University. The male Sprague-Dawley rats (Charles Rivers Labs, Portage, MI) (175-225 g body weight) underwent subtotal thyroidectomy and total parathyroidectomy under general anesthesia with isoflourane. Rats were fed rat chow (Harlan Teklad Global 2018, 1.01% calcium and 0.65% phosphorous) and given water *ad libitum*. They were housed at the Yale Animal Resource Center in accordance with the institutional policies. Serum calcium and PTH (1-84) levels were measured before the surgery and 7 days post-operatively to confirm their hypoparathyroid status. Serum PTH (1-84) levels were measured using rat intact PTH enzyme-linked immunosorbent assay kit (Immutopics, Inc., San Clemente, CA). Total serum calcium concentration was measured using a standard O-Cresolphthalein. Complexone colorimetric assay (Biotron Diagnostics, Hemet, CA). All measurements were performed three times and averaged.

Subcutaneous Implantation of Microspheres

To demonstrate the physiological effect of the PTH microspheres and thereby confirm the bioactivity of the PTH released from the microspheres, PTH microspheres were subcutaneously injected into hypoparathyroid rats and serum calcium levels were measured. Five hypoparathyroid rats were injected subcutaneously with 100 mg/kg of FITC-PTH containing microspheres in 1 mL of PBS with 0.5% tween 80. Rat blood was collected 1 day prior, immediately before the injection of microspheres, and 3 h post-injection. Three hypoparathyroid rats were injected subcutaneously with 100 mg/kg of PTH/Cal microspheres and serum calcium was sampled every 30 min for 3 h post-injection. All rats were sacrificed at the end of the study.