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PTH has the potential to rescue disturbed bone growth in achondroplasia

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Abstract

Introduction: Achondroplasia (Ach), the most common form of short-limb short stature, and related disorders are caused by constitutively active point mutations in the fibroblast growth factor receptor 3 (FGFR3) gene. Recent studies have provided a large body of evidence for the role of the proliferation and differentiation of chondrocytes in these disorders. Furthermore, a G380R mutation in FGFR3 ($FGFR3^{Ach}$), which results in achondroplasia, induces apoptosis in the chondrogenic cell line ATDC5. This is associated with a decrease in the expression of PTHrP, which shares the same receptor with PTH, and it is significant that PTHrP rescues these cells from apoptosis.
Methods: Fetuses derived from transgenic mice expressing $FGFR3^{Ach}$ under the control of the type II collagen promoter (AchTG) or from wild-type mice were obtained on the 15th day of pregnancy. The femurs were collected from these specimens and cultured for 4 days with PTH. The effects of PTH treatment were then determined by morphometric and histological analyses, *in situ* hybridization of type X collagen mRNA, and the TUNEL assay.
Results: AchTG femurs showed suppressed growth compared with wild type (0.29 ± 0.10 mm vs. 0.46 ± 0.06 mm, respectively; $p < 0.05$), particularly in cartilage. PTH treatments improved the growth velocity in the femurs of the AchTG (0.50 ± 0.06 mm; $p < 0.01$ vs. control). This was associated with the inhibition of both differentiation and apoptosis in chondrocytes.
Conclusions: Our data suggest that PTH inhibits differentiation and apoptosis in chondrocytes and improves bone growth. These effects thus counterbalance the effects of FGFR3 mutations. PTH therefore is a potential therapeutic agent for achondroplasia.

Keywords: Achondroplasia; Fibroblast growth factor receptor 3; Parathyroid hormone; Apoptosis; Chondrocytes

Introduction

Fibroblast growth factor receptor 3 (FGFR3) has been identified as a critical regulator of enchondral bone growth [8]. Mutations in the coding sequence of the *FGFR3* gene have also been identified as the cause of achondroplasia (Ach) [17,20], thanatophoric dysplasia (TD) [23,24], and related disorders [4,5,22,25]. Ach is the most common form of genetic short stature and exhibits characteristic phenotypes of rhizomeric short limbs, relative macrocephaly, and exaggerated lumbar lordosis. TD, a common but more severe and lethal skeletal dysplasia during the neonatal period, consists of two phenotypes distinguishable by radiological and other clinical criteria and referred to as TD types I and II (TDI and TDII) [24].

FGFR3-deficient mice exhibit skeletal overgrowth, suggesting that FGFR3 is a negative regulator of long bone growth during enchondral formation [7,9]. Several *in vitro* studies have also demonstrated that mutations in *FGFR3*, which are responsible for Ach and TD, cause ligand-independent activation of the receptor [27,28]. The effects of activated FGFR3 signals on bone growth in Ach have been studied recently *in vivo* using transgenic [14] and cDNA knock-in [6,26] models. Mutations in *FGFR3* were found to cause short stature and to result in features that mimicked human Ach. These experiments also demonstrated that a gain of FGFR3 function causes Ach, leading to the inhibition of chondrocyte proliferation.

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In the growth plate, parathyroid hormone (PTH)-related peptide (PTHrP) is expressed in the perichondrium and in proliferating chondrocytes, whereas its receptor is found primarily in the proximal prehypertrophic layer [12]. Mice in which the *pthrp* gene is disrupted die immediately after birth and show skeletal dysplasia and short limbs due to accelerated chondrocyte differentiation [10,13]. However, although transgenic mice that overexpress *pthrp* in proliferative chondrocytes also have shorter and thicker limbs, this is a result of decelerated terminal chondrocyte differentiation and delayed mineralization [2,29].

In a recent study, we showed that both Ach- and TDII-type mutant FGFR3 induce apoptotic changes in ATDC5 cells, a mouse chondrogenic cell line [11]. We also found that the expression of *pthrp* was markedly suppressed in ATDC5 cells expressing Ach or TDII mutant FGFR3, and that treatment with PTHrP, which functions in chondrocyte differentiation through the PTH/PTHrP receptor, blocks the apoptotic response in these cells [30]. These findings indicate that the acceleration of FGFR3 signaling, caused by these ACH- or TDII-inducing mutations, induces apoptosis in chondrocytes via the suppression of PTHrP [31].

In the present study, we evaluate the effects of PTH, which shares the same receptor with PTHrP and has been available for clinical usage, upon bone growth suppression caused by mutant FGFR3 in mouse organ cultures.

Materials and methods

Organ cultures

The generation of the Achondroplasia mouse model (AchTG), which expresses activated FGFR3 in the growth plate under the control of *col2a1* promoter and enhancer sequences, has been described previously [14]. To obtain fetuses from AchTG and wild-type mice (WT), WT female mice were mated with AchTG male mice. On day 15 of pregnancy, the bilateral femurs were dissected aseptically from the fetuses. Fetuses whose femurs were significantly different from the average expected size were discarded. The femurs were maintained in an intact state, and care was taken not to damage the perichondrium. Organ cultures of the femurs were then established using a previously described suspension culture technique [32] in a chemically defined medium (Biggers' BGJb medium, Life Technologies, Rockville, MD, USA) containing 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich). No serum was added to the medium, and four to six explants were incubated in each bottle. Each bottle contained 10 ml culture medium and was sealed airtight with a rubber stopper and metal clamp. The bottles were then flushed for 2 min with a gas mixture of 95% atmosphere and 5% CO_2 using a 24-gauge syringe needle. A second needle was used to balance the gas pressure in the bottles, which were incubated at 37 °C on a roller device (Taitec Co., Saitama, Japan) at 20–25 rpm. The culture bottles were flushed every 24 h with the same gas mixture. On the second day of organ culture, the medium was replaced. These fetal mouse femur cultures were maintained for 4 days with or without 1.0×10^{-8} M recombinant human PTH (1–34) (rhPTH) (Eli Lilly and Company, Indianapolis, IN, USA).

Morphometric analysis

Before and after the 4-day culture, the femurs were measured using a linear ocular scale and mounted on a dissecting microscope at 25× magnification. The distance between the head and inner malleolus of each femur was measured as the total length, and the longitudinal lengths of the proximal cartilage (PC), distal cartilage (DC), and calcified bone (CB) were also determined by measuring the length of the developing light zone of chondrocytes as well as that of the dark zone of calcification (Fig. 1A).

Tissue preparation

The fetal mouse femurs were fixed with 4% PFA after the lengths had been measured. These specimens were then embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Sections 10 μm thick were cut from the embedded specimens with a microtome, collected on silan-coated slide glasses, and stained with hematoxylin/eosin. For the *in situ* hybridization and TUNEL assays, specimens were embedded in OCT compound (Miles Laboratories, Elkhart, IN, USA), and then snap-frozen with liquid nitrogen. Sections 10 μm thick were cut from the OCT compound-embedded specimens with a cryostat and collected on poly-L-lysine-coated slide glasses.

In situ hybridization

Digoxigenin (DIG)-labeled single-stranded RNA probes were prepared with a DIG Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. A mouse *col10a1* cDNA fragment

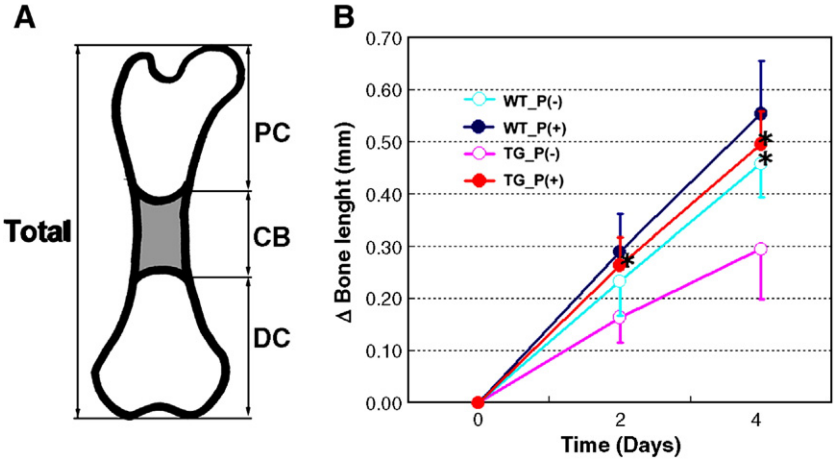

Fig. 1. Organ cultures of fetal mouse femurs. (A) Schematic representation of a cultured fetal mouse femur. Total: total length. PC: proximal cartilage portion. CB: calcified bone. DC: distal cartilage portion. (B) Time course of the elongation of the total length of the femurs during a 4-day culture. P(+/−): with or without rhPTH. ∗: $p < 0.05$ vs. TG P(−).