Development of Novel Therapy for Achondroplasia: Use of Parathyroid Hormone

Yoshitaka Yamanaka¹, Hiroyuki Tanaka¹, Daisuke Harada¹, Koso Ueda¹ and Yoshiki Seino²
¹Okayama University, Okayama, ²Osaka Kosei Nenkin Hospital, Osaka, Japan

Abstract. Achondroplasia (ACHs) and thanatophoric dysplasia (TD) are representative diseases of micromelic short stature that are elicited by a constitutively activated FGFR3 caused by point-mutation in the receptor molecule. Based on the mutated point in the receptor, there are several types of diseases of which the severity varies. In the chondrogenic cell line (ATDC5), the signaling cascade in the fibroblast growth factor 3 (FGFR3) was silenced by an overexpression of the mutated FGFR3 that resulted in decreased expression of the mRNA of the parathyroid hormone-related peptide (PTHrP); and also apoptosis was induced. The expression levels of PTHrP were inversely correlated with the severity of the disease; and the replacement of PTHrP prevented the apoptotic changes in the cell lines. In the following studies, however, we selected rhPTH that possesses a high homology and had been clinically used in the treatment of osteoporosis. In the organ culture, rhPTH remarkably elongated the proximal and distal cartilages of the ACH transgenic mice (ACHtg) and expressed collagen X similarly to the wild mice. An in vivo study of rhPTH significantly increased the growth of the long bones of ACHtg in comparison with those of the control (saline). The future prospects of parathyroid hormone (PTH) therapy of ACH are to be discussed.

Key words: achondroplasia, fibroblast growth factor receptor 3 (FGFR3), growth plate, parathyroid hormone related protein (PTHrP), parathyroid hormone (PTH)
calcification into the matrix of cartilage secreting the follicular vesicles. After migrating through these 3 zones, the fully differentiated chondrocytes are substituted by the bone tissues; thus, the bones are elongated.

**Achondroplasias and FGFR3 Mutation**

The molecule of FGFR3 is comprised of 6 domains: 3 immunoglobuline-like extracellular domains (each possesses S-S binding), one transmembrane domain, and 2 of paired tyrosine kinase intracellular domains. When the point mutation occurs in the transmembrane domain (G380R), ACH is elicited. And in the patients with TDI and TDII, the mutations are seen in the extracellular domain close to the transmembrane domain (Y373C) and in the intracellular 2nd tyrosine kinase (TK2) domain (K650E), respectively. On the other hand, in the patients with hypochondroplasia (HCH), a mild type of achondroplasia, and also in the patients with, the so-called severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), a severe type, the point mutations (K650N and K650M, respectively) are also seen in the TK2 domain. The difference in the severity of the phenotypes is considered to depend upon the codons (Fig. 1). Although the SADDAN is the severest type of achondroplasia, it is also known that those patients can survive throughout their lives if the appropriate care including respiration care is given.

At first, we prepared the mutants of FGFR3, in which the mutations were induced in the codons related to TDI and ACH in the extracellular and transmembrane regions as well as in the intracellular 2nd tyrosine kinase domain that is related to HCH, TDII and SADDAN. After transfecting the vectors of these mutants into the wild type cells, the protein expressions were observed by means of the immunoblot. When 2 kinds of proteins, immatured and matured, are expressed in this study, the latter and the former appear as the bottom band and as the upper band, respectively. And when the mutations in the extracellular (TDI) or transmembrane (ACH) domain are transfected, 2 bands always appeared just as with the wild types, while with the transfection of the mutations in the intracellular TK2 domain (HCH, TDII and SADDAN), only the immature protein was expressed.

Then, we explored the localization of these proteins by means of the FGFR3 immunofluorescent staining. As a result, it was clarified that in the case of TDI and ACH, in those, the mutations had occurred in the extracellular or transmembrane domains; and the FGFR3 was localized on the cell membrane just as in the wild type. On the other hand, in the case of HCH, TDII, and SADDAN, in those, the mutations occurred in the intracellular domains; and FGFR3 was not expressed on the membrane but in the endoplasmic reticulum (ER).
**Signaling Cascade in FGFR3**

Figure 2 summarizes the intracellular signaling cascade of FGFR3, where the roles of tyrosine are especially important for the signaling. From these functions of the tyrosine, we have selected the three major pathways, autophosphorylation, Stat 1 activation, and PKC (protein kinase C) activation by PLCγ (phospholipase Cγ) for our investigation.

Since the autophosphorylation of FGFR3 is the pathway localized on the membrane, the mutations of this pathway exist on the membrane or outside of it. Actually, the basal phosphorylations of the FGFR3 are high in the patients with TDI and ACH. In TDI patients, the autophosphorylation has already been enhanced when the FGF1, the ligand of FGFR3, is applied; on the contrary, in ACH patients, the dephosphorylation seems to be delayed. On the other hand, the diseases such as HCH, TDII, and SADDAN in which the abnormalities are localized in the intracellular domain (TK2) of the FGFR3, or in the endoplasmic reticulum, the phosphorylation activities are increased along with the severity of the disease. The phosphorylation activities were ligand independent and were not changed even when the ligand (FGF1) was applied in the system.

Consequently, weak phosphorylations of the Stat 1 (signal transducer and activators of transcription 1) were observed in TDI and TDII and strong ones in SADDAN. Regarding the activation of PLCγ, it was immunoprecipitated in the cell lysates and immunoblotted by the anti-FGFR3 antibody. The bounds of the PLCγ with FGFR3 were stronger in the severer diseases. In other words, in the severer diseases such as TDII and SADDAN, more activations of the phosphorylation in PLCγ were recognized. We have also obtained the findings on the activated sites on the FGFR3 molecules and on the stereostructures of the wild type and the mutated 2nd tyrosine kinase domain.

Aiming at a comprehensive investigation on the proteins expressed in the mutated FGFR3, the protein from the wild type chondrogenic cell line (ATDC5) and the protein from the ATDC5, in which the TDII mutated FGFR3 were overexpressed, were compared. As a result, there were clear differences in their constitutions, binding characteristics, and the phosphorylation status between these proteins.

Summerizing these findings, the severities of the ACH diseases are closely correlated with the degree of the autophosphorylation of the FGFR3s. The localization of the mutations can be divided into the membranous ones and of the ER, the mutations at the K650N localized in the ER; while, the mutations in ACH and TDI as well as the wild type FGFR3 are localized on the membrane. Accordingly, an anti-FGFR3 neutralizing antibody would be effective for the membranous type, ACH and TD1, but not for the ER type such as HCH, TDII, and SADDAN.

**Therapeutic Approach on Achondroplasia**

As is well known, the patients with achondroplasia suffer from short stature. Until recently, we had no choice in improving the short stature of achondroplasia except by surgical lengthening. Although growth hormone (GH) therapy has currently been applied, the efficacies are not as remarkable as in the patients with GH
deficiency. The reasons have been clarified from the histological findings in the growth plate of the mice, wild type and achondroplasia. In comparison with the wild type, the thickness and the 3 kinds of zones in the growth plate, especially the proliferative zone targeted by GH, are extremely thinned. And also because of the decrease in the numbers of the cells in the proliferative zone, the therapeutic efficiency of GH is markedly diminished.

Such being the case, we selected the parathyroid hormone related-peptide (PTHrP) as the candidate for a new therapeutic regimen of achondroplasia. There were a couple of reasons for this selection; the PTHrP deleted mice are short-limbed and their long bones are thick and short, and this resembles to achondroplasia. The sites of expression of PTHrP and FGFR3 in the growth plate are almost overlapped in the resting zone and the proliferative zone in the growth plate. And clinically, both the metaphyseal chondrodysplasias, Jansen-type and Blomstrand-type, are elicited by the point-mutations of the PTH/PTHrP receptors. Although the former type, a kind of metaphyseal chondrodysplasia, and the latter, a chondrodysplasia, are based on a gain-of-function mutation and loss-of-function mutation, respectively, their phenotypes are akin to those of achondroplasia.

At the beginning, the expression of PTHrP in the wild-type ATDC5 as well as in the cells of ACH-type and TDII-type were examined by means of the Northern Blot; and the results were that the expression levels of PTHrP were inversely correlated with the severity of the diseases. In a series of these studies it was clarified that the apoptosis that had been highly observed in the ACH and TDII cells, were rescued by the replacement of PTHrP. Then, we investigated the expressions of Bax (apoptosis inducer) and Bcl-2 (apoptosis suppressor) and compared the apoptotic changes with the BAX/Bcl2 ratios. In comparison with the wild type cell, those ratios were significantly increased in the ACH and TDII cells; but by the PTHrP replacement; the ratios were also decreased significantly (Fig. 3). These results clearly indicated that due to the cellular circumstances in which the expressions in PTHrP are decreased by a constitutively activated abnormal FGFR3, the level of Bcl-2 is lowered, so that the apoptosis is being enhanced. But with the replacement of PTHrP, the apoptosis are improved by an increase of the Bcl-2 expression and rescued from the apoptosis.

In the USA, the PTH from Eli Lilly has been used in the treatment of osteoporosis and has obtained good clinical results, not to mention the PTH and PTHrP that are in high homology and share the same receptor. When we consider any future clinical developments in this field, it is practically much more convenient to use PTH instead of PTHrP. Such being the case, we used...
recombinant human PTH (rhPTH) in the following organ culture and with *in vivo* studies.

**Fundamental Studies with rhPTH and Future Prospects**

A transgenic achondroplasia model mouse is short-limbed and has a large round head. In our organ culture study, the femur and the tibia were taken out from the fetal wild type and transgenic mice at 15 days after mating and cultured for 4 days with and without rhPTH; the length of each bone was measured on Day-2 and -4, and finally the measured portion of the bone was divided into 3 portions, the proliferative cartilage, calcified bone, and distal cartilage. The results showed that the growth disturbances in the transgenic mice were improved by replacement of rhTPH; and this effect was clearer in the tibias. More importantly, the effects were much more remarkable in the distal and proximal cartilage portions than in the calcified bone. These effects were also supported by the immunohistology of the bones targeting the collagen type X, a kind of all-differentiation marker indicating a hypertrophic chondrocyte. Collagen type X is expressed when the differentiations are progressed in order to increase the proliferation of the chondrocytes while slowing-down the differentiation. Actually, the increase in the expression of collagen X was observed in both the wild type and transgenic mice groups.

Then, an *in vivo* study was performed. Wild type female mice were mated with ACH-transgenic male mice, and then we obtained their offspring. To these offspring and the age-matched wild type neonatal mice, rhPTH 1000 µg/kg (high dose), 100 µg/kg (low dose) or saline was injected subcutaneously, consecutively from Day-1 to Day-21 for 22 days. On Day-21, the mice were sacrificed, and the length of the femur and tibia were measured; and they were also analyzed histologically. In the high dose group, the PTH significantly increased the length of the long bones in the ACH-transgenic mice, while in the wild type mice, PTH decreased the length significantly in comparison with the respective control groups. In 5 mice out of 7 ACH transgenic mice in the saline group, paralysis in the lower half of the body due to a spinal canal stenosis was seen; and they ultimately died. Although similar results were obtained in the low dose study in which the spinal canal stenosis was seen in 5 out of the 7 mice in the saline group and 2 out of the 4 mice in the PTH group, respectively; because of this event, the dose
of PTH must be more carefully scrutinized. The histological investigation showed that in the ACH-transgenic mice, rhPTH treatment increases the thickness of the growth plate and also increases cell numbers in the proliferative zone suggesting that PTH therapy improves the response to GH therapy by expanding the target area of GH.

Apart from achondroplasia, abnormalities in the bones such as brachydactyly in patients with pseudohyopoparathyroidism are elicited by growth plate closure induced by silencing in the PTH signal. Changing back to achondroplasia, it can be considered that a short stature occurs in the patients because of the deficiency in the PTH signal in the chondrocytes due to a weak expression of the PTHrP. Such being the case, in achondroplasia in which the expression of Bcl2 is decreased because of the point mutation of FGF3 and apoptosis in the chondrocyte is enhanced, as one of the treatments through the increment of Bcl-2 by the replacement of the PTH signal by PTHrP. In another series of studies, we have also been studying the suppression of apoptosis by increasing the bioavailability of Bcl-2 in applying IGF-1 instead of GH (Fig. 4).

References


