DEVELOPMENT OF RECOMBINANT PARATHYROID HORMONE AS A THERAPY FOR OSTEOPOROSIS : EVALUATION OF EFFICACY AND SAFETY

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BONE AND OSTEOPOROSIS

Skeleton of human body performs essential functions in life; it provides a framework for the body, protects internal organs from injury, serves as a mineral reservoir for the mineral homeostasis, and permits locomotion. Bone is chemically composed of two main parts, organic matrix and minerals, and anatomically comprised of 80% of cortical bone and 20% of trabecular cancellous bone. Cortical bone is compact and surrounds cancellous bone, contributing to structural integrity. Trabecular bone is most commonly found in the vertebrae, the pelvis and the ends of the long bones. It is metabolically more active than cortical bone and thus more responsive to skeletal changes including remodeling signals. It is composed of interconnecting lattice with an architecture designed to resist mechanical loads. The effect of osteoporosis is much more significant on trabecular bone than on cortical bone, as evidenced by the fact that osteoporotic fractures in the long bones tend to be in the proximal femur and distal radius which have greatest proportion of cancellous bone.

Bone remodeling is a lifelong renewal process of bone by which the structural and mechanical integrity of the skeleton is preserved. This also plays a role in the calcium homeostasis. Each year 10-30% of the adult bone is replaced by remodeling. Normal bone remodeling proceeds in a highly regulated cycle that involves a complex interplay of systemic hormones, local growth factors and cytokines, and mechanical stimuli (1, 2). The remodeling process consists of four distinct events: activation, resorption, reversal, and formation (2).

Osteoporosis is a metabolic bone disease which results from a disturbance in the normal bone remodeling, tilting the balance to bone resorption over formation. This disease is characterized by reduced amount of bone mass and deteriorated bone micro-architecture, which causes decreased physical strength of the skeleton, consequently increasing susceptibility to fractures. Osteoporosis is categorized into two groups depending on the cause of the disturbance: the primary osteoporosis that is mainly caused by the loss of gonadal function and aging, and the secondary osteoporosis which is associated with other specific diseases (3). Osteoporosis has a worldwide prevalence. 30-40% of postmenopausal women, and the elderly persons at the age of 70 or older will experience at least one osteoporotic fracture during their lives (4). The annual fracture rate due to osteoporosis in the United States is estimated to be 1.3 million, including 300,000 new cases of osteoporotic hip fractures (5). The impact of

osteoporosis on the health care problem is well appreciated; as menifested in the statistics, six months after a hip fracture only 25% of patients are fully recovered, whereas 50% need assistance even for routine daily activities and the rest 25% rely on long-term nursing care (6). This inflicts annual health care cost of over 10 billion dollars in the United States alone. It is obvious that as the aged population increases, the incidence of osteoporosis-related bone fractures will also rise.

Therapies for osteoporosis can be divided into two categories, one that inhibits bone resorption and the other that stimulates bone formation. Drugs of the former category can prevent further bone loss and hence are most appropriate for prophylactic purposes. One major drawback, however, of these drugs is that they are of little use for the patients with 'established osteoporosis' where bone density has fallen below the fracture threshold. Unfortunately, the most current osteoporosis drugs fall into this category, which is represented by estrogens, calcitonin, and bisphosphonates (6). In recent years there have been wider interests and higher investments on the bone and mineral research, starting to unveil the underlying mechanisms for the pathogenesis of osteoporosis. Although it is yet far from the complete understanding of the disease, accumulation of knowledge in the field of bone and mineral research will undoubtedly lead to more effective measures to deal with osteoporosis, both preventive and therapeutic. Several new drugs that are based on the underlying mechanisms for bone resorption are being developed (7-13). Most of these drugs are in early stage of development and thus should await the verification of their efficacy in human trials.

It is obvious that ideal therapy for osteoporosis can actually restore the bone lost from the disease. In this case, it is mandatory that the newly formed bone should be similar in architecture to the normal bone. Several drugs that may fulfill these requirements are under extensive tests in clinical human trials, among which fluoride and parathyroid hormone (PTH) are the front runners. Development of parathyroid hormone as a promising new therapy that can actually increase the bone density while maintaining the mechanical strength will be discussed below in more detail.

PTH THERAPY FOR OSTEOPOROSIS

PTH is a peptide of 84 amino acid residues synthesized in and secreted from parathyroid gland. It is a major regulator of calcium and skeletal homeostases, acting primarily through its receptor on target cells in bone and kidney. PTH has been traditionally considered to be catabolic for bone. It induces mobilization of calcium from bone by stimulating bone resorption. Receptors for PTH have been identified from bone cells of osteoblast lineage and kidney cells. They represent the first members of a new subfamily of G protein-coupled receptor, which transmit signals through both adenylate cyclase and phospholipase C. Studies of structure and function have defined the amino terminal domain ranging from residues 1 through 34 of PTH (PTH(1-34)) is necessary and sufficient for full biological activity of full-length PTH (PTH(1-84)) (14,15). Binding of PTH(1-84) or PTH(1-34) to its receptor can activate

both adenylate cyclase and phospholipase C in the same cell (16). The amino-terminal residues are crucial for activation of adenylate cyclase, since deletion of these residues dramatically reduces the activation of adenylate cyclase but maintains the stimulating activity of phospholipase C (15-18).

The non-physiological activity of PTH on bone metabolism, that is stimulation of bone formation by exogenous administration of PTH intermittently at low doses, has mainly been demonstrated in *in vivo* animal studies (19-27). Rats with osteopenia induced by ovariectomy share many similar characteristics with patients with postmenopausal osteoporosis. These include: increased bone turnover with resorption exceeding formation; an initial rapid phase of bone loss followed by a much slower phase; greater loss of cancellous than cortical bone; decreased absorption of calcium; and similar skeletal response to therapy with estrogen, bisphosphonates, calcitonin, PTH, and exercise. All of early studies were performed using parathyroid extract, whereas most recent data were obtained using synthetic PTH(1-34) due to limited availability of the full-length PTH. Recent success in overexpression of PTH(1-84) using recombinant DNA technology enabled use of the full-length PTH in the animal studies (28-30).

The histomorphometric evaluations showed the positive effects of daily injection of human PTH (hPTH) on such parameters as cancellous bone volume, bone formation rate, mineral apposition rate, mineralizing surface, and trabecular thickness, demonstrating the anabolic activity of hPTH(1-84) and the amino-terminal fragments of hPTH. The bone formed by the intermittent administration of hPTH is likely to be of normal quality as assessed by biomechanical testing of such parameters as maximum load, stress, and stiffness (27,31,32). Similar effects were observed not only with ovariectomized rats but also with senile rats (32). Taken together, these results confirm that daily injection of hPTH can restore bone, especially trabecular bone, lost either from menopause or from aging without sacrificing the bone quality. The anabolic effect of hPTH on bone has been compared to those of estrogen and bisphosphonates, all proving the better effectiveness of hPTH (26,27).

The anabolic effects of PTH on bone demonstrated in animal studies have led to the human trials of hPTH administration to osteoporotic patients, both men and women (33-35). All the data available from human studies were done with the amino-terminal fragments of hPTH (hPTH(1-34) or hPTH(1-38)). A few clinical studies using hPTH(1-84) are underway and thus the results will be available shortly. Results of small multicenter trial using daily injections of 500 U of synthetic hPTH(1-34) for 6-24 months showed striking increases in new bone accretion, cancellous bone volume, and osteoid covered cancellous surfaces (36). Effectiveness of intermittent administration of PTH in the prevention of bone loss due to estrogen deficiency was demonstrated in women with endometriosis who were being treated with nafarelin (an analogue of gonadotropin-releasing hormone) (37).

The mechanism whereby intermittent PTH therapy exerts anabolic effects on bone is not currently defined. Possible involvement of insulin-like growth factor I (IGF-I) in the anabolic action of PTH was first suggested by Canalis *et al.* (38) showing in the fetal rat calvaria model that only

intermittent but not continuous treatment of PTH increased local production of IGF-I. This hypothesis was further supported by Watson *et al.* (39), who used *in situ* hybridization techniques to show that restoration of bone mass by PTH was associated with increased osteoblast IGF-I gene expression. Of the two signal transduction pathways, adenylate cyclase and phospholipase C, stimulated by PTH, the former pathway is likely to be responsible for the *in vivo* bone formation activity of PTH, as shown in the studies using ovariectomized rats that analogs of PTH that stimulated adenylate cyclase pathway were able to increase bone mass, whereas fragments that only activated phospholipase C pathway failed to do so (40-42).

DEVELOPMENT OF RECOMBINANT HUMAN PTH PROCESS

The ability of PTH that stimulates restoration of bone of normal quality, as demonstrated in many animal and human studies, have led many drug companies to the development of hPTH as an ideal management for osteoporosis, both preventive and therapeutic. Two forms of hPTH, hPTH(1-34) and hPTH(1-84), are being tested in human trials. We are currently developing the full-length hPTH using recombinant DNA technology. As stated above, the amino-terminal residues of PTH are critical in transmitting signal to the adenylate cyclase pathway and this signaling is likely indispensible for the anabolic action of the peptide. Processing of the amino-terminal methionine of foreign proteins overexpressed in *E. coli* is often incomplete, leaving unprocessed protein with an additional methionine at the amino terminus. In addition, the size of peptides such as hPTH(1-84) is in many cases not large enough for efficient expression in *E. coli*. These properties of hPTH(1-84) led us to the development of a fusion expression strategy for efficient and economic expression of the peptide in *E. coli*. In choosing a fusion partner for hPTH(1-84), two points were considered, effectiveness in aiding the expression of the target protein and ease and efficiency of cleavage of the target protein from fusion partner. In terms of the expression efficiency, we maximized the expression level of hPTH(1-84) by using a fusion partner of as smallest size as possible without compensating for the expression level of the fusion protein. The effectiveness of cleaving target protein off from its fusion partner is one of the most important factors that determine the economy of a recombinant protein process. For the recovery of target proteins such as PTH whose integrity of the amino-terminal amino acid sequence is crucial for activity, use of enzymatic cleavage method over the chemical method is desirable because of the more pronounced specificity of the former than the latter method. For this reason, we chose a proteolytic enzyme, urokinase, for the cleavage of the PTH fusion protein, since commercial-scale production process for urokinase has already been established in one of our affiliates. Based on the substrate specificity of urokinase determined using chromogenic peptide substrates (43), we introduced by site-directed mutagenesis several different amino acid residues in the P3 subsite of the fusion protein substrate and optimized the site for the cleavage by urokinase. Culture of *E. coli* harboring the expression plasmid for PTH fusion protein was optimized in

terms of the amount of cell mass and the level of expression. The PTH fusion protein isolated as inclusion body from the recombinant *E. coli* was solubilized and then refolded. Condition for the cleavage of PTH from the fusion protein was optimized in terms of ratio of substrate to protease, reaction temperature and period, and buffer composition. PTH separated from the fusion protein was further purified by using ion exchange and reversed phase column chromatographies. Purity and identity of the purified hPTH(1-84) (rhPTH(1-84)) were confirmed by several physicochemical techniques. The *in vitro* bioactivity of the purified rhPTH(1-84) comparable to that of synthetic hPTH(1-84) was demonstrated by cAMP stimulation and competitive receptor binding assays using a rat osteosarcoma cell line, UMR 106, which possesses receptors for PTH (44).

EVALUATION OF BONE FORMATION EFFICACY AND SAFETY OF rhPTH(1-84)

Efficacy of rhPTH(1-84) in bone formation was demonstrated in animal studies using ovariectomized (OVX) rats. In one study, 6 month old female Sprague-Dawley rats were either ovariectomized or sham operated. The ovariectomized rats were allowed to develop osteopenia for 6 weeks. The rats received daily either placebo or an active compound for 4 weeks. The dose groups were : Sham/placebo control (n=10), OVX/placebo control (n=10), OVX/low dose rhPTH(1-84) (n=10), OVX/high dose rhPTH(1-84) (n=10), OVX/synthetic hPTH(1-34) (n=10), OVX/etidronate (n=10), and OVX/17β-estradiol. DXA scans of the femur were performed using Lunar DPX-L and associated small animal software prior to surgery, prior to the commencement and after the completion of dosing. Terminal measurements included histomorphometric assessment of the tibia using OsteoMeasure image analysis software and mechanical testing of the femur and lumbar vertebrae.

Treatment with rhPTH(1-84) restored DXA parameters of the distal femur to values exceeding the Sham control values, whereas antiresorptive controls did not restore the parameters. Histomorphometric evaluation of the tibia was performed in terms of static and dynamic parameters for bone formation. Trabecular area and width were significantly increased by daily dosing of rhPTH(1-84) compared to the Sham control and OVX rats. Mineral apposition and bone formation rates were markedly increased to supra-normal levels in the group treated with the high dose of rhPTH(1-84). The improvements with the treatment with rhPTH(1-84) in the bone density and the histomorphometric parameters were accompanied by the increase in the mechanical strengths of the cancellous bone as measured by maximum load and stress.

Safety of the rhPTH(1-84) was demonstrated in the preclinical toxicity studies including mutagenicity, safety pharmacology, acute and 28-day subacute toxicities, and toxicokinetics using mice, rats, rabbits, and cynomolgus monkeys. Dose-range finding experiment for the subacute toxicity study revealed remarkable species variance in the maximum tolerable level with the small animals much more tolerable than the large animals to the higher doses of rhPTH(1-84).

In summary, efficacy and safety of rhPTH(1-84) produced and purified from recombinant *E. coli* have been confirmed in the preclinical studies and further human clinical trials are underway for the development of rhPTH(1-84) as an osteoporosis drug capable of restoring bone density and quality.

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