Effects of different 1-34 parathyroid hormone dosages on fibroblast growth factor-23 secretion in human bone marrow cells following osteogenic differentiation

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Abstract

The importance of fibroblast growth factor (FGF)-23 as part of a hormonal bone-kidney-axis has been well established. Lately, FGF-23 has been suggested as an independent risk factor of death in patients on chronic hemodialysis. Hyperparathyroidism is a common feature of advanced kidney failure or end-stage renal disease. The independent effect of elevated parathyroid hormone (PTH) levels on FGF-23 secretion is still a matter of debate and has not yet been studied in an *in vitro* model of human bone marrow cells (BMC) during osteogenic differentiation. BMC from three different donors were cultivated for 4 weeks in cell cultures devoid of vitamin D either without 1-34 PTH or with PTH concentrations of 10 or 100 pmol/L, respectively. After 28 days, protein expression of the cells was determined by immunocytochemical staining, whereas real time-polymerase chain reaction served to analyze gene expression of several osteoblastic (osteocalcin, RANKL, Runx-2 and ostase) and osteoclastic markers (RANK, TRAP-5b). The concentrations of FGF-23, ostase and TRAP-5b were determined by ELISA at weeks 2, 3 and 4. We found a basal expression of FGF-23 with no increase in FGF-23 secretion after stimulation with 10 pmol/L 1-34 PTH. Stimulation with 100 pmol/L PTH resulted in an increase in FGF-23 expression (14.1±3.6 pg/mL with no PTH, 13.7±4.0 pg/mL with 10 pmol/L, P=0.84 and 17.6±3.4 pg/mL with 100 pmol/L, P=0.047). These results suggest a vitamin D and PTH-independent FGF-23 expression in human BMC after osteogenic stimulation. As only higher PTH levels stimulated FGF-23 expression, a threshold level might be hypothesized.

Introduction

Fibroblast growth factor (FGF)-23 is composed of 251 amino acids and has a molecular weight of 26 K-Dalton. It was first isolated in the antero-lateral thalamic nucleus of the brain.[1] FGF-23 was shown to be a critical pathogenic factor in several rare genetic disorders or tumor-induced osteomalacia (TIO).[2,3]

The main source of synthesis is thought to be in bone cells of the osteoblast-osteocyte lineage.[4] One of its major physiological actions is maintaining a normal phosphate and vitamin D balance. The hormone binds to a family of FGF-receptors requiring the transmembrane protein klotho as a co-factor to facilitate receptor activation.[5]

Animal studies in mice as well as experiments in rat and bovine parathyroid cell cultures have disclosed major effects of FGF-23 in target tissues.[6-9] The net effect of FGF-23 expression leads to increased urinary phosphate excretion and diminished calcitriol and PTH synthesis.

In contrast to the effects of FGF-23 in target tissues, the physiological stimuli of FGF-23 secretion have not been elucidated as clearly so far. 1-25-hydroxyvitamin D increases FGF-23 expression as part of a hormonal feedback loop. This effect is mediated via vitamin-D-receptor (VDR) action. Mice lacking VDR display markedly decreased FGF-23 levels.[10] The role of phosphate in humans on FGF-23 expression is still subject to controversial debates.[11,12] On the contrary, PTH has long been recognized as inducing elevations in 1-25-hydroxyvitamin D *via* induction of 25-hydroxyvitamin D-1a-hydroxylase (Cyp27b1) in the kidneys, exerting the opposite effect on 1-25-hydroxyvitamin D synthesis as opposed to FGF-23. In a transgenic mouse model of primary hyperparathyroidism it was postulated that PTH exerts a direct effect on FGF-23 expression in bone cells of mice calvaria and that osteoblast activation might be important in the regulation of FGF-23.[13] After parathyroidectomy FGF-23 levels decreased to normal levels in this animal study but changes in calcium, phosphate and calcitriol were also noted, potentially confounding the effect of PTH on FGF-23 secretion. However, an effect of PTH on FGF-23 secretion could not be proven definitively in humans suffering from primary hyperparathyroidism,[14] which might point to the existence of potential species differences. Furthermore, FGF-23 has been proposed to represent an independent risk factor of mortality in end-stage renal disease patients.[15] Many patients on renal replacement therapy or with advanced renal insufficiency develop secondary hyperparathyroidism. Therefore, an independent effect of PTH on FGF-23 secretion would be of relevant clinical interest. So far the physiological role of chronically elevated PTH levels on FGF-23 secretion in osteoblasts independent of vitamin D hormones has not yet been studied in a *human* cell model *in vitro.* Therefore, this study sought to investigate the effect of three different dosages of 1-34 PTH fragment (0, 10 and 100 pmol/L) on FGF-23 expression in a cell culture lacking of vitamin D of *human* bone marrow cells (BMC) during osteogenic differentiation *in vitro*.

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Materials and Methods

Cell culture

The approval of the institutional board review of the Heinrich-Heine Universität Düsseldorf, Germany, was granted prior to the beginning of the study. Human bone marrow was obtained by Jamshidi vacuum aspiration from the posterior iliac crest of three different donors each with normal kidney function (40- and 33-year-old male and 21-year-old female individuals). The bone marrow cells were cultivated in Dulbecco's modified Eagle's low glucose medium (DMEM, PAA Laboratories, Cölbe, Germany) with 20% fetal bovine serum (PAA), 100 U/mL penicillin, 100 mg/mL streptomycin (PAA) in culture flasks and incubated in 5% CO_2 at 37°C as described earlier.[16] The medium was changed every three days. After reaching confluence, the cells were harvested

with EDTA/trypsin and transferred into 24-well culture plates (Nunc, Wiesbaden, Germany) in a density of 5000 human bone marrow cells/cm^2. These cell cultures were cultivated for 28 days either without PTH, 10 pmol/L or 100 pmol/L 1-34 PTH in cell culture medium (DMEM) with osteogenic supplements (0.1 µmol/L dexamethasone, 50 µmol/L ascorbic acid phosphate, 20 mmol/L glycerolphosphate, Sigma, Taufkirchen, Germany) under standard cell culture conditions. Defined amounts of cell supernatants were harvested twice a week. Therefore, the medium was incubated with the cells for 3 or 4 days, respectively. These media were pooled according to the incubation times of 1, 2, 3 or 4 weeks and used for the ELISA measurement. Since the culture media contained 20% FCS, serum was included in the samples. Cell viability curves were calculated to exclude a confounding effect of PTH on cell proliferation (Figure 1).

Immunocytochemical staining

After 28 days of PTH stimulation, cell monolayers were stained with several antibodies, as described previously,[17] to detect the following antigens: CD-34 and CD-105 [monoclonal mouse antibodies (AB), DAKO Cytomation, Hamburg, Germany, dilution 1:20], osteocalcin (polyclonal goat AB, Santa Cruz, CA, USA, dilution 1:200), FGF-23 (polyclonal goat AB, Santa Cruz, dilution 1:250), receptor-activator of NFkB (RANK) (polyclonal goat AB, Santa Cruz, dilution 1:250) and receptor-activator of NFkB-ligand (RANKL) (polyclonal rabbit AB, Santa Cruz dilution 1:200). A biotin-labelled anti-rabbit-IgG combined with a streptavidin-horseradish-peroxidase (HRP)-complex (Vector) was used as a second antibody. Finally, 3-3-diaminobenzidine (DAB, Sigma) served as substrate for HRP. Alkaline phosphatase activity (ALP) was detected by ALP Kit (Vector Laboratories, Burlingame, CA, USA). For semi-quantitative evaluation of the samples by light microscopy (magnification: 40-fold), we used the following score system: negative (-), less than 10 positive cells (+), 10-50 positive cells (++), more than 50 positive cells (+++) (Figure 2).

Real time-polymerase chain reaction

m-RNA was isolated and a one-step RT-PCR was performed according to the manufacturers protocols (RNeasy® kit and OneStep RT-PCR kit, Quiagen, Hilden, Germany). GAPDH served as housekeeping mRNA. Table 1 shows which primers were used.

ELISA-detection of FGF-23, Ostase and TRAP 5b

Three sandwich-ELISAs were performed according to the manufacturers instructions [FGF-23: Immutopics Inc, San Clemente, CA, USA; Ostase and TRAP-5b: Immundiagnostic Systems Ltd. (IDS), Boldon, UK] for quantification of FGF-23, ostase (bone-specific alkaline phosphatase) and the active isoform 5b of the tartrate-resistant acid phosphatase (TRAP-5b) in cell culture supernatants. The concentrations were calculated using standard curves following the manufacturers' respective assay protocols. The FGF-23 assay by immutopics was used to measure intact FGF-23 in human serum, plasma and other biological fluids.

Photometric detection of PO42-

Phosphate concentration was measured by its reaction with ammonium molybdate and sulphuric acid forming an anorganic phosphormolybdate-complex, which can be detected at 340 nm (DiaSys Diagnostic Systems). The concentration was calculated using standard curves as described by the manufacturer.

Data collection

Concentrations of phosphate in mg/dL, TRAB-5b in U/L, ostase in µg/L and FGF-23 in

Table 1. Oligonucleotides used for polymerase chain reaction amplification.

Gene product	5' -> 3'	3'->5'	Source
GAPDH	ctcaagatcatcagcaatgcc	gatggtacatgacaaggtgc	NM_002046
Osteocalcin	agtccagcaaaggtgcagc	ggccgtagagcgccgat	NM_199173
RANKL	cagagcgcagatggatcct	gtaccaagaggacagactca	NM_003701
CD-34	catcacagaaacgacagtcaa	ctgccttgatgtcacttagg	NM_001773.1
CD-105	ggccgcacgctcgagtg	acatgagcagctccgg	NM_000118
Runx-2	ccttcaaggtggtagccct	gcctggggtctgtaatctg	NM_004348
TRAP5b	ctgtcctggctcaagaaacag	catagtggaagcgcagatagc	NM_001611.2

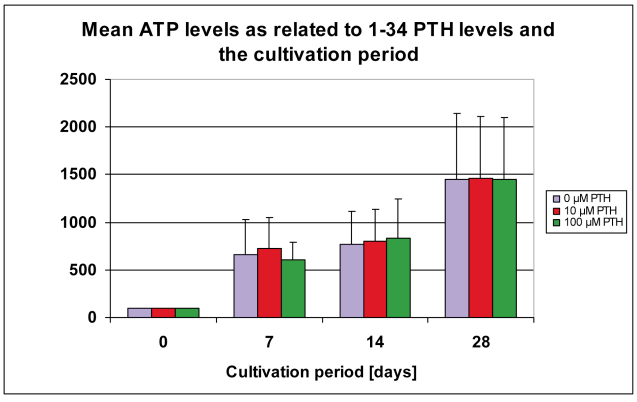

Figure 1. Depicted are cell viability data of human bone marrow cell cultures of each donor during osteogenic stimulation showing no PTH-induced effect on cell proliferation. Each column represents three measurements (one for each donor per PTH concentration). ATP levels in nM and PTH in µM. The Wilcoxon paired rank sum test was applied for statistical analysis (0 *versus* 10 µM PTH P=0.808, 0 *versus* 100 µM PTH P=0.936 and 10 *versus* 100 µM PTH P=0.870).

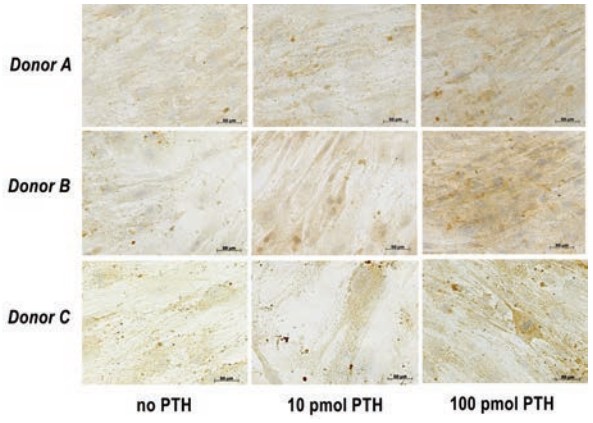

Figure 2. Immunocytochemical stainings (polyclonal FGF-23 goat antibody, Santa Cruz, dilution 1:250) of BMC after 28 days of osteogenic stimulation. Magnification 40fold: negative (-), less than 10 positive cells (+), 10-50 positive cells (++), more than 50 positive cells (+++). The absence of the hematopoetic marker CD34 proved little if any differentiation of BMC towards hematopoetic cell lineages (data not shown).

pg/mL were determined once a week for week 2, 3 and 4 in the cell culture supernatants in each donor.

Statistics

The Wilcoxon paired rank sum test was used to compare data from weeks 2, 3 and 4. A P-value ≤0.050 was considered significant.

Results

For all three patients a positive staining for CD 105 could be detected without any influence of increasing PTH-concentrations. While only very few cells were stained positive, the majority of the cells were negative for the hematopoetic marker CD 34 independent on the 1-34 PTH concentration added. The transcription factor Runx-2 and the osteoblast products RANKL and osteocalcin were identified in all experimental approaches without any noticeable influence of PTH. In contrast, elevated ostase levels were detected in 2 out of 3 patients. Here, bone marrow cells at higher PTH concentrations produced significantly increased ostase concentrations. The average dose-dependent levels of ostase (µg/L) were 5.75±1.76 without PTH, 7.25±0.29 with 10 pmol/L PTH and 13.29±1.82 with 100 pmol/L PTH respectively. The osteoclastic receptor RANK was present in all cell cultures and the levels were independent from the PTH concentration. However, the amount of the osteoclastic product TRAP-5b (U/L) increased with higher PTH concentration ranging from 2.98±0.72 without PTH, 3.36±1.0 with 10 pmol/L PTH and 4.74±0.50 with 100 pmol/L PTH respectively. As further evidence of at least a partial differentiation towards the osteoclastic lineage, RANK was detected semi-quantitatively in immunohistochemistry (RANK data not shown). FGF-23 (pg/mL) concentrations were similar following incubation with 0 and 10 pmol/L 1-34 PTH ranging from 14.1±3.6 without PTH to 13.7±4.0 with 10 pmol/L PTH (P=0.84). In contrast, stimulation with 100 pmol/L PTH led to an increase in FGF-23 levels to 17.6±3.4 pg/mL (P=0.047 and 0.040) (Figure 3). Individual cell cultures of each donor also demonstrated an increase in FGF-23 levels during incubation of the cell cultures with 100 pmol/L PTH at weeks 2, 3 and 4 (Figure 4). Phosphate levels were significantly increased due to the stimulation protocol, yet were stable at these levels at all time points in all cell cultures; 37.6±2.1 mg/dL (no PTH), 37.6±2.5 mg/dL (10 pmol/l PTH) and 38.5±1.6 mg/dL (100 pmol/L PTH).

Discussion

FGF-23 has been described as a pathogenic factor in various genetic syndromes and plays a major role in phosphate and vitamin D metabolism in patients suffering from chronic kidney disease.[18] We used 1-34 PTH concentrations of 10 and 100 pmol/L, as these levels are frequently encountered in patients with chronic kidney disease or end-stage renal disease on hemodialysis. Our *in vitro* results suggest a basal FGF-23 production in BMC after osteogenic differentiation independent of changes in vitamin D and PTH levels. Furthermore, a dose-dependent stimulatory effect of 1-34 PTH on FGF-23 secretion might be suggested. Based on the presence of Runx-2 and osteocalcin, osteoblastic differentiation could be identified in all cultures. We could also detect expression of RANK as evidence of an osteogenic differentiation towards the osteoclastic lineage. There was no correlation between these proteins and the PTH concentrations. Detectable increases in the marker proteins ostase and TRAP-5b imply an increased bone metabolism for both osteoblasts and osteoclasts, depending on the PTH concentration. So far, PTH has been postulated to exert an indirect effect on osteoclast activity *via* osteoblastic stimulation of the RANKL-RANK pathway. The increased osteoclast activity did not seem to be signaled via the RANKL-RANK pathway since both markers did not correlate with the three different PTH concentrations. In our model, only higher 1-34 PTH concentrations led to an increase in FGF-23 levels in all three of the donor cell cultures over a period of four weeks. Vitamin D or any other vitamin D derivatives were not part of the stimulation protocol, suggesting a vitamin D-independent and possibly dose-dependent effect of high PTH dosages on FGF-23 expression. We could also measure a basal FGF-23 secretion in the cell cultures not stimulated with exogenous 1-34 PTH, possibly due to elevated phosphate levels in the culture milieu. This is in line with a stimulatory effect of phosphate on FGF-23 production, although a study by Miyagawa and co-workers did not show an

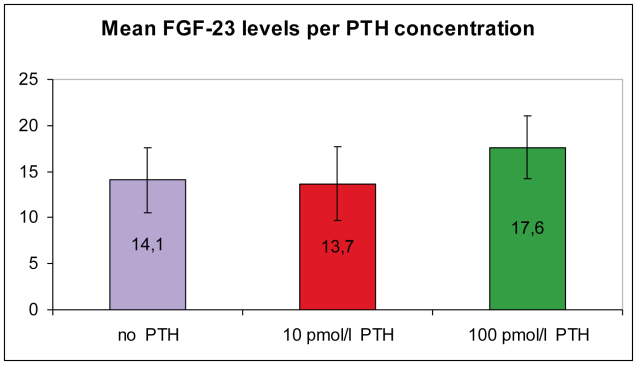

Figure 3. Mean FGF-23 in pg/mL (ELISA) concentrations and STD of weeks 2, 3 and 4 during osteogenic stimulation of BMC from three different donors with 0, 10 and 100 pmol/L 1-34 PTH. Each column represents nine measurements (three measurements in each donor). The Wilcoxon paired rank sum test was applied for statistical analysis (0 *versus* 10 pmol/L PTH P=0.840, 0 *versus* 100 pmol/L PTH P=0.047 and 10 *versus* 100 pmol/L PTH P=0.040).

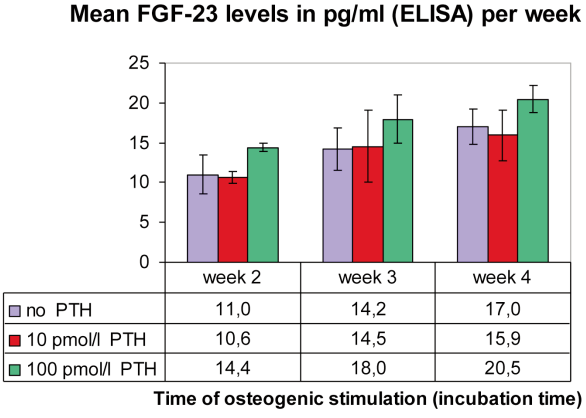

Figure 4. Mean FGF-23 concentrations in pg/mL (ELISA) and STD at weeks 2, 3 and 4 as related to different concentrations of 1-34 PTH (pmol/L) in BMC of three different human donors. Each column represents three measurements (one for each donor).

effect of phosphate on FGF-23 expression in an in vitro model of 10 week old mice osteocytes.[19] Therefore, changes in phosphate levels were avoided to minimize any potential confounding on FGF-23 expression. The steady increase of FGF-23 levels in the cell cultures of all three donors, might potentially be attributed to a constantly growing differentiation of BMC towards an osteoblastic lineage after osteogenic stimulation. It has been known for a long time that full-length (1-84) PTH is processed after secretion and the accumulation of different PTH fragments in ESRD patients is well documented. Although discussed controversially, some PTH fragments have been suggested to exert physiological effects in different cell systems. A 7-34 PTH fragment has been described but was not shown to exert physiological effects at least on bone cells.[20] We chose 1-34 PTH fragment instead of full-length 1-84 PTH in our cell culture model to measure only the biologic intact molecule and minimize any potential confounding on FGF-23 secretion by unexpected degradation of 1-84 PTH or actions of PTH fragments.

Due to the close relationship between PTH, calcitriol and phosphate the effects of PTH on FGF-23 secretion have been discussed controversially so far.

Studies in rats after parathyroidectomy or induction of renal failure have demonstrated a positive effect of PTH on FGF-23 secretion.[21,22] However, different other investigations in rodents could not demonstrate a stimulatory effect of PTH on FGF-23 secretion.[23-25]

Human studies have been more in line with a stimulatory effect of PTH on FGF-23 production so far. An experimental approach using intravenous infusions of 1-34 PTH over several hours in 20 healthy human subjects was in line with a physiological stimulation of FGF-23 by calcitriol, but did not shed more light on the potential independent effect of PTH on FGF-23 secretion.[26] Kobayashi *et al.* investigated FGF-23 secretion patterns in 50 patients with primary hyperparathyroidism.[27] In this study, FGF-23 levels also declined after parathyroidectomy on the first postoperative day, but in contrast to different other investigations on the regulation of FGF-23 secretion,[28,29] phosphate and calcitriol levels were not associated with changes in FGF-23 levels. Instead, calcium seemed to be positively correlated with increased FGF-23 levels suggesting different secretion patterns in patients with primary hyperparathyroidism and patients with advanced renal failure. Another clinical approach, using a biointact FGF-23 assay, investigated the effect of parathyroidectomy in 15 patients on dialysis and found a significant fall of FGF-23 postoperatively.[30] However, phosphate levels in this study fell also, which might potentially confound the effect of PTH on FGF-23 secretion in this study. In another study in postmenopausal women, daily subcutaneous injections of (1-34) PTH resulted in an increase in FGF-23 levels within 3 months of initiating therapy.[31]

Nevertheless, opposite to the aforementioned studies in humans, a recent investigation by Gutiérrez and coworkers, applying a 6 hour infusion protocol of (1-34) PTH in healthy adult volunteers, not only failed to demonstrate an increase in FGF-23 levels, but also described a significant decline in FGF-23 concentrations despite of increasing calcitriol levels.[32]

Limitations and strengths

There are several limitations to our study. Firstly, our cell *milieu* contained supra-physiological, though stable phosphate levels. Secondly, we investigated BMC only from three healthy donors. Therefore, further studies using this approach with more subjects and different PTH concentrations are clearly warranted. Thirdly, PHEX and Dmp1, other proposed local regulators of FGF-23, have not been studied in our current model as well as the possible effects of post-secretory proteolytic processing of FGF-23 by *e.g.* furin-like convertase or the glycosyl transferase. Nevertheless, we tried to minimize any confounding by measuring only full-length intact FGF-23 levels as this molecule is thought to be the only biologically active variant.

Suggested major strengths of our *in vitro* model are the use of human cell lines to avoid confounding by potential species differences and the definite lack of vitamin D compounds in the culture milieu to investigate an independent effect of PTH on FGF-23 expression.

Conclusions

FGF-23 is expressed in human bone marrow cells during osteogenic differentiation over four weeks *in vitro* independently of 1-34 PTH and vitamin D. Compared to stimulation with no PTH and only moderately elevated PTH levels, stimulation with high dosages of 1-34 PTH increases FGF-23 expression, suggesting a dose-dependent PTH effect on FGF-23 production. Further studies are necessary to elucidate an independent effect of PTH on FGF-23 secretion and different PTH concentrations might be tested.

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