

# Circulating Insulin-like Growth Factor Binding Protein-4 (IGFBP-4) is not Regulated by Parathyroid Hormone and Vitamin D *in vivo*: Evidence from Children with Rickets

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## ABSTRACT

**Objective:** Insulin-like growth factor binding protein-4 (IGFBP-4), inhibits IGF actions under a variety of experimental conditions. Parathyroid hormone (PTH), 1,25-hydroxy(OH)vitamin D, IGF-I, IGF-II and transforming growth factor (TGF)- $\beta$  are the major regulators of IGFBP-4 production *in vitro*. However, little is known about the *in vivo* regulation of circulating IGFBP-4 in humans.

**Methods:** We measured serum concentrations of calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), PTH, vitamin D, IGF-I, IGFBP-3, and IGFBP-4 in infants (n=22) with nutritional rickets before and after treatment of rickets with vitamin D (300 000 U single dose po).

**Results:** The mean $\pm$ SD age of the patients was 1.3 $\pm$ 1.6 years (range 0.2-3). Serum Ca and P increased, whereas ALP and PTH decreased after treatment (Ca from 6.6 $\pm$ 1.4 to 9.5 $\pm$ 1.6 mg/dL, P from 3.9 $\pm$ 1.4 to 5.4 $\pm$ 0.8 mg/dL, ALP from 2590 $\pm$ 2630 to 1072 $\pm$ 776 IU/mL and PTH from 407 $\pm$ 248 to 27.4 $\pm$ 20.8 ng/dL, respectively). Vitamin D levels were low (7.8 $\pm$ 2.5 ng/mL) and increased after treatment (18.1 $\pm$ 4.0 ng/mL, p<0.001). Serum IGF-I and IGFBP-3 levels both increased after treatment (IGF-I: 13.5 $\pm$ 12.2 vs. 23.7 $\pm$ 14.2 ng/mL, p<0.001 and IGFBP-3: 1108 $\pm$ 544 vs. 1652 $\pm$ 424 ng/mL, p<0.001). However, serum IGFBP-4 levels did not change significantly after treatment (18.8 $\pm$ 8.0 vs. 21.5 $\pm$ 4.8 ng/mL). No correlation between PTH and IGF-I, IGFBP-3 or IGFBP-4 was detected. Significant correlations were observed between PTH and ALP (r=0.53, p<0.05), and between IGF-I and IGFBP-3 (r=0.46, p<0.05).

**Conclusion:** The results demonstrate that contrary to *in vitro* studies, circulating IGFBP-4 levels are not influenced by secondary hyperparathyroidism in vitamin D deficiency rickets since IGFBP-4 levels did not change after normalization of PTH with vitamin D treatment.

**Key words:** IGF-I, IGFBP-3, IGFBP-4, PTH, rickets, Vitamin D, bone

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## Introduction

*In vitro* and *in vivo* studies emphasize that insulin-like growth factor binding protein-4 (IGFBP-4) may play an important role in modulating IGF actions in bone. IGFBP-4 is the major IGFBP produced by human osteoblasts and has been shown to be a potent inhibitor of IGF-stimulated cell proliferation (1,2). This effect is further modified by IGFBP-4 protease under variety of experimental conditions (3). Parathyroid hormone (PTH), 1, 25-hydroxy(OH)vitamin D, IGF-I, IGF-II and transforming growth factor (TGF)- $\beta$  are known regulators of IGFBP-4 production *in vitro* in human bone cells (4-7). However, little is known about the physiological regulation of circulating IGFBP-4 in humans. It was found that plasma IGFBP-4 levels correlated with bone mineral density in growth hormone (GH)-deficient adults (8). The increased circulating IGFBP-4 levels are found in elderly women with hip and spine fractures. Increase in IGFBP-4 was correlated with increased PTH in these women (9). This observation supported the role of PTH in the regulation of IGFBP-4. On the other hand, in subjects with primary hyperparathyroidism due to adenoma or hyperplasia, IGFBP-4 levels were found to be subnormal (10). Thus, human studies are inconclusive regarding the role of PTH in regulation of circulating IGFBP-4. To further investigate the

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role of PTH in regulation of IGFBP-4, we wanted to measure serum IGFBP-4 levels in situations, where hyperparathyroidism is more prominent and reversible. Vitamin D deficiency rickets is a perfect condition to test this hypothesis since secondary hyperparathyroidism is severe and is corrected rapidly after initiation of Vitamin D therapy. Thus, to investigate the role of PTH in the regulation of IGFBP-4 levels, we prospectively measured serum IGF-I, IGFBP-3, IGFBP-4 and PTH concentrations in infants with nutritional rickets before and after treatment.

## Methods

Patients with rickets were recruited from the outpatient clinics of Pediatric Hospitals after obtaining informed consent from the parents/guardian of each child. Diagnosis of rickets was established according to clinical, biochemical and radiological findings. Children who had a history of prematurity, renal, liver or intestinal disease or evidence of any of these disorders on physical examination or laboratory testing were excluded. Altogether, 22 infants with a mean age of  $1.3 \pm 1.6$  years were included in the study. Blood samples were obtained before and 3 months after treatment of rickets with vitamin D. The treatment was achieved by giving a single oral dose of 300 000 U of Vitamin D (stoss-therapy). Serum from the blood samples was separated within 2 h of the collection and was stored at  $-20$  °C until assayed. Serum calcium (Ca), phosphate (P), and total alkaline phosphatase (ALP) levels were determined by automatic analyzer. Serum intact PTH was measured by a two-site immunoradiometric assay (Allegro). Serum 25-(OH)Vitamin D levels were measured by chemiluminescence using Nichols Advantage competitive binding assay (San Juan Capistrano, California, USA). Serum IGF-I was determined by IRMA (DSL-5600 active, Diagnostics System laboratories, Webster, TX, USA) after separation of IGFs from IGFBPs by acid-ethanol extraction and neutralization

as described previously (11). Including the extraction step, the intraassay coefficient of variation (CV) was 5%, whereas the interassay CV was 12 %. Serum concentrations of IGFBP-3 were also measured by IRMA (DSL-6600) (11). Intraassay CV was 6% and interassay CV was 16%.

Serum IGFBP-4 was measured by an ELISA (DSL active IGFBP-4) assay according to the manufacturer's directions. For all measurements, interassay variability was less than 9% and intraassay variability was less than 7%. All blood samples were measured in duplicate.

## Statistics

Paired t-test was used to evaluate the differences in parameters examined before and after treatment with Vitamin D. Simple regression was used to analyze the relationships between the study parameters.

## Results

The mean ( $\pm$ SD) age of the patients was  $1.3 \pm 1.6$  (range 0.2 -3.0) years at the beginning of the study. Table 1 summarizes the findings on biochemical indices of rickets and IGF-I, IGFBP-3 and IGFBP-4 levels before and after treatment with vitamin D. These findings show that the patients had severe vitamin D deficiency with low blood levels of Ca and P, and very high levels of ALP and PTH. These values were almost completely normalized 3 months after treatment. Serum IGF-I and IGFBP-3 levels both increased after treatment for rickets, while serum IGFBP-4 levels did not change significantly ( $18.8 \pm 8.0$  vs.  $21.5 \pm 4.8$  ng/ml).

Correlation analyses demonstrated no relationship between PTH and IGF-I, IGFBP-3 or IGFBP-4 levels. Significant correlations were detected between PTH and ALP ( $r=0.53$ ,  $p<0.05$ ), and also between IGF-I and IGFBP-3 ( $r=0.46$ ,  $p<0.05$ ) before treatment, and before and after treatment combined.

**Table 1.** Serum calcium, phosphorus, alkaline phosphatase, PTH, 25-OH vitamin D, IGF-I, IGFBP-3 and IGFBP-4 levels before and after treatment in patients with rickets

	Before treatment	After treatment	P
Calcium (mg/dL)	6.6 $\pm$ 1.4	9.5 $\pm$ 1.6	<0.001
Phosphorus (mg/dL)	3.9 $\pm$ 1.4	5.4 $\pm$ 0.8	<0.01
Alkaline phosphatase (IU/mL)	2590 $\pm$ 2630	1072 $\pm$ 776	<0.05
PTH (ng/dL)	407 $\pm$ 248	27.4 $\pm$ 20.8	<0.001
25-(OH) vitamin D (ng/mL)	7.75 $\pm$ 2.49	18.12 $\pm$ 3.98	<0.001
IGF-I (ng/mL)	13.5 $\pm$ 12.2	23.7 $\pm$ 14.2	<0.001
IGFBP-3 (ng/mL)	1108 $\pm$ 544	1652 $\pm$ 424	<0.001
IGFBP-4 (ng/mL)	18.8 $\pm$ 8.0	21.5 $\pm$ 4.8	NS

PTH: parathyroid hormone  
NS: nonsignificant

## Discussion

The results of this study demonstrate that circulating IGFBP-4 levels are not influenced by secondary hyperparathyroidism in vitamin D deficiency rickets since IGFBP-4 levels did not change after normalization of PTH with vitamin D treatment. This observation is contradictory to the data obtained in *in vitro* studies suggesting regulation of IGFBP-4 by PTH and/or Vitamin D.

*In vitro* studies demonstrated a stimulatory effect of PTH on IGFBP-4 production. Treatment of SaOS-2 cells with PTH for 3 hours caused a 3.3-fold increase in IGFBP-4 mRNA levels, which was determined by reverse transcription-polymerase chain reaction (7). 1.25 (OH)<sub>2</sub> D<sub>3</sub> increases the secretion of IGFBP-4 by human osteoblast-like cells (5). Data regarding regulation of serum IGFBP-4 in humans are scarce. Using Western ligand blot analysis, Rosen et al (9) showed that serum IGFBP-4 levels are higher in elderly women with hip fractures and elevated PTH levels compared with age-matched controls. It was speculated that an increased local production of IGFBP-4 would inhibit the IGF stimulatory actions on bone synthesis potentiating the effect of PTH on bone resorption in these patients. However, in a recent study, serum IGFBP-4 levels were positively correlated with only radial bone mineral density (BMD), but not with lumbar or femoral BMD and vertebral fractures (12).

Honda et al (13) also found a weak correlation (r=0.26) between serum IGFBP-4 and PTH in healthy adults and in elderly individuals. They suggested that secondary hyperparathyroidism, which occurs as a consequence of age, could induce the inhibition of osteoblast proliferation by production of IGFBP-4 in the locale of bone-remodeling sites. Although much remains to be learned, our observation of no correlation in a more severe secondary hyperparathyroid state suggests that this conclusion is not valid. Consistent with our findings, Jehle et al (14), using Western blot found that IGFBP-4 levels in patients with primary hyperparathyroidism are comparable to those in controls. Similarly, Van Doorn et al (10), using RIA, found subnormal IGFBP-4 levels in subjects with primary hyperparathyroidism due to adenoma or hyperplasia, supporting what we observed in a secondary hyperparathyroid state in the present study.

Unlike previous cross-sectional studies, the present study is the first one to prospectively analyze serum IGFBP-4 levels in a high PTH state and after PTH levels have been decreased. We have seen no significant change in IGFBP-4 levels after the dramatic reduction of PTH in the subjects, while a slight increase was observed in IGF-I and IGFBP-3 levels. It is likely that the observed correlation between IGFBP-4 and PTH in the previous cross-sectional studies

was influenced by some covariants, such as age. In fact, serum IGFBP-4 levels correlated more strongly with age than with PTH levels (13).

The increase in both IGF-I and IGFBP-3 levels after the treatment of rickets may be due to a direct stimulatory effect of vitamin D and/or to improvement of nutritional status. However, none of the patients in this series were in a malnourished state. Similar to our findings, increased consumption of milk in elderly subjects for 3 months resulted in a decline in both PTH and ALP by 9%, in a significant rise in IGF-I by 10% and in a nonsignificant fall in IGFBP-4 by 1.9% (15). This observation is consistent with the findings in our study, where IGFBP-4 levels did not change significantly despite dramatic decline in PTH and ALP levels.

We conclude that circulating IGFBP-4 levels in children with rickets are not regulated by PTH or vitamin D, since the levels did not undergo significant change despite a 20-fold decrease in PTH levels after treatment with vitamin D. This finding is not in line with the data obtained from elderly persons and from *in vitro* studies. It is likely that the paracrine and endocrine regulators of IGFBP-4 are different.

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