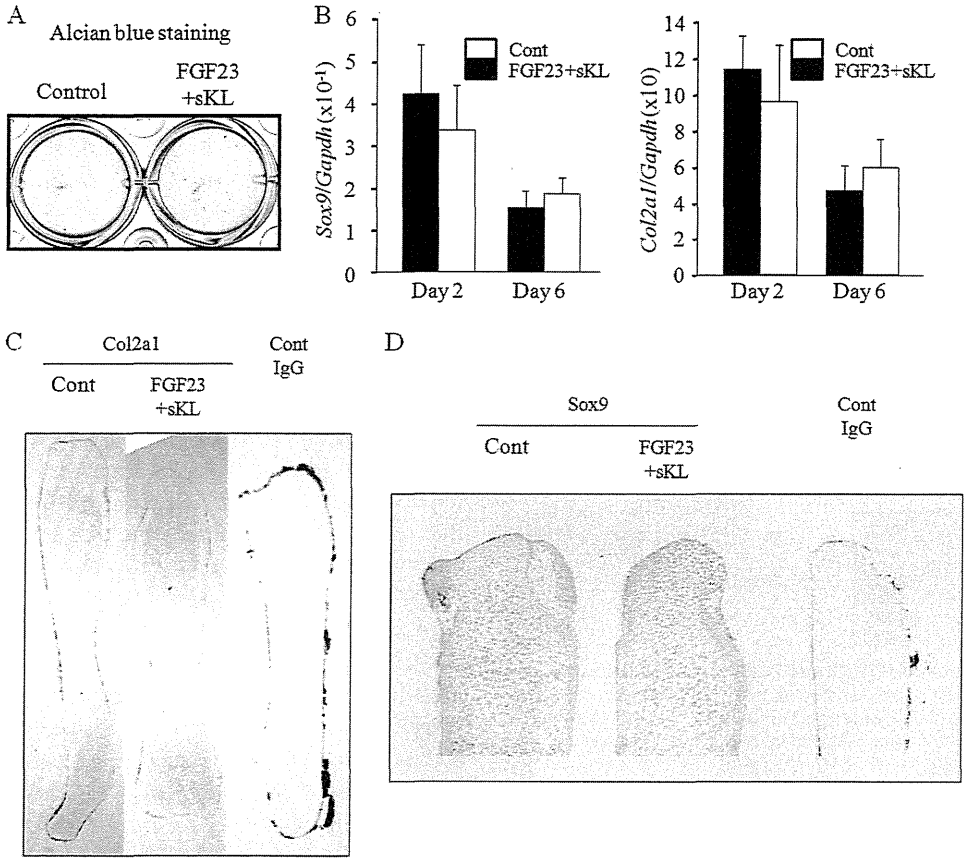

FIGURE 7. **FGF23/sKL does not affect the expression of Sox9 and Col2a1.** *A* and *B*, primary chondrocytes were grown to confluence and treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 6 days. Alcian blue staining was performed on day 6 (*A*), and expression levels of *Sox9* and *Col2a1* were determined by real-time RT-PCR on day 2 and 6 (*B*) ($n = 4$). *C* and *D*, metatarsal rudiments were isolated from E 15.5 embryos and cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Expression levels of Col2a1 (*C*) and Sox9 (*D*) were analyzed by immunohistochemistry ($n = 3–4$). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E.

these observations, immunohistochemical analysis showed that Ihh expression was lower in metatarsals treated with FGF23/sKL than that in controls (Fig. 9*B*). These data led us to speculate that decreases in Ihh expression may at least in part mediate the inhibitory effect of FGF23/sKL on metatarsal growth. To test this hypothesis, we treated metatarsals with FGF23/sKL in the presence or absence of conditioned-media obtained from Ihh-overexpressing HEK293 cells (Ihh-CM). Addition of Ihh-CM to the culture media did not affect the longitudinal growth of metatarsals, but partially rescued the impaired longitudinal growth of metatarsals by FGF23/sKL (Fig. 9*C*). In line with this, the inhibitory effect of FGF23/sKL on metatarsal growth was weaker in metatarsals treated with cyclopamine, an antagonist for Ihh signaling pathway (supplemental Fig. S3). These data indicate that decreased expression of Ihh is at least in part responsible for FGF23/sKL-induced impairments in metatarsal longitudinal growth.

Administration of sKL Impairs Chondrocyte Proliferation in Hyp Mice—To understand the *in vivo* role of FGF23/sKL signaling in chondrocyte biology, we utilized *Hyp* mice, a murine model for XLH, and intraperitoneally administered sKL into these mice. *Hyp* mice at postnatal day 10 already exhibited higher circulating FGF23 levels than those of wild-type (WT) littermate controls (Fig. 10*A*). Administration of sKL did not show any influence on body weight gain in wild-type or *Hyp* mice (Fig. 10*B*, supplemental Fig. S4*A*), but caused a shortening of the longitudinal length of the tibia in *Hyp* mice (Fig. 10*C*), whereas sKL did not affect the length of the tibia in WT mice (supplemental Fig. S4*B*). Histological analysis of the tibial growth plate revealed that the length of the proliferating zone was lower in sKL-treated *Hyp* mice than that in saline-treated *Hyp* mice (Fig. 10*D*), which was accompanied by a decline in the BrdU index in sKL-treated *Hyp* mice (Fig. 10*E*). In contrast to *Hyp* mice, sKL did not alter these parameters in WT mice (supplemental Fig. S4, *C* and *D*). To exclude the possibility that shortening of the proliferating zone is a consequence of altered phosphate metabolism, we analyzed expression levels of genes involved in phosphate metabolism in the femur and the kidney. As shown in Fig. 10*F* and *G* and supplemental Fig. S4*E*, expression of *Fgf23* in the femur and expressions of *Cyp27b1*, *Cyp24a1*, *Slc34a1*, and *Slc34a3* in the kidney were comparable between *Hyp* mice treated with sKL or saline. Consistent with this, circulating levels of phosphate were not different between *Hyp* mice treated with sKL or saline (Fig. 10*H* and supplemental Fig. S4*F*).

DISCUSSION

Recent advances in our understandings highlight the multifaceted nature of FGF23 function beyond its pivotal roles in the regulation of phosphate and vitamin D metabolism. For exam-

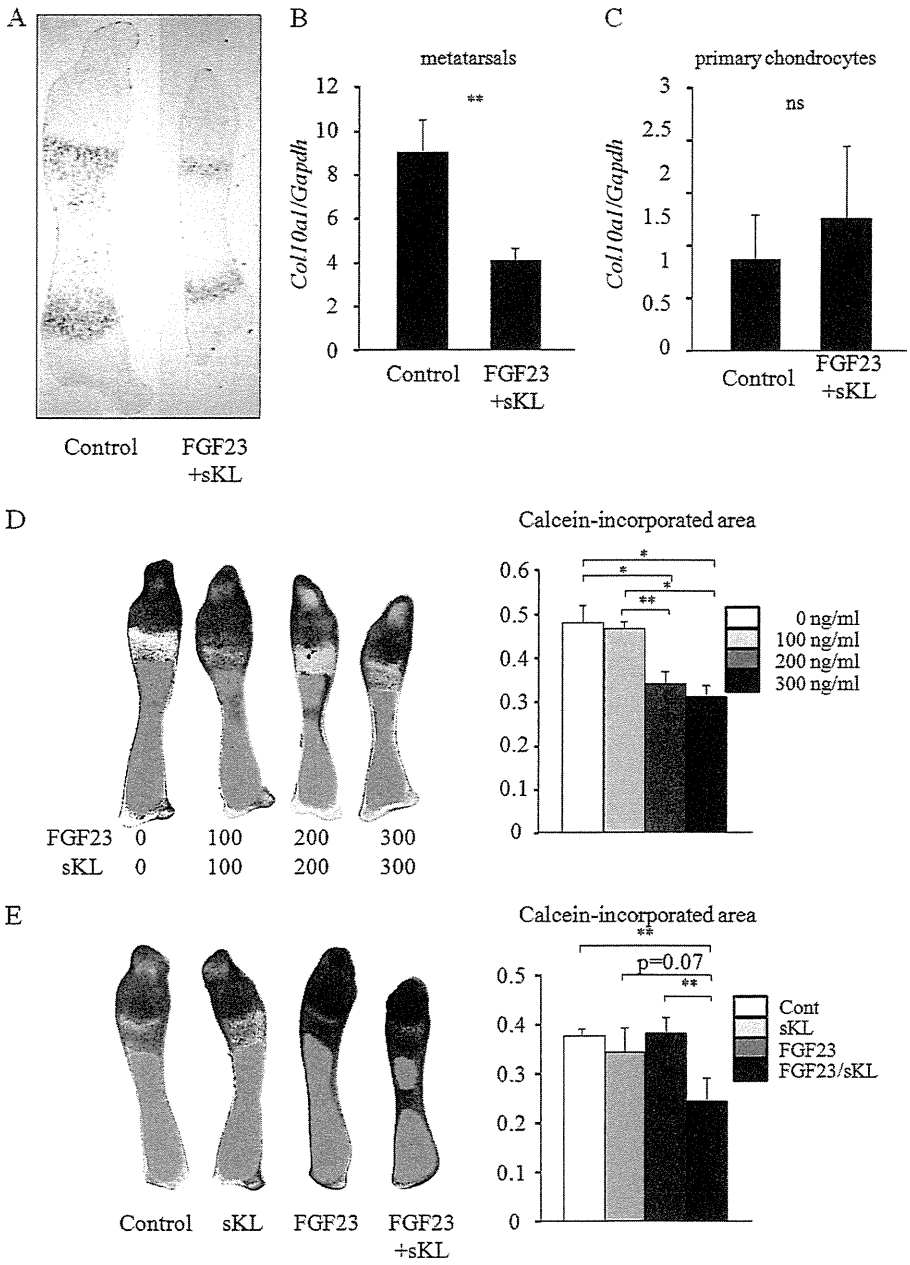

FIGURE 8. **Maturation of hypertrophic chondrocyte is impaired in metatarsals treated with FGF23/sKL.** *A* and *B*, metatarsal rudiments were isolated from E 15.5 embryos and cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Expression levels of *Col10a1* were analyzed by *In situ* hybridization (*A*) and real-time-RT-PCR (*B*) ($n = 3$). *C*, primary chondrocytes were treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 6 days. Expression levels of *Col10a1* were determined by real-time RT-PCR ($n = 3$). *D* and *E*, metatarsals were cultured in the presence of various concentrations of FGF23 and sKL (*D*), or FGF23 (300 ng/ml), sKL (300 ng/ml), or both (*E*) for 12 days. On day 12, the calcein incorporated area was visualized and quantified ($n = 3$–4). *RZ*: resting zone, *PZ*: proliferating zone, *HZ*: hypertrophic zone. The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, $p < 0.01$; **, $p < 0.05$. *ns*, not significant.

ple, FGF23 has been recently shown to be responsible for the development of left ventricular hypertrophy through activating calcineurin-NFAT signaling pathway in mice (26). Non-canonical activity of FGF23 could be operative as well in chondrocytes as evidenced by the previous study in which *Fgf23* and *Slc34a1* genes were deleted in mice (10). The lack of *Slc34a1* in *Fgf23*-deficient mice did not correct the decreased number of hypertrophic chondrocytes in *Fgf23*-deficient mice despite of the correction of serum phosphate levels, suggesting the presence of phosphate-independent action of FGF23 in chondrocytes; however, the precise mechanisms of phosphate-independent function of FGF23 in chondrocyte biology remain to be elucidated.

Initially, we demonstrated *in vitro* that FGF23 can mediate its signals in the presence of sKL. As previously reported, α-Klotho expression was extremely low in chondrogenic cells. In line with this, FGF23 alone could not activate ERK or induce *Egr1* expression in chondrogenic cells. Since there is an increasing amount of evidence that demonstrates the biological function of sKL in mice (12–15), we assessed the functional interaction between FGF23 and sKL in chondrogenic cells. In the current study, we utilized ~130 kDa of sKL produced by ectodomain

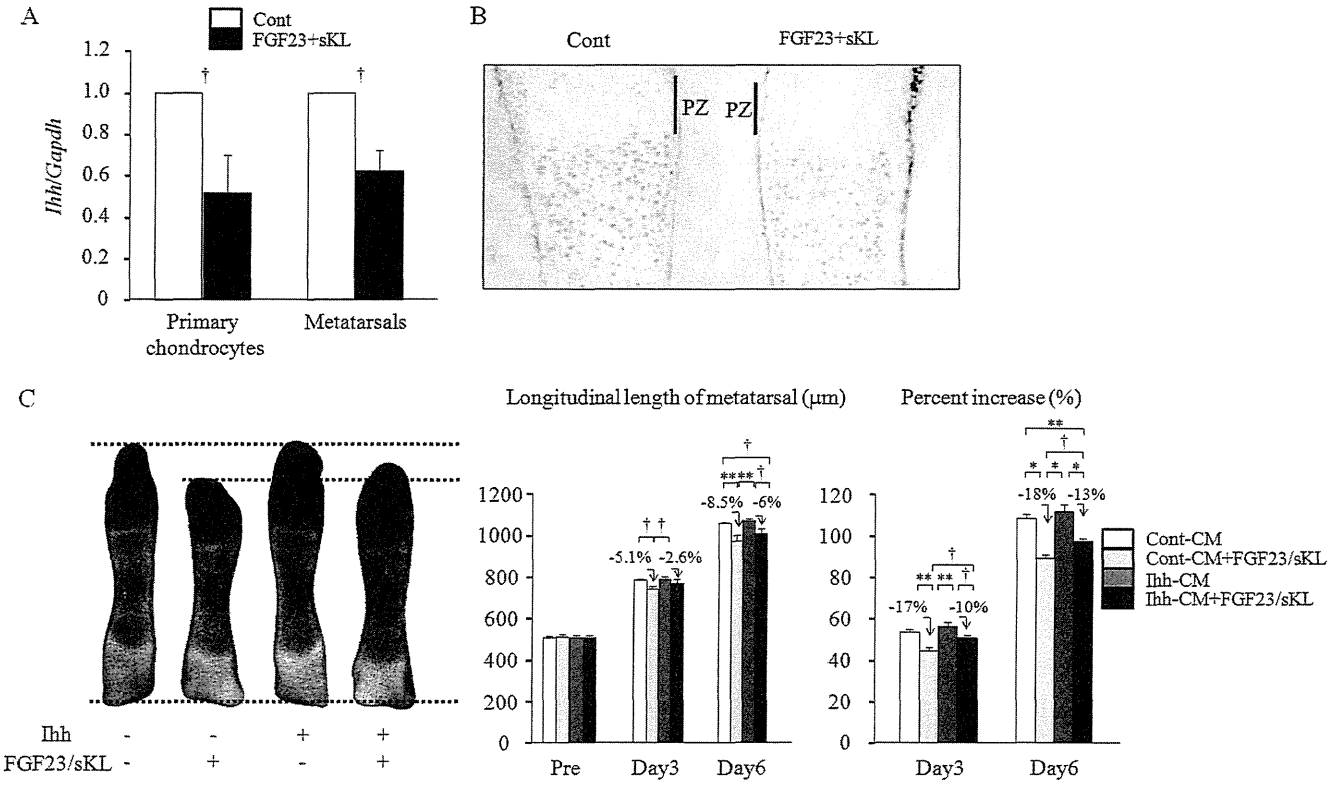

FIGURE 9. **Indian Hedgehog partly mediates the suppressive effect of FGF23/sKL on the longitudinal growth of metatarsals.** *A* and *B*, primary chondrocytes were treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 2 days. Metatarsal rudiments were cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Expression levels of *Ihh* were determined by real-time RT-PCR ($n = 4$) (*A*) and immunohistochemistry ($n = 3$) (*B*). *C*, metatarsals were treated with FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days in the presence of 10% of Ihh conditioned media (Ihh-CM) or control conditioned media (Cont-CM) and longitudinal lengths and percent increases in metatarsals were measured ($n = 3–4$). Conditioned media obtained from HEK293 cells overexpressing empty vector were used as Cont-CM. *PZ*: proliferating zone. The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, $p < 0.001$; **, $p < 0.01$; †, $p < 0.05$.

shedding based on a previous report showing that this type of sKL is the predominant sKL in human circulation (27). Interestingly, *in vitro* studies revealed that FGF23 exerted its signals in the presence of sKL in chondrogenic cells. The precise mechanisms whereby sKL mediates FGF23 signaling still need to be elucidated, but the finding that sKL forms a protein complex with FGF23 may raise the possibility that sKL may allow FGF23 to reach and bind to its specific receptors by making a complex with FGF23 in the circulation. Indeed, co-immunoprecipitation analysis revealed that the binding of FGF23 to FGFR3 was enhanced when sKL was present. However, it is still unclear as to whether FGF23/sKL complex is present in the circulation and further analyses are required to determine the significance of this complex in the *in vivo* physiological conditions.

Next, to elucidate the significance of FGF23/sKL signaling in chondrocyte biology, we introduced an *ex vivo* metatarsal organ culture system, which is a widely used procedure to recapitulate *in vivo* bone growth. Using this method, we found a unique phenotype with respect to chondrocyte proliferation such that FGF23/sKL suppressed proliferation in the proliferating zone. The finding that FGF23/sKL impaired chondrocyte proliferation in the proliferating zone led us to speculate that FGF23/sKL may recognize FGFR3 as its receptor because an activating mutation in the *Fgfr3* gene has been shown to result in impaired chondrocyte proliferation (28–30). Based on this, we analyzed whether FGFR3 is involved in FGF23/sKL-mediated suppression of metatarsal growth and provided evidence that FGFR3 is at least in part involved in the FGF23/sKL-mediated action on metatarsal growth. However, given the fact that FGF23/sKL showed a non-significant suppression on the linear growth of metatarsals infected with adenovirus containing microRNA specific for *Fgfr3*, we could not exclude the possibility of the involvement of other types of FGF receptors, although it is still possible that residual expression of FGFR3 may contribute to the suppressive action in FGFR3-knocked down metatarsals. Similar to FGFR3, FGFR1 is abundantly expressed in the growth plate predominantly in perichondrium and hypertrophic chondrocytes, whereas FGFR3 exhibits abundant expression in resting and proliferating chondrocytes, with less expression in hypertrophic chondrocytes (31). The expression profile of FGFR2 is also different from FGFR3, and FGFR2 is mainly expressed in mesenchymal condensation (31). Although previous *in vitro* studies demonstrated the binding of FGF23 to multiple FGF receptors (8, 9), the compartment-specific expression profile of FGFRs in the growth plate would confer a specific binding partner for FGF23 in the presence of sKL such that FGF23 suppresses chondrocyte proliferation in the proliferating zone through the activation of FGFR3.

Multiple pathways have been shown to mediate the effect of FGFR3 activation with respect to the suppression of chondrocyte proliferation. Ihh has been implicated to be the downstream target of FGFR3 activation (21–23) and is well known to play a critical role in chondrocyte proliferation (24, 25). Based on these findings, we tested our speculation that Ihh may be the

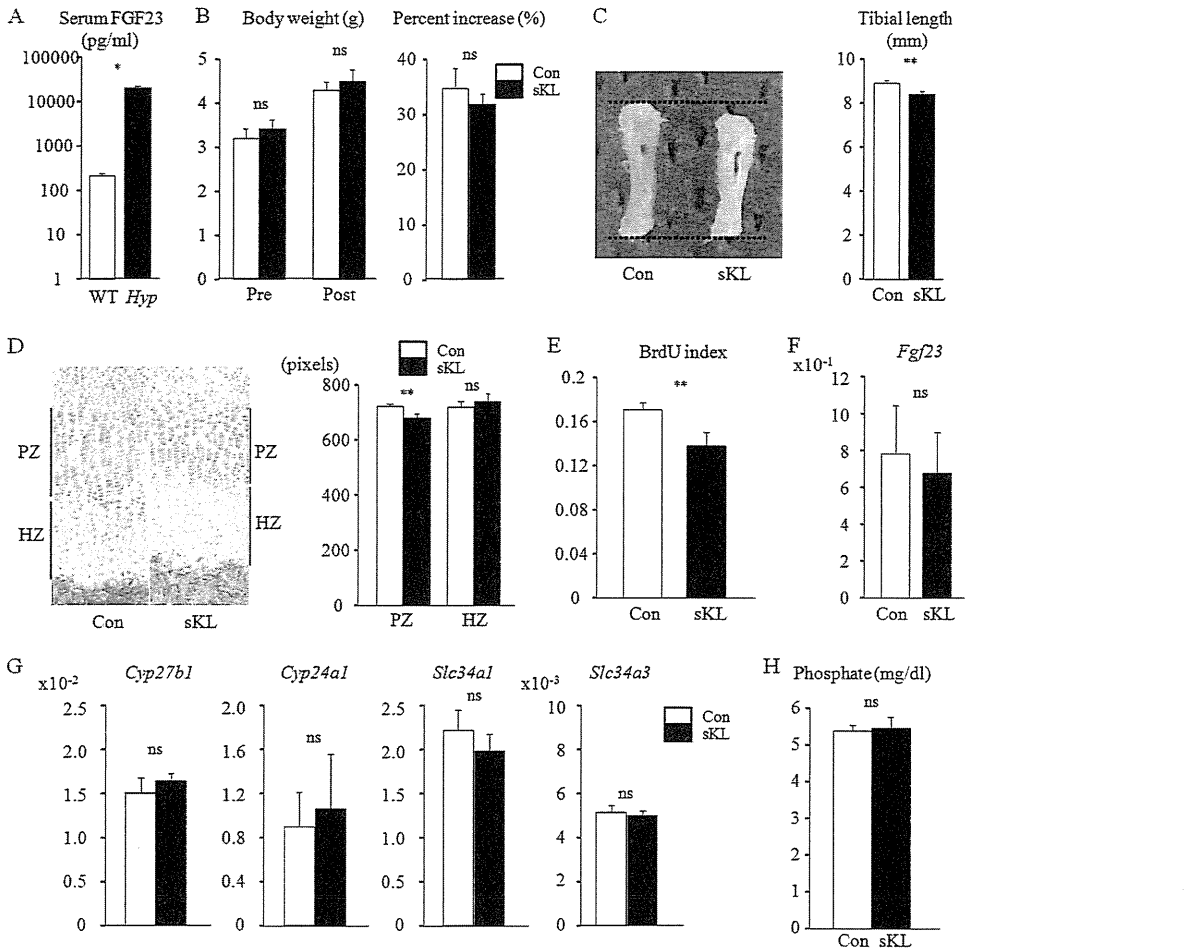

FIGURE 10. **Administration of sKL impairs chondrocyte proliferation in *Hyp* mice.** sKL (0.02 mg/kg) was intraperitoneally administered into *Hyp* mice for 3 days from postnatal day 7 and samples were collected on postnatal day 10. *A*, plasma FGF23 concentrations on postnatal day 10 in wild-type and *Hyp* mice (n = 3–4). *B*, body weights of *Hyp* mice on postnatal day 7 and day 10 and percent increases in body weight were calculated (n = 6–7). *C*, gross appearance of the tibia isolated on postnatal day 10 and tibial lengths were determined (n = 6–7). *D*, growth plates of tibiae were histologically analyzed by Hematoxylin and Eosin staining and the lengths of proliferating zones (*PZ*) and hypertrophic zones (*HZ*) were calculated (n = 6–7). *E*, BrdU staining was performed and the ratio of the number of BrdU-positive cells over total cells in the proliferating zone was determined (BrdU index) (n = 6–7). *F*, expression of *Fgf23* in the femur was determined by real-time RT-PCR (n = 3). *G*, expression levels of *Cyp27b1*, *Cyp24a1*, *Slc34a1*, and *Slc34a3* in the kidney were analyzed by real-time RT-PCR (n = 3–5). *H*, plasma levels of phosphate were measured (n = 6–7). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, $p < 0.001$; **, $p < 0.05$. *ns*, not significant.

downstream target of FGF23/sKL signaling and found that Ihh expression was reduced in the presence of FGF23/sKL and addition of Ihh protein partially reversed the impaired growth of metatarsals treated with FGF23/sKL. Since Ihh protein cannot fully rescue the growth impairment induced by FGF23/sKL signaling, other signals are likely involved in the action of FGF23/sKL on the suppression of chondrocyte proliferation. Because STAT1 has been shown to be activated in response to FGFR3 activation, which in turn results in the inhibition of chondrocyte proliferation (32), a signaling pathway through STAT1 activation may be involved in the FGF23/sKL-induced impairment in chondrocyte proliferation.

In XLH patients, it is well recognized that administration of phosphate and calcitriol is effective to improve linear growth, but is not sufficient to fully reverse impaired growth despite the correction in biochemical markers and rachitic changes (17, 33). This evidence may suggest the existence of factor(s) modulating the linear growth of XLH patients in addition to abnormal phosphate metabolism, and the current findings that FGF23 suppresses chondrocyte proliferation in the presence of sKL may at least in part explain the reason why the correction in serum phosphate levels by administration of phosphate and calcitriol cannot fully regain impaired growth in XLH patients. If this mechanism is operative, the blockade of FGF23 signaling as a strategy for the treatment of XLH patients would be very promising because suppressing FGF23 signaling may have an additional benefit such as enhancing chondrocyte proliferation beyond its capacity to correct phosphate and vitamin D metabolism. Indeed, recent *in vivo* animal studies have provided evidence for the striking effectiveness of the blockade of FGF23 signaling pathways by the anti-FGF23 neutralizing antibody in the improvement of rickets and growth retardation in *Hyp* mice (34).

The significance of sKL in chondrocyte biology in humans remains largely unknown. Recent development in the ELISA system to detect human sKL has revealed that significant amounts of sKL are present in the human circulation (35); however, it is still controversial as to whether sKL in the circulation has any biological functions despite evidence demonstrating the biological function of sKL in animal models (12–14). Nev-

ertheless, the finding that sKL levels are greater in fetal life and childhood than that in adults may suggest that sKL may have more pronounced effects during these times (35–37). Since sKL levels in XLH patients have been shown to be comparable to those of control subjects (36), elevated FGF23 levels in these patients may have a significant impact on the signaling pathways exerted by FGF23/sKL. Our *in vivo* results using supraphysiologic concentrations of sKL may not be used to reach a definitive conclusion regarding the role of physiologic concentrations of sKL in the regulation of chondrocyte biology, but these findings may underline the significance of sKL, especially in fetal life and childhood when sKL levels are elevated and chondrocyte proliferation and maturation are actively operative.

In summary, we demonstrated that FGF23 possessed a non-canonical function where FGF23 suppressed chondrocyte proliferation and maturation in cooperation with sKL independent of phosphate metabolism, and this effect was partly mediated through FGFR3 and involved the suppression of Ihh expression. These lines of evidence add to our growing knowledge regarding signaling networks exerted by FGF23 and provide insights into the unrecognized function of FGF23 signaling that could be important for chondrocyte biology.

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Original Article

Treatment of Hypophosphatemic Rickets with Phosphate and Active Vitamin D in Japan: A Questionnaire-based Survey

Makoto Fujiwara[1], Noriyuki Namba[1], Keiichi Ozono[1], Osamu Arisaka[2], Susumu Yokoya[3], and Committee on Drugs, Japanese Society for Pediatric Endocrinology*

[1]*Department of Pediatrics, Osaka University Graduate School of Medicine, Osaka, Japan*

[2]*Department of Pediatrics, Dokkyo Medical University School of Medicine, Tochigi, Japan*

[3]*Department of Medical Subspecialties, National Center for Child Health and Development, Tokyo, Japan*

Abstract. Hereditary hypophosphatemic rickets represented by X-linked hypophosphatemic rickets (XLH) is a rare disorder characterized by hypophosphatemia, elevated alkaline phosphatase (ALP) and undermineralization of bone. Active vitamin D and phosphate are administered to correct hypophosphatemia and elevation of ALP. Overtreatment with phosphate leads to secondary hyperparathyroidism, and a large dose of active vitamin D has a risk of hypercalciuria. To understand the situation concerning treatment of patients with hereditary hypophosphatemic rickets in Japan, we conducted a questionnaire survey of pediatric endocrinologists. Answers were obtained from 53 out of 68 hospitals where the pediatric endocrinologists worked. One hundred and thirty-five patients were treated in 28 hospitals during November 2009 and May 2010; 126 patients suffered from hereditary hypophosphatemic rickets, and 9 had hypophosphatemia caused by other miscellaneous reasons. The distribution of patient age was as follows: 27 (21%) were between 6 mo and 6 yr of age, 39 (31%) were between 6 and 12 yr of age, and 60 (48%) were more than 12 yr of age. Active vitamin D was given to 123 patients, and phosphate was given to 106 patients. As for the dose of phosphorus, 37.2–58.1 mg/kg/d was given divided into 2 to 6 aliquots. There were various control targets of treatment, including serum phosphate, serum ALP, rachitic change, urinary Ca/Cr, parathyroid hormone and growth. It is very important to avoid side effects of these treatments. No evidence is available about the optimal dose of phosphate or number of administrations in the treatment of patients with hypophosphatemic rickets. Although there is a recommendation for clinical management of patients with hypophosphatemic rickets, we should set a clinical guideline for it in Japan.

Key words: hypophosphatemia, phosphaturia, rickets, active vitamin D, phosphate

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Correspondence: Dr. Keiichi Ozono, Department of Pediatrics, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
E-mail: keioz@ped.med.osaka-u.ac.jp

* Member list of the Department of Molecular Endocrinology, National Research Institute for Child Health and Development: Osamu Arisaka, Susumu Yokoya, Shin Amemiya, Toshiaki Tanaka, Tomonobu Hasegawa, Hiroyuki Tanaka, Syohei Harada.

Introduction

Rickets is a disorder of calcification in chondrocytes and bone characterized by accumulation of unmineralized bone, termed osteoid. Characteristic X-ray findings such as cupping, flaring, and fraying strongly suggest

rickets, although metaphyseal dysplasia must be ruled out. The main causes of rickets are vitamin D deficiency and hereditary hypophosphatemic rickets. Hypophosphatemia and elevated levels of alkaline phosphatase (ALP) are associated with both vitamin D-deficient and hereditary hypophosphatemic rickets.

Hereditary hypophosphatemic rickets is classified mainly into 4 entities based on mode of inheritance and urinary excretion of calcium (1, 2). Recently, the genes responsible for these forms of hereditary hypophosphatemic rickets have been identified. Autosomal dominant hypophosphatemic rickets (ADHR, MIM 193100) is a rare disease characterized by low levels of serum phosphate and elevated levels of ALP and phosphaturia and is inherited in an autosomal dominant fashion. In 2000, genetic analysis of families with the disease successfully identified that the *FGF23* (fibroblast growth factor 23) gene is responsible for the disease (3). Now FGF23 is recognized as a hormone that plays a central part in regulation of the serum phosphate concentration, and its abnormality is involved in many cases of hypophosphatemic rickets (4, 5). In ADHR, since the mutant FGF23 is resistant to degradation, its concentration is elevated in serum. Thus, this mutation is a gain-of-function type. FGF23 works as a phosphaturic factor after binding to FGFR1 and its co-receptor, klotho, in the kidney and reduces serum phosphate concentrations (6). In addition, FGF23 decreases the production of 1, 25-dihydroxyvitamin D [1,25$(OH)_2$D] in renal tubules (7). In turn, 1, 25$(OH)_2$D and phosphate increase the expression of FGF23 (8). Therefore, administration of active vitamin D and phosphate may exert biphasic effects, i.e., acute increase in phosphate levels followed by decrease in phosphate levels associated with an increase in FGF23 levels.

Autosomal recessive hypophosphatemic rickets (ARHR1, MIM 241520) is also a rare disease in which hypophosphatemia and rickets are observed. The causal gene is *DMP1* (dentine matrix protein 1), and its expression is observed in osteocytes and osteoblasts (9). *ENPP1* is a newly identified causal gene (ARHR2, MIM 613312) (10, 11). The *ENPP1* gene encodes ectonucleotide pyrophosphatase/phosphodiesterase 1 and is also responsible for generalized arterial calcification of infancy (12). Although the mechanism remains obscure, FGF23 is elevated in both types of ARHR and reduces serum phosphate concentrations (13). In Japan, two single families are reported to have abnormalities in each of these gene (14, 15).

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH, MIM 241530) is a rare autosomal recessive disease characterized by hypophosphatemia and hypercalciuria. It is caused by *SLC34A3*, which encodes the type IIc sodium-dependent phosphate co-transporter (NaPi-IIc), a transporter for reabsorption of phosphate in the proximal renal tubules (16–18). The administration of phosphate alone ameliorates hypophosphatemia and hypercalciuria in HHRH.

X-linked hypophosphatemic rickets (XLH, MIM 307800) is the most frequent and prototype form of hypophosphatemic rickets in pediatric practice. In 1995, the gene responsible for the disease was identified as *PHEX* (phosphate regulating gene with homologies to endopeptidases on the X chromosome) (19). To date, over 200 mutations have been found in the *PHEX* gene and listed in the PHEXdb, PHEX Locus Database (http://www.phexdb.mcgill.ca). Patients with XLH are treated with active vitamin D and phosphate buffer. However, phosphate buffer is not available as a prescribed medicine in Japan. In addition, treatment with vitamin D and phosphate buffer is not an absolute cure for the disease, though a recommendation for treatment has been published (20).

Hypophosphatemic rickets is also caused by impaired function of renal tubules and tumors that produce FGF23. Malfunction of renal tubules sometimes involves reabsorption of essential nutrients or minerals other than phosphate and is called Fanconi syndrome (MIM 134600, 613388, or acquired). The acquired form

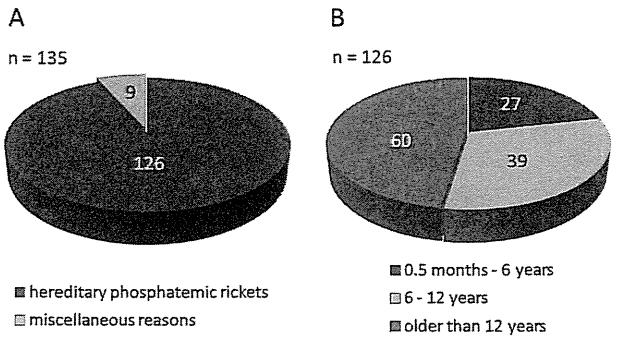

Fig. 1 Patient background. (A) The cause of hypophosphatemia in all patients. (B) The ages of the patients with hereditary hypophosphatemic rickets.

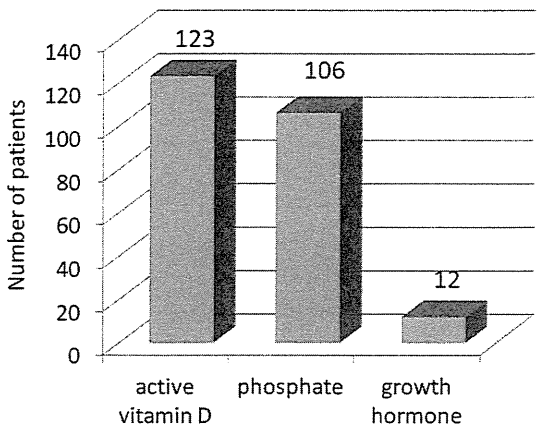

Fig. 2 Treatment for patients with hereditary hypophosphatemic rickets.

is called tumor-induced osteomalacia (TIO) and is rare in childhood (21–23).

We attempted to clarify how hypophosphatemic rickets is actually treated in Japan. To this end, we sent questionnaires concerning the experience of treatment of patients with hypophosphatemic rickets and the actual procedures.

Material and Methods

We sent questionnaires by mail to 68 hospitals where 80 pediatric endocrinologists approved by the Japanese Society of Pediatric Endocrinology worked in 2010. Survey subjects are patients who show hypophosphatemia for more than 6 mo. The questionnaire includes the number patients, patient profiles such as age and sex, hereditary pattern, type of medicine, and dose of phosphate including minimum and maximum dose and frequency.

Results

Responses to the questionnaire were obtained from 53 out of 68 (78% of total) hospitals to which the questionnaires were sent. A total of 135 patients were treated in 28 (53% of response) hospitals during November 2009 and May 2010; 126 patients suffered from hereditary phosphatemic rickets, and 9 had hypophosphatemia caused by other miscellaneous reasons (Fig. 1-A). In this paper, we focused on the 126 patients who had hereditary hypophosphatemic rickets. Patient profiles were as follows: 27 (21%) patients were between 6 mo and 6 yr of age, 39 (31%) patients were between 6 and 12 yr of age, and 60 (48%) patients were more than 12 yr of age (Fig. 1-B). Active vitamin D and phosphate were administered to 123 and 106 patients, respectively. Twelve patients were treated with growth hormone (Fig. 2). The means of the minimum and maximum doses of phosphorus were 37.2 and 58.1 mg/kg/d, respectively, and the dosed were administered in 2 to 6 aliquots (Table 1). Efficacy of the treatment was monitored by various factors including serum phosphate, ALP, intact PTH, urinary Ca/Cr, radiologic features, and growth. In particular, serum phosphate levels were monitored by 18 physicians. The target levels were set between 2 and 3.5 mg/dl. Serum ALP was also used as a marker by 17 physicians. The target levels varied from normal to 2,000 IU/l. In addition, 7 physicians employed intact PTH with target levels varying from normal to twice the normal level.

Table 1 Phosphorus dose and dose frequency

Dose (mg/kg/d)

	Minimum dose	Maximum dose
Mean	37.2	58.1
Range	15–100	30–120

Dose frequency

Physicians that adjust dose frequency: 17

	2 times/d	3 times/d	4 times/d	5 times/d	6 times/d
Minimum	3	9	4	1	–
Maximum	–	1	10	2	4

Physicians that use a fixed frequency: 7

2 times/d	3 times/d	4 times/d	5 times/d	6 times/d
1	–	5	–	1

Discussion

Hereditary hypophosphatemic rickets is often associated with bone deformity, bone pain and growth retardation. Bone deformity sometimes requires surgery for correction. At present, there is no curative therapy for XLH, and active vitamin D and phosphate are administered to correct hypophosphatemia and elevation of ALP (24). However, normalization of the serum phosphate concentration is difficult due to elevation of FGF23, leading to increased excretion of phosphate into urine (25, 26). Insufficient treatment is associated with growth retardation (27). On the other hand, overtreatment with phosphate leads to secondary hyperparathyroidism, and large doses of active vitamin D increase the risk of hypercalciuria (20). Though a recommendation for XLH treatment has been published, it is far from complete cure. Moreover, since phosphate is not a prescribed medicine in Japan, the buffer has to be prepared in the hospital dispensary.

To understand the situation concerning treatment of patients with hereditary hypophosphatemic rickets in Japan, we conducted a questionnaire survey among pediatric endocrinologists. The percentage of the patients with XLH covered by this questionnaire is unclear, but in Japan, it is rare that pediatric nephrologists alone treat patients with XLH.

In the survey, 103 to 106 (82 to 84%) of 123 patients with hereditary hypophosphatemic rickets were treated with both active vitamin D and phosphate. At least 17 (13%) of the patients with hereditary hypophosphatemic rickets were treated with active vitamin D only. Twelve (10%) of the patients with hereditary hypophosphatemic rickets were treated with growth hormone, probably because they had short stature and growth hormone deficiency. The criteria for adjusting the dose of active vitamin D or phosphate buffer were various. One problem is that both serum phosphate and ALP values are age dependent, and normalization of serum phosphate levels and ALP was difficult. X-ray findings are not quantitative, and growth is long term. Thus, these indices are difficult to use in the short term. It is also critical to avoid side effects of the treatment. Thus, the doses of active vitamin D and phosphate should be reduced when hypercalciuria and secondary hyperparathyroidism are observed, respectively.

No information is available concerning the most effective dose of phosphate and how many times it should be administered in the treatment

of patients with hypophosphatemic rickets. In Pediatric Endocrinology and Inborn Errors of Metabolism (28), 40–100 mg/kg/d, divided into 4 to 6 doses, is recommended. However, adherence tends to become poor when short intervals are selected. In this survey, 37.2–58.1 mg/kg/d of phosphorus divided into 3 to 4 doses was most common. Thus, most physicians seemed to treat XLH patients within the recommended way of treatment in the actual clinical setting.

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Serum Fibroblast Growth Factor 23 Is a Useful Marker to Distinguish Vitamin D-Deficient Rickets from Hypophosphatemic Rickets

Takuo Kubota[a, b] Taichi Kitaoka[a] Kohji Miura[a] Makoto Fujiwara[a]
Yasuhisa Ohata[a] Yoko Miyoshi[a] Keiko Yamamoto[a] Shinji Takeyari[c]
Takehisa Yamamoto[c] Noriyuki Namba[a] Keiichi Ozono[a]

[a]Department of Pediatrics, Graduate School of Medicine, and [b]First Department of Oral and Maxillofacial Surgery, Graduate School of Dentistry, Osaka University, and [c]Department of Pediatrics, Minoh City Hospital, Osaka, Japan

Key Words
Vitamin D · Fibroblast growth factor 23 · Rickets · Hypophosphatemia · Children

Abstract
Background/Aims: Vitamin D-deficient rickets (DR) has recently re-emerged among developed countries. Vitamin D deficiency can influence biochemical results of patients with fibroblast growth factor 23 (FGF23)-related hereditary hypophosphatemic rickets (HR), making differential diagnosis difficult. In the present study we evaluated the utility of serum FGF23 levels in the diagnosis of DR and during its treatment. ***Methods:*** The study group comprised 24 children with DR and 8 children with HR. Serum FGF23 levels and bone metabolism-related measurements were assessed. ***Results:*** Serum FGF23 levels in patients with DR were less than 19 pg/ml, while those in patients with HR were more than 57 pg/ml. There were significant differences in serum levels of calcium, phosphate, parathyroid hormone, and 1,25-dihydroxyvitamin D, as well as tubular maximum phosphate reabsorption per glomerular filtration rate between patients with DR and HR, but these values were not fully mutually exclusive. In addition, serum FGF23 and phosphate levels were increased following treatment. ***Conclusion:*** Serum FGF23 level is the most critical biochemical marker for distinguishing DR from HR and might be a good indicator of biochemical response to the intervention. Serum FGF23 levels show utility for the diagnosis of DR and in the assessment of its response to treatment.

Introduction

Rickets is caused by defective mineralization in the growth plate of cartilage and in the matrix of bone in a growing child [1, 2]. Bowed legs, enlargement of the wrists and knees, rachitic rosary, craniotabes, growth retardation, delayed initiation of walking, and waddling gait are often associated with rickets. Diagnosis of rickets requires radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone.

The most common cause of rickets is vitamin D deficiency, although genetic or acquired disorders of the gut, liver, kidney, and metabolism of vitamin D can cause rickets [2]. Increased numbers of patients with vitamin D deficiency have been reported among children in recent years throughout the world [3–5], including Japan [6–9]. Circulating 25-hydroxyvitamin D [25(OH)D] concentration is the best clinical indicator of vitamin D repletion in the body. Vitamin D deficiency is diagnosed by the mea-

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1663–2818/14/0000–0000$39.50/0

E-Mail karger@karger.com
www.karger.com/hrp

Keiichi Ozono, MD, PhD
2-2 Yamadaoka
Suita, Osaka 565-0871 (Japan)
E-Mail keioz@ped.med.osaka-u.ac.jp

surement of serum 25(OH)D concentration below 20 ng/ml in adults [10, 11]. In addition, many experts have commonly proposed a cutoff value of 20 ng/ml for serum 25(OH)D concentration to designate vitamin D deficiency in children [12, 13]. Treatment of vitamin D deficiency with native vitamin D or active vitamin D is effective for the correction of rickets [8, 14].

X-linked hypophosphatemic rickets (HR) is the most common form of heritable rickets and is manifested by fibroblast growth factor 23 (FGF23) excess and renal phosphate wasting [15, 16]. Clinical and radiographic features are mostly similar to vitamin D-deficient rickets (DR). Biochemical findings include hypophosphatemia and low-to-normal circulating 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. Serum concentrations of parathyroid hormone (PTH) are usually normal or modestly elevated in some cases. Other forms of FGF23-related hereditary HR have been described, including an autosomal dominant form caused by mutations in FGF23 and autosomal recessive forms caused by mutations in dentin matrix protein 1 and in ectonucleotide pyrophosphatase/phosphodiesterase 1. The prevalence of these forms of HR appears much less than that of X-linked HR. Serum FGF23 concentrations are increased in patients with HR [17, 18]. FGF23 decreases serum phosphate concentrations by the inhibition of renal proximal tubular phosphate reabsorption and the suppression of 25(OH)D-1α-hydroxylase [19]. Vitamin D and phosphate are necessary for the treatment of HR [15, 20].

In collaboration with other institutes, we previously reported on the diagnostic utility of serum FGF23 measurement in patients with hypophosphatemia [21]. However, it remains unclear whether serum FGF23 measurement is useful for differentiating DR and HR, especially in the case of comorbidity of HR plus vitamin D deficiency. Thus, in the current study, we report the diagnostic utility of serum FGF23 measurements to distinguish patients with DR from those with HR.

Subjects and Methods

Subjects

This study included 32 patients who attended Osaka University Hospital or Minoh City Hospital (Osaka, Japan) from January 2003 through June 2012 and who were diagnosed with DR or HR based on clinical, laboratory, and radiographic findings, as well as clinical course. In detail, the diagnostic criteria of DR included radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone, high serum levels of alkaline phosphatase (AP) and PTH, and low 25(OH)D levels. Vitamin D deficiency was defined as serum 25(OH)D levels less than 20 ng/ml [13]. The diagnosis of DR was confirmed by no recurrence of rickets after discontinuation of treatment. The diagnostic criteria of HR included radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone, low serum phosphate concentrations, and tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR), high AP levels, and normal levels of PTH, 1,25$(OH)_2$D, and 25(OH)D. Although 2 patients did not meet the criteria of HR due to low 25(OH)D levels, they were diagnosed with HR because of high FGF23 levels and resistance to α-calcidol treatment. Other disorders which could develop rickets were excluded, including malabsorption, liver and renal tubular diseases, parathyroid disorders, type I and II vitamin D-dependent rickets, hypophosphatasia, primary disorders of bone matrix, drug-induced mineralization defects, and tumors. Twenty-four patients (11 boys, 13 girls) were diagnosed with DR and 8 (2 boys, 6 girls) with HR. Seven of the 8 patients with HR were sporadic, while 1 patient inherited HR from her mother. Physical examinations were made, and blood and urine samples were taken. Radiography demonstrated rachitic signs in the metaphysis of a long bone in all the patients. Complaints, feeding type before solid food, restricted and/or unbalanced diet, and sunlight exposure were evaluated for DR patients. Dietary content and sun exposure were based on information obtained from parents or guardians. When patients were not given some foods because of concern about allergy, it was considered as a restricted diet. When patients did not take certain foods, it was considered as an unbalanced diet. Playing outside twice a week or less was regarded as insufficient sun exposure. Laboratory data without serum FGF23 levels of 3 DR patients and those with serum FGF23 levels of 2 HR patients were included in previous publications by our group [9, 21]. Measurement of serum FGF23 levels was approved by the institutional review board of Osaka University Hospital and written informed consent was obtained from the parents or guardians of the patients. Patients with DR were treated with α-calcidol suspension because neither cholecalciferol nor ergocalciferol suspension is available on prescription or on the market in Japan.

Measurements

Laboratory measurements included serum levels of calcium (reference range: 8.4–10.0 mg/dl), phosphate (4.2–6.2 mg/dl for the age of 1 year), AP (353–1,009 U/l for the age of 1 year), PTH (10–60 pg/ml), 1,25$(OH)_2$D (20–60 pg/ml), 25(OH)D (the lower limit, 20 ng/ml [13]), and FGF23 (10–50 pg/ml for adults [21]), as well as TmP/GFR (2.7–6.3 mg/dl for the ages 1–24 months [22]) and urine calcium/creatinine ratio (U-Ca/Cr). TmP/GFR was calculated from the formula: TmP/GFR = serum phosphate – urine phosphate × serum creatinine/urine creatinine [23]. Serum 25(OH)D levels were measured in 3 out of 8 with patients with HR. Serum FGF23 levels were measured by an ELISA method that recognizes only full-length biologically active FGF23 (Kainos Laboratories, Japan). The lowest reportable value of FGF23 was 10 pg/ml. Serum 25(OH)D levels were measured by a competitive immunoluminometric direct assay (LIAISON 25OH Vitamin D TOTAL Assay; DiaSorin, USA, 20 samples) and by competitive protein-binding assays (Mitsubishi Chemical Medience, Japan, 6 samples; BML, Japan, 1 sample) because of differences of assay costs.

Statistics

Data were analyzed by a Mann-Whitney U test, ROC analysis, or paired t test using JMP (SAS Institute, USA) and SPSS (IBM SPSS, USA) statistical software.

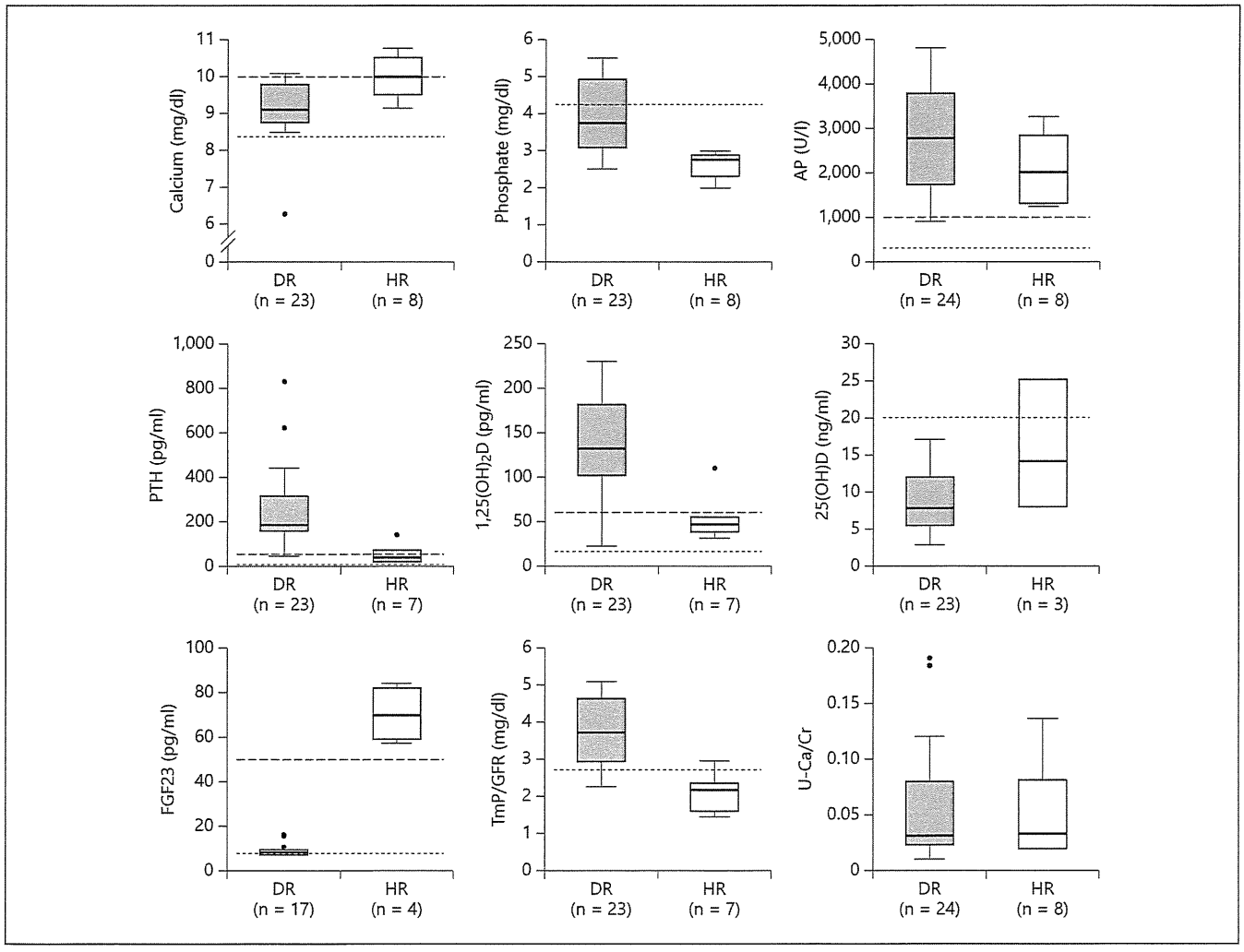

Fig. 1. Biochemical measurements of patients with DR and HR before treatment. Note that only the serum FGF23 level is exclusive between DR and HR. The data are presented as box plots. $p < 0.01$ for calcium, phosphate, PTH, $1,25(OH)_2D$, FGF23, and TmP/GFR. Dashed and dotted lines are the upper and lower limit of reference ranges, respectively.

Results

Clinical Features of DR Patients

Clinical features, including complaints, feeding type before solid foods, restricted and/or unbalanced diet restriction, and sunlight exposure were evaluated in the 24 DR patients. Complaints consisted of bowed legs (n = 18, 75%), elevated serum AP level (n = 5, 21%), and convulsions (n = 1, 4%). Feeding type before solid food was exclusively breast milk (n = 21, 89%) and breast plus formula milk (n = 1, 4%). Twelve patients (50%) had a restricted and/or unbalanced diet. There were 6 patients (25%) with insufficient sun exposure.

Characteristics of DR and HR Patients

There were no differences in age, height, and weight between DR and HR patients. Age was 17 ± 7 (mean ± SD) months, height was −1.3 ± 1.5 SD score (SDS), and weight was −0.5 ± 1.3 SDS in DR patients compared to age 21 ± 8 months, height −1.9 ± 1.0 SDS, and weight −0.1 ± 0.8 SDS in HR patients.

Utility of Serum FGF23 Levels to Distinguish HR and DR Patients

Laboratory findings of DR and HR patients were determined before treatment (fig. 1). Serum calcium concentration was lower in patients with DR than those

Table 1. ROC analysis of biochemical data for the diagnosis of DR and HR

Measurement	AUC	SE	95% CI	p
FGF23	1.00	0.00	1.00, 1.00	<0.01
25(OH)D	0.82	0.11	0.61, 1.04	0.09
Calcium	0.57	0.20	0.17, 0.96	0.72
Phosphate	0.14	0.09	−0.03, 0.32	0.06
$1,25(OH)_2D$	0.12	0.11	−0.10, 0.34	<0.05
PTH	0.11	0.08	−0.05, 0.27	<0.05
TmP/GFR	0.02	0.03	−0.04, 0.09	<0.05

Serum FGF23 level is the most significant measurement for distinguishing patients with DR from those with HR on ROC analysis (1 = DR, 0 = HR).

with HR, although they stayed within the reference range (8.4–10.0 mg/dl) in most patients with DR (fig. 1). Serum phosphate concentration was higher in patients with DR than those with HR. Some patients with DR had reference serum phosphate concentrations (4.2–6.2 mg/dl for the age of 1 year). Serum levels of PTH and $1,25(OH)_2D$ and TmP/GFR were increased in patients with DR compared to those with HR. While there were significant differences in serum levels of calcium, phosphate, PTH, and $1,25(OH)_2D$, as well as TmP/GFR, between patients with DR and with HR, a clear overlap of the data existed between the two groups. Of note, a patient with HR had an obvious increase in serum PTH (145 pg/ml) and $1,25(OH)_2D$ (110 pg/ml). All serum 25(OH)D levels in patients with DR were less than 18 ng/ml. Patients with HR had serum 25(OH)D levels of 8.2, 14.1, and 25.2 ng/ml before the treatment, indicating the presence of vitamin D deficiency in at least 2 patients with HR. Serum FGF23 levels were different and, notably, exclusive between patients with DR and HR. Serum FGF23 levels in all patients with DR were 18 pg/ml or less, while 72% had levels less than 10 pg/ml (reference range for adults: 10–50 pg/ml [21]). In contrast, serum FGF23 levels in patients with HR were 58 pg/ml and more. These results indicate that the measurement of serum FGF23 levels is useful to distinguish patients with DR from those with HR. There were no differences in serum AP levels and U-Ca/Cr between patients with DR and HR. Furthermore, ROC analysis showed the AUC of serum FGF23 had a more significant p value among the measurements, including TmP/GFR, PTH, and $1,25(OH)_2D$ (table 1). These results suggest that serum FGF23 measurements may be more useful compared to measurements including TmP/GFR, PTH, and $1,25(OH)_2D$ to discriminate patients with DR from those with HR.

Response of Serum FGF23 Levels to Intervention among DR Patients

Laboratory data of patients with DR were determined in the period before intervention and 1–3 months following treatment using α-calcidol combined with lifestyle advice for adequate sun exposure and diet (fig. 2). Both data before and 1–3 months after intervention were obtained in 6 patients with DR. Serum levels of FGF23, as well as phosphate, PTH, and AP, were significantly changed after intervention compared to those before. In addition, percent increases in FGF23 levels after intervention were positively correlated with those in serum phosphate concentrations ($p < 0.05$, $r = 0.87$; data not shown), and tended to be inversely correlated with percent decreases in PTH levels ($p = 0.06$, $r = -0.79$). Serum calcium and $1,25(OH)_2D$ levels, TmP/GFR, and U-Ca/Cr were not obviously changed by intervention. These results suggest that serum FGF23 level might be a good indicator of biochemical response to treatment and for lifestyle advice to patients with DR.

Discussion

Our study demonstrated that serum FGF23 is clearly suppressed in infants with DR. Thus, it is suggested that FGF23 is a useful marker to distinguish DR from HR, although not all patients with DR require FGF23 measurement. Indeed, serum levels of calcium, phosphate, PTH, and $1,25(OH)_2D$, as well as TmP/GFR, are also useful to distinguish DR from HR; however, only serum FGF23 levels were mutually exclusive between DR and HR. In addition, serum FGF23 levels were increased following treatment with α-calcidol and lifestyle advice on sun exposure and diet, suggesting that serum FGF23 level might be a good indicator of biochemical response to intervention. At least 2 patients with HR had low serum 25(OH)D levels and one of them had high PTH and $1,25(OH)_2D$ levels. These results suggest hypovitaminosis D may influence the biochemical data of patients with HR and make diagnosis of HR difficult. On the other hand, FGF23 remained high in patients with HR even when hypovitaminosis D is a complicating factor.

Serum PTH levels in patients with HR are usually normal or modestly elevated in some case [16]. However, given that patients with HR may have low serum 25(OH)D

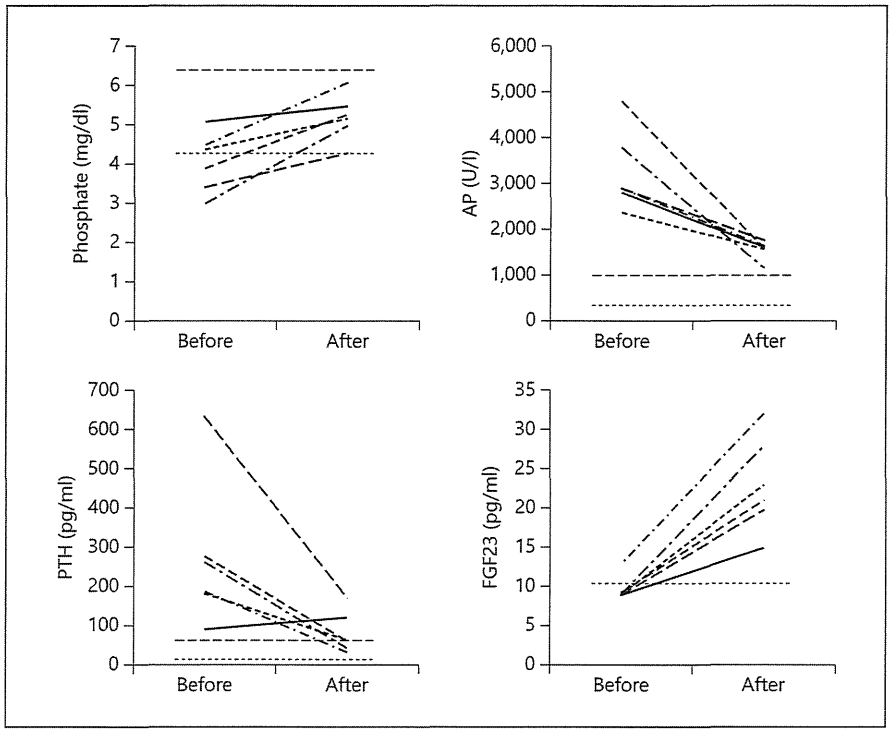

Fig. 2. Response of biochemical measurements to intervention in patients with DR. Serum levels of phosphate, PTH, AP, and FGF23 were significantly changed after the intervention compared to those before. $p < 0.01$ for phosphate, AP, and FGF23; $p < 0.05$ for PTH. Dashed and dotted lines are the upper and lower limit of reference ranges, respectively.

levels, clinicians should bear in mind that high serum PTH levels do not preclude a possibility of HR. In this study, there was a clear and exclusive difference in serum FGF23 levels between infants with DR and HR. These results indicate that a finding of the decrease in serum FGF23 levels is useful for differentiating DR from HR.

It is unclear to what extent serum FGF23 levels are affected in patients with DR. This study showed that serum FGF23 levels were 18 pg/ml or less in 18 infants with DR and was below 10 pg/ml in 72% of these. We previously described a maximum serum FGF23 level of 23.9 pg/ml in hypophosphatemic patients with vitamin D deficiency, Fanconi syndrome, and Cushing's syndrome whose ages ranged from 1 to 75 years [21]. Other researchers have reported that serum intact FGF23 levels were 23.4 pg/ml (mean) in adult females with vitamin D deficiency, a 36% reduction compared to those [36.7 pg/ml (mean)] in healthy control subjects [24]. Serum phosphate concentrations were not reduced in patients with vitamin D deficiency, while bone mineralization was not assessed. In our study, the mean serum FGF23 levels in 18 infants with DR was 8.4 pg/ml, a 71% reduction compared to those [28.9 pg/ml (mean)] in healthy control adults [25], although there could be a difference in FGF23 levels between healthy infants and adults. Thus, serum FGF23 levels in patients with vitamin D deficiency might vary depending on age, serum phosphate concentration, or defective bone mineralization. FGF23 levels less than 19 pg/ml might be useful to distinguish patients with rickets due to vitamin D deficiency from those with rickets due to FGF23 excess who might also be vitamin D deficient.

Serum FGF23 levels in patients with HR were 70.8 ± 11.5 pg/ml (mean ± SD) with a minimum value of 58 pg/ml. This is consistent with previous reports [25, 26] and also our previous study which suggested that FGF23 levels more than 30 pg/ml with hypophosphatemia indicate the presence of excessive FGF23-related diseases such as HR [21].

The nutritional and lifestyle survey of our patients showed that a majority with DR had been exclusively fed with breast milk before solid foods and that some patients had a restricted and/or unbalanced diet and limited sun exposure. Limited vitamin D intake and sun exposure are causes of DR without intrinsic diseases [13]. Supplementation with vitamin D for infants is recommended in many countries [27]. However, no recommendation for vitamin D supplementation is given in Japan. Considering patients with DR were mostly fed exclusively with breast milk, a recommendation concerning vitamin D

supplementation for infants is necessary in Japan to decrease the prevalence of DR.

Patients with DR exhibited an increase in serum PTH and $1,25(OH)_2D$ levels. Although $1,25(OH)_2D$ and PTH are thought to induce the expression of FGF23 in bone [17, 28], this is not the case in our study. The finding of the striking reduction of FGF23 levels in patients with DR indicates hypovitaminosis D and/or a chronic decrease in serum phosphate levels might have more influence on the decreased FGF23 expression, thereby overcoming any increase caused by 1,25(OH)2D and PTH. On the other hand, the patient with HR complicated by vitamin D deficiency displayed an increased FGF23 level, suggesting that the intrinsic genetic abnormality may have more of an impact on FGF23 expression than that caused by vitamin D deficiency. Further study is necessary to elucidate the mechanism(s) regulating FGF23 synthesis in osteocytes.

Serum FGF23 levels were increased after intervention using α-calcidol combined with lifestyle advice. The increases in FGF23 levels were accompanied by increases in serum phosphate concentrations, raising the possibility that increased serum phosphate could increase FGF23. Although FGF23 regulates serum phosphate by inhibiting renal tubular reabsorption, the effects of phosphate on FGF23 remain unclear [29]. However, it is known that $1,25(OH)_2D$ is an important systemic regulator of FGF23 that induces FGF23 expression. Thus, in our study, $1,25(OH)_2D$ derived from α-calcidol might directly increase FGF23. Another possible mechanism for the increased FGF23 is that lifestyle advice for adequate sun exposure and diet might improve hypovitaminosis D although 25(OH)D levels were not measured after intervention.

Our study has several limitations. First, the sample size is small. However, our results indicate that serum FGF23 levels are distinctly decreased in patients with DR compared to those with HR. Second, we did not have a control group composed of healthy infants, although it does not seem ethical to obtain blood samples from healthy infants. Third, genetic analysis was not performed in patients with HR. However, X-linked HR is the most common type of HR and other forms of HR are much less prevalent.

In summary, our study revealed that the measurement of serum FGF23 levels was highly useful for distinguishing infants with HR from those with DR, as serum FGF23 levels were exclusive between DR and HR. It is also suggested that serum FGF23 levels might be a good indication of biochemical response to the intervention in patients with DR.

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Extremely low flow tracheal gas insufflation of helium-oxygen mixture improves gas exchange in a rabbit model of piston-type high-frequency oscillatory ventilation

Atsushi Baba[1†], Tomohiko Nakamura[2*], Tetsuya Aikawa[3†] and Kenichi Koike[1†]

* Correspondence: tnakamura@naganoch.gr.jp
†Equal contributors
[2]Division of Neonatology, Nagano Children's Hospital, Toyoshina, Azumino City, Nagano 399-8288, Japan
Full list of author information is available at the end of the article

Abstract

Objective: The purpose of this study was to show the effects of the tracheal gas insufflation (TGI) technique on gas exchange using helium-oxygen mixtures during high-frequency oscillatory ventilation (HFOV). We hypothesized that a helium-oxygen mixture delivered into the trachea using the TGI technique (0.3 L/min) would enhance gas exchange during HFOV.

Methods: Three rabbits were prepared and ventilated by HFOV with carrier 70% helium/oxygen or 70% nitrogen/oxygen gas mixture with TGI in a crossover study. Changing the gas mixture from nitrogen70% to helium70% and back was performed three times per animal with constant ventilation parameters.

Results: Compared with the nitrogen-oxygen mixture, the helium-oxygen mixture of TGI reduced $PaCO_2$ by 7.6 mmHg ($p < 0.01$) and improved PaO_2 by 14 mmHg ($p < 0.01$). Amplitude during TGI was significantly lower with the helium-oxygen mixture than with the nitrogen-oxygen mixture ($p < 0.01$) and did not significantly affect mean airway pressure.

Conclusions: This study demonstrated that a helium-oxygen mixture delivered into the trachea using the TGI technique would enhance CO_2 elimination and improve oxygenation during HFOV.

Background

In spite of advances in approach and therapeutic benefits of conventional mechanical ventilation in respiratory failure in the neonatal intensive care unit (NICU), ventilator-induced lung injury remains a major problem. This is particularly relevant in patients who need aggressive maintenance of pressures and FiO_2 for adequate oxygenation. In such patients, high-frequency oscillatory ventilation (HFOV) has been considered advantageous for maintaining oxygenation using higher mean airway pressure with minimal risk of complication. During HFOV, tidal volume (Vt) and associated swings in alveolar pressure are very small [1,2]. HFOV has been used in a variety of clinical situations, including neonatal respiratory distress syndrome (RDS), congenital diaphragmatic hernia (CHD), meconial aspiration syndrome (MAS), air leak syndrome and other [3-5]. HFOV showed a number of different mechanisms in addition to bulk convection that have been