Inverse Correlation between the Changes of Lumbar Bone Mineral Density and Serum Undercarboxylated Osteocalcin after Vitamin K₂ (Menatetrenone) Treatment in Children Treated with Glucocorticoid and Alfacalcidol

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Abstract. We have reported that alfacalcidol plus menatetrenone, a vitamin K₂ with four isoprene units (menaquinone-4), treatment is useful for improving bone problems in children with skeletal unloading. The aim of this study was to evaluate the effect of menatetrenone on bone metabolism in long-term glucocorticoid-treated children with alfacalcidol treatment. Twenty children who had been treated with fixed dosages of prednisolone and alfacalcidol (0.03 µg/kg/day) for 24 weeks were enrolled in a prospective pilot study, and assigned to receive alfacalcidol (0.03 µg/kg/day) or alfacalcidol (0.03 µg/kg/day) plus menatetrenone (approximately 2 mg/kg/day). Bone biochemical markers and bone mineral density (BMD) were measured at baseline and after the 12-week treatment. In the group receiving alfacalcidol plus menatetrenone, serum carboxylated osteocalcin (OC) ($p=0.0022$) and lumbar BMD ($p=0.0029$) increased and serum undercarboxylated OC ($p=0.0004$) decreased significantly in comparison to the group receiving alfacalcidol; further, the change of lumbar BMD showed an inverse correlation to the change of serum undercarboxylated OC ($r=-0.744$, $p=0.0134$) and positive correlations to the baseline values of bone turnover markers such as serum levels of intact OC, bone-specific alkaline phosphatase and type I procollagen carboxyl extension peptide and urinary levels of deoxypyridinoline and N-telopeptide of type I collagen. No adverse effect was observed. This is a small short-term study, but its results suggest that menatetrenone effectively and safely increases lumbar BMD probably through carboxylation of OC in long-term prednisolone-treated children with alfacalcidol treatment who have a high bone turnover. Randomized double-blind controlled trials are needed to confirm our findings.

Key words: Glucocorticoid, Osteoporosis, Bone mineral density, Vitamin K, Vitamin D
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Introduction

Bone disorder is a serious adverse effect of long-term

glucocorticoid therapy, and glucocorticoid-induced osteoporosis occurs more severely in children than in adults [1]. Bisphosphonates are efficacious for glucocorticoid-induced osteoporosis in adults [2], however its effect and safety in children are unknown. There are very few reports on the management of glucocorticoid-induced osteoporosis in children, and no effective treatment is established.

Recently, it has been reported that the key patho-

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genetic mechanisms of long-term glucocorticoid-induced osteoporosis are the inhibition of osteoblastogenesis and the promotion of apoptosis of osteoblasts and osteocytes [3]. Vitamin D metabolites such as alfacalcidol have been reported to be efficacious for glucocorticoid-induced osteoporosis, and this effect seems to be linked with an increase of bone formation [4]. However, the effect of vitamin D metabolites is insufficient [3].

Vitamin K, which plays essential roles in bone metabolism [5], increases bone formation [6]. Recently, menatetrenone, a vitamin K₂ with four isoprene units (menaquinone-4), has been reported to increase metacarpal bone mineral density (BMD) [7] and to prevent vertebral fractures [8] in osteoporotic patients. In addition, it has been reported that menatetrenone inhibits apoptosis of human osteoblasts [9] and prevents glucocorticoid-induced bone loss [10, 11]. Menatetrenone promotes 1,25-dihydroxy vitamin D₃-induced osteocalcin (OC) accumulation and mineralization in human osteoblasts [12, 13], and alfacalcidol plus menatetrenone has a synergistic effect on ovariectomy-induced bone loss in rats [14]. Recent clinical studies suggest that a vitamin D metabolite plus menatetrenone treatment increases bone formation [15–17]. In the present prospective pilot study, we attempted to investigate whether menatetrenone effectively and safely improves lumbar BMD in long-term glucocorticoid-treated children with alfacalcidol treatment.

Materials and Methods

Subjects and treatment

The study was carried out prospectively at Yamaguchi University Hospital with groups of patients being treated during the same time period, from July to October in 1998, because a circannual rhythm was observed for serum undercarboxylated OC [18]. The protocol was approved by the Institutional Review Board at Yamaguchi University Hospital, and all children and/or their parents gave their written informed consent. Patients were eligible if (i) they were ambulatory growing Japanese children aged between 4 and 14 years, (ii) they had been treated with oral prednisolone for over a year, (iii) their disease conditions, dosages of prednisolone,

walking activities measured using a pedometer (HJ-003, Omron Co., Kyoto, Japan) and calcium intakes had not changed for the preceding 24 weeks, and (iv) they had received alfacalcidol (0.03 μ g/kg/day) for the preceding 24 weeks. Patients were excluded if (i) they had abnormalities of liver or kidney function, or on a spinal radiograph, (ii) they had diseases or had taken medications known to affect bone metabolism, or (iii) their disease conditions, dosages of prednisolone, walking activities or calcium intakes changed during the treatment. Patients were assigned to receive alfacalcidol (0.03 μ g/kg/day) or alfacalcidol (0.03 μ g/kg/day) plus menatetrenone (approximately 2 mg/kg/day). Patients with reduced walking activity and lower calcium intake, which are great disadvantages, were assigned to the group receiving alfacalcidol plus menatetrenone. Alfacalcidol was given once after dinner, and menatetrenone was given in 2 divided doses after breakfast and dinner daily. Patients took these medicines orally within 30 minutes after a meal.

Measurements of bone biochemical markers and bone mineral density

Bone biochemical markers and BMD were measured at baseline and after the 12-week treatment. Venous blood and urine samples were collected between 0900h and 1000h after an overnight fast, and stored at -80°C . All bone biochemical markers were measured at Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan) including serum intact OC (immunoradiometric assay, Mitsubishi Chemical Co., Tokyo, Japan), serum carboxylated and undercarboxylated OC (enzyme-linked immunosorbent assay, Takara Biomedicals, Shiga, Japan), serum bone-specific alkaline phosphatase (enzyme immunoassay, Metra Biosystems, Mountain View, CA), serum type I procollagen carboxyl extension peptide (radioimmunoassay, Orion Diagnostica, Helsinki, Finland), serum and urinary calcium (o-cresolphthalein complexone method), plasma intact parathyroid hormone (immunoradiometric assay, Nichols Institute, Capistrano, CA), urinary deoxypyridinoline (high-performance liquid chromatography), urinary N-telopeptide of type I collagen (enzyme-linked immunosorbent assay, Ostex International, Seattle, WA). Human OC contains three residues of γ -carboxyglutamic acid at positions 17,

21 and 24 of the amino acid sequence, and the carboxylated OC kit recognizes the position 17 alone whereas the undercarboxylated OC kit recognizes the position 21 and 24 [8, 19]. Anteroposterior second to fourth lumbar BMD was measured by dual energy X-ray absorptiometry using the array mode of QDR-4500A (Hologic, Waltham, MA). The precision error for anteroposterior lumbar BMD in children was less than 1.0%.

Statistical analysis

The data are presented as mean $\pm$ SD. To compare the two groups at baseline, Fisher's exact test was done for sex, puberty, and vitamin D receptor genotype, and Student's or Welch's *t* test, or Mann-Whitney U test were done for other characteristics. The change from baseline was assessed as the percentage change for each individual patient, and the Mann-Whitney U test was done to compare the percentage changes after the treatment in the two groups. In the group receiving alfacalcidol plus menatetrenone, Pearson correlated analysis was done for the baseline values as well as the percentage changes of the parameters, and forward stepwise regression analysis was carried out between the percentage changes of lumbar BMD and other parameters. All these analyses were performed with a significance of two-sided $p < 0.05$.

Results

Baseline characteristics

A total of 20 patients were enrolled at baseline, and 2 patients withdrew because their disease conditions and dosages of prednisolone changed. The baseline characteristics of the patients are summarized in Table 1. The group receiving alfacalcidol plus menatetrenone had significantly reduced walking activity ($p=0.0156$) and lower calcium intake ($p=0.0183$) than the group receiving alfacalcidol. Vitamin D receptor genotype, which may be associated with the effect of alfacalcidol [20], was similar in the two groups.

Changes of bone biochemical markers

In the group receiving alfacalcidol plus menatetrenone, plasma menaquinone-4 increased markedly (153.02 ± 230.58 nmol/L), and serum carboxylated OC increased and serum undercarboxylated OC decreased in all patients (Fig. 1). The changes in bone biochemical markers are shown in Table 2. The group receiving alfacalcidol plus menatetrenone showed significantly increased serum carboxylated OC ($p=0.0022$), decreased serum undercarboxylated OC ($p=0.0004$), and decreased serum calcium corrected by serum albumin ($p=0.0144$) in comparison to the group receiving alfacalcidol. Plasma intact parathyroid hormone as well as urinary bone resorption markers in the group receiving alfacalcidol plus menatetrenone were lower than those in the group receiving alfacalcidol, though the differences were not significant. In the group receiving alfacalcidol plus menatetrenone, the change of the corrected serum calcium was correlated to the changes of plasma intact parathyroid hormone ($r=-0.748$, $p=0.0128$) and urinary deoxypyridinoline ($r=0.666$, $p=0.0357$).

Change of bone mineral density

The group receiving alfacalcidol plus menatetrenone showed significantly increased lumbar BMD ($p=0.0029$) in comparison to the group receiving alfacalcidol (Table 2). In the group receiving alfacalcidol plus menatetrenone, the change of lumbar BMD showed an inverse correlation to the change of serum undercarboxylated OC (Fig. 2; $r=-0.744$, $p=0.0134$), while it showed no correlation to the change of serum carboxylated OC ($r=0.067$, $p=0.8531$). In addition, the change of lumbar BMD was positively correlated to the baseline values of bone turnover markers in the group receiving alfacalcidol plus menatetrenone (Table 3). By forward stepwise regression analysis on the change of lumbar BMD in the group receiving alfacalcidol plus menatetrenone, the changes of serum carboxylated OC, undercarboxylated OC and the corrected calcium were selected, and the applied coefficient R-square was 0.824. All the explanatory variables were squared to explain the dependent variable.

Table 1. Baseline characteristics of the patients

Treatment	alfacalcidol (n=8)	alfacalcidol plus menatetrenone (n=10)
Age (years)		
Total	10±3, range 4–14	9±3, 5–14
Boys	7±4, 4–11	8±4, 5–13
Girls	11±1, 9–14	10±3, 5–14
Sex (no.)		
Boys	2	3
Girls	6	7
Puberty (no.)		
Pre	3	6
Post	5	4
Body mass index (kg/m ²)	18.6±2.2	18.3±3.8
Walking activity (steps/day)	15326±5712	9707±4073 ^{a)}
Calcium intake (mg/day)	659±167	486±141 ^{b)}
Prednisolone therapy		
Duration (months)	33±15, 16–57	37±13, 15–57
Dose (mg/kg/day)	0.18±0.10, 0.06–0.34	0.22±0.05, 0.12–0.29
Vitamin D receptor genotype (no.)		
bb	6	7
Bb	2	3
Serum		
Intact osteocalcin (μg/L)	26.1±14.2	27.9±22.1
Carboxylated osteocalcin (μg/L)	12.4±3.8	14.8±9.5
Undercarboxylated osteocalcin (μg/L)	22.2±18.5	19.8±18.0
Bone-specific alkaline phosphatase (U/L)	83.5±49.7	88.0±42.9
Type I procollagen carboxyl extension peptide (μg/L)	192.8±80.2	245.3±98.8
Calcium corrected by serum albumin (mg/dL)	9.3±0.4	9.7±0.4
Inorganic phosphorus (mg/dL)	4.3±0.7	4.7±0.7
25-Hydroxy vitamin D (nmol/L)	90.1±20.7	100.3±26.0
1,25-Dihydroxy vitamin D (pmol/L)	88.9±20.8	99.3±35.9
Plasma		
Intact parathyroid hormone (ng/L)	13.5±3.5	15.3±6.9
Vitamin K ₁ (nmol/L)	1.11±1.00	0.84±0.40
Menaquinone-4 ^{c)} (nmol/L)	0.79±0.45 (n=4)	0.61±0.54 (n=4)
Urinary		
Deoxypyridinoline (pmol/μmol creatinine)	22.4±12.3	29.5±17.0
N-telopeptide of type I collagen (nmol bone collagen equivalents/mmol creatinine)	434.0±346.3	541.9±446.1
Calcium (mg/mg creatinine)	0.21±0.12	0.20±0.16
Lumbar bone mineral density (g/cm ²)	0.704±0.147	0.643±0.149
Underlying disease (no.)		
Juvenile rheumatoid arthritis	1	3
Systemic lupus erythematosus	1	2
Dermatomyositis	0	2
Muscular dystrophy	1	0
Myasthenia gravis	1	0
Autoimmune hepatitis	1	0
Lymphoid interstitial pneumonia	0	1
IgA nephropathy	0	1
Nephrotic syndrome	2	1
Membranoproliferative glomerulonephritis	1	0

Mean ± SD.

^a p=0.0156, ^b p=0.0183 vs the group receiving alfacalcidol (Student's *t* test).^c Plasma menatetrenone <0.23 nmol/L in 4 patients of the group receiving alfacalcidol and 6 patients of the group receiving alfacalcidol plus menatetrenone.

Fig. 1. Serum carboxylated osteocalcin (OC) (A) and serum undercarboxylated OC (B) of each patient at baseline and at 12 weeks in the group receiving alfacalcidol plus menatetrenone ($n=10$).

Adverse effects

None of the patients had any fracture, and no adverse effect was observed during the 12-week treatment.

Discussion

This is the first report showing the effect of vitamin K on bone metabolism in glucocorticoid-treated chil-

dren. We analyzed children whose disease conditions and dosages of prednisolone had not been changed from 24 weeks before the study to the end of 12-week study period, in order to evaluate strictly the effect of menatetrenone. Fixed disease conditions and dosages of glucocorticoids would be needed for a strict evaluation of treatment. The 12-week short treatment period was determined, because, in children, (i) disease conditions and dosages of glucocorticoids are generally not maintained for a long duration and (ii) the change of BMD is very rapid. The change from baseline was assessed as the percentage change for each individual patient, because the values of bone biochemical markers and BMD in children are dependent on many factors such as sex, age, pubertal status and underlying disease.

In the group receiving alfacalcidol plus menatetrenone, lumbar BMD increased significantly in comparison to the group receiving alfacalcidol and the change of lumbar BMD showed an inverse correlation to the change of serum undercarboxylated OC, although the group receiving alfacalcidol plus menatetrenone had lower walking activity and calcium intake. These results suggest that menatetrenone improved lumbar BMD probably through carboxylation of OC. In contrast, there was no correlation between the changes of lumbar BMD and those of serum carboxylated OC. The reason for the lack of correlation is unknown, but it is possibly due to the stimulated incorporation of OC into bone matrix, or because the carboxylated OC kit detected the carboxylation of OC with less sensitivity than the undercarboxylated OC kit, that is, the carboxylated OC kit recognizes position 17 alone whereas the undercarboxylated OC kit recognizes positions 21 and 24 [8, 19]. OC has been reported to stimulate bone mineral maturation [21], therefore menatetrenone may act mainly on mineralization. This effect may be dependent on the inhibition of apoptosis of human osteoblasts by menatetrenone [9], because menatetrenone promotes 1,25-dihydroxy vitamin D₃-induced mineralization in human osteoblasts [13]. The change of lumbar BMD was positively correlated to the baseline values of bone turnover markers, and menatetrenone may increase lumbar BMD in conditions with a high bone turnover. Recently, not only vitamin K but also insulin-like growth factor (IGF)-I was reported to modulate carboxylated OC synthesis [22]. Glucocorticoids decrease the expression of

Table 2. Percentage changes after the treatment in bone biochemical markers and bone mineral density

Treatment	alfacalcidol (n=8)	alfacalcidol plus menatetrenone (n=10)
Serum		
Intact osteocalcin	+10.0±32.6	+16.3±68.8
Carboxylated osteocalcin	-3.5±38.0	+111.1±82.0 ^{a)}
Undercarboxylated osteocalcin	+13.7±49.4	-87.0±12.4 ^{b)}
Bone-specific alkaline phosphatase	-4.0±17.3	+0.5±13.7
Type I procollagen carboxyl extension peptide	-8.0±14.4	+2.1±23.3
Calcium corrected by serum albumin	+1.1±3.5	-3.2±3.0 ^{c)}
Inorganic phosphorus	-1.5±12.9	-12.6±7.7
25-Hydroxy vitamin D	-12.6±25.3	-20.4±8.8
1,25-Dihydroxy vitamin D	-2.8±29.4	+7.7±32.8
Plasma		
Intact parathyroid hormone	+4.2±43.8	-20.6±29.8
Vitamin K ₁	+51.8±121.1	+42.7±112.0
Urinary		
Deoxypyridinoline	-3.6±25.5	-13.8±24.6
N-telopeptide of type I collagen	+17.7±89.4	+3.1±41.6
Calcium	+86.3±212.0	+54.0±102.5
Lumbar bone mineral density	-1.3±2.4	+4.1±3.2 ^{d)}

Mean±SD. ^a p=0.0022, ^b p=0.0004, ^c p=0.0144, ^d p=0.0029 vs the group receiving alfacalcidol (Mann-Whitney U test).

Fig. 2. Correlation between the percentage changes of lumbar bone mineral density (BMD) and serum undercarboxylated osteocalcin (OC) ($r = -0.744$, $p = 0.0134$) in the group receiving alfacalcidol plus menatetrenone ($n = 10$).

IGF-I and IGF-binding proteins (IGFBPs) such as IGFBP-3 and IGFBP-5 which enhance IGF activity [23]. Therefore, menatetrenone may promote carboxylated OC synthesis which is suppressed by decreased IGF-I activity.

In the present study as well as a recent clinical study [17], serum intact OC showed no significant difference between the groups receiving a vitamin D metabolite and a vitamin D metabolite plus menatetrenone, although menatetrenone [8, 17, 24] has been reported to increase serum OC. OC synthesis is transcriptionally stimulated by vitamin D and its post-translational carboxylation is dependent on vitamin K [5], and a possible interaction between vitamins D and K is indicated by the finding that vitamin D improved the carboxylation status of OC [18]. Vitamin D metabolites are known to increase serum OC which is decreased by glucocorticoids [25]. Vitamin D receptors themselves have been suggested to be γ -carboxyglutamic acid proteins [26], therefore the increase of serum intact OC induced by vitamin K may be associated with a vitamin D receptor-mediated effect. This may be the reason why serum intact

Table 3. Correlation between the percentage change of lumbar bone mineral density and the baseline values of bone turnover markers in the group receiving alfacalcidol plus menatetrenone (n=10)

	r	p value
Serum		
Intact osteocalcin	0.633	0.0482
Bone-specific alkaline phosphatase	0.681	0.0280
Type I procollagen carboxyl extension peptide	0.906	<0.0001
Urinary		
Deoxypyridinoline	0.811	0.0028
N-telopeptide of type I collagen	0.621	0.0543

OC did not increase in the present study.

Menatetrenone inhibits bone resorption [27, 28], and this process does not seem to be mediated through carboxylation. The decrease of the corrected serum calcium could be induced, in part, by decreased bone resorption, and the inverse correlation between the corrected serum calcium and plasma intact parathyroid hormone would result from the action of parathyroid hormone. In addition, menatetrenone has been reported to increase intestinal calcium absorption [29]. The decrease of plasma intact parathyroid hormone may result from a vitamin D receptor-mediated effect. These might be another mechanism by which menatetrenone could improve lumbar BMD.

This is a small short-term study, but its results suggest that menatetrenone effectively and safely improves lumbar BMD probably through carboxylation of OC in long-term prednisolone-treated children

with alfacalcidol treatment who have a high bone turnover. Randomized double-blind controlled trials are needed to confirm our findings.

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