levels in patients with ADHR change with time and are not always high [18]. In some patients with ADHR, hypophosphatemia disappears with normalization of circulatory FGF23. Therefore, the regulatory mechanisms of FGF23 production seem to be disrupted when patients with mutations in *FGF23* show hypophosphatemia and high FGF23 levels. Increased FGF23 levels have been also reported in hypophosphatemic patients with McCune-Albright syndrome and linear sebaceous nevus syndrome [19,20]. Recently, it was shown that mutations in family with sequence similarity 20, member C (*FAM20C*) cause FGF23-related hypophosphatemic disease with dental anomalies and ectopic calcification [21].

In addition to these genetic disorders, FGF23 also causes hypophosphatemic rickets/osteomalacia in acquired diseases. Typical examples are TIO and hypophosphatemic osteomalacia by administration of intravenous iron. It has been shown that certain preparations of intravenous iron such as saccharated ferric oxide and iron polymaltose cause hypophosphatemic osteomalacia. Evaluation of these hypophosphatemic patients showed that FGF23 was high [22,23]. Because FGF23 levels in hypophosphatemic patients with vitamin D deficiency and Fanconi syndrome are rather low [24], these results suggest that intravenous iron caused hypophosphatemia by increasing FGF23 levels. In addition, single injection of iron polymaltose in patients with iron-deficiency anemia increased FGF23 and impaired tubular reabsorption of phosphate [25]. Therefore, these iron preparations seem to cause hypophosphatemia by high FGF23 levels. However, the administration of dextrin citrato-iron (III) complex caused neither high FGF23 nor hypophosphatemia [23]. Therefore, it is not clear how these iron preparations cause increased levels of FGF23.

TIO

TIO is a paraneoplastic syndrome usually caused by slow-growing mesenchymal tumors. Most tumors responsible for TIO are now pathologically classified as phosphaturic mesenchymal tumor, mixed connective tissue variant [26]. It is not uncommon for patients with TIO to complain of severe muscle weakness and bone pain that profoundly affect quality of life. However, this disease can be cured by complete removal of responsible tumors, indicating that some humoral factor causes this disease. As in other FGF23-related hypophosphatemic diseases, patients with TIO show hypophosphatemia with impaired proximal tubular phosphate reabsorption. In addition, serum $1,25(OH)_2D$ is low to low normal. Furthermore, FGF23 is elevated in most patients with TIO and rapidly decreases after successful removal of responsible tumors [27,28]. All these results indicate that FGF23 is the principal humoral factor causing TIO.

Several other factors obtained from tumors in patients with TIO have been reported to show activities that inhibit phosphate transport in kidney cells, cause hypophosphatemia *in vivo* or impair mineralization of bone. These include secreted frizzled-related protein 4, matrix extracellular phosphoglycoprotein and FGF7 [29-31]. However, none of these humoral factors have been shown to be elevated in patients with TIO. Therefore, it is unlikely that one of these humoral factors works as a principal agent for the development of TIO. Still, it is possible that these factors work together with FGF23 and contribute to at least some aspects of TIO or other FGF23-related hypophosphatemic diseases.

ISSUES IN THE DIAGNOSIS OF TIO

As mentioned above, TIO is a curable disease by complete resection of the responsible tumors. Therefore, it is of pivotal clinical importance to locate the causative tumors in patients with TIO. However, the responsible tumors for TIO are often small and exist within bone, making them difficult to find. Several systematic imaging studies including skeletal survey by magnetic resonance imaging (MRI), or [^{18}F]fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT), have been used for the detection of the responsible tumors for TIO [32,33]. In addition, somatostatin receptor scintigraphy has been shown to be useful in at least some patients with TIO because mesenchymal tumors often express various types of somatostatin receptors [34-36]. However, TIO is not a common disease and there are no large scale studies examining the utility of these imaging studies in the detection of responsible tumors for TIO.

Functional tumors like aldosterone-producing adenomas and adrenocorticotropic hormone-producing pituitary adenomas can be localized by venous sampling. This method is based on the assumption that the responsible tumor is the major or only source of the hormone in the patient. For example, in a patient with primary aldosteronism by single adenoma, aldosterone production from the contralateral adrenal gland is suppressed, resulting in a greater difference in aldosterone levels between adrenal veins. In patients with TIO, FGF23 levels rapidly decrease after complete removal of responsible tumors [37]. In our experience, FGF23 decreased to lower

than the lower limit of the reference range in successfully operated patients and became undetectable in five out of six patients (Fig. 1). These results suggest that FGF23 production from normal bone is suppressed and the causative tumors are the main or only sources of FGF23 in patients with TIO, raising the possibility that venous sampling is useful for the detection of responsible tumors. Other studies have also examined whether or not it is possible to identify the responsible tumors using the fact that TIO is caused by secretion of FGF23 from these tumors. We previously demonstrated a patient with hypophosphatemic osteomalacia who had noticed a subcutaneous tumor in the right inguinal region [37]. We suspected that this tumor was the cause of his hypophosphatemic osteomalacia. However, the patient reported that the tumor had been present for many years before the onset of symptoms. Therefore, we wanted to prove that the tumor was actually producing FGF23 and causing TIO in this patient. We collected venous samples from all of the major veins through a catheter inserted from the right femoral vein and measured FGF23 levels. The results showed that there was some difference in FGF23 levels in the veins of this patient, and FGF23 levels were higher in the draining and adjacent vein to the tumor. These results suggested that systemic venous sampling is useful for the identification of tumors responsible for TIO.

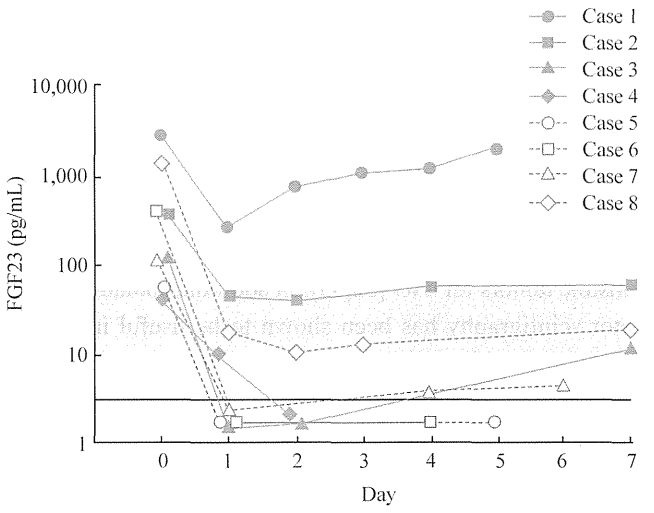

Fig. 1. Changes in fibroblast growth factor 23 (FGF23) levels after removal of responsible tumors for tumor-induced rickets/osteomalacia (TIO). Responsible tumors for TIO were operated on day 0 and FGF23 levels were followed for up to 7 days. FGF23 was measured by an enzyme-linked immunosorbent assay that detects full-length FGF23 with a detection limit of 3 pg/mL (The horizontal line). The shaded area indicates the reference range for FGF23 (10 to 50 pg/mL). In six patients who were cured by the operation, FGF23 became undetectable in five patients within 2 days. In addition, FGF23 was below the lower limit of the reference range on day 2 in patient 8. FGF23 increased after the initial drop in patient 1 and 2, and hypophosphatemic osteomalacia was not cured in these patients.

Since then, we have prospectively conducted systemic venous sampling in patients with suspected TIO [38]. We collected up to 22 samples from all the major veins in each patient and measured FGF23 levels. If there was a difference in FGF23 levels, subsequent imaging studies such as CT and MRI were conducted aimed at the region with high FGF23 levels. Using this method, we were able to identify responsible tumors in eight out of ten patients with suspected TIO in this series. However, FGF23 levels were virtually the same throughout the body in two patients and we could not find the tumors in those two patients. Fig. 2 shows another example of the results of venous sampling. FGF23 was high in this patient's left external iliac vein and a tumor was found in the left femoral head.

Results of systemic venous sampling in 14 patients were also reported by another group [39]. They divided patients into two groups, with or without suspicious sites by prior imaging studies including octreotide scintigraphy, FDG-PET/CT, MRI,

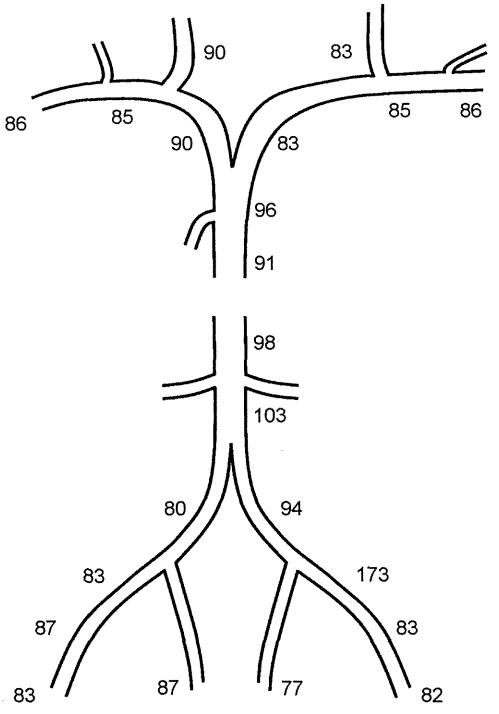

Fig. 2. Fibroblast growth factor 23 (FGF23) levels obtained in venous sampling. FGF23 was high in patient's left external iliac vein and a tumor was found in the left femoral head. The unit of FGF23 is pg/mL.

and CT. They concluded that sampling is useful for patients with suspicious sites in order to confirm that the detected tumors are responsible for TIO. However, they indicated that this sampling is not useful in the absence of suspicious lesions by imaging studies. They have conducted venous sampling after imaging studies and this approach is different from ours. However, they could not find any difference in FGF23 levels in four patients without suspicious lesions, similar to two patients in our series. Therefore, it is clear that systemic venous sampling is not a perfect way to localize the responsible tumors for TIO. However, it is not yet known which imaging study is best for the detection of causative tumors for TIO. While octreotide scintigraphy has been shown to be useful in some cases, the sensitivity and specificity of this scintigraphy is unknown, partly because of the rarity of TIO. In addition, this scintigraphy is not available in several countries, including Japan. Therefore, several methods should be used to identify the responsible tumors and we believe that venous sampling is useful for at least some patients with suspected TIO as shown in several case reports [40-43].

CONCLUSIONS

TIO has been a difficult disease for clinicians. There was no specific biochemical marker for this disease before the identification of FGF23. The identification and the establishment of an assay method for FGF23 made the diagnosis and follow-up of patients with TIO easier. Still, while the definitive diagnosis of TIO depends on finding the causative tumors, there are no standard or perfect ways of finding them. Even if the responsible tumors are found, it is possible that these tumors cannot be completely removed because of the location of the tumors or the condition of the patients. While medical treatment with neutral phosphate and active vitamin D improves the symptoms of affected patients to some degree, these medications can be associated with adverse events such as diarrhea and secondary-tertiary hyperparathyroidism. In this respect, it is interesting to see whether the inhibition of FGF23 activity is useful for patients with hypophosphatemic rickets/osteomalacia caused by excessive activity of FGF23 (Table 1). Several methods such as anti-FGF23 antibodies, the C-terminal fragment of FGF23 that compete with intact FGF23 for the binding to the Klotho-FGF receptor complex, an inhibitor of extracellular signal-regulated kinase, and inhibition of FGF receptor have been shown to antagonize the actions of FGF23 and increase phosphate level in animals [44-48]. However, there are no data on the utility of these methods in humans except for anti-FGF23 antibody [49]. Clearly, better diagnostic methods for the localization of causative tumors and treatments are needed for patients with TIO.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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Active vitamin D possesses beneficial effects on the interaction between muscle and bone

Ken-ichiro Tanaka [a], Ippei Kanazawa [a,*], Toru Yamaguchi [a], Shozo Yano [b], Hiroshi Kaji [c], Toshitsugu Sugimoto [a]

[a] Internal Medicine 1, Shimane University Faculty of Medicine, Izumo, Japan
[b] Department of Laboratory Medicine, Shimane University Faculty of Medicine, Izumo, Japan
[c] Department of Physiology and Regenerative Medicine, Kinki University Faculty of Medicine, Osakasayama, Japan

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ABSTRACT

Vitamin D deficiency and advanced glycation end products (AGEs) are suggested to be involved in the pathogenesis of osteoporosis and sarcopenia. However, the effects of vitamin D and AGEs on myogenesis and the interaction between muscle and bone remains still unclear. We previously showed that osteoglycin (OGN) is secreted from myoblasts and stimulates osteoblastic differentiation, suggesting that it plays important roles in the interaction between muscle and bone. The aim of this study is thus to examine the effects of vitamin D and AGEs on myoblastic differentiation of C2C12 cells and osteoblastic differentiation of osteoblastic MC3T3-E1 cells through OGN expression. 1α,25-dihydroxyvitamin D_3 (1,25D) and eldecalcitol, an active vitamin D analog, induced the expression of MyoD, myogenin and OGN, and these effects were abolished by vitamin D receptor (VDR) suppression by siRNA in C2C12 cells. Moreover, conditioned medium from 1,25D-pretreated C2C12 cells stimulated the expression of type 1 collagen and alkaline phosphatase in MC3T3-E1 cells, compared to control medium from 1,25D-untreated C2C12 cells. In contrast, conditioned medium from VDR-suppressed and 1,25D-pretreated C2C12 cells showed no effects. AGE2 and AGE3 suppressed the expression of MyoD, myogenin and OGN in C2C12 cells. Moreover, 1,25D blunted the AGEs' effects. In conclusion, these findings showed for the first time that active vitamin D plays important roles in myogenesis and muscle-induced osteoblastogenesis through OGN expression. Active vitamin D treatment may rescue the AGEs-induced sarcopenia as well as – suppressed osteoblastic differentiation via OGN expression in myoblasts.

1. Introduction

The number of patients with aging-related diseases, such as sarcopenia and osteoporosis, has been increasing rapidly worldwide in recent years. Indeed, it is reported that more than 30% in elderly people over the age of 80 years suffered from sarcopenia and/or osteoporosis [1]. A number of studies on the causes and treatments of sarcopenia have been performed. Several clinical studies have shown that vitamin D deficiency is associated with an increased risk of falls, and that vitamin D supplementation reduces the risk in vitamin D-deficient patients [2–5]. 1α,25-dihydroxyvitamin D_3 (1,25D) enhanced myoblastic differentiation through modulating growth factors and fast-myosin heavy chain expression in mouse myoblastic C2C12 cells [6,7]. On the other hand, there are also numerous studies on the effects of vitamin D on bone cells such as osteoblasts, osteoclasts, and osteocytes. Regarding the effects of vitamin D on bone formation, several studies reported that 1,25D exerted direct effects on osteoblasts and induced their differentiation and mineralization via the vitamin D receptor (VDR) [8,9]. These findings indicate that vitamin D may be essentials to muscle and bone strength.

Cumulative evidence has shown that there is a positive correlation between lean body mass and bone mineral density (BMD), suggesting that muscle and bone are related to each other [10]. Although the mechanism of the interaction between muscle and bone is still unclear, we recently showed that osteoglycin (OGN), which is the seventh member of the small leucine-rich proteoglycans and was initially isolated from bovine bone as an inducer of matrix mineralization [11], was a crucial humoral factor linking muscle to bone [12]. OGN overexpression in myoblastic cells

* Corresponding author. Address: Department of Internal Medicine 1, Shimane University Faculty of Medicine, Izumo 693-8501, Japan. Fax: +81 853 23 8650.
E-mail addresses: ken1nai@med.shimane-u.ac.jp (K.-i. Tanaka), ippei.k@med.shimane-u.ac.jp (I. Kanazawa), yamaguch@med.shimane-u.ac.jp (T. Yamaguchi), syano@med.shimane-u.ac.jp (S. Yano), hkaji@med.kindai.ac.jp (H. Kaji), sugimoto@med.shimane-u.ac.jp (T. Sugimoto).

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induced osteoblast phenotype and mineralization in osteoblastic cells, suggesting that OGN is an important molecule for the interaction between muscle and bone tissues. However, regulatory factors of OGN are not reported so far.

It has been shown that the risk of osteoporotic fracture is increased in diabetic patients [13]. Advanced glycation end products (AGEs) are produced by non-enzymatic reactions of carbohydrates with proteins especially in diabetic status and elderly people [14,15]. AGEs adversely affect bone: we showed that the combination of high glucose and AGE2 or AGE3 inhibited the mineralization of osteoblastic MC3T3-E1 cells, and that AGE2 or AGE3 inhibited the osteoblastic differentiation and mineralization of mouse stromal ST2 cells [16–19]. On the other hand, several studies have shown that sarcopenia is associated with diabetes mellitus [20–23]. Previous studies showed that serum AGE levels were significantly correlated with weak grip strength and walking disability in elderly women [24,25]. These findings suggest that AGEs adversely affect both bone and muscle tissues, causing osteoporosis and sarcopenia, respectively, in type 2 diabetes. However, there are no studies on the direct effects of AGEs on myoblasts and osteoinductive factors derived from muscle.

In this study, we thus examined the roles of active vitamin D in the interaction between muscle and bone. In addition to 1,25-dihydroxyvitamin D_3 (1,25D), 25-hydroxyvitamin D_3 (25D), and 24,25-dihydroxyvitamin D_3 (24,25D), we used eldecalcitol (ELD), which is a novel analog of active vitamin D and recently became available for clinical use, because there are no studies on its direct effects on myoblasts. Moreover, we investigated whether or not active vitamin D can rescue the AGEs' adverse effects on myoblastic differentiation.

2. Materials and methods

2.1. Materials

Recombinant 1,25D, 25-hydroxyvitamin D_3 (25D), and 24,25-dihydroxyvitamin D_3 (24,25D), and ELD are kindly provided by Chugai Pharma. Anti-β-actin antibody was obtained from Sigma–Aldrich Corp (St. Louis, MO). Anti-Alkaline Phosphatase (ALP), anti-OGN, anti-myogenin, anti-MyoD, anti-VDR antibodies, VDR siRNA, and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-type 1 collagen (Col1) antibody was from Calbiochem and Ingenex Corp. (San Diego, CA, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

Mouse myoblastic C2C12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). The cells were cultured in DMEM with 2% horse serum for 6 days to differentiate into myotube. Mouse osteoblastic MC3T3-E1 cells were provided by Dr. H. Komada (Ohu Dental Collage, Koriyama, Japan). The cells were cultured in α-MEM with 10% FBS and 1% penicillin–streptomycin. The medium was changed twice a week.

2.3. Conditioned medium collection

After reached cell confluent, C2C12 cells were incubated with or without 1,25D for 2 days. After that, the cells were washed and incubated in DMEM without FBS and 1,25D for 24 h. Then, the medium was collected and stored at −80 °C. The medium from vitamin D-untreated cells was used as controls. The conditioned medium was added at 20% of final concentration in α-MEM when effects of the conditioned medium on MC3T3-E1 cells were examined.

2.4. Preparation of AGEs

AGE2, AGE3, and nonglycated BSA were prepared as previously described [16–18]. AGE2 and AGE3 were prepared by incubating 50 mg/mL BSA (Sigma, St. Louis, MO) with 0.1 M DL-glyceraldehyde (Nacalai Tesque, Kyoto, Japan) and 0.1 M glycolaldehyde (Sigma), respectively, under sterile conditions in 0.2 M phosphate buffer (pH 7.4) containing 5 mM diethylene-triamine-pentaacetic acid (DTPA) at 37 °C for 7 days. Nonglycated BSA was incubated under the same conditions except for the absence of DL-glyceraldehyde or glycolaldehyde as a negative control. Then low molecular weight reactants and aldehydes were removed using a PD-10 column chromatography and dialysis against phosphate-buffered saline (PBS).

2.5. Protein extraction and Western blot analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1% Triton X-100 and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20 and 3% dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma–Aldrich Corp.) and an enhanced chemiluminescence detection system, LAS-4000 IR multi color (FUJIFILM). The results depicted in each figure are representative of at least three independent cell preparations. Each experiment was repeated three times.

2.6. RNA extraction and real-time PCR

Total RNA was prepared from cells using Trizol reagent (Invitrogen, San Diego, CA). cDNA was synthesized using a SuperScript-III cDNA synthesis kit (Invitrogen). Specific mRNA was quantified by using an ABI PRISM 7000 sequence detection system (Applied Biosystems Inc.) with SYBR Premix Ex TaqTM II (Perfect Real Time) kits (TaKaRa) according to the manufacturer's standard protocol. The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows:

GAPDH, 5′-GTGTACATGGTTCCAGTATGAGTCC-3′ and 5′-AGTGAGTTGTCATATTTCTCGTGGT-3′; OGN, 5′-TGCTTTGTGGTCACATGGAT-3′ and 5′-GAAGCTGCACACAGCACAAT-3′; Myogenin, 5′-GCTGCCTAAAGTGGAGATCCT-3′ and 5′-GCGCTGTGGGAGTTGCAT-3′; MyoD, 5′-GACGGCTCTCTCTGCTCCTT-3′ and 5′-AGTAGAGAAGTGTGCGTGCT-3′.

2.7. Transfection of small interfering RNA (siRNA)

Mouse VDR siRNA or control siRNA were transfected into C2C12 cells with LipofectAMINE (Invitrogen). Six hours later, the cells were fed with fresh medium containing 10% FBS, and the transfected cells were harvested for 48 h and were used for the experiments.

2.8. Statistics

All experiments were repeated at least three times. Data are expressed as mean ± S.E. Statistical analysis was performed using analysis of variance. A P value <0.05 was taken to indicate a significant difference.

3. Results

3.1. Effects of vitamin Ds on the expression of OGN and myoblastic differentiation in myoblastic cells

We first examined the effects of 1,25D on the expression of OGN and myoblastic differentiation in myoblastic C2C12 cells. As shown in Fig. 1A, 10^{-10} M 1,25D markedly increased the expressions of OGN and myogenin proteins after 48-h incubation in these cells. Moreover, 1,25D increased the expressions of OGN, myogenin, and MyoD proteins (10^{-11}M–10^{-8}M) (Fig. 1B). Next, we examined the effects of vitamin D on the expressions of OGN, myogenin, and MyoD. As shown in Fig. 1C, 10–10 M ELD as well as 1,25D increased the expressions of OGN, myogenin, and MyoD proteins, although 10^{-10}M 25D or 24,25D did not affect them. Moreover, 10^{-10}M 1,25D and ELD significantly increased the mRNA levels of OGN, myogenin, and MyoD although 10^{-10}M 25D or 24,25D did not affect them (Fig. 1D–F).

3.2. Effects of a reduction in endogenous VDR by siRNA on 1,25D- and eldecalcitol-induced OGN expression in myoblastic cells

We examined the effects of a reduction in endogenous VDR by siRNA on 1,25D- and ELD-induced OGN expression in C2C12 cells. We confirmed that the level of VDR protein was suppressed by VDR siRNA transfection by Western blot analysis (Fig. 2A). As shown in Fig. 2B, a reduction in endogenous VDR by siRNA suppressed 1,25D- and ELD-induced expression of OGN protein. Moreover, a reduction in endogenous VDR by siRNA significantly suppressed 1,25D- and ELD-induced the expression of OGN mRNA (Fig. 2C).

3.3. Effects of conditioned medium from 1,25D-pretreated and/or VDR siRNA-transfected myoblastic cells on osteoblast phenotype

We examined the effects of conditioned medium from 1,25D-pretreated and/or VDR siRNA-transfected C2C12 cells on osteoblast phenotype of MC3T3-E1 cells. Before using the conditioned medium, we confirmed that the level of OGN protein was increased in conditioned medium from 1,25D-pretreated C2C12 cells compared with control medium (Fig. 3A). The conditioned medium from 1,25D-pretreated C2C12 cells increased the expressions of Col1 and ALP proteins compared with controls in MC3T3-E1 cells (Fig. 3B). Moreover, the expressions of Col1 and ALP proteins were partially suppressed by conditioned medium from 1,25D-pretreated C2C12 cells knocked down by VDR siRNA, compared with the conditioned medium from 1,25D-pretreated C2C12 cells without VDR silencing (Fig. 3C).

3.4. Effects of AGEs as well as co-incubation with 1,25D and AGEs on myoblastic differentiation and the expression of OGN in myoblastic cells

We investigated the effects of AGE2 or AGE3 on myoblastic differentiation in C2C12 cells. As shown in Fig. 4A, 200 μg/mL

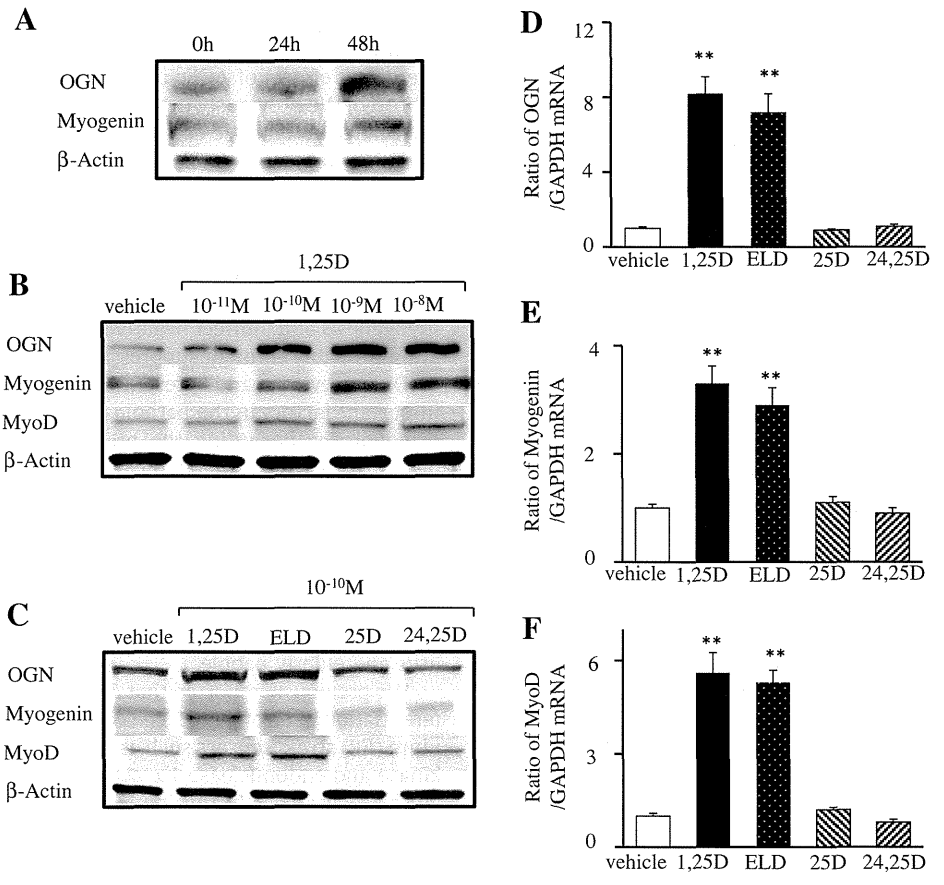

Fig. 1. 1,25D and ELD induced the expression of OGN and myoblastic differentiation in myoblastic cells. (A) Total proteins were extracted from 10^{-10}M 1,25D-pretreated C2C12 cells after 0-, 24-, or 48-h incubation. (B) Total proteins were extracted from 10^{-11} to 10^{-8}M 1,25D-pretreated C2C12 cells after 48-h incubation. (C) Total proteins were extracted from 10^{-10}M 1,25D- or ELD-pretreated C2C12 cells after 48-h incubation. (D-F) Total RNAs were extracted from 10^{-10}M 1,25D-, ELD-, 25D-, or 24,25D-pretreated C2C12 cells after 24-h incubation. Western blot analysis was performed with anti-OGN, myogenin, MyoD, or β-actin antibodies. Real-time PCR was performed and data were expressed as the GAPDH mRNA ratio. $^{**}P < 0.01$ relative to control.

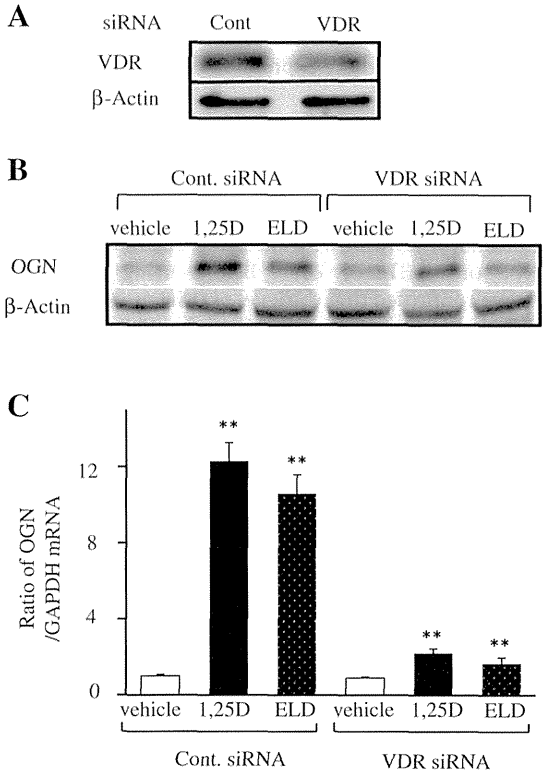

Fig. 2. A reduction in endogenous VDR by siRNA suppressed activated vitamin D-induced OGN expression in myoblastic cells. (A) Total proteins were extracted from control siRNA- or VDR siRNA-transfected C2C12. (B) Total proteins were extracted from 10^{-10}M 1,25D- or EDL-pretreated C2C12 cells for 48 h with control siRNA- or VDR siRNA-transfection. (C) Total RNAs were extracted from 10^{-10}M 1,25D- or ELD-pretreated C2C12 cells for 24 h with control siRNA- or VDR siRNA-transfection. Western blot analysis was performed with anti-VDR, anti-OGN or β-actin antibodies. Real-time PCR was performed and data were expressed as the GAPDH mRNA ratio. $^{**}P < 0.01$ relative to vehicle with control siRNA-transfection or with VDR siRNA-transfection.

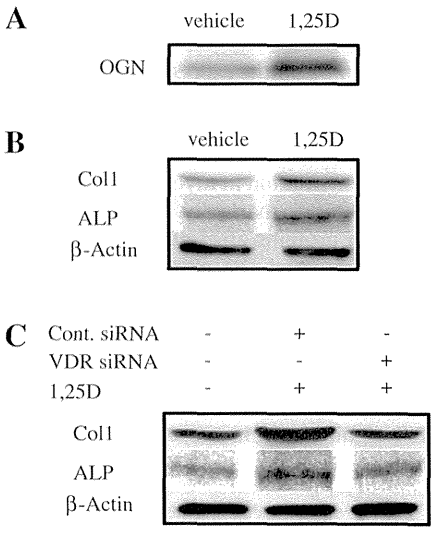

Fig. 3. Conditioned medium from 1,25D-pretreated myoblastic cells induced osteoblast phenotype in MC3T3-E1 cells. After reached cell confluent, C2C12 cells were incubated with or without 1,25D for 2 days. After that, the cells were washed and incubated in DMEM without FBS and 1,25D for 24 h. Then, the medium was collected. (A) Western blot analysis was performed with anti-OGN antibody using 10 μL medium and loading buffer. (B) MC3T3-E1 cells were cultured with 20% of each conditioned medium for 48 h. Total protein from these cells was extracted, and Western blot analysis was performed with anti-Col1, ALP, or β-actin antibodies. (C) Conditioned medium was obtained from the cultures of control siRNA- or VDR siRNA-transfected C2C12 cells with or without 1,25D.

AGE2 or AGE3 markedly suppressed the expressions of MyoD and myogenin (Fig. 4A) as well as OGN protein (Fig. 4B) in C2C12 cells. Moreover, AGE2 or AGE3 significantly inhibited the mRNA expression of MyoD, myogenin, and OGN (Fig. 4C–E). Finally, we examined the effects of 1,25D on AGEs-suppressed myoblastic differentiation and the expression of OGN in C2C12 cells. As shown in Fig. 4F, 10–10 M 1,25D recovered AGE2- or AGE3-suppressed expressions of MyoD and myogenin proteins. Moreover, 1,25D recovered AGE2- or AGE3-suppressed the levels of OGN protein (Fig. 4G).

4. Discussion

The interaction between muscle and bone has recently attracted widespread attention. We previously found that OGN is a humoral molecule derived from myoblast and might induce the osteogenic differentiation [12]. Since vitamin D is well-known to be an important factor for myoblastic differentiation and muscle strength [7,26], we hypothesized that vitamin D might affect OGN expression in myoblasts. Along with the markers of myogenesis, the expression of OGN mRNA and protein was increased by 1,25D and ELD in C2C12 cells. And, 1,25D- and ELD-induced OGN expression was partially but significantly canceled by VDR silencing by siRNA. In addition, the conditioned medium from 1,25D-pretreated C2C12 cells increased Col1 and ALP expression in MC3T3-E1 cells. This is the first evidence that active vitamin D is involved in the interaction between muscle and bone probably via OGN expression.

Although receptor for AGEs (RAGE) is expressed in muscle [27], the direct effects of AGEs on myoblastic differentiation were not reported. We previously demonstrated that AGE2 and AGE3, which are related to diabetic complications [14,15], inhibited the differentiation of stromal cells into osteoblasts and the mineralization of osteoblastic cells through RAGE expression [16,17], suggesting that AGEs directly affect osteoblast function and bone formation. In the present study, we showed that AGE2 and AGE3 suppressed the myoblastic differentiation and the expression of OGN in C2C12 cells, suggesting that AGEs have direct negative effects on myogenesis and indirect negative effects on osteoblastic differentiation probably through suppressing OGN expression in myoblasts.

Our previous and present studies suggest that AGEs are important factors in the pathology of not only sarcopenia but also osteoporosis. Regarding the prevention and treatment of these diseases, the agents that abolish the effects of AGEs should be clinically important. Thus, we tested the effects of co-incubation with 1,25D and AGEs on myoblastic differentiation and OGN expression. While AGEs decreased the expressions of MyoD, myogenin and OGN, 1,25D markedly increased them under the presence of AGEs. These findings indicate that 1,25D treatment might be useful to prevent sarcopenia and osteoporosis associated with diabetes mellitus and elderly people.

We previously reported that AGEs inhibited the osteoblastic differentiation of stromal ST2 cells by suppressing endoplasmic reticulum (ER) stress sensors such as inositol-requiring transmembrane kinase and endonuclease 1α, activating transcription factor 6, and old astrocyte specifically induced substance, which leads to the accumulation of abnormal proteins [18]. Previous studies also showed that the administration of AGE precursors or AGEs increased ER stress and apoptosis in mice chondrocytes or human aortic endothelial cells [28,29]. In humans, elevated RAGE, ER stress marker glucose-regulated protein 78, and cell-cycle regulator p21 were all positively correlated with enhanced senescence-associated-β-galactosidase activity in patients with diabetic nephropathy [30].

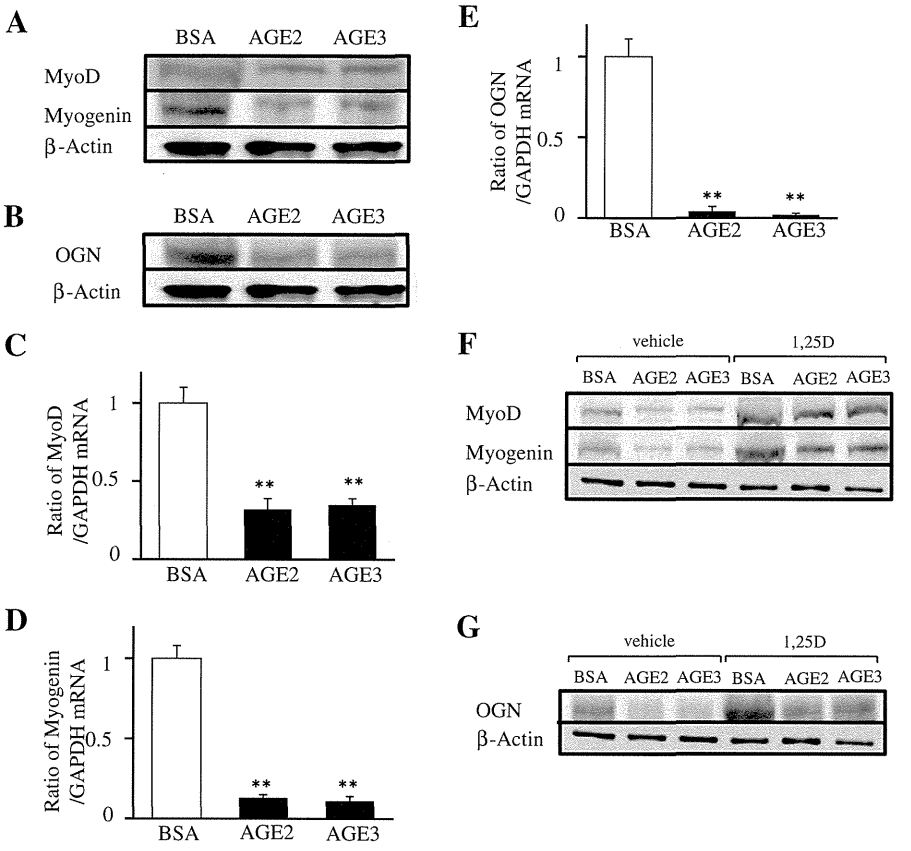

Fig. 4. AGEs suppressed the myoblastic differentiation and the expression of OGN in myoblastic cells, while 1,25D recovered the AGEs' effects. (A and B) Total proteins were extracted from 200 μg/mL AGE2- or AGE3-treated C2C12 cells after 48-h incubation. Western blot analysis was performed with anti-MyoD, myogenin, anti-OGN, or β-actin antibodies. (C-E) Total RNA was extracted from 200 μg/mL BSA-, AGE2-, or AGE3-treated C2C12 cells after 24-h incubation. Real-time PCR was performed and data were expressed as the GAPDH mRNA ratio. $^{**}P < 0.01$ relative to control. (F and G) Total proteins were extracted from 200 μg/mL BSA-, AGE2-, or AGE3-treated C2C12 cells after 48-h incubation with or without pretreatment of 10^{-10}M 1,25D for 24 h. Western blot analysis was performed with anti-MyoD, myogenin, OGN or β-actin antibodies.

In contrast, Riek et al. showed that vitamin D was a natural ER stress reliever that induced an anti-inflammatory monocyte/macrophage phenotype in type 2 diabetic patients [31,32]. Although further studies are needed to clarify the mechanism, AGEs and vitamin D might modulate the myoblastic differentiation and the expression of OGN through ER stress.

In this study, we found for the first time that vitamin D regulates OGN expression, leading to stimulate osteoblastic differentiation indirectly. However, to confirm our results, further experiments are necessary. We used C2C12 cells as a model for myoblastic cells because the cells are frequently used to examine the function and differentiation of myoblast *in vitro*. Although the cells were obtained by serial passage of myoblasts cultured from muscle tissue of C3H mice after a crush injury [33], they might not be identical to natural muscle cells *in vivo*. Therefore, we need further *in vivo* experiments and clinical studies in future. Furthermore, several molecules linking muscle to bone were previously reported. For example, we reported that Tmem119 is an important local inducer of muscle ossification [34], and that FAM5C acts as another humoral factor to stimulate osteoblastic differentiation [35]. Further studies focused on these would be interested.

In conclusion, 1,25D and ELD increased the differentiation of C2C12 cells and the expression of OGN, leading to the differentiation of MC3T3-E1 cells, suggesting that these agents might be useful to prevent and treat not only osteoporosis but also sarcopenia. Moreover, we found that, in contrast to active vitamin D, AGEs suppressed the myoblastic differentiation and the expression of OGN, suggesting that AGEs accumulation may be involved in sarcopenia and osteoporosis by suppressing myoblastic differentiation and OGN expression. In addition, 1,25D might be useful to prevent sarcopenia and osteoporosis by direct or indirect fashions through muscle tissues.

Conflict of interest

None of the authors has any conflict of interest.

Acknowledgments

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Original article

Changes in bone mineral density, bone turnover markers, and vertebral fracture risk reduction with once weekly teriparatide

Shiro Tanaka
Graduate School of Medicine and Public Health, Kyoto University, Kyoto, Japan

Tatsuhiko Kuroda
Asahi Kasei Pharma Corporation, Tokyo, Japan

Toshitsugu Sugimoto
Shimane University Faculty of Medicine, Shimane, Japan

Toshitaka Nakamura
National Center for Global Health and Medicine, Tokyo, Japan

Masataka Shiraki
Research Institute and Practice for Involutional Diseases, Nagano, Japan

Address for correspondence:
Masataka Shiraki MD PhD, Department of Internal Medicine, Research Institute and Practice for Involutional Diseases, 1610-1 Meisei, Misato, Azumino, Nagano 399-8101, Japan.
Tel: +81 263-77-2134; Fax: +81 263-77-6963;
ripid@fc4.so-net.ne.jp

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Abstract

Objective:
We aimed to evaluate the surrogacy of bone mineral density and bone turnover markers for incident vertebral fracture using data from 237 patients treated with once weekly 56.5 μg teriparatide or placebo.

Methods:
This analysis was conducted using data from the Teriparatide Once-Weekly Efficacy Research trial, a randomized, double-blind, placebo-controlled trial for patients with severe osteoporosis in Japan. A total of 237 subjects (placebo group, $n=130$; teriparatide group, $n=107$) were assessed at baseline and at 72 weeks. Main outcome measures included estimation of the treatment effects of once weekly teriparatide on vertebral fracture risk reduction using percentage changes in lumbar bone mineral density and bone turnover markers.

Results:
The percentage change in lumbar bone mineral density was 6.69% in the teriparatide group compared with 0.28% in the placebo group ($p<0.01$). One incident vertebral fracture occurred in the teriparatide group compared with 16 in the placebo group. The unadjusted and adjusted hazard ratios of the teriparatide group compared with the placebo group were 0.07 (95% confidence interval: 0.01 to 0.56) and 0.64 (95% confidence interval: 0.06 to 6.36), respectively. The proportion of treatment effect explained by changes in lumbar bone mineral density was 83% (Freedman's method) and 66% (Chen's method). There were no notable changes in hazard ratios if we adjusted for bone turnover markers.

Conclusions:
Most of the vertebral fracture risk reduction with once weekly 56.5 μg teriparatide is explained by changes in lumbar bone mineral density rather than changes in bone turnover markers.

Introduction

Bone mineral density (BMD) is the major risk factor for fracture in patients with osteoporosis[1]. Changes in BMD are a potential surrogate marker for fracture endpoints in clinical trials, and using BMD values reduces costs and accelerates the pace of drug development[2]. Changes in BMD are also used as a treatment goal for osteoporosis[3]. The surrogacy of BMD for anti-fracture efficacy has been intensively investigated in patients treated by antiresorptive agents[4–8], although results are conflicting. Therefore, the surrogacy of each drug should be investigated individually. However, little is known about the use of BMD as a surrogate endpoint in patients treated with teriparatide. To our knowledge, only the

Fracture Prevention Trial performed a posthoc analysis that showed that increases in lumbar BMD after daily teriparatide accounted for 30% to 41% of the observed reduction in the risk of vertebral fracture[9]. Some researchers have explored non-BMD determinants of fracture risk that explain the remaining fracture risk reduction, such as levels of type I collagen cross-linked N-telopeptides (NTX) and type I collagen cross-linked C-telopeptides (CTX)[10–12].

Once weekly 56.5 μg teriparatide (human PTH 1-34) was shown to be efficacious for increasing lumbar BMD by 6.7% at 72 weeks and reducing the risk of vertebral fracture by 80% in postmenopausal women and elderly men with osteoporosis in the Teriparatide Once-Weekly Efficacy Research (TOWER) trial[13]. In this analysis, we aimed to evaluate the surrogacy of BMD and bone turnover markers for vertebral fracture using data from 237 patients from the TOWER trial. Bone turnover markers included serum levels of bone alkaline phosphatase (BAP), osteocalcin, procollagen type 1 amino-terminal propeptide (P1NP), and urinary levels of NTX.

Patients and methods

Ethics statement

This analysis was conducted with data from the TOWER trial, which was a randomized, multicenter, double-blind, placebo-controlled trial in Japan[13]. The protocol of the TOWER trial was approved by the Institutional Review Boards at each participating institution and was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice (GCP). Written informed consent was obtained from all participants prior to their participation in the study.

Patients and intervention

The original eligibility criteria of the TOWER trial included men and postmenopausal women between 65 and 91 years of age with primary osteoporosis, who had up to five prevalent vertebral fractures, and low BMD (T-score <-1.67) at the lumbar spine (L2 to L4), femoral neck, total hip, or distal radius. Patients were randomly assigned to receive weekly subcutaneous injections of placebo ($n=290$) or 56.5 μg of teriparatide ($n=288$) for 72 weeks. All patients received daily oral supplements of calcium 610 mg, vitamin D 400 IU, and magnesium 30 mg. A total of 237 patients were included in this analysis after excluding patients whose measurements of lumbar BMD at baseline and end of study were not available.

Data collection

At baseline, age, height, weight, and vertebral fracture number and grade were investigated. The lumbar spine BMD was measured at baseline and end of the study using dual-energy x-ray absorptiometry (DXA) with QDR (Hologic, Bedford, MA, USA) or DPX (GE Healthcare, Fairfield, CT, USA). A central facility performed quality assurance of the longitudinal adjustment by calibrating each machine with standardized phantoms. All DXA measurements were analyzed at a central site by a radiologist blinded to treatment group assignment.

Serum and urine samples were obtained under nonfasting conditions at baseline and at 4, 12, 24, 48, and 72 weeks. Bone turnover markers were measured centrally in a single batch at a validated institution (Mitsubishi Chemical Medience, Tokyo, Japan). Serum levels of osteocalcin were measured with an immunoradiometric assay (BGP-IRMA Mitsubishi; Mitsubishi Chemical Medience); P1NP was measured by RIA (Orion Diagnostica, Espoo, Finland); NTX was measured with an enzyme-linked immunoassay (Osteomark; Inverness Medical Innovations Inc., Waltham, MA, USA). The coefficients of variation for DXA and details of other data collection and methods of bone turnover markers appear in the original trial publication[13].

Vertebral fracture from Th4 to L4 was defined by a semi-quantitative and quantitative morphometric method using X-ray films of the thoracic and lumbar spine taken at baseline, 24, 48, and 72 weeks[14]. The assessment of incident vertebral fractures was conducted by an independent committee of three experts who were blinded to treatment. Incident vertebral fracture was defined as a vertebral fracture that was normal (semi-quantitative grade 0) at baseline of the original trial.

Statistical analysis

The primary endpoint was time from randomization to incidence of new, radiographically confirmed vertebral fractures (Th4 to L4). Hazard ratios (HRs) and 95% confidence intervals (CIs) for the incidence of vertebral fracture were estimated by Cox regression. We evaluated the association between vertebral fracture and changes in BMD and bone turnover markers using two statistical criteria for surrogate endpoints, namely Freedman's proportion of treatment effect explained (PTE)[15] and Chen's PTE[9]. Freedman's PTE is defined as $\mathrm{PTE}=1-\alpha/\beta$, where α is a log HR for treatment effect adjusted for surrogates and β is a log HR for treatment effect unadjusted for surrogates. Chen's PTE is defined as $(A-B)/(A-C)$, where A is the fracture risk of patients in the placebo group with a change in lumbar BMD observed in the placebo group at 72 weeks, B is the fracture risk of patients in the placebo group with a change in lumbar BMD observed in

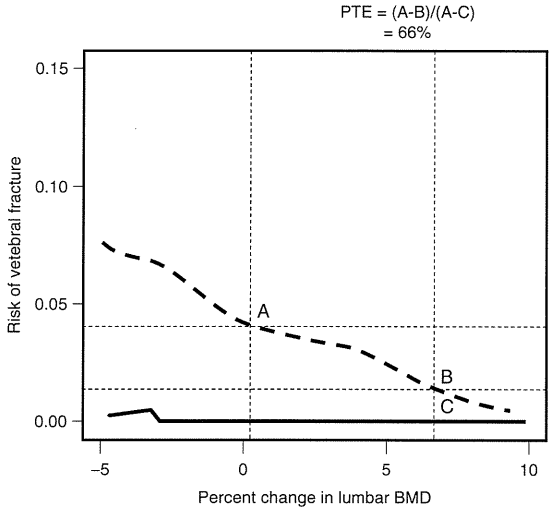

Figure 1. Proportion of treatment effect explained by Chen's method. Solid line: teriparatide group; dashed line: placebo group. Proportion of treatment effect explained (PTE) is defined by $(A-B)/(A-C)$[8]. A: The fracture risk of patients in the placebo group with a change in lumbar bone mineral density (BMD) of placebo group level at 72 weeks. B: The fracture risk of patients in the placebo group with a change in lumbar BMD of teriparatide group level at 72 weeks. C: The fracture risk of patients in the teriparatide group with a change in lumbar BMD of teriparatide group level at 72 weeks.

the teriparatide group at 72 weeks, and C is the fracture risk of patients in the teriparatide group with a change in lumbar BMD observed in the teriparatide group at 72 weeks, respectively (Figure 1). The vertebral fracture risks at the three points (A, B, and C) were estimated as a spline function, a smooth curve of vertebral fracture risk depending on percentage change in lumbar BMD using generalized additive models. All p-values were two-sided, and the significance level was 0.05. All statistical analyses and data management were conducted at a central data center using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

There were no significant differences in age, body mass index, BMD, and bone turnover markers between groups (Table 1). Longitudinal profiles of BMD and bone markers in percentage change from baseline at 4, 12, 24, 48, and 72 weeks are presented in Figure 2. BMD increased from 0.709 ± 0.123 at baseline to 0.755 ± 0.133 g/cm^2 at 72 weeks in the teriparatide group, representing a 6.69% increase. In the placebo group, BMD increased from 0.721 ± 0.117 at baseline to 0.724 ± 0.128 g/cm^2 at 72 weeks, representing a 0.28% increase. Differences between treatment groups were significantly different ($p<0.01$). Serum BAP decreased from 33.9 ± 12.9 to 24.8 ± 7.8 U/L in the teriparatide group and 32.9 ± 11.6 to 27.4 ± 8.8 U/L in the placebo group ($p<0.01$). Serum P1NP decreased from 52.5 ± 25.1 to 38.5 ± 18.1 ng/mL in the teriparatide group and 51.7 ± 20.8 to 45.6 ± 20.0 ng/mL in the placebo group ($p<0.01$). Serum osteocalcin decreased from 7.69 ± 2.60 to 7.56 ± 2.37 ng/mL in the teriparatide group and 8.02 ± 3.30 to 7.62 ± 2.73 ng/mL in the placebo group ($p=0.79$). Urinary NTX decreased from 42.9 ± 21.5 to 35.4 ± 18.2 nmolBCE/mmolCr in the teriparatide group and increased from 41.4 ± 23.3 to 47.8 ± 25.4 nmolBCE/mmolCr in the placebo group ($p<0.01$). Serum NTX increased from 13.5 ± 4.8 to 15.4 ± 6.3 nmolBCE/L in the teriparatide group and 13.7 ± 5.3 to 17.3 ± 6.6 nmolBCE/L in the placebo group ($p<0.01$).

Table 1. Baseline characteristics of 237 patients with osteoporosis.

	Placebo group ($n=130$)		Teriparatide group ($n=107$)	
	Mean	SD	Mean	SD
Age (years)	74.7	5.6	74.4	5.5
Men (number [%])	6	[5.6%]	4	[5.6%]
Body mass index (kg/m^2)	23.1	3.2	23.1	3.2
Lumbar BMD (g/cm^2)	0.721	0.117	0.709	0.123
Serum BAP (U/L)	32.9	11.6	33.9	12.9
Serum osteocalcin (ng/mL)	8.0	3.3	7.7	2.6
Serum PINP (ng/mL)	51.7	1.8	52.5	2.4
Serum NTX (nmol BCE/L)	13.7	5.3	13.5	4.8
Urinary NTX (nmol BCE/mmol Cr)	41.4	23.3	42.9	21.5

BMD, bone mineral density; BAP, bone specific alkaline phosphatase; P1NP, procollagen type 1 amino-terminal propeptide; NTX, type I collagen cross-linked N-telopeptides.

During a follow-up of 72 weeks, one incident vertebral fracture was observed in the teriparatide group and 16 incident vertebral fractures were observed in the placebo group. The HR of the teriparatide group compared with the placebo group was 0.07 (95% CI: 0.01 to 0.56, $p=0.01$). Table 2 shows the HRs of teriparatide unadjusted and adjusted for percentage change in BMD at the lumbar spine and the criteria for surrogacy using Freedman's PTE. As shown, the unadjusted and adjusted HRs were 0.07 (95% CI: 0.01 to 0.56) and 0.64 (95% CI: 0.06 to 6.36), respectively. Freedman's PTE was 83%, indicating that most of the effects of teriparatide for fracture risk reduction were explained by the percentage change in lumbar BMD. The results were similar if we used absolute changes in lumbar BMD (Freedman's PTE = 76%). Figure 1 shows the spline curves for vertebral fracture risks according to treatment groups estimated by generalized additive models. The vertebral fracture risk in the teriparatide group was consistently low, whereas that in

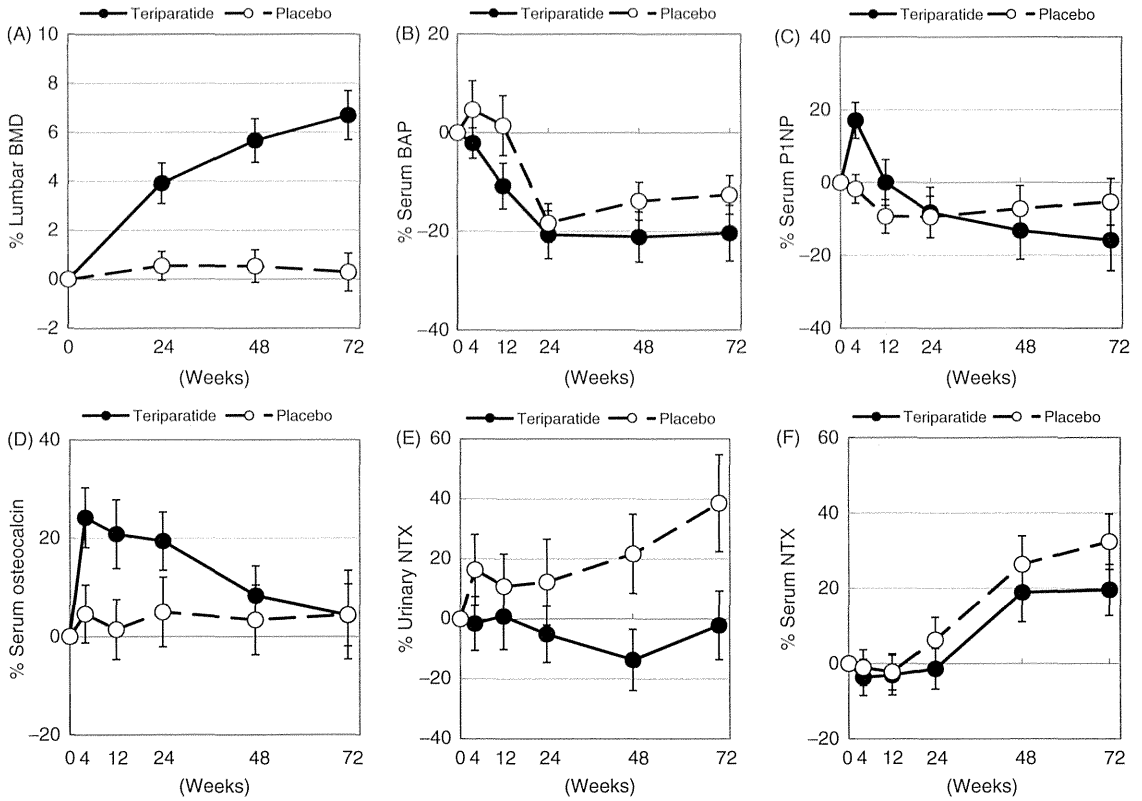

Figure 2. Changes by group from baseline to 72 weeks in lumbar BMD and bone turnover markers. Solid lines: teriparatide group; dashed lines: placebo group. (A) BMD; (B) bone-specific alkaline phosphatase (BAP); (C), procollagen type 1 amino-terminal propeptide (P1NP); (D) osteocalcin; (E) and (F), type I collagen cross-linked N-telopeptides (NTX).

Table 2. Proportion of treatment effect explained by lumbar BMD.

	Unadjusted				Adjusted for lumbar BMD			
	HR	95% CI		*p*	HR	95% CI		*p*
Teriparatide vs. placebo	0.07	0.01	0.56	0.01	0.64	0.06	6.36	0.70
Change in lumbar BMD per 5%					0.32	0.13	0.77	0.01
PTE*						83%		

HR, hazard ratio; CI, confidence interval; BMD, bone mineral density; PTE, proportion of treatment effect explained.
*PTE was calculated as one minus log adjusted hazard ratio for treatment divided by log unadjusted hazard ratio for treatment.

the placebo group decreased as the percentage change in lumbar BMD increased. The vertebral fracture risks at the three points were $A = 0.04$, $B = 0.0135$, $C = 0.00$, respectively (Chen's PTE = 66%). On the other hand, adjustment for each bone marker alone instead of BMD did not increase the HRs of teriparatide toward null, yielding PTEs for bone markers of approximately 0%. Table 3 shows the HRs of teriparatide further adjusted for bone markers and Freedman's PTEs for each adjustment factor. The HR of teriparatide increased from 0.64 (Table 2) to 0.83 (Table 3) if we adjusted for serum P1NP additionally, and Freedman's PTE increased from 83% to 93%. There were no notable changes in HRs for other bone turnover markers.

Discussion

Changes in BMD are a potential surrogate marker for anti-fracture efficacy in long-term treatment of osteoporosis. Because the surrogacies of change in BMD to anti-fracture efficacy differ by drug[4–8], the relationship should be evaluated in each drug.

Table 3. Proportion of treatment effect explained by combinations of lumbar BMD and bone markers.

	Serum BAP				Serum osteocalcin			
	HR	95% CI		p	HR	95% CI		p
Teriparatide vs. placebo	0.73	0.07	7.57	0.79	0.65	0.06	6.57	0.72
Change in lumbar BMD per 5%	0.18	0.06	0.54	<0.01	0.31	0.12	0.78	0.01
Change in bone markers per 5%	0.76	0.62	0.93	0.01	1.00	0.93	1.06	0.90
PTE*		88%				84%		
	Serum P1NP				Urinary NTX			
	HR	95% CI		p	HR	95% CI		p
Teriparatide vs. placebo	0.83	0.08	8.47	0.97	0.51	0.05	4.93	0.56
Change in lumbar BMD per 5%	0.18	0.06	0.57	<0.01	0.28	0.11	0.70	0.01
Change in bone markers per 5%	0.89	0.79	1.01	0.07	0.96	0.90	1.03	0.27
PTE*		93%				74%		

BMD, bone mineral density; BAP, bone specific alkaline phosphatase; HR, hazard ratio; CI, confidence interval; PTE, proportion of treatment effect explained; P1NP, procollagen type 1 amino-terminal propeptide; NTX, type I collagen cross-linked N-telopeptides.
*Four separate bivariate Cox regression models were fitted to each combination of BMD and bone markers. PTEs were calculated as one minus log adjusted hazard ratio for treatment divided by log unadjusted hazard ratio for treatment.

The current analysis of data from a randomized placebo-controlled trial in Japan demonstrated that most of the vertebral fracture risk reduction by once weekly 56.5 μg teriparatide is explained by the change in lumbar BMD: 83% using Freedman's method and 66% using Chen's method. These estimates for PTE at the vertebra are not only higher than those seen in a previous study using antiresorptive agents such as alendronate (16%)[6] or risedronate (18%)[16] but are also higher than with strontium (no significant association)[8] or with daily teriparatide (30 to 41%)[9].

The major difference between the current study and previous studies of patients treated with teriparatide[9,13,17] is the dose and schedule (once weekly 56.5 μg vs. daily 20 μg). Another difference between these studies is in the patients' characteristics, that is, the TOWER trial comprised Japanese patients with a mean age and BMI of 76 years and 23 kg/m^2, respectively, while the previous study was conducted mainly with Western patients who were slightly younger and more obese. Furthermore, the relative risk for once weekly 56.5 μg teriparatide treatment observed in the TOWER trial was 0.20, which is lower than that seen with 20 μg teriparatide daily (RR = 0.47), alendronate (RR = 0.52), or risedronate (RR = 0.64)[17–20]. These factors have the potential to influence the relationship between change in lumbar BMD and vertebral fracture risk reduction. Moreover, once weekly teriparatide administration is associated not only with an increase in BMD but with improved bone material properties and collagen deterioration, as observed in ovariectomized monkeys[21]. The bone quality improvements produced by new bone with once weekly teriparatide may contribute to a higher correlation of fracture reduction.

The explained values adjusted for bone formation markers (P1NP and BAP) only slightly increased the PTE value from the change of BMD. It was reported that greater reductions of bone turnover markers in response to bisphosphonate treatment were associated with a lower risk of spinal, hip, and non-spinal fractures[12], but the relationship between changes in bone turnover markers and fracture risk reduction has not been seen with bone formation agents such as teriparatide. Changes in bone turnover markers with teriparatide may not contribute to fracture risk reduction.

Several limitations warrant mention. First, in the original TOWER trial, a total of 542 participants were evaluated for fracture incidence, and incident vertebral fracture occurred in seven cases (2.7%, 7/261) in the teriparatide group and 37 cases (13.2%, 37/281) in the placebo group. However, not all study sites measured BMD using DXA. Therefore, the number of participants in this analysis was limited. Second, the PTE value to incident hip fracture was not evaluated in this analysis. In the original TOWER trial, only one hip fracture in the teriparatide group and three hip fractures in the placebo group were observed, although measurement of BMD by DXA was not carried out in sites where fractures were observed.

Conclusion

We conclude that most of the vertebral fracture risk reduction by once weekly 56.5 μg teriparatide is explained by the change in lumbar BMD.

Transparency

Declaration of funding
This study was supported by the Asahi Kasei Pharma Corporation.

Declaration of financial/other relationships

S.T. received a research grant from Asahi Kasei Pharma Corporation. T.K. is an employee of Asahi Kasei Pharma Corporation. T.S. received research grants and consulting fees from the following pharmaceutical companies: Asahi Kasei Pharma and Daiichi Sankyo. T.N. received research grants and/or consulting fees from the following pharmaceutical companies: Chugai, Teijin, Asahi Kasei Pharma, and Daiichi Sankyo. M.S. received consulting fees from the following pharmaceutical companies: Chugai, Daiichi Sankyo, Asahi Kasei Pharma, Teijin, and MSD.

CMRO peer reviewers may have received honoraria for their review work. The peer reviewers on this manuscript have disclosed that they have no relevant financial relationships.

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ORIGINAL ARTICLE

Once-weekly teriparatide reduces the risk of vertebral fracture in patients with various fracture risks: subgroup analysis of the Teriparatide Once-Weekly Efficacy Research (TOWER) trial

Tetsuo Nakano · Masataka Shiraki · Toshitsugu Sugimoto · Hideaki Kishimoto · Masako Ito · Masao Fukunaga · Hiroshi Hagino · Teruki Sone · Tatsuhiko Kuroda · Toshitaka Nakamura

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Abstract Once-weekly teriparatide (human parathyroid hormone [1–34]) (56.5 μg for 72 weeks) injections provided a vertebral fracture risk reduction in Japanese osteoporotic patients evaluated in the Teriparatide Once-Weekly Efficacy Research (TOWER) trial. Using data from the TOWER trial, a subgroup analysis was performed to study the efficacy of once-weekly teriparatide for a variety of baseline clinical risk factors in placebo ($n = 281$) and teriparatide ($n = 261$) groups. Significant fracture risk reductions were observed in the subgroups of individuals aged <75 years [relative risk (RR) 0.06, $p = 0.007$] and ≥75 years (RR 0.32, $p = 0.015$). A significant risk reduction was observed among patients with prevalent vertebral fracture in the subgroup with 1 (RR 0.08, $p = 0.015$) or ≥2 (RR 0.29, $p = 0.009$) prevalent vertebral fractures, and in those with grade 3 deformity (RR 0.26, $p = 0.003$). Significant risk reduction was observed in the subgroup with lumbar bone mineral density (BMD) < −2.5 SD (RR 0.25, $p = 0.035$). In the teriparatide group, no incident fracture was observed in the subgroups with a prevalent vertebral fracture number of 0, with grade 0–2 vertebral deformity, or with lumbar BMD ≥2.5 SD. Significant risk reduction was observed in all of the bone turnover marker and estimated glomerular filtration rate subgroups. In conclusion, once-weekly 56.5 μg teriparatide injection reduced the vertebral fracture risk in patients with varying degrees of fracture risk, age, vertebral fracture number and grade, bone turnover level, and renal function.

T. Nakano (✉)
Tamana Central Hospital, 1950 Naka, Tamana, Kumamoto 865-0064, Japan
e-mail: nakano@tamana-chp.jp

M. Shiraki
Research Institute and Practice for Involutional Diseases, 1610-1 Meisei, Misato, Azumino, Nagano 399-8101, Japan

T. Sugimoto
Internal Medicine 1, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan

H. Kishimoto
Nojima Hospital, 2714-1 Sezaki-cho, Kurayoshi, Tottori 682-0863, Japan

M. Ito
Medical Work-Life-Balance Center, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

M. Fukunaga
Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan

H. Hagino
School of Health Science and Rehabilitation Division, Faculty of Medicine, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan

T. Sone
Department of Nuclear Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan

T. Kuroda
Project for Bone Metabolic Disease, Asahi Kasei Pharma Corporation, 1-105 Kanda, Jinbocho, Chiyoda-ku, Tokyo 101-8101, Japan

T. Nakamura
National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

Keywords Teriparatide · Once-weekly injection · Osteoporosis · Vertebral fracture

Introduction

The risk of fragility fracture depends upon the patient's age, sex, body mass index, lifestyle parameters, bone mineral density, and history of prior fracture [1, 2]. These factors independently increase the fracture risk, and patients who have more than one risk factor may be at risk to develop severe osteoporosis. They should be treated immediately with a powerful drug for fracture prevention that rapidly increases bone mineral density (BMD). Previous clinical trials have indicated that the risk reductions from treatment with anti-resorptive agents may depend on baseline patient characteristics such as age, BMD, and prevalent fracture status [3, 4].

Daily teriparatide (20 μg) injections have demonstrated anti-fracture efficacy with a bone anabolic mechanism [5]. Daily teriparatide injection has also provided an increased BMD in the Japanese population [6]. Increased BMD can be expected with teriparatide treatment, regardless of the baseline bone turn over marker status [7]. The fracture risk reduction effects were different depending on the baseline fracture risk factors such as age, BMD status, or prevalent vertebral fracture number and grade [8–11].

Recently, another regimen of once-weekly (56.5 μg) teriparatide usage provided both rapid and powerful anti-fracture efficacy in the Teriparatide Once-Weekly Efficacy Research (TOWER) trial [12]. The power in relative risk reduction on incident vertebral fracture with weekly teriparatide injection was 80.0 % when compared to placebo. However, the risk reduction for each baseline clinical fracture risk was not clear.

We completed a subgroup analysis of data from the TOWER trial to address these issues.

Materials and methods

These subgroup analyses were conducted with data from the TOWER trial, which was a randomized, multi-center, double-blind, placebo-controlled trial in Japan [12]. The protocol of the TOWER study was approved by the institutional review boards at each participating institution and was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all participants prior to their participation in the study.

Subjects and intervention

The TOWER trial subjects included men and postmenopausal women with primary osteoporosis between 65 and 91 years of age, who had one to five vertebral fractures, and low BMD (T-score < -1.67) at the lumbar spine (L2–L4), femoral neck, total hip, or distal radius. Subjects were randomly assigned to receive weekly subcutaneous injections of placebo ($n = 290$) or 56.5 μg of teriparatide ($n = 288$) for 72 weeks. All subjects received daily oral supplements of calcium 610 mg, vitamin D 400 IU, and magnesium 30 mg.

Baseline data collection

Subject age, height, weight, vertebral fracture number and grade were recorded at the start of the trial. The lumbar spine BMD and hip BMD was measured using dual-energy X-ray absorptiometry: QDR (Hologic, Bedford, MA) or DPX (GE Healthcare, Fairfield, CT). Serum and urine samples at the start of this trial were obtained under non-fasting conditions. Serum osteocalcin was measured with immunoradiometric assay (BGP-IRMA Mitsubishi; Mitsubishi Chemical Medience, Tokyo). Procollagen type I amino-terminal propeptide (P1NP) was measured by RIA (Orion Diagnostica, Espoo, Finland), and urinary cross-linked *N*-telopeptide of type I collagen (NTX) was measured with enzyme-linked immunoassay (Osteomark; Inverness Medical Innovations Inc., Waltham, MA). Estimated glomerular filtration (e-GFR) rate was calculated by the Modification of Diet in Renal Disease formula: e-GFR (mL/min/1.73 m^2) = $186.3 \times \text{Creatinine}^{-1.154} \times \text{Age}^{-0.203} \times 0.742 \times 0.881$ [13].

Outcome

Vertebral fracture from T4 to L4 was defined by a semi-quantitative (SQ) and quantitative morphometric method using X-ray pictures of the thoracic and lumbar spine taken at baseline, 24, 48, and 72 weeks [14]. Incident vertebral fracture assessment was conducted by an independent committee of three experts who were blinded to the treatment. Incident vertebral fracture was defined as a vertebral fracture that was normal (SQ grade 0) at baseline of the original trial.

Statistical analysis

The baseline clinical risk factor characteristics between the teriparatide group and the placebo group was compared with a Mann–Whitney *U*-test. The subgroup categories were: age (above or below 75 years old); prevalent vertebral fracture number (0, 1, ≥2); prevalent vertebral fracture grade of 0 (normal), 1 (mild), 2 (moderate), 3 (severe); lumbar BMD (−2.5 SD, ≥−2.5 SD); bone turnover markers (<median, ≥median); and e-GFR (<70 mL/min/1.73 m^2, ≥70 mL/min/1.73 m^2).