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Histomorphometric analysis of minimodeling in the vertebrae in postmenopausal patients treated with anti-osteoporotic agents

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ABSTRACT

Minimodeling is a type of focal bone formation that is characterized by the lack of precedent bone erosion by osteoclasts. Although this form of bone formation has been described for more than a decade, how anti-osteoporotic agents that are currently used in clinical practice affect the kinetics of minimodeling is not fully understood. We performed a bone morphometric analysis using human vertebral specimens collected from postmenopausal patients who underwent spinal surgery. Patients were divided into three groups according to osteoporosis medication; non-treated, Eldecalcitol (ELD, a vitamin D derivative that has recently been approved to treat patients with osteoporosis in Japan)-treated, and bisphosphonate-treated groups. Five to six patients were enrolled in each group. There was a trend toward enhanced minimodeling in ELD-treated patients and suppressed of it in bisphosphonate-treated patients compared with untreated patients. The differences of minimodeling activity between ELD-treated and bisphosphonate-treated patients were statistically significant. The present study suggests that ELD and bisphosphonates have opposite effects on minimodeling from one another, and show that minimodeling also takes place in vertebrae as has been described for the ilium and femoral head in humans.

1. Introduction

Osteoporosis is characterized by reduced bone mass and the loss of microarchitectural integrity in bone tissue, ultimately leading to bone fragility and increased susceptibility to fracture (Kanis, 1994). Osteoporosis-related fractures in elderly patients severely compromise their quality of life and activities of daily living (ADL) and impose a major burden on society and the medical economy, especially in developed countries (Egermann et al., 2005). Therefore, it is imperative to learn more about the pathology of and prophylaxis for osteoporosis and osteoporosis-related fractures.

Minimodeling is a form of trabeculae modeling and is so termed based on the miniature nature of this process in vivo (Frost, 1990). In conventional remodeling, bone formation is preceded by bone resorption, and, therefore, the cement lines are usually irregularly shaped. In contrast, in minimodeling, bone formation occurs on quiescent bone surfaces and thus creates smooth cement lines (Frost, 1990; Jee et al., 2007; Kobayashi et al., 2003). The nature and function of minimodeling are not fully understood; however, it is likely to be an adaptive mechanism to strengthen the skeletal microstructure in response to mechanical stress (Frost, 1990). Past studies have shown that minimodeling can be enhanced in patients and animals who are treated with bone anabolic agents, such as PTH (teriparatide) (Lindsay et al., 2006; Ma et al., 2006) and prostaglandin E2 (Zhou et al., 2001; Yao et al., 1999). On the other hand, the potential effects of more common anti-osteoporotic agents, such as vitamin D3 and bisphosphonates (BPs) on minimodeling in humans have remained unaddressed.

In the present study, we aimed to evaluate the potential effects of a new vitamin D3 analog, Eldecalcitol (2β-(3-hydroxypropyloxy)-1,25-dihydroxyvitamin D3, henceforth referred to as ELD), and BPs on minimodeling in humans using surgical specimens that were collected from postmenopausal patients who underwent spinal surgery. In accordance with past reports (Jee et al., 2007; Kobayashi et al., 2003), minimodeling was readily observed also in the human vertebral specimens that were examined in the present study. Most importantly, we found that patients who had been treated with ELD prior to surgery tend to have increased minimodeling activity compared with the control patients and those treated with BPs. Our data suggest that ELD potentially activates minimodeling in postmenopausal patients while BPs have negative or little impact on this form of bone formation, and that each anti-osteoporotic agents have distinct effects on minimodeling in vivo.

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2. Materials and methods

2.1. Study design, subjects, and ethics statement

Of the female patients with lumbar degenerative disorder who underwent lumbar spinal surgery at our institution from October 2012 to July 2014, we enrolled those who confirmed by interview that they had reached menopause >3 years prior. Patients were excluded if they had primary hyperparathyroidism; Cushing's syndrome; premature menopause due to hypothalamic, pituitary or gonadal insufficiency, poorly controlled diabetes mellitus (HbA1c over 8.0%); or other causes of secondary osteoporosis. Patients were also excluded if they had taken glucocorticoids or hormone replacement therapy in the past 3 months or had clinically significant hepatic, cardiac, or malignant disorders. Sixteen patients were eligible for this study. Ten of these patients were taking osteoporosis medications. Five of these ten patients had taken BPs (BP group), while the other five had solely taken ELD (ELD group), for >6 months. Six patients had no history of osteoporosis medication (Ctrl group). Bone specimens were collected from the lamina of the lumbar vertebra that was removed during surgery. The present study was approved by the Institutional Review Board of Keio University School of Medicine (approval number, 20,130,109) and informed consent was obtained from each patient.

2.2. Measurement of BMD

The BMD of the L2-L4 vertebrae and bilateral femurs was measured prior to surgery by dual-energy X-ray absorptiometry (Prodigy; GE Healthcare, Tokyo, Japan). The mean value of the bilateral femoral neck BMD was used for statistical analysis.

2.3. Serum and urinary bone metabolic markers

The levels of serum PTH-intact and tartrate-resistant acid phosphatase-5b (TRACP-5b) were measured by enzyme immunoassay. Serum homocysteine and hydroxyproline were measured by high-performance liquid chromatography. Urinary type 1 collagen N-telopeptide (NTX) and deoxypyridinoline (DPD) were measured by an enzyme immunoassay. The intact amino-terminal pro-peptide of type 1 collagen (P1NP) was measured by a radioimmunoassay. Serum was collected and analyzed prior to surgery.

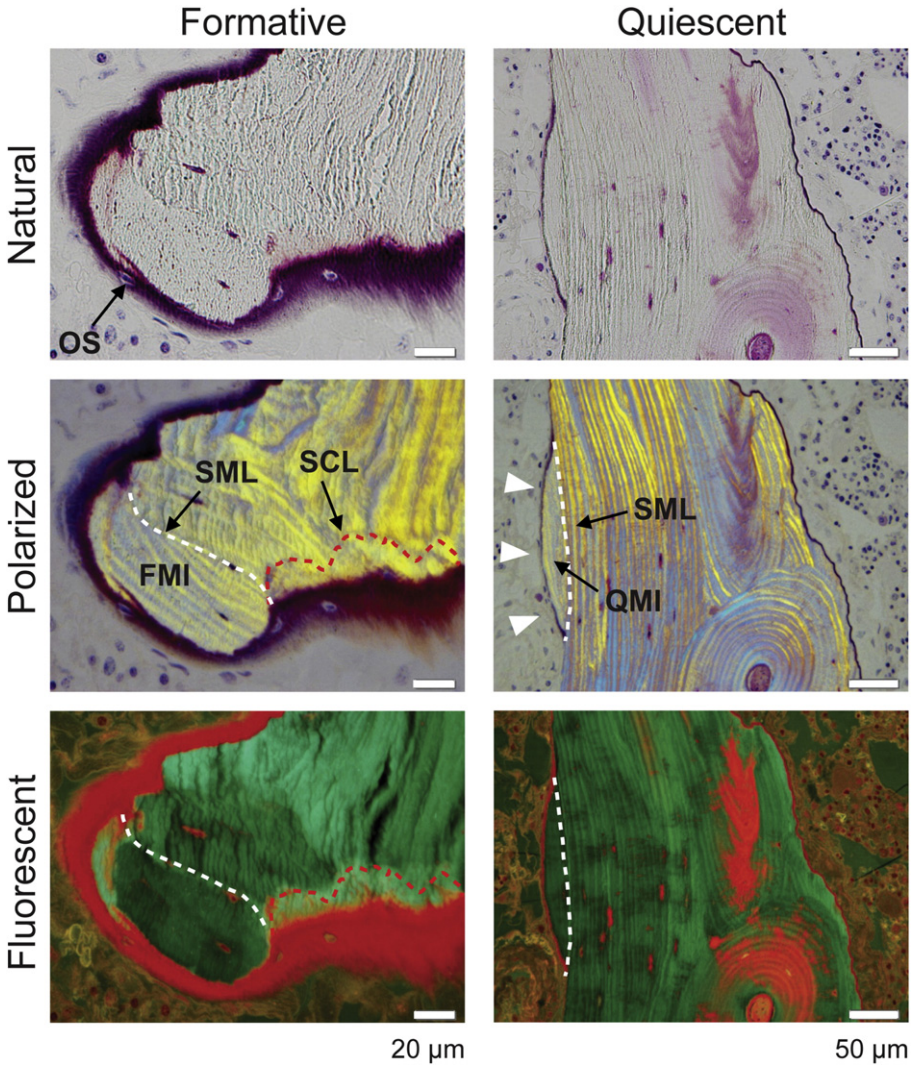

Fig. 1. Minimodeling sites on trabeculae. Conventional (a), polarized (b), and fluorescent (c) light micrographs of a trabecula with minimodeling in a lamina bone in a lumbar spine biopsy specimen from a 75-year-old woman who underwent lumbar decompression surgery for lumbar spinal canal stenosis. Note that bone surface is covered with osteoid in the formative minimodeling site (left panels); whereas, no apparent osteoid is found in the quiescent minimodeling site (right panels). OS, osteoid surface; QS quiescent surface; FMI, formative minimodeling; QMI, quiescent minimodeling; SCL, smooth cement line; SCL scalloped cement line.

Table 1
Baseline demographic subject data.

Group		Ctrl	ELD	BP	*p*-Value
N		6	5	5	–
Age (years)		77.7 ± 8.6	79.2 ± 4.3	80.0 ± 2.9	n.s.
BMI (kg/m^2)		23.4 ± 4.4	21.4 ± 3.9	22.9 ± 3.7	n.s.
Diagnosis		DS;1, LCS;5	DS;2, LCS;3	LCS;5	n.s.
Surgical method		Dec;5, Fus;1	Dec;4, Fus;1	Dec;5	n.s.
Surgery time (min)		94.8 ± 57.7	83.2 ± 49.3	61.2 ± 23.1	n.s.
EBL (g)		166.8 ± 268.6	136.0 ± 146.2	62.0 ± 83.5	n.s.
DEXA (g/cm^2)	Lumbar	0.95 ± 0.09	1.04 ± 0.27	1.12 ± 0.21	n.s.
	Femoral neck	0.67 ± 0.18	0.66 ± 0.05	0.73 ± 0.09	n.s.
DEXA (YAM%)	Lumbar	85.3 ± 8.0	92.6 ± 23.8	99.4 ± 18.6	n.s.
	Femoral neck	73.5 ± 18.9	73.3 ± 5.8	81.0 ± 9.3	n.s.

BMI body mass index, *EBL* estimated blood loss, *YAM* young adult mean, *DS* degenerative spondylolisthesis, *LCS* lumbar spinal canal stenosis, *Dec*; decompression, *Fus* fusion, *n.s.* not significant.

2.4. Bone histomorphometry

Surgical specimens were collected from the lamina of the 4th or 5th lumbar spine using a bone chisel (6 or 10 mm in width), fixed in 70% ethanol, and stained with Villanueva bone stain. No histological abnormality suggestive of metabolic bone diseases such as osteomalacia was found in any of the specimens. A bone histomorphometric analysis was performed by direct tracing using a digitizer. The histomorphometric parameters were analyzed using Bone Histomorphometric System software (System Supply, Nagano, Japan). Minimodeling sites were identified by the formation of mineralized bone on a smooth cement line, which indicates the lack of preceding bone resorption (Frost, 1990; Kobayashi et al., 2003; Ubara et al., 2005; Yajima et al., 2007). Minimodeling sites were further grouped into formative and quiescent minimodeling sites. Formative minimodeling sites were defined as minimodeling sites with an osteoid surface (Fig. 1). Those sites without an osteoid surface were defined as quiescent minimodeling sites. The number and bone volume (BV) of minimodeling sites were measured in each specimen. The parameters of the bone histomorphometric analysis are presented according to the standardized nomenclature (Dempster et al., 2013). All of the histomorphometric analyses were performed in a blinded manner.

2.5. Statistical analysis

The data are presented as the means ± SD. The differences between the groups were examined by one-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference, statistical differences between individual groups were evaluated with Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant. GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA) was used for all of the statistical analyses.

Table 3
Structural indices of the surgical specimens.

	Ctrl	ELD	BP	*p*-Value
BV/TV (%)	18.4 ± 5.1	10.8 ± 3.0	17.8 ± 5.4	n.s.
OV/BV (%)	2.07 ± 2.22	1.78 ± 1.22	2.24 ± 3.19	n.s.
Tb.Th (μm)	158.1 ± 43.6	110.0 ± 30.3	123.3 ± 18.8	n.s.
Tb.N	1.17 ± 0.15	1.02 ± 0.26	1.45 ± 0.36	n.s.
Tb.Sp (μm)	713.5 ± 119.7	949.5 ± 301.4	610.5 ± 173.5	n.s.

BV/TV bone volume/tissue volume, *OV/BV* osteoid volume/bone volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation.
n.s. not significant.

3. Results

3.1. Baseline demographic data of the subjects

There were no significant differences in the baseline demographic details between the three groups with respect to age, body mass index, type of lumbar degenerative disorder, surgical method, surgery time, estimated blood loss, and BMD of the lumbar and femoral neck (Table 1). Preoperative symptoms, neurological status, and ADL restriction as assessed by the JOA score (Hirabayashi et al., 1981) were not significantly different between these three groups (data not shown).

3.2. Serum and urinary biochemical parameters

The serum P1NP level, a bone formation marker, was significantly higher in the Ctrl group than in the BP group (Table 2). Bone resorption markers (serum TRACP-5b, urinary DPD, and NTX) in the Ctrl group were all above the normal range, but they were not significantly different from those in the ELD and BP groups. These results indicate that bone turnover was highly enhanced in the Ctrl group, and the high bone turnover was suppressed in the patients who had been treated with osteoporosis medications (ELD and BP groups). There were no statistically significant differences in the other parameters between the three groups.

3.3. Bone histomorphometric analysis

Consistent with the BMD data, there were no significant differences in the structural indices between the three groups (Table 3). Osteoblastic and osteoclastic indices tended to have higher values in the Ctrl group compared with those in the ELD and BP groups, indicating that bone turnover is enhanced in untreated patients (Table 4). However, only the Ob.S/OS ratio was significantly different between the Ctrl and BP groups.

Minimodeling sites were identified in 14 of 16 specimens (88%). Two specimens that lacked apparent minimodeling sites were found in the Ctrl and BP groups. Formative minimodeling sites were found in 5 specimens in the Ctrl group (83%), 5 in the ELD group (100%), and 3 in the BP group (60%). Quiescent minimodeling sites were identified

Table 2
Serum and urinary biochemical parameters.

Group		Ctrl	ELD	BP	*p*-Value
Serum	Ca (mg/dl)	9.2 ± 0.5	9.0 ± 0.4	9.7 ± 0.4	n.s.
	IP (mg/dl)	3.7 ± 0.5	3.6 ± 0.6	3.6 ± 0.5	n.s.
	PTH (pg/ml)	52.3 ± 23.4	38.2 ± 17.8	39.4 ± 11.9	n.s.
	Intact P1NP (μg/L)	51.4 ± 13.1	39.8 ± 16.3	23.4 ± 10.1	(Ctrl vs BP) 0.026
	TRACP-5b (mU/dl)	460.5 ± 151.5$^{\#}$	376.2 ± 134.7	272.8 ± 132.7	n.s.
Urine	DPD (nmol BCE/mmol Cr)	10.7 ± 4.3$^{\#}$	6.2 ± 2.1	6.5 ± 1.5	n.s.
	NTX (nmol BCE/mmol Cr)	61.4 ± 25.2$^{\#}$	45.8 ± 8.6	41.2 ± 40.9	n.s.

P1NP amino-terminal pro-peptide of type 1 collagen, *TRACP-5b* tartrate-resistant acid phosphatase-5b, *DPD* deoxypyridinoline, *NTX* type 1 collagen N-telopeptide.
n.s. not significant, # values outside the normal range.

Table 4
Osteoblastic and osteoclastic indices of the surgical specimens.

	Ctrl	ELD	BP	*p*-value
OS/BS (%)	17.66 ± 17.69	13.34 ± 8.12	13.97 ± 16.84	n.s.
Ob.S/OS (%)	55.96 ± 18.52	34.87 ± 12.28	31.06 ± 6.39	(Ctrl vs BP) 0.036
Ob.S/BS (%)	12.16 ± 14.84	4.43 ± 3.33	3.91 ± 4.11	n.s.
N.Ob/BS (N/mm)	5.36 ± 5.34	2.41 ± 1.65	2.18 ± 2.04	n.s.
N.Ob/OS (N/mm)	30.17 ± 8.36	18.72 ± 4.65	20.43 ± 5.27	n.s.
ES/BS (%)	4.81 ± 3.44	4.04 ± 3.99	2.13 ± 2.09	n.s.
Oc.S/BS (%)	0.68 ± 0.70	0.50 ± 0.58	0.25 ± 0.26	n.s.
Oc.S/ES (%)	15.61 ± 13.48	8.32 ± 5.06	9.79 ± 5.24	n.s.
N.Oc/BS (N/mm)	0.13 ± 0.12	0.12 ± 0.13	0.07 ± 0.06	n.s.
N.Oc/ES (N/mm)	3.20 ± 2.68	2.41 ± 1.25	4.48 ± 3.31	n.s.

OS/BS osteoid surface/bone surface, *Ob.S/OS* osteoblast surface/osteoid surface, *Ob.S/BS* osteoblast surface/bone surface, *N.Ob/BS* number of osteoblasts/bone surface, *N.Ob/OS* number of osteoblasts/osteoid surface, *ES/BS* eroded surface/bone surface, *Oc.S/ES* osteoclast surface/eroded surface, *Oc.S/BS* osteoclast surface/bone surface, *N.Oc/BS* number of osteoclasts/bone surface, *N.Oc/ES* number of osteoclasts/eroded surface.
n.s. not significant.

in 3 specimens in the Ctrl group (50%), 4 in the ELD group (80%), and 3 in the BP group (60%). In addition, we found that bone lacunae in the BP group specimens were often devoid of osteocytes (Fig. 2). As previously shown in mice treated with a bisphosphonate (alendronate) (Tsuboi et al., 2016), this finding suggests that bisphosphonates not only exhibit a negative impact on osteoclasts but also on osteocytes in humans.

The number of formative minimodeling sites/BV (N.F.MI/BV) in ELD group was significantly higher than in BP group, whereas, there was no difference in the number of quiescent minimodeling sites/BV (N.Q.MI/BV) between these two groups (Fig. 3A). N.F.MI/BV in ELD group was also higher than in Ctrl group, but the difference did not reach statistical significance. The formative minimodeling BV/BV (F.MI.BV/BV), which reflects the bone formation activity through minimodeling, in ELD group was significantly higher than in BD or Ctrl group (Fig. 3B). On the other hand, there was no difference in the quiescent minimodeling BV/BV (Q.MI.BV/BV) among three groups. Similar trend was also found in the minimodeling osteoid volume/BV (MI.OS/BV) and in the formative minimodeling bone volume/the number of formative minimodeling sites (F.MI.BV/N.F.MI, which reflects the bone forming activity of each minimodeling sites), where these indices in ELD group were significantly higher than those in BP group (Fig. 3C and D). Taken together, these observations suggest that the number of minimodeling sites and bone formation through minimodeling were enhanced in patients who were treated with ELD, but were suppressed in those treated with BPs.

4. Discussion

Our data showed that minimodeling is readily observed in the vertebral tissues collected from postmenopausal patients, and that the patients treated with ELD have increased bone formation through minimodeling compared with those with BPs. These observations suggest that a vitamin D analog ELD and BPs have apparently opposite effects on minimodeling in vivo, where ELD increases and BPs suppresses minimodeling-based bone formation. Although it has been shown that ELD can promote osteoblast differentiation and focal bone production through minimodeling in rats (de Freitas et al., 2011) and primates (Saito et al., 2015; Smith et al., 2013), the potential effects of ELD on minimodeling in humans has remained to be elucidated. The present study supports the notion that ELD positively regulates minimodeling in humans, and suggest that ELD improves trabecular

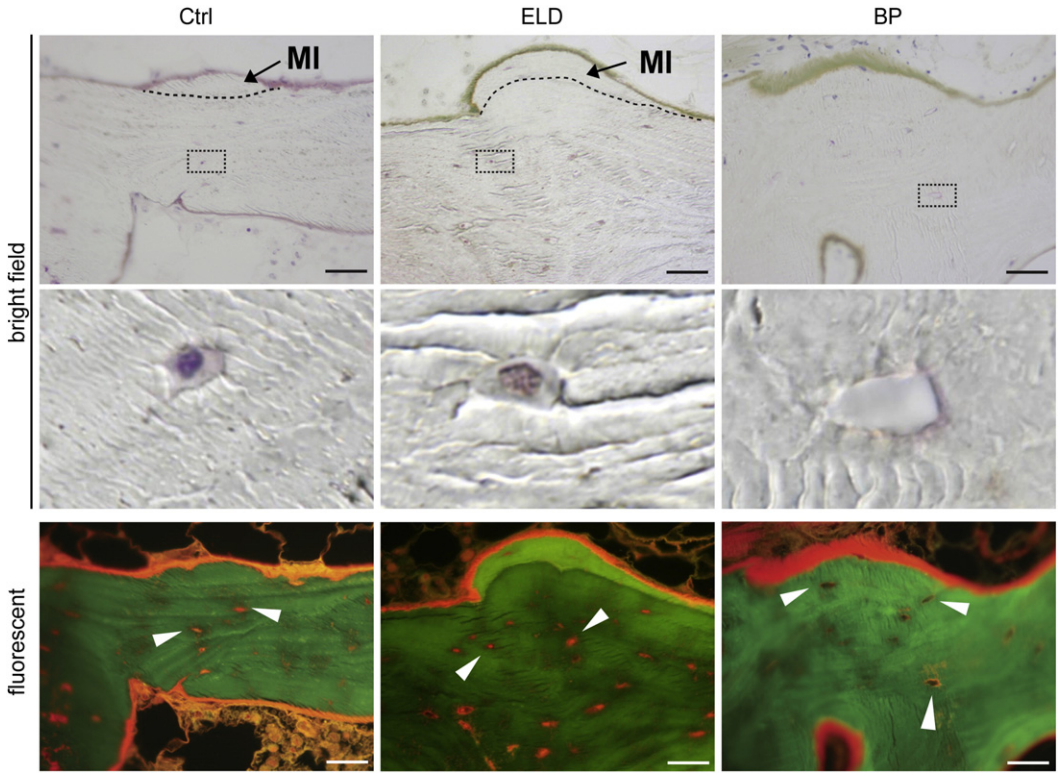

Fig. 2. Minimodeling sites in human specimens. Representative images of trabeculae of a Ctrl patient and patients treated with ELD or BP are shown (upper and middle panels, bright light images; lower panels, fluorescent images). In minimodeling, bone is formed on a smooth cement line (dotted line). Middle panels represent the magnified images of the boxed area in the upper panels, showing the presence (Ctrl and EDL groups) or absence (BP group) of an osteocyte in a bone lacuna. Lacunae containing osteocytes show bright red fluorescent signal (arrowheads); whereas those lacking osteocytes show very low fluorescence signal (right-lower panel). MI, minimodeling. Bars, 30 μm.

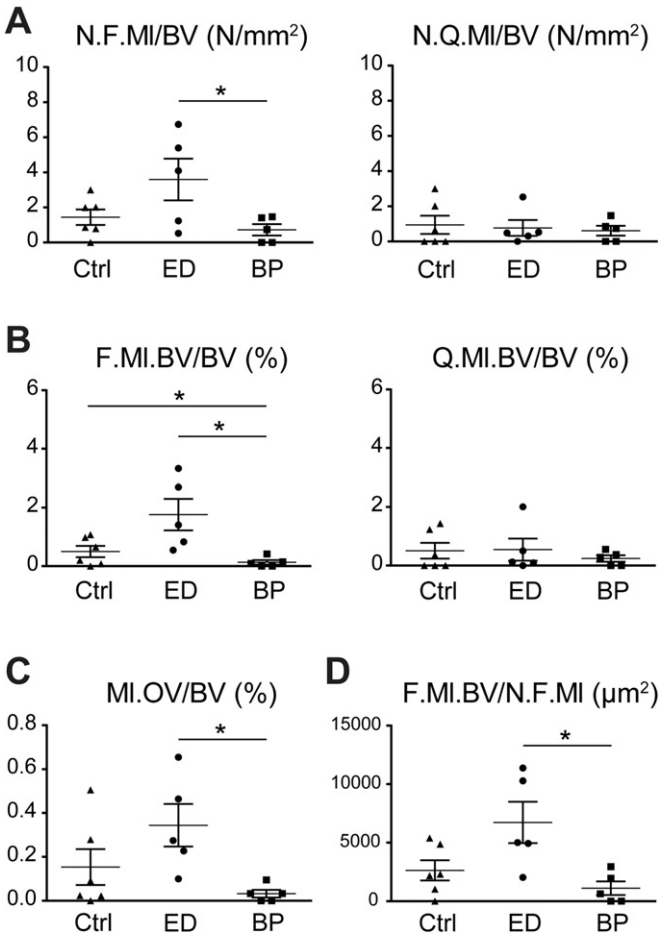

Fig. 3. Histomorphometric analysis of minimodeling. Bars indicate means ± SD. *$p < 0.05$.

microstructure by enhancing de novo bone formation in humans, thereby potentially reducing the risk of fragile fracture.

The concept of minimodeling is not new (Kobayashi et al., 2003; Takahashi et al., 1964; Frost, 1995); however, controversy still remains regarding how readily minimodeling occurs under physiological conditions in humans. In rats, a major proportion of bone formation occurs through minimodeling in their growth period (<6 months old) (Chow et al., 1993). In humans, Frost et al. showed that 3.3% of cement lines in the trabecular bone of bone specimens that were collected from 157 healthy human subjects did not exhibit scalloping and hypothesized that focal de novo bone formation (minimodeling) occurs in humans throughout life (Kobayashi et al., 2003; Takahashi et al., 1964; Frost, 1995). In agreement, Kobayashi et al. performed in vivo fluorochrome labeling and identified minimodeling in 63% of the bone specimens that were collected from menopausal patients (Kobayashi et al., 2003). This study, however, could potentially have underestimated the number of minimodeling sites because minimodeling sites were restricted to newly formed bone sites with fluorochrome incorporation. In agreement with the observations by Frost and Takahashi (Kobayashi et al., 2003; Takahashi et al., 1964; Frost, 1995), minimodeling-based bone formation was identified in 5 of 6 untreated patients and 86% of all the patients who were enrolled in the present study. Furthermore, the extent of minimodeling in our study was comparable with the results of Kobayashi's study (Kobayashi et al., 2003). Although some studies have reported the absence of minimodeling-based formation in human samples (Lindsay et al., 2006; Ma et al., 2006), our data are in agreement with the idea that minimodeling is not an exceptionally rare event in postmenopausal osteoporotic patients.

The nature and function of minimodeling are not fully understood. Previous studies have shown that bone anabolic agents, including PTH, PGE_2, and vitamin D analogs, stimulate bone formation through minimodeling in animals (Zhou et al., 2001; Yao et al., 1999; Saito et al., 2013) and humans (Lindsay et al., 2006; Ma et al., 2006). In studies using rodents, it has been shown that vitamin D analog-induced minimodeling can potentially increase trabecular connectivity (Erben, 2001). Recently, this observation was further confirmed in primates that were treated with ELD (Saito et al., 2015). Saito et al. showed that the trabecular connectivity of the lumber vertebra from OVX monkeys that were treated with ELD showed a higher node-to-node strut length and a lower terminus-to-terminus strut length compared with those in non-treated OVX monkeys. Based on these observations, the authors concluded that ELD treatment leads to an increase in trabecular connectivity in primates and may improve the biomechanical properties of the lumbar vertebrae. Although there are no data to support whether this biomechanical improvement also occurs in humans, it is tempting to speculate that the enhanced bone formation through minimodeling in patients who were treated with ELD contributed, at least in part, to the reduced rate of fracture incidence (Matsumoto et al., 2011).

Unlike other studies that use iliac bone specimens, we used the basal part of the lamina in the present study. Although it is not common to use the lamina for histomorphometric analysis, we were able to collect intact bone tissues with relative ease. It should also be noted that we had sampled other posterior parts of the vertebrae in our preliminary experiments and found the basal part of the lamina to be the least affected by the degenerative changes in the vertebrae. Most importantly, because the specimens were collected from bone tissues that are usually disposed of after surgery, there was virtually no disadvantage to or burden on the patients who were enrolled in the study.

There are several limitations in the present study. Because osteoporosis medications are often prescribed in combination, relatively few postmenopausal patients who underwent lumbar surgery were treated with a single drug in our patient pool. Moreover, lumber degenerative disorders affect women less frequently than men. For these reasons, we could only enroll 5–6 eligible patients in each group out of a total of approximately 150 patients who underwent spinal surgery at our institute during the study period. Furthermore, the subjects in our study all had lumbar degenerative disorder, which may have indirectly affected the systemic bone metabolism due to the decreased physical activity. Therefore, the results of the present study have to be interpreted with caution. In addition, we found no statistically significant differences in the bassline BMD among the subject groups, irrespective of the presence or absence of anti-osteoporotic medication prior to surgery (Table 1). This could have possibly derived from various factors, including the relatively small number of subjects, the relatively short period of medication usage (please note that ELD became available in 2011 in Japan), and the differences in the BMD among the patients when the medication was initiated. Nevertheless, the serum and urinary biochemical parameters were all within the normal range in patients that had been treated with ELD or BP; whereas, there was an increase in the values of TRACP-5b, DPD, and NTX in patients without any medication (Ctrl) (Table 2). The data indicate that the treatment with ELD or BP had been successful and effective in suppressing bone turnover rate, and supports the efficacy of the treatment in these patients.

In summary, despite the limited number of patients enrolled in the present study, our data suggest that a vitamin D analog ELD induces minimodeling-based bone formation in postmenopausal osteoporotic patients, whereas, BPs have suppressive effects on minimodeling. We would also like to emphasize herein that the histomorphometric analysis was fully done in a blind manner so as to eliminate any potential bias in evaluating the data. Consistent with the hypothesis raised by Frost and Takahashi (Jee et al., 2007; Kobayashi et al., 2003; Frost, 1995), our observations indicate that minimodeling is a physiologically relevant event that occurs throughout life. However, the nature and functions of minimodeling in humans remain to be elucidated. Further clinical investigations are warranted to clarify the physiological role of minimodeling and its potential contributions in skeletal homeostasis.

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