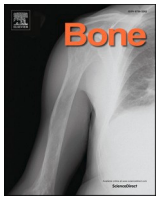
Bone 152 (2021) 116095

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone



Full Length Article

Anti-Siglec-15 antibody suppresses bone resorption by inhibiting osteoclast multinucleation without attenuating bone formation

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ARTICLE INFO

Keywords:
Siglec-15
Osteoclast
Osteoporosis
Anti-resorptive agent
Two-photon microscope
Bone coupling

ABSTRACT

Anti-resorptive drugs are widely used for the treatment of osteoporosis, but excessive inhibition of osteoclastogenesis can suppress bone turnover and cause the deterioration of bone quality. Sialic acid-binding immunoglobulin-like lectin 15 (Siglec-15) is a transmembrane protein expressed on osteoclast precursor cells and mature osteoclasts. Siglec-15 regulates proteins containing immunoreceptor tyrosine-based activation motif (ITAM) domains, which then induce nuclear factor of activated T-cells 1 (NFATc1), a master transcription factor of osteoclast differentiation. Anti-Siglec-15 antibody modulates ITAM signaling in osteoclast precursors and inhibits the maturation of osteoclasts *in vitro*. However, *in situ* pharmacological effects, particularly during postmenopausal osteoporosis, remain unclear. Here, we demonstrated that anti-Siglec-15 antibody treatment protected against ovariectomy-induced bone loss by specifically inhibiting the generation of multinucleated osteoclasts *in vivo*. Moreover, treatment with anti-Siglec-15 antibody maintained bone formation to a greater extent than with risedronate, the first-line treatment for osteoporosis. Intravital imaging revealed that anti-Siglec-15 antibody treatment did not cause a reduction in osteoclast motility, whereas osteoclast motility declined following risedronate treatment. We evaluated osteoclast activity using a pH-sensing probe and found that the bone resorptive ability of osteoclasts was lower following anti-Siglec-15 antibody treatment compared to after risedronate treatment. Our findings suggest that anti-Siglec-15 treatment may have potential as an anti-resorptive therapy for osteoporosis, which substantially inhibits the activity of osteoclasts while maintaining physiological bone coupling.

1. Introduction

Bone is a highly dynamic tissue that continuously undergoes bone formation and bone resorption, which is performed and regulated by several cell types. This sequential process, called bone remodeling, is well-coordinated so that homeostatic bone metabolism and skeletal strength are maintained [1]. Various cells and cytokines modulate bone remodeling, and imbalances in their equilibrium can lead to pathological bone disorders, such as osteoporosis and osteopetrosis. Osteoporosis is characterized by the deterioration of bone microstructure and bone fragility, leading to a high risk of fracture [2]. Postmenopausal osteoporosis, induced by estrogen deficiency following menopause, is one of the most common subtypes of primary osteoporosis. This condition, in which bone resorption is dominant over bone formation, results in bone loss [3].

The master transcriptional regulator of osteoclast differentiation is nuclear factor of activated T-cells 1 (NFATc1) [4]. Receptor activator of nuclear factor-κB ligand (RANKL), which binds to receptor activator of NF-κB (RANK), is one of the signals that regulates NFATc1. Proteins containing immunoreceptor tyrosine-based activation motif (ITAM)

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https://doi.org/10.1016/j.bone.2021.116095
Received 22 March 2021; Received in revised form 6 June 2021; Accepted 27 June 2021
Available online 1 July 2021

domains also play important roles in NFATc1 regulation. ITAM domains are present on the transmembrane adaptor protein DNAX-activating protein 12 kDa (DAP12) and Fc receptor common γ chain (FcR-γ). Mice lacking both DAP12 and FcR-γ exhibit severe osteopetrosis and completely disrupted osteoclast differentiation [5,6], whereas DAP12-deficient mice exhibit mild osteopetrosis [7].

Sialic acid-binding immunoglobulin-like lectin 15 (Siglec-15) is a transmembrane protein that recognizes sialylated glycans and promotes osteoclast differentiation by binding to DAP12 [8,9]. Siglec-15 knockout mice exhibit mild osteopetrosis with no other apparent phenotype [10,11]. Thus, this molecule has been identified as a possible therapeutic target for bone-destructive diseases [12,13]. Treatment with monoclonal antibodies against Siglec-15 reduces the number of multinucleated osteoclasts while preserving the number of mononuclear osteoclasts *in vitro*, indicating the inhibitory effects of the antibodies on the multinucleation of osteoclasts during the terminal phase of differentiation. However, it is unclear whether anti-Siglec-15 antibodies impair the multinucleation of osteoclasts *in vivo*. Furthermore, anti-Siglec-15 antibody treatment increases bone mass in healthy mice [12] and juvenile rats [14,15], but it remains unknown whether anti-Siglec-15 antibody treatment affects the cellular dynamics involved in bone remodeling of adults with postmenopausal osteoporosis.

In this study, we investigated the effects of monoclonal antibodies targeting Siglec-15 on osteoclasts in a mouse model of ovariectomy-induced osteoporosis using intravital imaging with two-photon microscopy and bone morphometry.

2. Materials and methods

2.1. Mice

Female C57BL/6J mice were purchased from Japan Clea (Tokyo, Japan). The generation of Col1a1(2.3)-enhanced cyan fluorescent protein (ECFP) mice and tartrate-resistant acid phosphatase (TRAP)-tdTomato mice (C57BL6/background) has been described previously [16,17]. All mice were fed a standard diet (Oriental Yeast Co., Ltd., Tokyo, Japan; MF) and maintained under a 12-hour light/dark cycle in a specific pathogen-free animal facility at Osaka University (Osaka, Japan). All animal experiments were performed according to institutional animal experimental guidelines, using protocols approved by the Animal Experimental Committee of Osaka University.

2.2. Drug treatments

To induce osteoporosis, bilateral ovariectomy was performed in 8–12-week-old female mice. Mice were divided into the sham operation group and the ovariectomy group. Ovariectomized mice were further assigned to 3 groups: treatment with rat immunoglobulin G (IgG) antibody (FUJIFILM Wako Pure Chemical Corporation, 10 mg/kg, once every 2 weeks; control group), treatment with risedronate (EA Pharma Co., Ltd., Tokyo, Japan, 5 μg/kg, 2 times a week; RIS group), and treatment with the anti-Siglec-15 rat monoclonal antibody 32A1 (Daiichi Sankyo Co., Ltd., Tokyo, Japan, 0.1 to 10.0 mg/kg, once every 2 weeks; Siglec group). The generation of 32A1 was described previously [14,15].

2.3. Blood sampling and analysis

Mice were sacrificed 14 days after ovariectomy. Blood samples were collected from the inferior vena cava and centrifuged for 15 min at 1700 ×*g*. The concentrations of the following markers were measured by Oriental Yeast Co., Ltd.: aspartate aminotransferase (AST; Japan Society of Clinical Chemistry [JSCC] transferable method), alanine aminotransferase (ALT; JSCC transferable method), total bilirubin (T-Bil; enzymatic method), total protein (TP; Biuret method), albumin (Alb; BCG method), and creatinine (Cre; enzymatic method).

2.4. Micro-computed tomography

Mice were euthanized 28 days after ovariectomy, and quantitative bone morphometric analyses of the femur and fifth vertebra were then performed using micro-computed tomography (μCT, ScanXmate-RX; Comscantecno, Kanagawa, Japan). Scanning was performed using a source voltage of 90 kV and a source current of 200 μA. Visualization and data reconstruction were performed using TRI/3D-BON software (RATOC System Engineering, Tokyo, Japan).

2.5. Histomorphometric analysis

To label active bone formation, all mice were injected subcutaneously with tetracycline (20 mg/kg) and calcein (10 mg/kg) 5 days and 2 days prior to sacrifice, respectively. The dissected and 70% ethanol-fixed right femur and vertebra were treated with Villanueva bone stain and embedded in methacrylate (Wako Pure Chemical Industries, Osaka, Japan) without decalcification. Histomorphometric bone parameters were determined based on the standardized nomenclature for bone histomorphometry.

2.6. Intravital two-photon bone imaging

Mouse parietal bone marrow was observed by two-photon microscopy as described previously [16]. For cell deformation index (CDI) measurements, the imaging system consisted of an upright two-photon microscope (A1R-MP; Nikon, Tokyo, Japan) equipped with a 25× water-immersion objective (APO: numerical aperture [NA], 1.1; Nikon). The system was driven by a dual-laser (Chameleon Vision II Ti: Sapphire; Coherent, Santa Clara, CA, USA) tuned to 860 and 1040 nm. Fluorescence was detected by an external non-descanned detector (Nikon) with the following filters: 417/60 nm for the second harmonic generation, 480/40 nm for ECFP, and 583/22 for tdTomato. For bone-resorbing index (BRI) measurements, the imaging system was composed of an upright two-photon microscope (LSM 780 NLO; Carl Zeiss) equipped with a 20× water-immersion objective (WPlan-Apochromat, NA 1.0). This system was driven by a laser (Chameleon Vision II Ti: Sapphire; Coherent) tuned to 940 nm.

2.7. Cell deformation index analysis for quantifying osteoclastic activity

Osteoclast morphological changes were quantified using the image analysis software CL-Quant 2.30 (Nikon). Cell shapes were recognized semi-automatically by the software, and the CDI was calculated as the ratio of the cell areas that changed over 10 min. CDI values negatively correlate with the bone resorptive activity of osteoclasts [18].

2.8. Bone-resorbing index analysis

A pH-sensing chemical probe (pHocas-3) dissolved in phosphate buffered saline was injected subcutaneously at a dose of 5 mg/kg per day for 3 consecutive days before imaging. After raw images were processed by spectral unmixing, constant γ corrections were applied to all images using NIS Elements integrated software to enhance the signal-to-noise ratio: tdTomato, $\gamma = 1.0$; and pHocas-3, $\gamma = 2.5$. We assessed the bone-resorbing ability of osteoclasts after image acquisition. Osteoclast areas were binarized according to Otsu's thresholding method and automatically extracted from the original maximum intensity projection images. The mean pHocas-3 fluorescence intensities in osteoclast areas (pHocas-3 signals) and outside osteoclast areas (pHocas-3 noise) were measured. The bone-resorbing index (BRI) was determined by calculating the signal-to-noise ratio of pHocas-3 [18].

2.9. Statistics

Data were analyzed using GraphPad Prism software (GraphPad

Software Inc., San Diego, CA, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Holm-Sidak test for comparisons among groups. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Anti-Siglec-15 antibody treatment prevented bone loss following ovariectomy

To investigate the effects of anti-Siglec-15 antibody treatment on estrogen deficiency-induced bone loss, we examined the trabecular bone of the femur and fifth vertebra by μCT analysis at 4 weeks after ovariectomy. In this experiment, we administered control IgG (10.0 mg/kg) and 32A1 (0.3 mg/kg, 1.0 mg/kg, 3.0 mg/kg, 10.0 mg/kg) once every 2

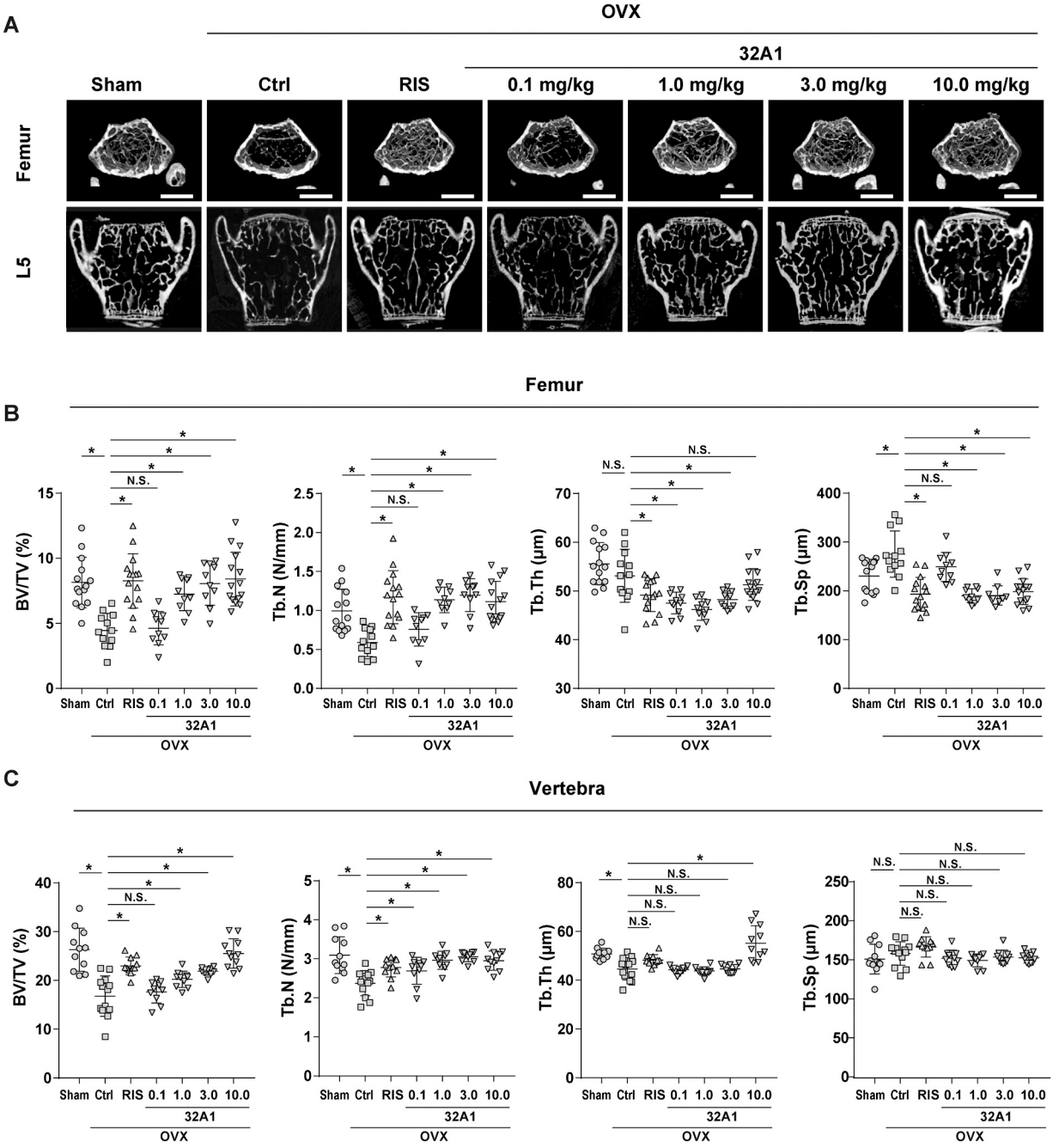

Fig. 1. Effects of anti-Siglec-15 therapy on trabecular bone. (A) Representative 3D-reconstructed micro-computed tomography (μCT) images of a distal femur and a coronal section of the fifth vertebra 4 weeks after antibody treatment. Scale bar: 1 mm. (B, C) Ratio of trabecular bone volume to total bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) of the distal femur and fifth vertebra. Data were analyzed statistically using one-way ANOVA analysis (*$P < 0.05$). Ctrl, ovariectomized group receiving control IgG; RIS, ovariectomized group receiving risedronate; 32A1, ovariectomized group receiving anti-Siglec-15 antibody in increasing concentrations (0.1 mg/kg, 1.0 mg/kg, 3.0 mg/kg, 10.0 mg/kg). Data are presented as the mean ± standard deviation.

weeks, and risedronate (5 μg/kg) twice per week.

Fig. 1A presents representative images of the reconstructed distal femur and a coronal section of the vertebra. Fig. 1B and C present the parameters describing bone microarchitecture (ratio of trabecular bone volume to total bone volume [BV/TV], trabecular thickness, trabecular number [Tb.N], and trabecular separation). The antibody control group had lower BV/TV and Tb.N compared to the sham group. A dose-dependent increase in BV/TV was observed in the Siglec group: when the concentration of 32A1 was >1.0 mg/kg, the BV/TV was significantly higher than in the control group, similar to the RIS group. These results suggest that anti-Siglec-15 antibody therapy for bone loss following ovariectomy is as effective as risedronate treatment. There were no side effects of 32A1 (10 mg/kg), such as weight loss (Supplementary Fig. 1A) or liver and kidney dysfunction (Supplementary Fig. 1B). Furthermore, we analyzed the effects of 32A1 on trabecular bone in control sham-operated mice, to investigate the effects of steady-state drug concentrations. In these mice, 32A1 (10 mg/kg) treatment also ameliorated the changes in bone mass of the femur and vertebra (Supplementary Fig. 2). Based on these results, 32A1 was administered at a concentration of 10 mg/kg in subsequent experiments.

3.2. Anti-Siglec-15 antibody inhibited osteoclast multinucleation in vivo

To further investigate the effects of anti-Siglec-15 antibody treatment *in vivo*, we examined the histomorphometry of trabecular bone in the third lumbar vertebra. Both risedronate and anti-Siglec-15 antibody treatments caused decreases in the number of osteoclasts and the surface of osteoclasts per bone surface (Fig. 2A). The number, and therefore generation, of multinucleated osteoclasts were higher in mice that had been subjected to ovariectomy (Fig. 2A). “Giant osteoclasts”, defined as those with more than 10 nuclei [19], were observed in the RIS group, but not in the Siglec group (Fig. 2B). The number of multinucleated osteoclasts was lower in the Siglec than RIS group (Fig. 2A). Both risedronate and anti-Siglec-15 antibody treatment reduced the number of mononuclear osteoclasts compared to the control group, but there was no significant difference between these two groups (Supplementary Fig. 3). In the control group, hook-shaped deep-bone resorption fossa were observed, which signified activated bone resorption. Both risedronate and anti-Siglec-15 antibody treatments reduced the eroded surface per bone surface ratio, but this effect was more prominent in the Siglec group (Fig. 2A). These findings indicated that anti-Siglec-15 antibody treatment had a more significant effect on bone resorption compared to risedronate under our experimental conditions. Therefore, the pharmacological effects of the anti-Siglec-15 antibody appear to

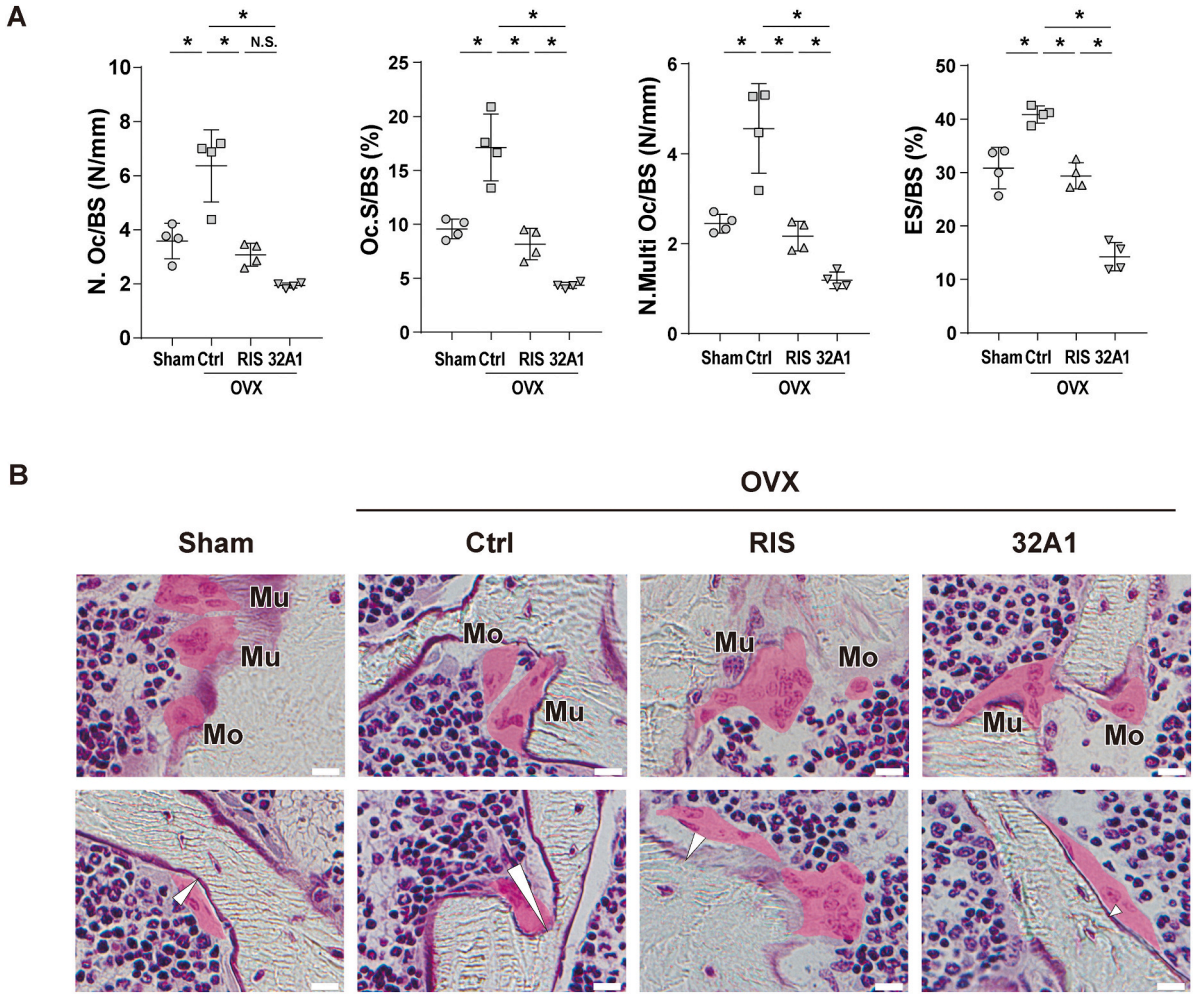

Fig. 2. Histomorphometric analysis of the third vertebrae of 12-week-old mice. (A) Ratio of osteoclast number to bone surface (N.Oc/BS), osteoclast surface to bone surface (Oc.S/BS), number of multinuclear osteoclasts per bone surface (N.Multi Oc/BS), eroded surface per bone surface (ES/BS). (B) Villanueva bone staining. Osteoclasts attached to eroded surfaces are shown. The white arrow indicates the depth of erosion. Mo, mononuclear osteoclast; Mu, multinuclear osteoclast. Scale bar: 10 μm. Ctrl, ovariectomized group receiving control IgG; RIS, ovariectomized group receiving risedronate; 32A1, ovariectomized group receiving anti-Siglec-15 antibody. Data were analyzed statistically using one-way ANOVA analysis (*$P < 0.05$). Data are presented as the mean ± standard deviation.

involve the inhibition of osteoclast multinucleation, which differs from the effect of risedronate *in vivo*.

3.3. Anti-Siglec-15 antibody treatment maintained bone formation more effectively than risedronate treatment

Anti-resorptive drugs also affect bone formation by inhibiting osteoclasts. Therefore, we further analyzed parameters related to bone formation. The number of osteoblasts per bone surface (N.Ob/BS) and the surface of osteoblasts per bone surface (Ob.S/BS) were lower in the RIS group than in the control group (Fig. 3A). In contrast, anti-Siglec-15 antibody treatment did not significantly reduce the N.Ob/BS or the Ob.S/BS (Fig. 3A). No significant differences in osteoid surface per bone surface were observed between the groups. However, osteoid volume per bone volume (OV/BV) was lower in the RIS group than in the control group. The Siglec group maintained similarly high values of OV/BV to the control group. The mineralizing surface per bone surface ratio, mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS) were lower in the RIS group than in the control group (Fig. 3A, B). However, the MAR and BFR/BS were higher in the Siglec group than in the RIS group (Fig. 3A). Taken together, these results indicate that anti-Siglec-15 antibody does not attenuate bone formation; this is in contrast to risedronate, which negatively affects bone formation.

3.4. Effects of antibody treatment on the motility and activity of osteoclasts

Next, we assessed how the inhibition of multinucleation affects cellular function in the mature osteoclasts of living mice using intravital imaging, a technique that enables the visualization of bone remodeling. Dynamic motility (amoeboid movement) was evaluated *via* CDI analysis (Fig. 4A, B). The CDI values were lower in the control and RIS groups than in the sham group. In contrast, the CDI values were the highest in

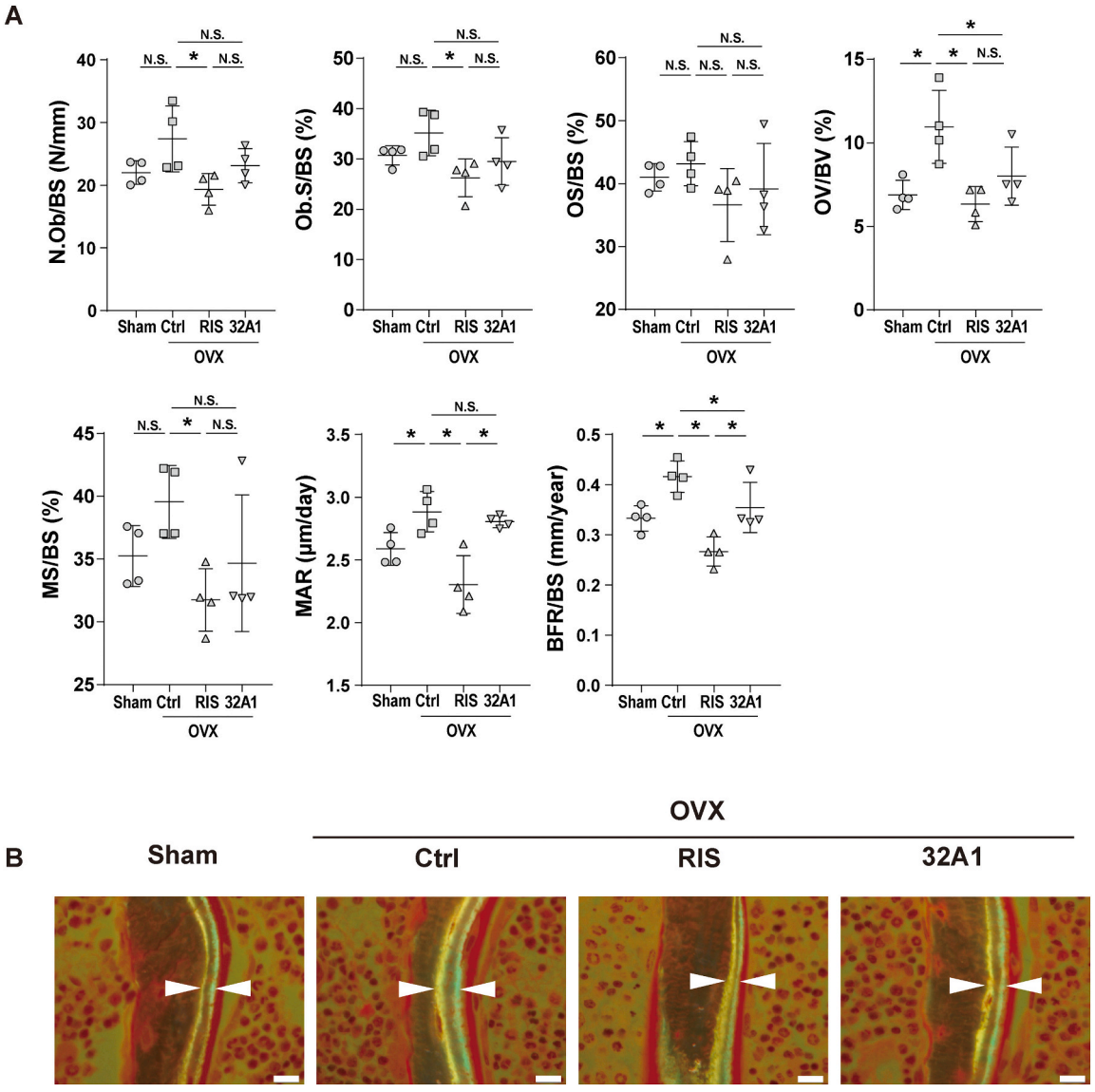

Fig. 3. Histomorphometric analysis of the third vertebrae of 12-week-old mice. (A) Number of osteoblasts per bone surface (N.Ob/BS), osteoblast surface per bone surface (Ob.S/BS), osteoid surface per bone surface (OS/BS), osteoid volume per bone volume (OV/BV), mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS). (B) Double-labeled bands fluorescently visualized using tetracycline (yellow) and calcein (green). Scale bar: 10 μm. Ctrl, ovariectomized group receiving control IgG; RIS, ovariectomized group receiving risedronate; 32A1, ovariectomized group receiving anti-Siglec-15 antibody. Data were analyzed statistically using one-way ANOVA (*$P < 0.05$). Data are presented as the mean ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

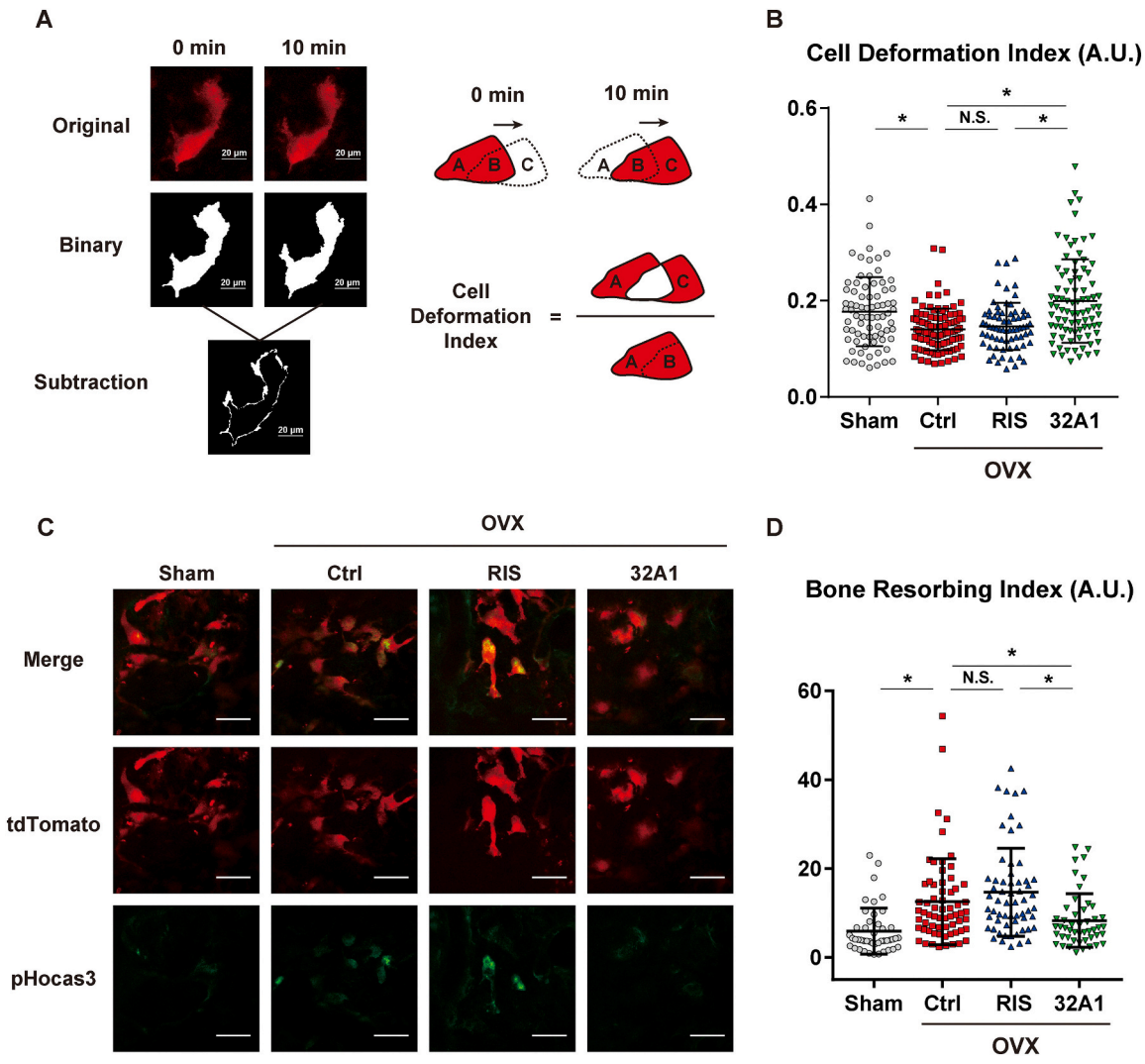

Fig. 4. Effects of each drug on the motility and acidification of osteoclasts. (A) The cell deformation index (CDI) was calculated as (A + C) / (A + B), where the ratio of the area changed during a 10-min period was divided by that of the previous time frame. (B) CDI of osteoclasts with and without osteoporosis after the administration of control IgG, risedronate, and anti-Siglec-15 antibody. Data points represent single cells collected from 3 to 4 mice/group. (C) Representative images of bone resorption activity in TRAP-tdTomato mice treated with the pH-sensing chemical probe (pHocas-3) (upper). Mature osteoclasts expressing TRAP-tdTomato signals (middle), and green fluorescent signals at a low pH (lower). Scale bar: 50 μm. (D) The bone-resorbing index (BRI) of osteoclasts. Data points represent single visual fields collected from 3 to 4 mice/group. Ctrl, ovariectomized group receiving control IgG; RIS, ovariectomized group receiving risedronate; 32A1, ovariectomized group receiving anti-Siglec-15 antibody. Data were analyzed statistically using one-way ANOVA analysis (*$P < 0.05$). Data are presented as the mean ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Siglec group compared to both the control and RIS groups. We further examined the bone resorptive activity of osteoclasts using a pH-sensing probe (Fig. 4C, D). The BRI values were higher in the control and RIS groups than in the sham group. In contrast, the BRI values were lower in the Siglec group than in the RIS group. These results suggest that anti-Siglec-15 antibody treatment strongly inhibits bone resorptive activity.

Regions of bone that exhibit osteoblast-osteoclast interactions are typically observed in the reversal phase of bone remodeling, where bone formation is active. Therefore, we further analyzed the crosstalk between osteoblasts and osteoclasts. Larger-sized osteoclasts were observed in the RIS group than in the Siglec group. Furthermore, the smaller-sized osteoclasts in the Siglec group were in contact with osteoblasts (Supplementary Fig. 4), suggesting that anti-Siglec-15 antibody treatment did not disturb cellular communications between osteoblasts and osteoclasts, in contrast to risedronate.

4. Discussion

Siglec-15 is involved in the maturation of osteoclasts by regulating ITAM signaling, which is necessary for the phosphorylation of NFATc1, a master regulator of osteoclast differentiation [10]. While Siglec-15 is localized intracellularly in human myeloid cells in the lymph nodes and spleen [20], it is expressed on the surface of osteoclasts [8,10,12], thus enabling the anti-Siglec-15 antibody to act as an anti-resorptive agent. In this study, we found that anti-Siglec-15 antibody treatment reduced ovariectomy-induced bone loss by inhibiting the generation of multinucleated osteoclasts and preserving bone formation.

Estrogen deficiency induces postmenopausal osteoporosis, which is the most common subtype of primary osteoporosis [21]. Although anabolic agents such as parathyroid hormone and anti-sclerostin antibodies strongly increase bone mass, the duration of administration is limited to 2 years and 1 year, respectively. As the predominant effect of estrogen deficiency is osteoclast activation, the administration of anti-resorptive agents to treat postmenopausal osteoporosis seems to be a

rational treatment strategy. However, evidence suggests that the long-term use of anti-resorptive drugs increases the risk of atypical femoral fracture and osteonecrosis of the jaw [22–24]. Although the pathophysiology of these diseases has not been fully elucidated, the attenuated bone turnover following the excessive suppression of bone resorption may be an important factor in their development [25]. Due to the increasing need for long-term treatments for postmenopausal osteoporosis, anti-resorptive agents that can preserve bone formation are urgently required. Previous studies showed that anti-Siglec-15 antibody treatment inhibited bone resorption *in vivo* in healthy mice and in juvenile mice subjected to steroid-induced osteoporosis without impairing their development [12,14,15]. Kameda et al. demonstrated that Siglec-15-null mice had resistance to bone loss following ovariectomy [13]. Therefore, we hypothesized that anti-Siglec-15 antibodies could also effectively treat bone loss induced by estrogen deficiency. In this study, we firstly found that anti-Siglec-15 antibody treatment protected against abrupt bone loss triggered by estrogen deficiency in mice.

An important benefit of anti-Siglec-15 antibody treatment is the specific inhibition of osteoclast maturation. The bone resorption activity of mononuclear osteoclasts is weak; multinucleation is necessary to generate mature functional osteoclasts [26,27]. Hiruma et al. found that treatment with polyclonal antibodies specific for mouse Siglec-15 markedly inhibited the differentiation of osteoclasts *in vitro*, particularly the generation of multinucleated osteoclasts [8]. Kameda et al. also reported that TRAP-positive cells derived from Siglec-15$^{-/-}$ cells were mostly mononuclear and could not form actin rings [13]. Consistent with these *in vitro* results, in the present study we firstly found that anti-Siglec-15 antibody treatment inhibited the multinucleation of osteoclasts *in vivo* compared to risedronate. Although anti-Siglec-15 antibody and risedronate treatments increased bone mass and reduced the number of osteoclasts to a similar extent, the number of multinucleated osteoclasts was significantly lower in the Siglec group than in the RIS group. Treatment with bisphosphonates can induce the generation of giant osteoclasts that have more than 10 nuclei, whereas most osteoclasts in the Siglec group had only 2–3 nuclei (data not shown). These results indicate that anti-Siglec-15 antibody treatment specifically inhibits multinucleation *in vivo*.

Osteoclasts produce various cell-to-cell contact-dependent and soluble coupling factors, which stimulate osteoblast differentiation and function [28–31]. This suggests that reductions in osteoclast number caused by anti-resorptive agents may attenuate bone formation [32]. The presence of osteoclasts themselves, not their bone resorptive activity, is important for the stimulation of bone coupling [29]. Therefore, agents that specifically inhibit the function of osteoclasts but do not reduce their number may exert positive effects on bone formation. In the present study, the Siglec group maintained its bone formation potential, whereas the RIS group exhibited relatively low bone formation activity. Furthermore, intravital imaging revealed higher osteoclast motility and lower osteoclast resorptive activity in anti-Siglec-15-treated mice compared to mice treated with risedronate. Some osteoclasts in the Siglec group were physically close to osteoblasts, whereas the osteoclasts in the RIS group were situated away from osteoblasts (Supplementary Fig. 1), suggesting that osteoclast motility may be associated with maintained coupling *via* cell-to-cell contact. However, the influence of non-resorbing mononuclear osteoclasts on bone coupling has not been fully elucidated [28–31]. Therefore, several key questions remain regarding how osteoclasts treated with anti-Siglec-15 antibody regulate the crosstalk between bone resorption and bone formation.

In this study, we discovered that anti-Siglec-15 antibody could strongly inhibit bone resorption while maintaining bone formation. This drug candidate may have potential as an effective treatment for postmenopausal osteoporosis.

CRediT authorship contribution statement

Hiroyuki Tsukazaki: Conceptualization, Data curation, Methodology, Validation, Visualization, Formal analysis, Investigation, Writing – original draft. **Junichi Kikuta:** Conceptualization, Methodology, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Tomoka Ao:** Conceptualization, Methodology, Investigation, Writing – original draft. **Akito Morimoto:** Conceptualization, Methodology, Writing – original draft. **Chie Fukuda:** Conceptualization, Methodology, Resources, Writing – review & editing. **Eisuke Tsuda:** Conceptualization, Methodology, Resources, Writing – review & editing. **Masafumi Minoshima:** Resources. **Kazuya Kikuchi:** Resources. **Takashi Kaito:** Writing – review & editing, Supervision. **Masaru Ishii:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgments

This work was supported by funding from CREST, Japan Science and Technology Agency, Japan (to M.I.), Grants-in-Aid for Scientific Research (S) from the Japan Society for the Promotion of Science, Japan (JSPS to M.I.); PRIME (Japan Agency for Medical Research and Development, Japan [AMED] to J.K.), a Grant-in-Aid for Young Scientists from JSPS (to J.K.), and grants from the Uehara Memorial Foundation, Japan (to M.I.), the Kanae Foundation, Japan for the Promotion of Medical Sciences (to M.I), the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Japan (to M.I.), and the Takeda Science Foundation, Japan (to M.I.).

Declaration of competing interest

C.F. and E.T. are employees of Daiichi Sankyo Co., Ltd. The other authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2021.116095.

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