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Donepezil prevents RANK-induced bone loss via inhibition of osteoclast differentiation by downregulating acetylcholinesterase

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Abstract

Objective: Donepezil, an inhibitor of acetylcholinesterase (AChE) targeting the brain, is a common medication for Alzheimer's disease. Interestingly, a recent clinical study found that administration of this agent is associated with lower

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risk of hip fracture independently of falling, suggesting its direct effect on bone tissues as well. AChE has been reported to be involved in osteoblast function, but the role of AChE on osteoclastogenesis still remains unclear. We analyzed the effect of AChE and donepezil on osteoclastogenesis *in vivo* and *in vitro*.
Methods: Cell-based assays were conducted using osteoclasts generated in cultures of murine bone marrow macrophages (BMMs) with receptor activator of nuclear factor-kappa B ligand (RANKL). The effect of donepezil was also determined *in vivo* using a mouse model of RANKL-induced bone loss.
Results: Recombinant AChE in BMMs cultured with RANKL further promoted RANKL-induced tartrate-resistant acid phosphatase (TRAP)-positive osteoclast differentiation. RANKL also upregulated AChE expression in BMMs. RNA interference-mediated knockdown of AChE significantly inhibited RANKL-induced osteoclast differentiation and suppressed gene expression specific for osteoclasts. AChE upregulated expression of RANK, the receptor of RANKL, in BMMs. Donepezil decreased *cathepsin K* expression in BMMs and the resorptive function of osteoclasts on dentine slices. Donepezil decreased RANK expression in BMMs, resulting in the inhibition of osteoclast differentiation with downregulation of c-Fos and upregulation of Id2. Moreover, administration of donepezil prevented RANKL-induced bone loss *in vivo*, which was associated with the inhibition of bone resorption by osteoclasts.
Conclusions: AChE promotes osteoclast differentiation *in vitro*. Donepezil inhibits osteoclast function *in vitro* and prevents bone loss by suppressing bone resorption *in vivo*, suggesting the possibility that donepezil reduces fracture risk in patients with Alzheimer's disease.

Keywords: Acetylcholinesterase, Osteoclast, Donepezil, Alzheimer's disease, Differentiation

1. Introduction

Bone is continuously renewed in a balanced alternation by osteoclasts and osteoblasts [1]. Hemopoietic cells of the monocytes/macrophage lineage differentiate into osteoclasts, which are capable of resorbing bone under the control of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), both of which are generated by osteoblastic cells in bone tissues [2]. The expression of RANK, the receptor of RANKL, in osteoclast precursors is required for RANKL-induced osteoclast differentiation. After RANKL binds to RANK, the transcription factor c-Fos is upregulated for activating nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*), the master regulator for osteoclast differentiation, leading to the expression of genes specific for osteoclasts, such as tartrate-resistant acid phosphatase (TRAP) and *cathepsin K* [3].

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Donepezil, which is an inhibitor of acetylcholinesterase (AChE) and thereby leads to increased concentrations of acetylcholine in the brain, is widely used for the treatment of patients with Alzheimer's disease [4]. Of note, recent clinical data suggested that donepezil administration dose-dependently reduced the increased risk of hip fracture independently of falling in patients with Alzheimer's disease [5]. Although donepezil can easily cross the blood-brain barrier and acts centrally in the brain, the half-life of donepezil in humans has been known to be around 3 days, suggesting that donepezil may act directly on bone cells as well [6]. Thus, it is possible to speculate that donepezil has a dual function in affecting bone mass peripherally as well as centrally.

AChE, which rapidly hydrolyzes the neurotransmitter acetylcholine in synapses and neuromuscular junction, has been detected in various cells such as hematopoietic, osteogenic and neoplastic cells [7] [8] [9] [10]. Several lines of evidence have shown that AChE has non-hydrolytic functions as well, besides its well-known action as a hydrolytic enzyme of acetylcholine [10]. Axonal and neurite growth is regulated by AChE without hydrolytic activity in chick nerve cells [11], and the neurite outgrowth-promoting effect of AChE was associated with a non-catalytic mechanism in chick sympathetic neurons [7]. The rate of oxygen consumption in macrophages is increased by stimulating AChE independent of catalytic activity [12].

Recent evidence has indicated that AChE is involved in the function of osteoblasts. For instance, AChE mRNA variant 3′ terminated with exon 6 expression is increased with osteoblast differentiation, whereas its suppression increases the proliferation rate in human osteosarcoma cells [13]. Furthermore, Genever and his colleagues demonstrated that AChE is a novel osteoblast-derived mediator of cell-matrix interactions [14] [15]. Although AChE is also expressed in macrophages and osteoclasts as well [16] [17], the function of AChE in osteoclasts has not been examined before.

We hypothesized that AChE may be directly involved in osteoclastic differentiation. In this report, we investigated whether AChE and donepezil affect differentiation and function of osteoclasts *in vitro*. To examine this possibility, cell-based assays were conducted using osteoclasts generated by murine bone marrow macrophages (BMMs) treated with RANKL. The effect of donepezil was also determined *in vivo* using a mouse model of RANKL-induced bone loss.

2. Methods

2.1. Animals

All mice (C57BL/6) were obtained from Tokyo Laboratory Animals Science, and were kept on a normal laboratory chow in an environmentally controlled

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clean room at the Department of Oral and Maxillofacial Surgery, Saitama Medical University. The experiments were conducted according to the institutional guidelines for ethical animal experiments. To obtain the mouse model of RANKL-induced bone loss, soluble RANKL (sRANKL, 1 mg/kg, Oriental Yeast, Japan) was injected intraperitoneally at 24 h intervals for 3 days into male mice (9-week-old) as previously described [18]. To evaluate the prophylactic effect of donepezil, donepezil (2 mg/kg, Sigma-Aldrich, USA) was injected before each injection of sRANKL three times at 24 h intervals intraperitoneally. This dose of donepezil was selected on the basis of previous studies [19] [20] [21]. The mice were killed 90 min after the last injection. Blood samples were collected at the time of sacrifice. Six mice were used in each group.

2.2. Cell culture and reagents

We have obtained TRAP-positive osteoclasts according to the established protocols [18]. We used BMMs derived from tibia of 10-week-old female mice. BMMs were cultured in α-minimal essential medium (MEM) containing 10% FBS incubated with 10 ng/ml of macrophage colony-stimulating factor (M-CSF, Peprotech, USA) for 2 days. BMMs were further cultured in the presence or absence of 100 ng/ml of RANKL. Donepezil or recombinant mouse acetylcholinesterase (rAChE, R&D, USA) at indicated concentrations were added at the same time with RANKL. The conditions of each experiments are as follows: 24 well plates at 300×10^4 cells per well or 48 well plates at 100×10^4 cells per well for TRAP staining and harvest of total RNA; 8-mm chamber slides at 1×10^4 cells per well for immunocytochemistry; 96 well plates at 4×10^3 cells per well for cell proliferation assay. For heat-inactivated AChE experiments, recombinant AChE was heated for 3 min at 100°C prior to adding to BMMs cultures.

2.3. TRAP staining

For TRAP staining, cells were fixed with 10% formalin for 5 min to stain for TRAP. Thereafter, they were re-fixed with ethanol:acetone (50:50 v/v) for 1 min and incubated in acetate buffer (pH 4.8) containing naphthol AS-MX phosphate (Sigma-Aldrich), fast red violet LB salt (Sigma-Aldrich), and 50 mM sodium tartrate at room temperature. TRAP-positive multinucleated cells with more than 3 nuclei were counted as osteoclasts. Results are representative of at least four independent experiments.

2.4. Cell proliferation assay

The cells were incubated in conditioned medium for the indicated times with donepezil or rAChE. The sample cells were quantified using a Cell Titer 96

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Aqueous One Solution Cell Proliferation Assay (Promega, USA), according to the manufacturer's instructions. The measurements are represented by means of at least three independent experiments, with each data point based upon six replicates.

2.5. Quantitative reverse-transcriptase polymerase chain reaction (qPCR)

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Japan) and following qPCR was conducted as previously described [18]. For qPCR of *AChE*, TaqMan-based detection was conducted using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). TaqMan Gene Expression Assays (Thermo Fisher Scientific) for AChE (Mm00477274_g1) and Mouse GAPDH (Mm03302249_g1) were used. For qPCR of genes related with osteoclast differentiation, we used SYBR green-based technique using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). The gene-specific primer pairs used are as follows: *NFATc1* sense, 5′-ATGCCAAATACAGCTTTCCAGTC-3′ and *NFATc1* antisense, 5′-CGTCTTCCACCTCCACGTCG-3′; *cathepsin K* sense, 5′-CCTGGAGGGCCAACTCAAG-3′ and *cathepsin K* antisense, 5′-ATCTCTCTGTACCCTCTGCAT-3′; *RANK* sense, 5′-AGAGGCATTATGAGCATCTCG-3′ and *RANK* antisense, 5′-GGAGTGCACTTAGAGGACAGGT-3′; *inhibitors of differentiation/DNA binding 2 (Id2)* sense, 5′-GACAGAACCAGGCGTCCA-3′ and *Id2* antisense, 5′-AGCTCAGAAGGGAATTCAGATG-3′; β-*actin* sense, 5′-AGAAGGACTCCTATGTGGGTGA-3′ and β-*actin* antisense, 5′-CATGATCTGGGTCATCTTTTCA-3′. For qPCR analysis, values were normalized to β-actin or GAPDH using the 2_ΔΔCt method. The measurements are represented by the means of at least three independent experiments, with each data point based upon triplicates.

2.6. Western blot analysis

Western blot analysis was performed as described previously [18]. We used anti-AChE rabbit polyclonal antibody (sc-11409, Santa Cruz, USA), anti-c-Fos rabbit monoclonal antibody (#2250, Cell Signaling, USA), and anti-Id2 rabbit polyclonal antibody (sc-489, Santa Cruz) as primary antibodies. Anti-rabbit IgG HRP-linked antibody (#7074, Cell Signaling) and donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz) were used as secondary antibodies. Anti-β-actin mouse monoclonal antibody (sc-47778, Santa Cruz) was used as a loading control. Similar independent experiments were repeated at least three times.

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2.7. Measurement of serum deoxypyridinoline (DPD) cross-links

The serum DPD cross-links was measured by Mouse DPD assay kit (CSB-E08401m, CUSABIO, China). The measurements are represented by the means of at least three independent experiments, with each data-point based upon six replicates.

2.8. Retroviral infection

To generate retrovirus stock, PLATINUM Select shRNA-mir Series retroviral vectors (pMX, pMX-shAChE, transOMIC technologies, USA) were transfected into the packaging cell line Plat-E using $CaCl_2$ [22]. Supernatant was collected from the virus culture media 48 h after transfection and filtered through a 0.45-μm filter. BMMs were incubated with virus supernatant for 12 h in the presence of polybrene (8 μg/ml). After removing the virus supernatant, BMMs were incubated in the presence of M-CSF and RANKL for 5 days.

2.9. High performance liquid chromatography (HPLC)

To measure serum concentration of donepezil, HPLC was used. 100 μl of the serum sample was gathered in 1.5 ml tube and 70 μl of CH3CN was carefully put in the ample solution. The solution was mixed well by using a sonicator. Then, the sample tube was left sitting for three hours on ice and the tube was centrifuged for three hours by 12,000 g. 100 μl of the supernatant was filtered by using a spin column cut by molecular weight of 10,000 and the filtered liquid centrifugal was evaporated. A dried sample was dissolved with 100 μl of Solvent A and the whole quantity of the solution was applied to HPLC. The condition for HPLC was as follows: solvent A (0.1% TFA in H_2O); solvent B (0.1% TFA in CH_3CN); column (Wako C18 for PTH amino acid); column temperature (35°C); flow rate (0.7 ml/min); wavelength of detection (271 nm). All samples were examined in duplicate assays.

2.10. Immunocytochemistry

For immunofluorescence, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed in phosphate-buffered saline (PBS), then permeated for 5 min on ice. After blocking with 1% BSA solution, anti-AChE rabbit polyclonal antibody (sc-11409, Santa Cruz, USA) as primary antibody was incubated with specimens. Then Alexa Fluor568 goat anti-rabbit IgG (#A11011, Thermo Fisher Scientific) was used as secondary antibody and subsequently mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories, USA). Similar independent experiments were repeated at least three times.

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To detect apoptosis of osteoclasts, the fluorescein-dUTP TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, USA) was conducted. Briefly, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then rinsed in PBS and then permeabilized for 5 min on ice before labelling with 50 μl of TUNEL reaction mixture and incubating at 37 °C for 1 h in a humidified chamber under parafilm coverslips. After washing with PBS, slides were mounted in MobiGLOW Mounting Medium. Cells were counted under fluorescence microscopy. Samples were run in six replicates for each condition tested.

2.11. Histological and histomorphometric analyses

Mice given soluble PBS, RANKL, and/or donepezil were injected with calcein (25 mg per kg of body weight; Sigma) intraperitoneally 4 days and 1 day before sacrifice. After vertebrae were fixed in 4% paraformaldehyde and dehydrated by ethanol, they were embedded in methylmethacrylate. The plastic blocks were trimmed and 4 or 7 μm-thick sections were prepared with a RM2255 (Leica Microsystems). Undecalcified sections of the lumbar vertebrae were stained for von Kossa, Toluidine Blue and TRAP staining. Static and dynamic histomorphometric analyses were performed using OsteoMeasure Analysis System (OsteoMetrics) following nomenclature defined by the American Society for Bone and Mineral Research (ASBMR). Bone volume/tissue volume (BV/TV; %), bone formation rate/bone surface (BFR/BS; $\mu m^3/\mu m^2$/year), mineral apposition rate (MAR; μm/year), osteoblast surface (Ob.S/BS; %) and osteoclast number/bone perimeter (No.Oc/B.Pm; per 100 mm) were analysed. Six mice were examined for each group.

2.12. Statistical analysis

Comparisons between two groups were analyzed using Student's t-tests and comparisons among three groups were analyzed using One-Way Analysis of Variance and Bonferroni/Dunn methods (#, $p < 0.05$; ##, $p < 0.01$). All values are represented as the mean ± S.E.M.

3. Results

3.1. AChE accelerates RANKL-induced osteoclast differentiation, and RANKL upregulates endogenous AChE concentration in BMMs

We hypothesized that AChE plays a vital role in osteoclast differentiation and function *in vitro*. To examine this hypothesis, recombinant AChE was added at 50, 100, and 200 ng/ml to BMMs cultures in the presence of RANKL. Cultures were conducted for 5 days in the presence of RANKL and M-CSF.

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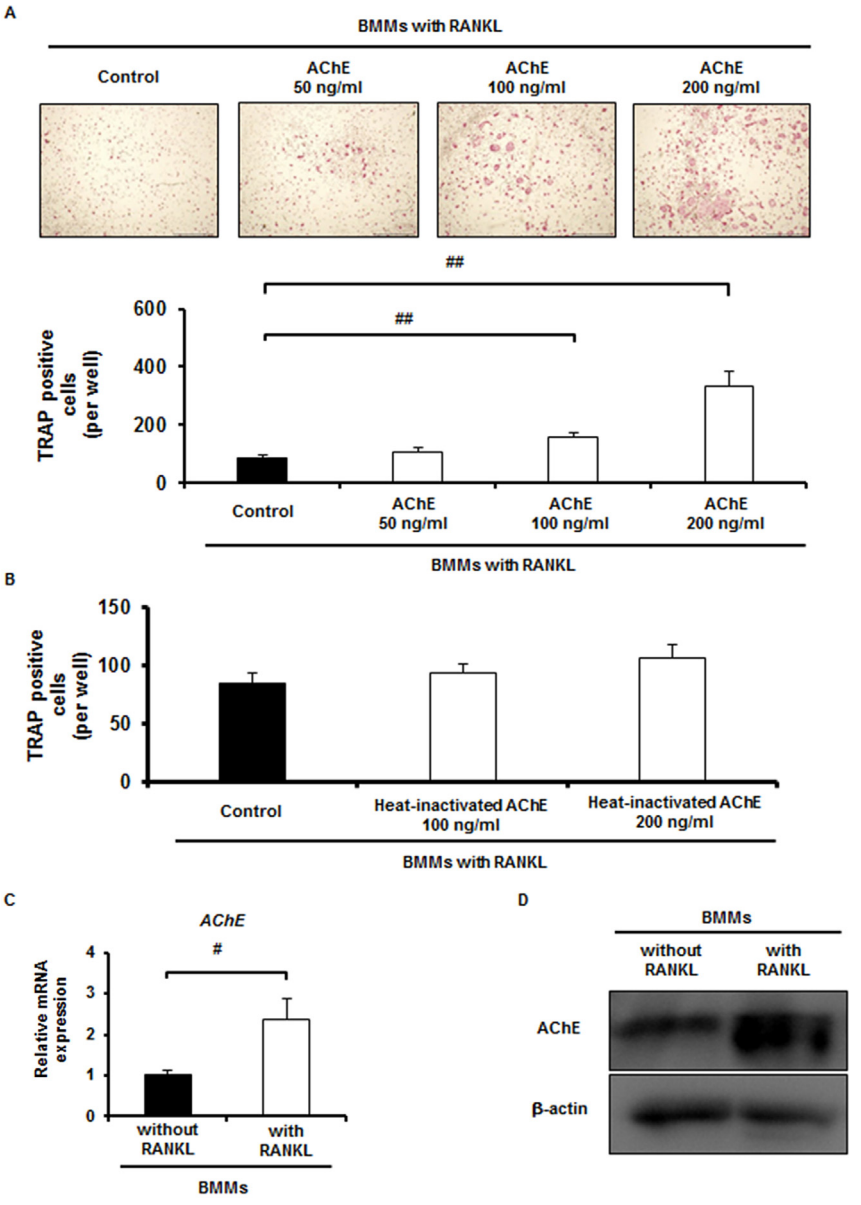

Fig. 1. AChE promotes RANKL-induced osteoclast differentiation of BMMs, and RANKL upregulates endogenous AChE. A, Effect of rAChE (0, 50, 100, and 200 ng/ml) on osteoclast differentiation of BMMs: (upper panel) TRAP staining (Scale bar, 1.0 mm), (lower panel) number of TRAP-positive multinucleated cells formed (more than 3 nuclei). Data are calculated from three repeated experiments. B, Effect of heat-inactivated rAChE (0, 100, and 200 ng/ml) on osteoclast differentiation of BMMs: number of TRAP-positive multinucleated cells formed (more than 3 nuclei). Data are calculated from three repeated experiments. C, Expression of AChE mRNA in BMMs in the presence or absence of RANKL for 24 h estimated by quantitative RT-PCR analysis. Data are calculated from three repeated experiments. D, Expression of AChE protein in BMMs in the presence or absence of RANKL for 48 h estimated by Western blot analysis. Data are calculated from three repeated experiments. rAChE, recombinant acetylcholinesterase; RANKL, receptor activator of nuclear factor-κB ligand; BMMs, bone marrow macrophages; TRAP, tartrate-resistant acid phosphatase; #, $p < 0.05$; ##, $p < 0.01$. Data are expressed as the means ± S.D.

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RANKL-induced TRAP-positive cell formation was significantly increased when rAChE was added at 100 and 200 ng/ml in a dose-dependent manner (Fig. 1A). Expression of both *NFATc1* and *cathepsin K* was also upregulated significantly by adding 100 ng/ml of rAChE (Supplementary Fig. S1A). To confirm that the increase of TRAP-positive cell formation is not due to the stimulation of cell proliferation of BMMs, we assessed proliferation assay of BMMs cultures. rAChE rather inhibited proliferation in BMMs cultures significantly (Supplementary Fig. S1B).

Supplementary material related to this article is found, in the online version, at http://dx.doi.org/10.1016/j.heliyon.2015.e00013.

We examined whether AChE promotes osteoclast differentiation independent of enzymatic action. Heat-inactivated rAChE was added at 100 and 200 ng/ml to the BMMs cultures treated with RANKL. Cultures were conducted for 5 days in the presence of RANKL and M-CSF. We found that heat-inactivated AChE did not promote RANKL-induced osteoclast formation (Fig. 1B). This result suggests that enzymatic activity of AChE is required for promoting RANKL-induced osteoclast differentiation.

We next conducted qPCR analysis to examine mRNA expression of AChE in BMMs in the presence or absence of RANKL. mRNA expression of AChE was significantly upregulated in BMMs in the presence of RANKL (Fig. 1C). Western blot analysis showed that the expression of AChE protein was also upregulated by RANKL stimulation (Fig. 1D). Immunofluorescence staining showed that RANKL increased accumulation of AChE protein in BMMs compared with the control group (Supplementary Fig. S1C). Short hairpin AChE (shAChE)-A, B or C was retrovirally delivered to BMMs and the mRNA expression level of AChE was verified by qPCR (Supplementary Fig. S2A). Among three subtypes of shAChE, the subtype shAChE-A most effectively suppressed mRNA expression of AChE. Knockdown of AChE-A in BMMs suppressed RANKL-induced TRAP-positive cell formation (Supplementary Fig. S2B) and *NFATc1* expression (Supplementary Fig. S2C). These results indicate that AChE derived from BMMs promotes RANKL-induced osteoclast differentiation. Interestingly, treatment of BMMs with AChE significantly upregulated mRNA expression of RANK, the receptor of RANKL, in a time-dependent manner (Supplementary Fig. S2D).

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3.2. Donepezil suppresses osteoclast differentiation

In order to block the signals of AChE in BMMs treated with RANKL, donepezil, a specific inhibitor of AChE, was used. As expected, when

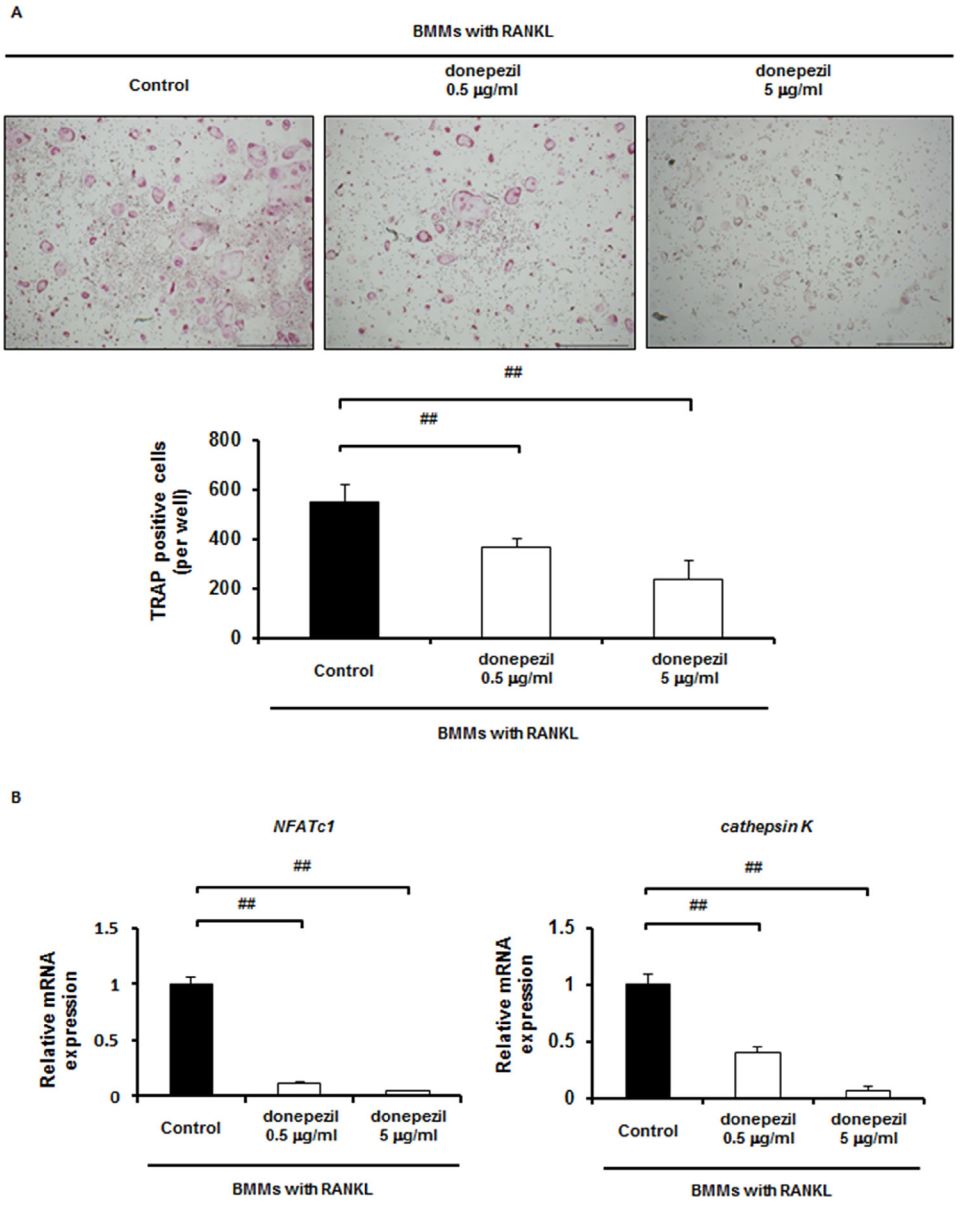

Fig. 2. Inhibition of osteoclast differentiation in BMMs by donepezil. A, Effect of donepezil (0, 0.5 and 5 μg/ml) on osteoclast differentiation of BMMs: (upper panel) TRAP staining, (lower panel) number of TRAP-positive multinucleated cells formed (Scale bar, 1.0 mm). Data are calculated from four repeated experiments. B, mRNA expression of osteoclast-specific genes in BMMs treated with RANKL with or without donepezil (0, 0.5 and 5 μg/ml) for 5 days estimated by qPCR analysis: (left panel) *NFATc1*, (right panel) *cathepsin K* . Data are calculated from three repeated experiments. #, $p < 0.05$; ##, $p < 0.01$. Data are expressed as the means ± S.D.

donepezil was added at 0.5 and 5.0 μg/ml to BMMs cultured for 5 days in the presence of RANKL, TRAP-positive cell formation was significantly decreased in a dose-dependent manner (Fig. 2A). Gene expression of both *NFATc1* and

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cathepsin K was also dose-dependently downregulated by donepezil (Fig. 2B). To confirm that the decrease of TRAP-positive cell formation is not due to the inhibition of cell proliferation, we assessed whether donepezil affects cell proliferation of BMMs. Donepezil rather increased cell proliferation in BMMs cultures (Supplementary Fig. S3A), suggesting that the suppressing effect of donepezil on osteoclast formation is simply not due to the inhibition of cell proliferation.

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3.3. Donepezil induces Id2 expression in osteoclast differentiation

To obtain an insight into what molecule or molecules are involved in the inhibitory effect of donepezil on osteoclast differentiation, we examined *Id2*, *the protein inhibitor of activated STAT3 (PIAS3)*, and *paired box protein 6 (Pax6)* mRNA expression, since these molecules have been reported to be involved in osteoclast differentiation as negative regulators [22] [23] [24]. The mRNA expression level of *Id2* in donepezil-treated BMMs was upregulated during osteoclast differentiation in a dose-dependent manner (Fig. 3A), whereas the levels of *PIAS3* and *Pax6* were not changed appreciably (data not shown). The level of Id2 protein was also upregulated in a similar manner (Fig. 3B). mRNA expression of Id2 was not changed at 9 h but it was greatly upregulated at 24 h after stimulation by donepezil (Supplementary Fig. S3B). Furthermore, the level of c-Fos protein, which plays an essential role for osteoclast differentiation [25], was downregulated by donepezil in a dose-dependent manner (Fig. 3B). We then examined whether donepezil affects RANK expression of BMMs, since mRNA expression of RANK was upregulated when BMMs were cultured with 100 ng/ml of rAChE (Supplementary Fig. S2D). Expectedly, mRNA expression of RANK in BMMs was downregulated by adding donepezil in a time-dependent manner (Fig. 3C). These results suggest that donepezil directly inhibits osteoclast differentiation, in accompanied with the Id2 upregulation and c-Fos downregulation.

It is not known whether AChE promotes RANKL-induced osteoclast formation by degradation of acetylcholine. If this is the case, acetylcholine will be produced by RANKL-treated BMMs and then reduce TRAP-positive cell formation. However, a previous report demonstrated that acetylcholine did not affect TRAP-positive cell formation but directly induced apoptosis in RANKL-treated BMMs cultures [17]. This suggests that acetylcholine is not produced by RANKL-treated BMMs. To test whether acetylcholine is produced by RANKL-treated BMMs, we added donepezil in the RANKL-treated BMMs cultures. Unexpectedly, donepezil did not induce apoptosis in RANKL-treated

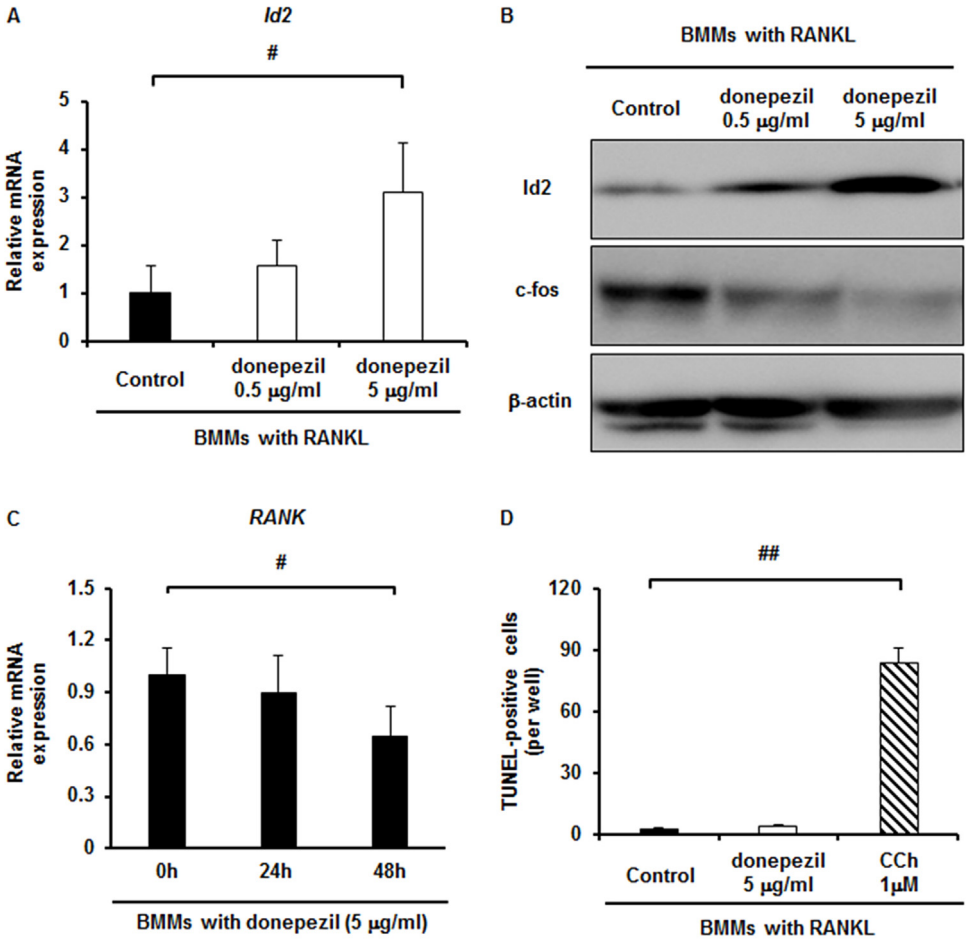

Fig. 3. Id2 upregulation by donepezil-dependent inhibition of osteoclast differentiation and donepezil does not induce apoptosis in osteoclast. A, The expression of Id2 mRNA in BMMs with donepezil (0, 0.5 and 5 μg/ml) in the presence of RANKL for 24 h estimated by qPCR analysis. Data are calculated from three repeated experiments. B, The expression of Id2 and c-Fos protein in BMMs with donepezil (0, 0.5 and 5 μg/ml) in the presence of RANKL for 36 h estimated by Western blot analysis. Data are calculated from three repeated experiments. C, Expression of RANK mRNA in BMMs with donepezil (5 mg/ml) for the indicated times estimated by qPCR analysis. Data are calculated from three repeated experiments. D, Detection of apoptotic cells in BMMs stimulated by donepezil (5 μg/ml) or carbamylcholine (CCh at 1 μM) in the presence of RANKL for 48 h. Data are calculated from three repeated experiments. RANKL, receptor activator of nuclear factor-κB ligand; BMMs, bone marrow macrophages; TRAP, tartrate-resistant acid phosphatase; Id2, inhibitors of differentiation/DNA binding 2; #, $p < 0.05$. Data are expressed as the means ± S.D.

BMMs at all (Fig. 3D). In contrast, carbamylcholine (CCh) which is the stable analogue of acetylcholine induced apoptosis (Fig. 3D). These results suggest that acetylcholine is not accumulated in the culture supernatant of RANKL-treated BMMs.

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3.4. Donepezil prevents RANKL-induced bone loss *in vivo* via suppression of osteoclastic bone resorption

Finally, because donepezil strongly inhibited RANKL-induced osteoclast differentiation *in vitro*, we examined whether donepezil affects bone mass in growing wild type mice in a short-term administration and has a preventive effect on the loss of bone mass in mice which are induced bone loss by short-term administration of RANKL. Donepezil administration three times for 3 days at 24 h intervals in 9-week-old mice exhibited high bone mass by inhibiting bone resorption (Fig. 4A–C). In RANKL-induced model, injections of soluble RANKL (1 mg/kg) three times at 24 h intervals induced bone loss in 9-week-old male mice (Fig. 4A–C). Donepezil administration also prevented RANKL-induced bone loss (Fig. 4A). Donepezil also decreased the number of osteoclasts *in vivo* (Fig. 4B) and serum concentration of DPD, a marker of bone resorption (Fig. 4C). In contrast, osteoblastic bone formation was not changed by donepezil administration. (Supplementary Fig. S4A–C). Donepezil was detected in serum on HPLC in mice injected with donepezil (Supplementary Fig. S4D). These findings suggest that donepezil directly prevents bone loss by suppressing osteoclastic bone resorption *in vivo*.

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4. Discussion

The present study demonstrates that AChE plays an important role for osteoclast differentiation and that donepezil inhibits osteoclast differentiation and function *in vitro,* which may be a cause at least in part for the RANKL-induced bone loss *in vivo* in mice treated with donepezil. Knockdown of endogenous AChE resulted in the inhibition of *NFATc1* mRNA expression and TRAP-positive cell formation. Furthermore, endogenous AChE mRNA expression was downregulated by donepezil. These results suggest that the endogenous level of AChE in BMMs is enhanced by AChE in a positive feedback loop to promote osteoclast differentiation. RANK mRNA expression was upregulated by AChE (Supplementary Fig. S2D) and downregulated by donepezil (Fig. 3C). This suggests that AChE modulates the sensitivity of BMMs to RANKL.

Heat-inactivated AChE did not promote RANKL-induced osteoclast differentiation (Fig. 1B). This suggests that the enzymatic activity of AChE is required for the promotion of osteoclast differentiation. Since acetylcholine has been known to be the target molecule of AChE, we examined the involvement of acetylcholine in the promotion of osteoclast differentiation by AChE. Bajayo et al. demonstrated that acetylcholine did not affect TRAP-positive cell

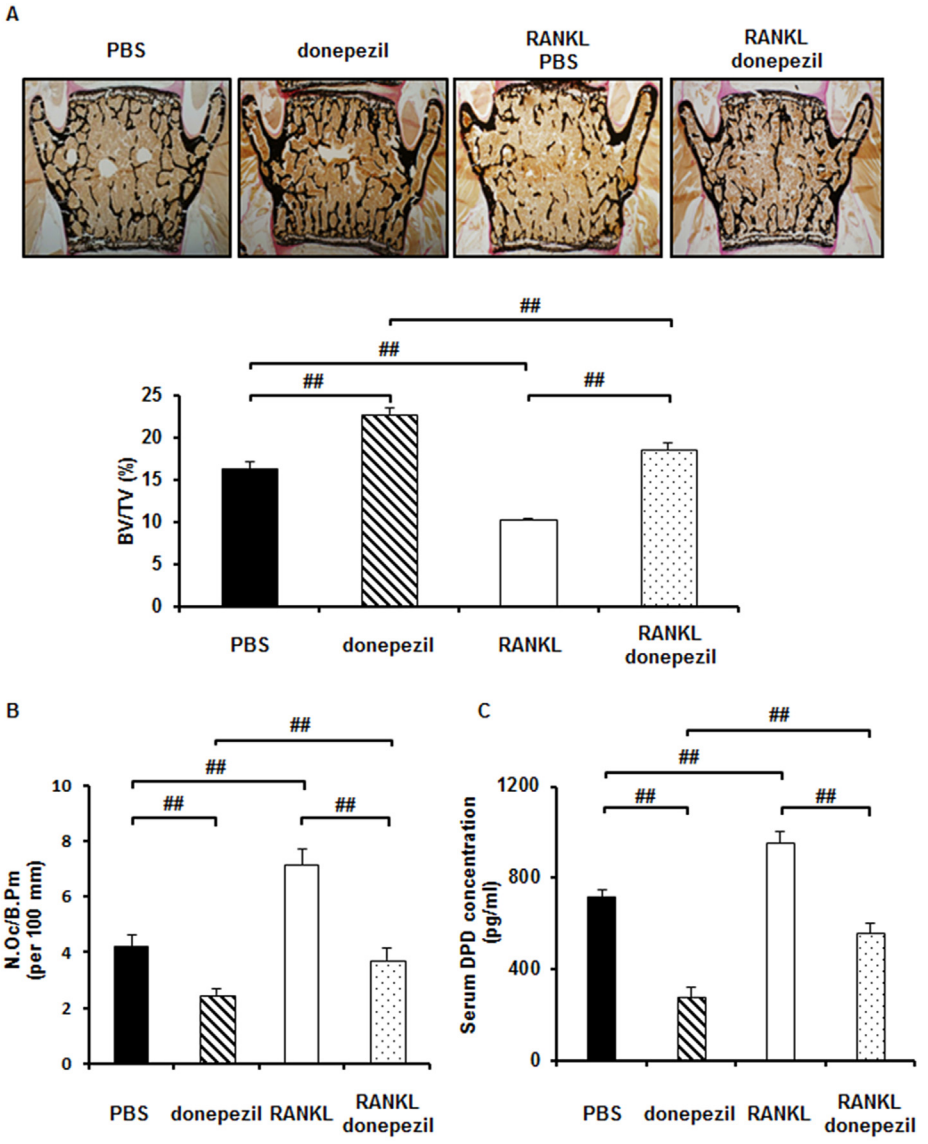

Fig. 4. Donepezil prevents RANKL-induced bone loss *in vivo* by suppressing osteoclastic bone resorption. Histomorphometric analyses of mice injected with PBS ($n = 6$), donepezil ($n = 6$), RANKL with PBS ($n = 6$), or RANKL with donepezil (2 mg/kg) ($n = 6$). A, (upper panel) von Kossa staining of mouse vertebra, (lower panel) bone volume/tissue volume (BV/TV; %). B, osteoclast number/bone perimeter (No.Oc/B.Pm; per 100 mm). C, Serum concentration of DPD measured by ELISA. Data are calculated from three repeated experiments. PBS, phosphate-buffered saline; RANKL, receptor activator of nuclear factor-κB ligand; DPD, deoxypyridinoline; ##, $p < 0.01$. Data are expressed as the means ± S.D.

formation but directly induced apoptosis in RANKL-treated BMMs cultures [17]. In this context, we expected that donepezil which blocks the activity of AChE causes accumulation of acetylcholine in the culture supernatant and subsequently induces apoptosis in RANKL-treated BMMs cultures without affecting osteoclast differentiation. Surprisingly, we found that donepezil did not

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induce TUNEL-positive cells, but inhibited TRAP-positive osteoclast formation (Fig. 3D, Fig. 2A). This suggests that acetylcholine is not accumulated in RANKL-treated BMMs cultures. Given these experimental results, the degradation of acetylcholine appears not to be required for the promotion of RANKL-induced osteoclast differentiation by AChE. We speculate that AChE promotes osteoclast differentiation via its enzymatic activity independent of the degradation of acetylcholine. Further investigation is needed for elucidating the unknown mechanisms.

From the physiological point of view, the increment of serum AChE concentration may give rise to detrimental effect for maintaining bone mass. Since the serum AChE activity is continuously increased with age [26], AChE may accelerate the risk of bone loss in elderly people. The AChE concentration in monocytes and macrophages was upregulated under inflammatory conditions. Thus, it is possible to consider that inflammation promotes bone resorption in part due to AChE production.

Our study also demonstrates that the inhibitory effect of donepezil on osteoclast differentiation is accompanied with Id2 upregulation. The Id proteins modulate RANKL-mediated osteoclastogenesis by inhibiting MITF binding to the promoter of osteoclast-associated immunoglobulin-like receptor [23]. Other molecules and compounds which can inhibit osteoclast differentiation with the modulation of Id2 expression have been reported. The small molecule harmine upregulates Id2 expression in parallel with the inhibition of osteoclast differentiation [27]. Interleukin-3 inhibits osteoclast differentiation with sustaining Id2 expression and downregulating c-Fos expression [28]. Therefore, it is interesting that c-Fos expression was downregulated during osteoclast differentiation by donepezil treatment.

It is possible that donepezil simply binds to RANKL and thereby reduces its bioavailability. However, as our data showed that donepezil reduces RANK expression of BMMs, we speculate that main contribution against the inhibitory effect of osteoclast differentiation by donepezil may be due to reduction of RANK expression. We also consider that the possibility of RANKL-independent inhibitory effect by donepezil may be small because donepezil attenuates RANK expression. On the other hand, since we did not estimate whether donepezil affects RANKL expression of osteoblasts, it is possible that donepezil modulates RANKL expression of RANKL-producing cells.

Furuya et al. demonstrated that anti-mouse RANKL-neutralizing monoclonal antibody significantly increased bone mineral density in the trabecular area on day 2, day 3, and day 4 in 6-week-old mice with reduction of a serum bone resorption marker and TRAP-positive cells by histological analysis [29]. This

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report demonstrates that the blockade of RANK-RANKL signaling only 2 days increases bone mass in growing mice. Consistent with this observation, our result demonstrated that donepezil significantly increased bone mass in trabecular area on day 3 in 9-week-old mice with reduction of serum bone resorption marker and TRAP-positive cells by histological observation. As we found that donepezil showed inhibitory effect of RANK expression, it is possible that the blockade of RANK-RANKL signaling by donepezil in growing mice may result in high bone mass.

Donepezil is the most common medication used for the treatment of Alzheimer's disease [30]. Patients with Alzheimer's disease have increased risk of serious falls and show higher incidence of hip fracture and lower hip bone mass [31] [32] [33]. Recently, a case-control study reported that the patients treated with donepezil were dose-dependently associated with the lower risk of hip fracture independently of falling [5]. Whether donepezil is a bone protective drug in patients with Alzheimer's disease is an interesting clinical project, but this hypothesis needs further basic as well as clinical investigations.

In conclusion, AChE plays a novel function in osteoclastogenesis directly. Donepezil prevents RANKL-induced bone loss. Further experiments are necessary to elucidate the molecular mechanism of AChE in osteoclastogenesis.

Declarations

Author contribution statement

Tsuyoshi Sato: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tetsuya Yoda: Conceived and designed the experiments.

Yuichiro Enoki, Yasushi Sakamoto, Kazuhiro Yokota, Masahiko Okubo, Naoki Hayashi, Toru Fukuda: Performed the experiments.

Michiko Usui, Shu Takeda: Analyzed and interpreted the data.

Toshihide Mimura, Yoshihiko Nakazato, Nobuo Araki, Yasushi Okazaki, Shoichiro Kokabu, Masahito Matsumoto: Contributed reagents, materials, analysis tools or data.

Tatsuo Suda: Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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