Biochemical and Biophysical Research Communications 527 (2020) 874–880

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Autophagy-dependent mitochondrial function regulates osteoclast differentiation and maturation

Shigeki Aoki [1, *], Kengo Shimizu [1], Kousei Ito

Laboratory of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba-city, Chiba, 260-8675, Japan

ARTICLE INFO

Article history:
Received 1 April 2020
Received in revised form
29 April 2020
Accepted 30 April 2020
Available online 16 May 2020

Keywords:
Osteoclasts
Autophagy
Mitochondria
Steroids
Osteoporosis
Bone remodeling

ABSTRACT

Bone homeostasis is maintained by bone remodeling, which involves continuous bone resorption by osteoclasts and bone formation by osteoblasts. Dysregulation of bone turnover, caused by osteoclast overactivation, causes destructive bone diseases. However, the mechanisms underlying the maintenance of osteoclast differentiation and activation are unclear. Herein, we examined the role of autophagy in the maintenance of osteoclast differentiation and maturation. We used *in vitro* and *in vivo* assays to evaluate relationships between mitochondrial activity and autophagy during osteoclast differentiation and maturation. Our results indicate that autophagy was enhanced during osteoclast differentiation and maturation, and autophagic activity was positively correlated with osteoclast activity and survival. Maintenance of mitochondrial function, which is critical during osteoclast differentiation and maturation, was controlled by autophagy. Continuous exposure of osteoclasts to glucocorticoids upregulated autophagic processes. Treatment with the autophagic inhibitor chloroquine suppressed prolonged survival of activated osteoclasts and attenuated excessive osteoclast activity. Our study shows that autophagy-dependent mitochondrial function plays an important role in osteoclast differentiation and maturation. Elucidating the mechanisms regulating autophagic activity in osteoclasts, and developing bone-tissue—specific inhibitors of autophagy, will lead to improved understanding of the pathologies involved in destructive bone diseases.

1. Introduction

Bone participates in organism maintenance, organ protection, physical activity, and hematopoiesis. Bone homeostasis is maintained by bone remodeling, which involves ongoing bone resorption by osteoclasts and bone formation by osteoblasts [1]. Disruption of normal bone turnover by osteoclast overactivation causes destructive bone diseases such as osteoporosis, rheumatoid arthritis, and bone-cancer metastases [1]. Osteoclast-targeting drugs, including bisphosphonates, are used against destructive bone diseases. Bisphosphonates, which enter osteoclasts by reacting with osteoclast-secreted acids, inhibit bone resorption by inducing osteoclast apoptosis [1]. Bisphosphonates are highly specific, and, thus, reduce the frequency of fracture occurrence, which is an efficacy endpoint in osteoporosis treatment [2]. However, bisphosphonates increase the risk of brittle bone disease, which may lead to side effects such as jaw osteonecrosis [3]. Long-term bisphosphonate use also increases the risk of atypical femoral fractures [3]. These undesirable side effects result from bisphosphonate-suppressed bone turnover; therefore, it is imperative to determine the mechanisms controlling bone remodeling and develop therapeutic agents that do not decrease bone turnover.

Osteoclasts are large multinucleated cells formed via differentiation and fusion of monocytes and macrophages. Osteoclast differentiation from bone marrow cells (BMs) is induced by co-stimulation of receptor activator of NF-κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [4]. A smooth transition from bone resorption phase (mediated by osteoclasts) to bone formation phase (mediated by osteoblasts) is critical for

Abbreviations: ALP, alkaline phosphatase; BMs, bone marrow cells; CQ, chloroquine diphosphate; Dex, dexamethasone; GST, glutathione S-transferase; M-CSF, macrophage colony-stimulating factor; mTORC1, mammalian target of rapamycin complex 1; RANK, receptor activator of NF-κB; RANKL, RANK ligand; ROS, reactive oxygen species; S6K1, p70 S6 kinase-1; sRANKL, ectodomain of RANKL; TRAP, tartrate-resistant acid phosphatase.

* Corresponding author. Laboratory of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba-city, Chiba, 260-8675, Japan.
E-mail address: aokishigeki@chiba-u.jp (S. Aoki).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbrc.2020.04.155
0006-291X/

maintenance of normal bone turnover. Recent studies have identified an exosome that expresses RANK on its surface and is secreted during osteoclast activation, spurring a hypothesis that bone formation is initiated by osteoblasts that receive this RANK-expressing exosome [5]. The discovery of this coupling factor, which mediates bone remodeling and homeostasis, has improved our understanding of the mechanisms involved in maintenance of bone turnover. However, after participating in bone remodeling, activated osteoclasts must undergo apoptosis. If bone resorption is prolonged by osteoclast overactivation, it may become the dominant phase, leading to bone destruction. However, the mechanisms underlying the maintenance of osteoclast differentiation and activation remain unclear.

Osteoclasts are non-proliferative, highly specialized cells that rapidly undergo apoptosis when terminally differentiated. Previous studies have shown that osteoclasts have high energy requirements and possess abundant mitochondria [6]. Osteoclast function and activity are regulated by mitochondria, which are critical organelles producing ATP via oxidative phosphorylation [6]. Thus, when bone resorption becomes predominant and bone turnover is imbalanced, osteoclasts show markedly enhanced mitochondrial function required to maintain this phenomenon.

Recent studies indicate that the maintenance of intracellular homeostasis involves autophagy, an intracellular process of self-degradation in which cellular contents, such as defective mitochondria and denatured proteins, are enveloped by an autophagosome membrane and degraded in lysosomes [7]. Various types of cancer cells also show upregulated autophagy when increased ATP levels, supplied by the mitochondria, are required for their survival; this process contributes to the maintenance of function and activity in these cells via mitochondria-mediated metabolism [8]. Macrophages, which are osteoclast precursors, can reduce mitochondrial membrane potential and cause apoptosis when intracellular autophagy is inhibited [9]. This suggests that osteoclast survival and activity are maintained by an ATP supply that is delivered via mitochondrial oxidative phosphorylation, and that mitochondrial function is regulated by autophagy.

In our present study, we examined the role of autophagy in osteoclast differentiation and maturation. We also used *in vitro* and *in vivo* analyses to evaluate the relationship between mitochondrial activity and autophagy during osteoclast differentiation and maturation.

2. Materials and methods

2.1. Mice

Male C57BL/6J mice (8-week-old) were purchased from the Charles River Laboratories (Kanagawa, Japan) and maintained in the experimental animal facility at Chiba University (Chiba, Japan). All procedures involving animals were approved by the Animal Care Committee of Chiba University.

2.2. Reagents

Chloroquine diphosphate (CQ), oligomycin, and dexamethasone (Dex) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and were dissolved in ultrapure water or dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan).

2.3. Osteoclast formation

Mouse bone marrow cells (BMs) were obtained from the tibiae of C57BL/6J mice as described previously [10]. BMs were cultured for 24 h in α-Minimum Essential Medium (αMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), and penicillin/streptomycin (Nacalai Tesque) in the presence of macrophage colony-stimulating factor (M-CSF; 10 ng/mL, TONBO Biosciences, San Diego, CA, USA). Afterwards, non-adherent cells (2.5×10^5 cells/cm^2) were incubated with the ectodomain of RANKL fused to glutathione S-transferase (GST-sRANKL; 100 ng/mL, Oriental Yeast, Shiga, Japan) and M-CSF (50 ng/mL) to induce osteoclastogenesis. Culture medium was changed every 3 days. The osteoclasts were then evaluated using tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich) according to the manufacturer's protocol. TRAP enzymatic activity in these osteoclasts was determined using the TRACP & ALP Assay Kit (TaKaRa, Shiga, Japan) following the manufacturer's protocol.

2.4. TUNEL assay and assessment of intracellular reactive oxygen species (ROS)

Apoptotic osteoclasts were visualized using a TUNEL staining kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI, USA) according to the manufacturer's instructions. Nuclei were stained with TO-PRO-3 iodide (Life Technologies). For measurement of intracellular ROS levels, osteoclasts were incubated with 10 μM 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCFDA, Life Technologies) for 30 min. Fluorescence was detected using Carl Zeiss LSM700 laser scanning confocal microscope (Prenzlauer, Berlin, Germany).

2.5. Evaluation of mitochondrial membrane potential

Osteoclasts were incubated for 30 min with 500 nM JC-1 (Life Technologies) dissolved in αMEM. JC-1 monomers can be detected at the excitation and emission wavelengths of 490 and 530 nm, and JC-1 polymers at 525 and 590 nm, respectively, using a Carl Zeiss LSM700 laser scanning confocal microscope.

2.6. Immunostaining

Osteoclasts were fixed in PBS containing 4% formaldehyde, permeabilized using PBS containing 0.1% Triton X-100, and then immunolabeled with an anti-LC3 primary antibody (Cell Signaling Technology, Beverly, MA, USA) and Alexa Fluor dye-conjugated secondary antibody (Life Technologies). Nuclei were stained with TO-PRO-3 iodide. Fluorescence was detected on a Carl Zeiss LSM700 laser-scanning confocal microscope.

2.7. Western blotting

Osteoclasts were lysed on ice using cell lysis buffer containing PBS (pH 7.4), 1% Triton X-100, and protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). Equal amounts of protein from each lysate were separated by SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, Berlin, Germany). After blocking the membrane, the blots were probed with specific primary antibodies (Cell Signaling Technology). Immunolabeled proteins were detected using an HRP-labeled donkey anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL prime detection reagent (GE Healthcare, Buckinghamshire, UK). Signals were visualized using an ImageQuant LAS 4000 system (GE Healthcare).

2.8. Dex-induced osteoporotic mouse model

To establish the mouse model of Dex-induced osteoporosis, C57BL/6J mice were injected subcutaneously with Dex (2 mg/kg) once daily for 35 days, as described previously [11]. On days 28–35,

CQ (50 mg/kg) was also co-administered intraperitoneally, and the mice were consequently examined for the effects of autophagic inhibition. On the day following the last injection, whole blood was collected from the mice, and sera were separated using Microtainer serum separator tubes (Becton Dickinson, San Jose, CA, USA). Serum TRAP5b and alkaline phosphatase (ALP) activities were evaluated using the MouseTRAP™ ELISA Immunoassay kit (IDS, Boldon, UK) and TRACP & ALP Assay Kit, respectively, according to the manufacturers' instructions.

2.9. Statistical analysis

All data are expressed as mean ± SD or mean ± SE of four independent osteoclast cultures or mice. Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA), followed by Bonferroni test where applicable.

3. Results

3.1. Mitochondria play an important role in osteoclast differentiation and maturation

To induce differentiation and maturation in osteoclasts *in vitro*, BMs isolated from C57BL/6J mice, were treated with GST-sRANKL and M-CSF. The degree of BM differentiation into active osteoclasts was evaluated by TRAP enzymatic activity. Our results indicate that osteoclast activity increased at 4–5 days after induction of differentiation, but decreased markedly on day 6 (Fig. 1A and B). A distinct increase in TUNEL-positive cells was observed at the end of osteoclast differentiation (Fig. 1C), suggesting that osteoclasts were undergoing apoptosis. Intracellular ROS accumulation tended to increase gradually in later stages of osteoclast differentiation and maturation (Fig. 1C). These results indicate that intracellular oxidative stress levels increased at the end of osteoclast differentiation, leading to cell death.

Osteoclasts have high-energy (ATP) requirements, and most of their functions are maintained via activity of their mitochondria [6]. Thus, we used JC-1 dye to evaluate mitochondrial activity in osteoclasts undergoing differentiation and maturation. Our results show that mitochondrial membrane potential increased with progressing osteoclast differentiation (day 5) and decreased at the terminal stage of osteoclast differentiation (day 6) (Fig. 1D). When osteoclasts undergoing maturation were exposed to oligomycin, which inhibits mitochondrial ATP synthase (Fig. 1E), their TRAP activity decreased in oligomycin-concentration—dependent manner (Fig. 1F). This finding suggests that ATP supply from the mitochondria also plays an important role in the formation and maintenance of mature osteoclasts. Considering that mitochondria with reduced membrane potential generally show increased ROS production [12], our results suggest that mitochondrial activity decreased with progressing osteoclast differentiation and maturation, and that oxidative stress in osteoclasts caused cell death at the terminal stage of their differentiation.

3.2. Autophagy, enhanced during osteoclast differentiation and maturation, regulates mitochondrial activity

Autophagy, an intracellular homeostatic mechanism that helps cells survive by supplying energy and removing defective proteins, also regulates mitochondrial function [8]. Thus, we examined changes in autophagic function during osteoclast differentiation and maturation. To investigate the autophagic function, we evaluated autophagic flux using the turnover of the autophagosome marker LC3. Osteoclasts exposed to CQ, which suppresses lysosomal degradation at the final stage of autophagy, exhibited marked LC3 puncta at 5 days after induction of differentiation, indicating enhanced autophagic activity (Fig. 2A). However, autophagic activity decreased on day 6 (Fig. 2A), which may have been linked with TRAP activity and mitochondrial function. Autophagic activity is negatively regulated by the mammalian target of rapamycin complex 1 (mTORC1) [13]. Indeed, upstream (Akt) and downstream (p70 S6 kinase-1, S6K1) expression of mTORC1 fluctuated with progressing osteoclast differentiation and maturation (Fig. 2B), corresponding with changes in autophagic activity. The expression of p62, which acts as substrate for autophagy by interacting with LC3, also decreased on day 5, and recovered on day 6, after induction of osteoclast differentiation (Fig. 2B). Therefore, it is possible that autophagic activity increased during osteoclast differentiation and maturation, and decreased at the terminal stage.

Next, when maturing osteoclasts were exposed to CQ, an inhibitor of autophagy (Fig. 2C), TRAP activity decreased in a CQ-concentration—dependent manner (Fig. 2D). This suggests that autophagy also plays an important role in the formation and maintenance of mature osteoclasts. To evaluate the association between enhanced autophagic function and mitochondrial activity during osteoclast differentiation and maturation, we examined mitochondrial membrane potential in osteoclasts exposed to CQ. Our results show that high mitochondrial activity, observed in the absence of CQ, was abolished by CQ exposure (Fig. 2E). These results also suggest that autophagy affected osteoclast differentiation and maturation via mitochondrial activation.

3.3. Steroid-mediated osteoclast overactivation is dependent on autophagic activity

Steroids are used to treat inflammation and immune disorders; however, glucocorticoids can induce osteoporosis via osteoclast overactivation [14]. Indeed, BMs exposed to the glucocorticoid Dex during their induced differentiation into mature osteoclasts, showed continued TRAP activity (Fig. 3A), similar to that involved in osteoclast overactivation. Next, we examined whether autophagy is also involved in maintaining osteoclast overactivation. The expression of the Akt-mTOR pathway, which negatively regulated autophagy even on day 6 post-induction of differentiation via Dex exposure, was attenuated (Fig. 3B); this suggests that autophagy was also involved in prolonging the survival of active osteoclasts. Therefore, we examined whether inhibition of autophagy in overactive osteoclasts can suppress the prolongation of osteoclast survival. When BMs were treated with CQ at 4 days after Dex was used to induce their differentiation into osteoclasts (Fig. 3C), the prolonged survival of active osteoclasts associated with exposure to Dex was suppressed (Fig. 3D and E). These results suggest that suppression of autophagy can, in turn, suppress osteoclast overactivation.

3.4. Chloroquine, an autophagy inhibitor, alleviates steroid-induced osteoporosis

Autophagy may be involved in osteoclast overactivation associated with Dex exposure. Inhibition of autophagy may suppress the prolonged survival of Dex-induced active osteoclasts. Thus, we examined whether inhibitors of autophagy can be used therapeutically against osteoporosis-like pathological conditions caused by the continuous administration of Dex. The mouse model of osteoporosis was established by administering Dex (2 mg/kg) to male C57BL/6J mice daily for 28 days(Fig. 4A) [11]. In addition, CQ (50 mg/kg) was co-administered to these mice daily for 7 days, starting at day 28 after the initiation of Dex administration (Fig. 4A). At the end of the treatment period, mouse sera were collected for evaluation of TRAP5b (used as index of bone resorption) and ALP

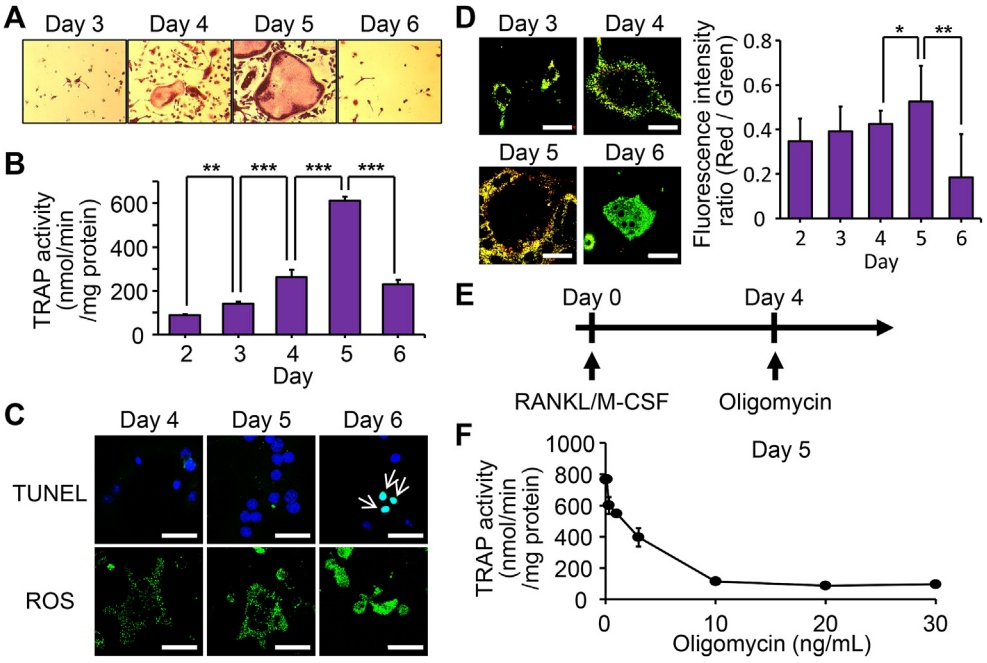

Fig. 1. Mitochondria play an important role in osteoclast differentiation and maturation. (A) shows TRAP-positive staining in bone marrow cells (BMs) differentiated using GST-sRANKL (RANKL) and M-CSF for 6 days. (B) TRAP activity of cells in (A) was evaluated using p-nitrophenyl phosphate as substrate. **P < 0.01, ***P < 0.001. (C) Fragmentation of cellular DNA in (A) was detected by TUNEL assay (green, upper panels). Nuclei were stained with TO-PRO-3 iodide (blue). White arrows indicate TUNEL-positive nuclei. Intracellular reactive oxygen species (ROS) of cells in (A) were detected by H_2DCFDA (green, lower panels). Scale bars, 30 μm. (D) BMs in (A) were stained using JC-1 and observed via confocal microscopy. Representative images show merged polymeric (red) and monomeric (green) JC-1 signals. Scale bars, 30 μm. The ratio of polymeric to monomeric JC-1 was then calculated. *P < 0.05, **P < 0.01. (E) BMs were differentiated into osteoclasts using RANKL and M-CSF, and were exposed to oligomycin at day 4 of differentiation. (F) BMs were cultured for 5 days with each oligomycin concentration per treatment protocol shown in (E). TRAP activity in these cells was evaluated using p-nitrophenyl phosphate as substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

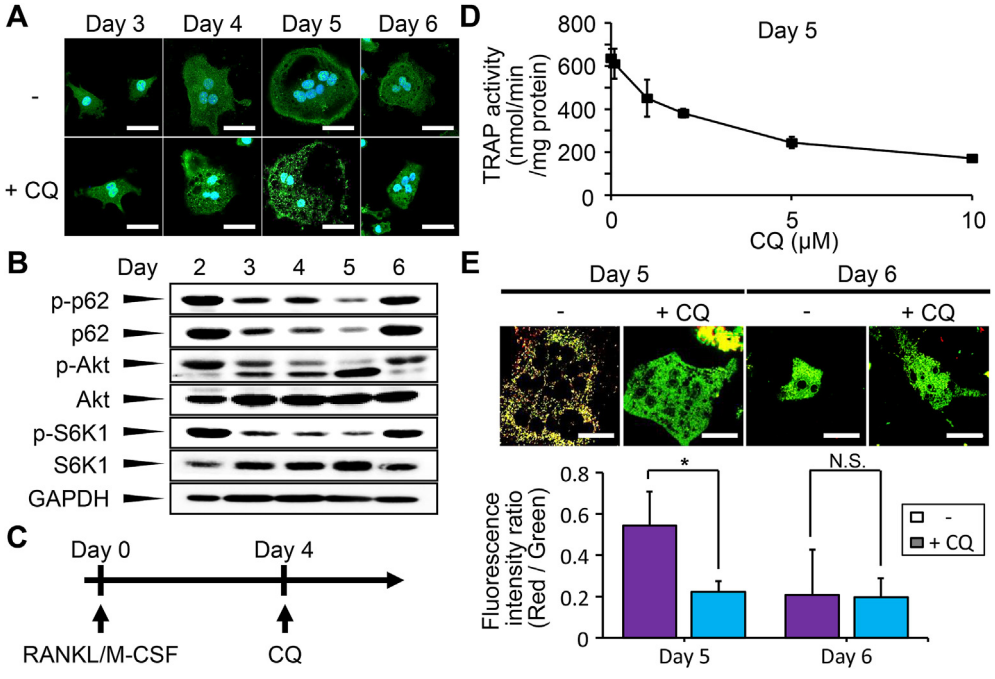

Fig. 2. Autophagy, enhanced during osteoclast differentiation and maturation, regulates mitochondrial activity. (A) Bone marrow cells (BMs) were differentiated using GST-sRANKL (RANKL) and M-CSF for 6 days. Autophagic activity was evaluated via its flux with/without chloroquine (CQ, 5 μM) for 2 h. LC3 was immunolabeled with an anti-LC3 antibody (green) and nuclei were stained using TO-PRO-3 iodide (blue). Scale bars, 30 μm. (B) Total cell lysates, prepared from cells in (A), were subjected to western blotting using the indicated antibodies. (C) BMs were differentiated into osteoclasts using RANKL and M-CSF, and exposed to CQ at day 4 of differentiation. (D) BMs were cultured for 5 days with each CQ concentration per treatment protocol shown in (C). TRAP activity in these cells was evaluated using p-nitrophenyl phosphate as substrate. (E) BMs, cultured with/without CQ (5 μM) for 5 or 6 days per treatment protocol shown in (C), were stained with JC-1 and observed via confocal microscopy. Representative images show merged polymeric (red) and monomeric (green) JC-1 signals. Scale bars, 30 μm. The ratio of polymeric to monomeric JC-1 was then calculated. *P < 0.05. N.S., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(used as index of osteogenesis) activity. Our results show that Dex administration increased serum TRAP5b activity, confirming that osteoporosis had been induced; our results also show that CQ co-administration significantly suppressed the elevated TRAP5b activity (Fig. 4B). Dex administration also reduced osteogenic activity, which remained unchanged by co-administration of CQ (Fig. 4C). These results suggest that autophagy was enhanced in overactive osteoclasts, and that inhibiting the autophagic function may have alleviates the overactivity of bone resorption observed in osteoporosis.

4. Discussion

Mitochondrial function plays an important role in the maintenance of osteoclast activity [6]. This study shows that mitochondrial activation and ATP supply from the mitochondria are essential for osteoclast differentiation and maturation (Fig. 1D–F). Although mitochondria are the major ATP producers, they also generate ROS as a by-product of ATP production, and ROS are harmful to cells [12]. Osteoclasts are activated by RANKL upon its interaction with RANK, a receptor expressed on osteoclast precursors. This process also generates oxidative stress via ROS [15]. While such oxidative stress may promote osteoclast formation [15], excessive accumulation of intracellular ROS induces cell death via oxidation and damage to intracellular proteins, lipids, and DNA. In this study, we observed intracellular ROS accumulation during osteoclast apoptosis, in the late phases of osteoclast differentiation and maturation (Fig. 1C). Excessive ROS accumulation generally impairs mitochondrial function [12], and our results indeed show a marked decrease in mitochondrial membrane potential in the late stages of osteoclast differentiation and maturation (Fig. 1D). These results suggest that intracellular ROS accumulation and decreased ATP supply from the mitochondria induced osteoclast death in the late stages of osteoclast activation. These findings indicate that a transient elevation in mitochondrial function during osteoclast differentiation and maturation is likely crucial for normal bone remodeling and maintenance of bone homeostasis.

Autophagy breaks down unnecessary cellular proteins and organelles to be used for cellular metabolism, and protects living bodies from various stressful environments [7]. Our present study revealed that autophagy was enhanced during osteoclast differentiation and maturation, and that autophagic activity was positively correlated with osteoclast activity and survival (Fig. 2A and B). Phosphorylation levels of Akt and S6K1 indicate that mTORC1 activity decreased with osteoclast differentiation and maturation (Fig. 2B). Autophagy is negatively regulated downstream of mTORC1, which controls nutrition and intracellular metabolism [13]; therefore, autophagic function is likely controlled by changes in mTORC1 activity, which is associated with osteoclast differentiation and maturation. In general, autophagy induced during nutrient starvation is mediated by the suppression of mTORC1 via ROS accumulation [16]. This suggests that during osteoclast differentiation and maturation, the mTOR signaling pathway was suppressed by ROS production that accompanies mitochondrial activation. Conversely, ROS may function as a second messenger upstream of MAPK, PI3K, and NF-κB in osteoclast differentiation [17]. Activation of mTOR and autophagic suppression that occurred at the end of osteoclast activation may have been caused by accumulated ROS (Fig. 2A and B). Intracellular ROS may also be eliminated by enhanced autophagy [18], indicating that the link between ROS-mediated signaling and autophagy is highly complex and requires more detailed studies.

Our study reveals that the maintenance of mitochondrial function, which is critical during osteoclast differentiation and maturation, was controlled by autophagy (Fig. 2C–E). Mitophagy, a specialized form of autophagy, is a mechanism that selectively decomposes injured mitochondria and promotes mitochondrial metabolism [19]. When oxidative phosphorylation in the

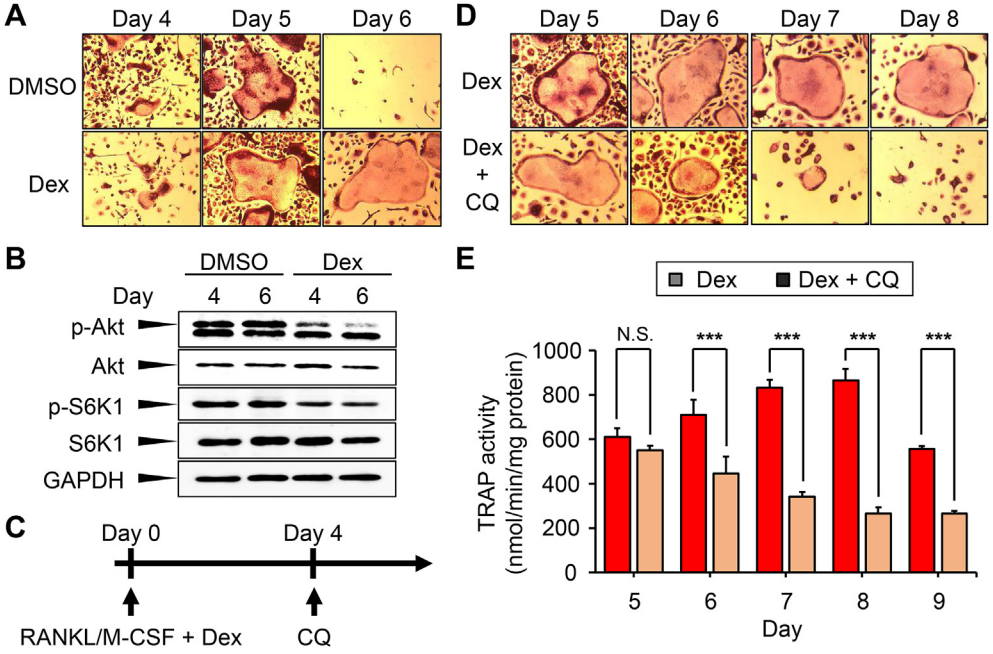

Fig. 3. Steroid-mediated osteoclast overactivation is dependent on autophagic activity. (A) shows TRAP-positive staining in bone marrow cells (BMs) differentiated using GST-sRANKL (RANKL) and M-CSF with/without dexamethasone (Dex, 1 μM) for 6 days. (B) Total cell lysates, prepared from cells in (A), were subjected to western blotting using the indicated antibodies. (C) BMs were differentiated into osteoclasts using RANKL and M-CSF plus Dex, and then exposed to chloroquine (CQ, 5 μM) at day 4 of differentiation. (D) BMs were cultured for 9 days with/without CQ per treatment protocol shown in (C), and TRAP-positive cells were stained. (E) TRAP activity of cells in (D) was evaluated using p-nitrophenyl phosphate as substrate. ***P < 0.001. N.S., not significant.

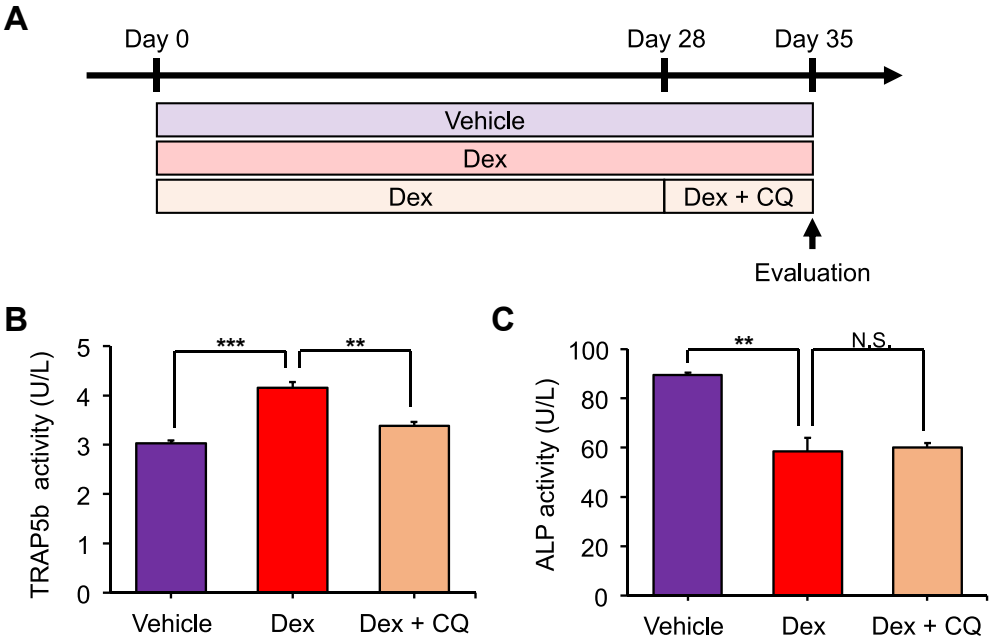

Fig. 4. Chloroquine, an autophagy inhibitor, alleviates steroid-induced osteoporosis. (A) Timeline for establishing the osteoporotic mouse model using dexamethasone (Dex, 2 mg/kg/day) and intraperitoneal injection of chloroquine (CQ, 50 mg/kg/day) is shown. (B) TRAP5b activity in mouse serum was evaluated using ELISA immunoassay. **P < 0.01, ***P < 0.001. (C) Alkaline phosphatase (ALP) activity in mouse serum was evaluated using p-nitrophenyl phosphate as substrate in alkaline buffer. **P < 0.01. N.S., not significant.

mitochondria is enhanced, Rheb of small GTPase accumulates in the outer mitochondrial membrane and induces mitophagy to supply ATP from the mitochondria [20]. Because increased oxidative phosphorylation in the mitochondria generates copious amounts of ROS and induces mitochondrial damage, mitophagy may play an important role in osteoclast differentiation and maturation. In addition to enhancing ATP production by providing an intermediate to the mitochondrial TCA cycle, autophagy also maintains mitochondrial function via mitophagy. Thus, autophagy is an important mechanism in osteoclast differentiation and maturation.

Factors that cause steroid-induced osteoporosis include continuous use of glucocorticoids [14]. Glucocorticoids inhibit osteoclast apoptosis, promote differentiation of osteoclast precursors into osteoclasts, and enhance bone resorption by prolongation of osteoclast life [14]. We found that continuous exposure of osteoclasts to glucocorticoids enhances autophagy. Also, our results show that treatment with an autophagic inhibitor CQ suppressed the prolonged survival of activated osteoclasts and attenuated excessive osteoclast activity (Figs. 3, 4). Glucocorticoids induce autophagy in multiple cell lines and are involved in promoting the cytoprotective autophagy described above [21]. These findings suggest that inhibitors of autophagy can suppress the overactive osteoclasts involved in steroid-induced osteoporosis. In general, cancer cells have higher autophagic activity than normal healthy cells. This is because cancer cells need an increased supply of metabolites to support their excessive growth and resistance to various stresses [22]. Therefore, clinical trials examining the activity of autophagy inhibitors in cancer are currently ongoing. Some of these studies suggest that CQ and hydroxychloroquine may show enhanced activity against cancer when used in combination with anticancer drugs [23]. Because osteoclast overactivation is observed in bone-cancer metastasis [1], autophagy inhibitors are expected to exert therapeutic effects on both osteoclast overactivation and bone-cancer metastasis. Additionally, because various destructive bone diseases are caused by osteoclast overactivation, autophagy inhibitors that act on bone tissue may alleviate the pathologies associated with these conditions.

Our study shows that autophagy-dependent mitochondrial function plays an important role in osteoclast differentiation and maturation. In steroid-induced osteoporosis model, excessive enhancement of autophagy was accompanied by the continuous activation of osteoclasts, which may progress bone destruction. In the future, we hope that elucidation of the regulation mechanism of autophagic activity in osteoclasts and the development of bone tissue-specific autophagy inhibitors will lead to a deeper understanding and improvement in the pathology of destructive bone diseases.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported by a Grant-in-Aid for Early-Career Scientists (19K16440) from the Japan Society for the Promotion of Science, and by a Grant-in-Aid from Chiba Foundation for Health Promotion & Disease Prevention.

References

[1] X.L. Xu, W.L. Gou, A.Y. Wang, Y. Wang, Q.Y. Guo, Q. Lu, S.B. Lu, J. Peng, Basic research and clinical applications of bisphosphonates in bone disease: what have we learned over the last 40 years? J. Transl. Med. 11 (2013) 303.
[2] M.J. Favus, Bisphosphonates for osteoporosis, N. Engl. J. Med. 363 (2010) 2027–2035.
[3] S.J. Wimalawansa, Optimum duration and safety of long-term use of potent anti-resorptive medications in osteoporosis, Expet Rev. Endocrinol. Metabol. 11 (2016) 329–348.
[4] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423 (2003) 337–342.
[5] Y. Ikebuchi, S. Aoki, M. Honma, M. Hayashi, Y. Sugamori, M. Khan, Y. Kariya, G. Kato, Y. Tabata, J.M. Penninger, N. Udagawa, K. Aoki, H. Suzuki, Coupling of bone resorption and formation by RANKL reverse signalling, Nature 561 (2018) 195–200.
[6] Y. Zhang, N. Rohatgi, D.J. Veis, J. Schilling, S.L. Teitelbaum, W. Zou, PGC1beta

organizes the osteoclast cytoskeleton by mitochondrial biogenesis and activation, J. Bone Miner. Res. : Off. J. Am. Soc. Bone Mineral Res. 33 (2018) 1114–1125.
[7] N. Mizushima, Autophagy: process and function, Gene Dev. 21 (2007) 2861–2873.
[8] M. Kawaguchi, S. Aoki, T. Hirao, M. Morita, K. Ito, Autophagy is an important metabolic pathway to determine leukemia cell survival following suppression of the glycolytic pathway, Biochem. Biophys. Res. Commun. 474 (2016) 188–192.
[9] S.K. Matta, D. Kumar, AKT mediated glycolytic shift regulates autophagy in classically activated macrophages, Int. J. Biochem. Cell Biol. 66 (2015) 121–133.
[10] N. Takahashi, N. Udagawa, S. Tanaka, T. Suda, Generating murine osteoclasts from bone marrow, Methods Mol. Med. 80 (2003) 129–144.
[11] T. Komori, Animal models for osteoporosis, Eur. J. Pharmacol. 759 (2015) 287–294.
[12] D.C. Liemburg-Apers, P.H. Willems, W.J. Koopman, S. Grefte, Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism, Arch. Toxicol. 89 (2015) 1209–1226.
[13] L. Yu, C.K. McPhee, L. Zheng, G.A. Mardones, Y. Rong, J. Peng, N. Mi, Y. Zhao, Z. Liu, F. Wan, D.W. Hailey, V. Oorschot, J. Klumperman, E.H. Baehrecke, M.J. Lenardo, Termination of autophagy and reformation of lysosomes regulated by mTOR, Nature 465 (2010) 942–946.
[14] H.J. Kim, H. Zhao, H. Kitaura, S. Bhattacharyya, J.A. Brewer, L.J. Muglia, F.P. Ross, S.L. Teitelbaum, Glucocorticoids suppress bone formation via the osteoclast, J. Clin. Invest. 116 (2006) 2152–2160.
[15] N.K. Lee, Y.G. Choi, J.Y. Baik, S.Y. Han, D.W. Jeong, Y.S. Bae, N. Kim, S.Y. Lee, A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation, Blood 106 (2005) 852–859.
[16] K. Bensaad, E.C. Cheung, K.H. Vousden, Modulation of intracellular ROS levels by TIGAR controls autophagy, EMBO J. 28 (2009) 3015–3026.
[17] V.J. Thannickal, B.L. Fanburg, Reactive oxygen species in cell signaling, Am. J. Physiol. Lung Cell Mol. Physiol. 279 (2000) L1005–L1028.
[18] T. Hirao, M. Yamaguchi, M. Kikuya, H. Chibana, K. Ito, S. Aoki, Altered intracellular signaling by imatinib increases the anti-cancer effects of tyrosine kinase inhibitors in chronic myelogenous leukemia cells, Canc. Sci. 109 (2018) 121–131.
[19] S.J. Goldman, R. Taylor, Y. Zhang, S. Jin, Autophagy and the degradation of mitochondria, Mitochondrion 10 (2010) 309–315.
[20] S. Melser, E.H. Chatelain, J. Lavie, W. Mahfouf, C. Jose, E. Obre, S. Goorden, M. Priault, Y. Elgersma, H.R. Rezvani, R. Rossignol, G. Benard, Rheb regulates mitophagy induced by mitochondrial energetic status, Cell Metabol. 17 (2013) 719–730.
[21] S. Aoki, M. Morita, T. Hirao, M. Yamaguchi, R. Shiratori, M. Kikuya, H. Chibana, K. Ito, Shift in energy metabolism caused by glucocorticoids enhances the effect of cytotoxic anti-cancer drugs against acute lymphoblastic leukemia cells, Oncotarget 8 (2017) 94271–94285.
[22] K.S. Choi, Autophagy and cancer, Exp. Mol. Med. 44 (2012) 109–120.
[23] G. Manic, F. Obrist, G. Kroemer, I. Vitale, L. Galluzzi, Chloroquine and hydroxychloroquine for cancer therapy, Mol. Cell. Oncol. 1 (2014), e29911.