Original Article

Inhibitory effect of chloroquine on bone resorption reveals the key role of lysosomes in osteoclast differentiation and function

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The lysosome is an acidic compartment containing certain hydrolytic enzymes necessary for the intracellular digestion of macromolecules. In terms of the activity of bone-resorbing osteoclasts, the secretion of lysosomal vesicles containing protons and matrix-degrading proteinases into the resorption lacunae is essential. Chloroquine (CQ), one of the lysosomotropic agents, has an immunosuppressive effect and is used for the treatment for rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). However, the direct effect of CQ on osteoclasts has not been reported. Here, we show that CQ suppresses the bone resorbing activity of osteoclasts by inhibition of the acidification in the lysosomes, as well as osteoclast differentiation *in vitro*. CQ treatment ameliorates the bone loss induced by RANKL injection in mice. These results suggest that CQ has a bone-increasing effect by inhibiting osteoclast differentiation and function. In addition, a lysosomal proton pump inhibitor bafilomycin A1 also inhibits osteoclast differentiation. Thus, this study revealed the importance of the lysosomes in osteoclast differentiation and function *in vivo* as well as *in vitro*, suggesting the therapeutic efficacy of immunosuppressive CQ in osteoclast-mediated bone loss or destruction.

Rec.5/7/2012, Acc.5/27/2012, pp222-231

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Key words chloroquine, lysosome, osteoclast, inflammatory bone disease

Introduction

Bone homeostasis is tightly regulated by the balanced activities of bone formation and bone resorption. Osteoclasts, which degrade bone matrix, are multinucleated cells, and these cells are involved in the dynamic bone remodeling which takes place in coordination with osteoblasts[1, 2]. An imbalance in bone remodeling caused by increased bone resorption over bone formation leads to the loss of bone mass in several bone disorders such as osteoporosis and rheumatoid arthritis (RA)[3, 4].

Osteoclasts attach to the mineralized matrix and degrade it by a secretion of protons and proteinases. The secretion of protons is mediated by the proton pump localized at the lysosome and the ruffled border membrane, which is generated by the fusion of lysosomal secretory vesicles with the plasma membrane that is in apposition to bone[5]. The lysosomal secretory vesicles contain proteinases, such as the cysteine proteinase cathepsin K and the matrix metalloproteinase MMP9, both of which are essential for the degradation of bone matrix proteins[6]. Thus, the lysosome plays a critically important role in osteoclastic bone resorption.

Osteoclast differentiation is regulated by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NFκB) ligand (RANKL), a tumor necrosis factor family cytokine[2, 7]. While M-CSF is crucial for the proliferation and survival of osteoclast precursor cells[8], RANKL stimulation is essential for differentiation. The signaling pathway mediated by the immunoglobulin (Ig)-like receptors, such as OSCAR, TREM-2, SIRPβ1 and PIR-A[9], is also essential for osteoclast differentiation in order to activate osteoclastogenic complex[10]. Finally, RANKL and Ig-like receptor signals are integrated by the master transcription factor of osteoclast differentiation, NFATc1[11]. This transcription factor induces the expression of molecules essential for the bone resorbing activity of osteoclasts, such as cathepsin K, MMP9, the H^+-ATPase subunits and carbonic anhydrase II[12].

Lysosomotropic agents chloroquine (CQ) and hydroxychloroquine (HCQ), both of which are derivatives of antimalarial drug quinacrine, penetrate acidic compartments such as the lysosome and raises the compartment pH to neutrality[13]. Although CQ and HCQ was originally developed as an anti-malarial drug, it has been shown that CQ and HCQ exert an anti-inflammatory effect and also inhibit antigen presentation in dendritic cells, cytokine production in macrophages, and calcium and Toll-like receptor signaling in B, T and other immune cells[14-18]. Thus, CQ and HCQ has become one of the most commonly prescribed drugs in the treatment of many rheumatic diseases, including RA[19], systemic lupus erythematosus (SLE)[20], palindromic arthritis[21] and psoriatic arthritis[22]. Administration of CQ or HCQ reportedly results in a slowing or even arrest of joint destruction in RA patients[23] as well as the increased bone mineral density (BMD) in SLE patients[24, 25]. Although the osteoclastic bone resorption in these diseases is enhanced, the direct effect of CQ on osteoclasts, in which the lysosomal function is crucial for their activity, has yet to be elucidated.

In this study, we investigated the effects of CQ on osteoclast function and differentiation. CQ suppresses both the differentiation and bone resorbing activity of osteoclasts. This study demonstrates the importance of the lysosome in the function as well as the differentiation of osteoclasts.

Materials and methods

1)Mice

C57BL/6JJc1 mice (8-week-old) were purchased from CLEA Japan. All of the animals were maintained in a specific-pathogen free environment and all of the animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

(1)*In vitro* osteoclast culture

In vitro osteoclast differentiation has been described previously[10]. The bone marrow-derived macrophages (BMMs) were cultured in the osteoclastogenic medium containing 50 ng/ml RANKL (Peprotech) and 10 ng/ml M-CSF in the presence of CQ (Sigma-Aldrich) or Bafilomycin A1 (BafA1) (Tocris Bioscience) for 3 days. TRAP-positive cells with more than three nuclei ($TRAP^+$ MNCs) were counted.

(2)*In vitro* pit formation assay

Bone resorption activity of osteoclasts was assessed using a pit formation assay performed as previously described[26]. For the pit formation assay, osteoclasts were cultured on dentin slices with or without CQ in a 96-well plate. The resorbed pit area was measured using an image analyzing software, Image J.

2)TUNEL staining of mature osteoclasts

Manifestations of cell death were sought using fluores-

cent *in situ* terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL staining) using *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics). The cells were analyzed using a fluorescence microscope (Leica DMI3000B, Leica Microsystems Ltd) with an appropriate filter set. The experiment was performed in duplicate on three independent occasions in a 96-well plate.

3)Cell proliferation and apoptotic assays

Cell proliferation and apoptotic assays are performed described previously[10]. BMMs were cultured in the osteoclastogenic medium with or without CQ for 24 h. Before 1 h of 24 h the BrdU (Roche Diagnostics) is added to the medium. Cells were labeled with FITC-conjugated anti-BrdU antibody (BD Pharmingen). Apoptosis of osteoclast precursor cells was evaluated 24 h after RANKL stimulation with or without CQ using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics) based on a TUNEL technique. In both experiments, cells were analyzed by flow cytometry, FACS Canto II and analyzed with Diva software (BD Biosciences). In Fig. 3C, the number of nucleus was counted for the analysis of the cell viability after TRAP staining.

4)Acridine orange staining

Intracellular acidification was determined by acridine orange (AO) quenching assay. Osteoclasts were exposed to the lysosomotropic weak base, AO (Wako Pure Chemical Industries, Ltd.) for staining as described previously[27]. The accumulation of AO into lysosomes was visualized using a fluorescence microscope (Leica DMI3000B, Leica Microsystems Ltd).

5)Immunoblot analysis

Immunoblot analysis was performed as previously described[28]. Cells were solubilized in the lysis buffer (1% Nonidet P-40 in 150 mM NaCl, 20 mM Tris-HCl pH7.5, 1 mM EDTA, 10 mM NaF, 2 mM PMSF), supplemented with the complete protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were subjected to immunoblot analysis using specific antibodies for NFATc1 (Santa Cruz Biotechnology) and β-actin (Sigma-Aldrich).

6)RANKL-induced bone loss model

GST-RANKL recombinant protein was purified from BL21 cells as described previously[29]. After purification using glutathione-Sepharose 4B and Mono-Q (GE Healthcare), the protein was further purified with ToxinEraser™ Endotoxin Removal Kit (GenScript). We confirmed that endotoxin level is less than 50 EU/mg protein. Eight-week-old mice were intraperitoneally injected with 1 mg/kg of GST-RANKL three times at intervals of 24 h as previously reported[29]. CQ was injected 1 h before the every GST-RANKL injection. After the last injection, all of the mice were sacrificed and subjected to microcomputed tomography (microCT) analysis[30].

7)Statistical analysis

Statistical analysis was performed using Student's *t*-test (*p<0.05, **p<0.01, ***p<0.005, n.s.: not significant, throughout the paper). All data are expressed as means ± SEM.

Results

1)CQ inhibits osteoclastic bone resorption

We evaluated the effect of CQ on the bone resorbing activity of mature osteoclasts by pit formation assay. CQ significantly inhibited the formation of pits in a dose-dependent manner (Fig.1A, B). Since it has been reported that CQ induces cell apoptosis in various types of cells, we analyzed the survival of CQ-treated osteoclasts by TUNEL assay. Although the cell survival was not affected at lower concentrations, the number of TUNEL-positive cells was slightly but significantly increased at 7.5 μM and 10 μM (Fig.1C). However, the pit area per osteoclast, which shows the bone resorbing activity in a single cell, was dramatically decreased (Fig.1D). These results indicate that CQ mainly affects the bone resorbing activity of osteoclasts, and slightly affects the survival of osteoclasts. AO staining revealed that the CQ treatment results in a raising of the pH in osteoclasts (Fig.1E), suggesting that CQ inhibits bone resorption through the neutralization of the protons in the lysosome. These observations indicate a significant role for lysosomes in the bone resorbing activity of osteoclasts.

2)Lysosomal function and osteoclast differentiation

Next we examined the effect of CQ on osteoclast differentiation *in vitro*. Interestingly, the formation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts was suppressed by CQ in a dose-dependent manner (Fig.2A, B). Consistent with this, the expression of NFATc1, the

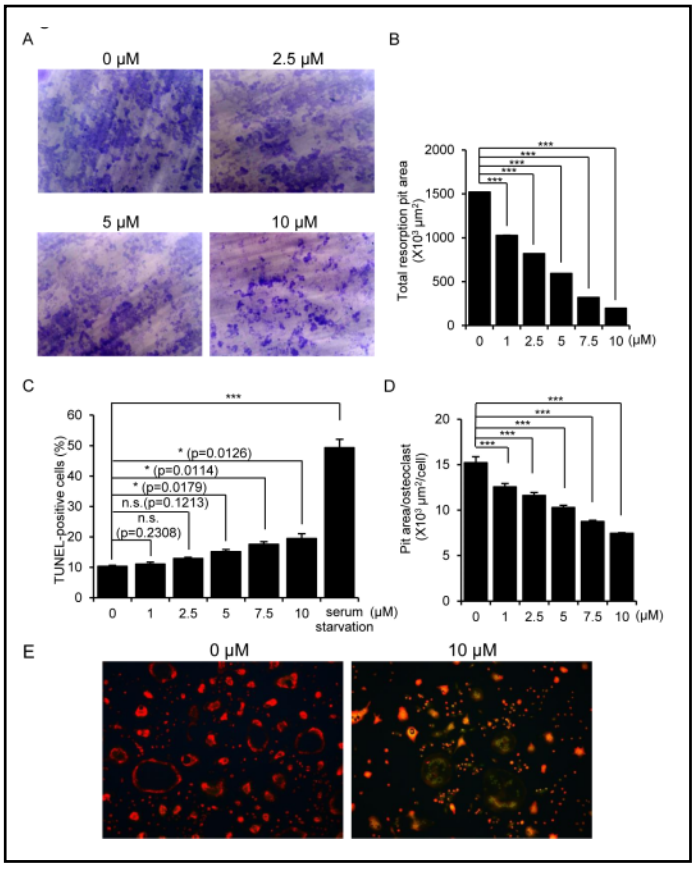

Fig.1 Chloroquine suppresses osteoclastic bone resorption

(A)Effects of CQ on the bone resorbing activity of mature osteoclasts.
(B)Total pit area resorbed by osteoclasts.
(C)Effect of CQ on the survival of mature osteoclasts.
(D)Total pit area per osteoclast.
(E)Effects of CQ on the acidification of lysosomes in osteoclasts.

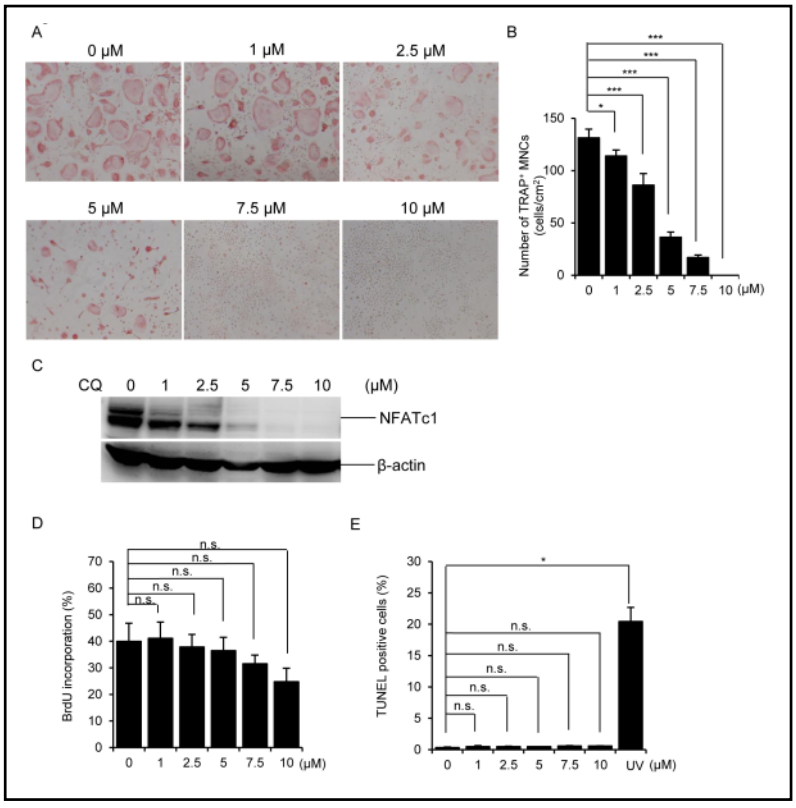

Fig.2 Inhibition of osteoclast differentiation by CQ

(A)The formation of TRAP⁺ osteoclasts induced by RANKL and M-CSF in the presence of CQ.
(B)The number of TRAP⁺ osteoclasts in (A).
(C)Expression level of NFATc1 in (A).
(D)Proliferation of the CQ-treated osteoclast precursor cells.
(E)Survival of the CQ-treated osteoclast precursor cells.

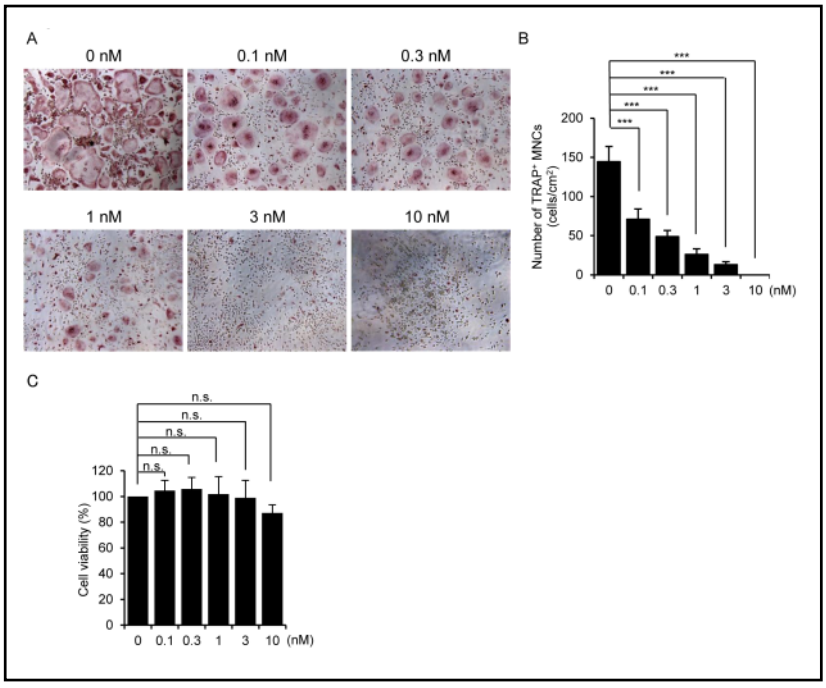

Fig.3 Inhibition of osteoclast differentiation by BafA1

(A)The formation of TRAP⁺ osteoclasts induced by RANKL and M-CSF in the presence of BafA1.

(B)The number of TRAP⁺ osteoclasts in (A).

(C)Cell viability of the BafA1-treated cells.

master transcription factor of osteoclast differentiation, was inhibited by CQ treatment (Fig.2C). We also analyzed the cell proliferation and survival of osteoclast precursor cells by BrdU incorporation and TUNEL assays. Although CQ tends to suppress the proliferation in a dose-dependent manner, we found no statistically significant difference in the proliferation (Fig.2D) and the survival (Fig.2E). These results indicate that CQ does not affect the survival significantly but slightly affects the proliferation in osteoclast precursor cells. To confirm the importance of lysosomal acidification in osteoclast differentiation, we tested the effect of another lysosome inhibitor, BafA1, which selectively inhibits a vacuolar-type H^+-ATPase. Similar to CQ, BafA1 also inhibits osteoclast differentiation in a dose-dependent manner (Fig.3A, B) without any defects in the cell survival (Fig.3C). These results suggest that lysosomal function has a critical role in osteoclast differentiation.

3)CQ protects against the bone loss induced by RANKL injection in mice

We investigated whether CQ has a preventive effect on the bone loss associated with enhanced osteoclastic bone resorption. We treated 8-week-old female mice that had been intraperitoneally injected with GST-RANKL at 24 h intervals for 3 days. In this model, exogenous RANKL mediates osteoclast differentiation and stimulates osteoclasts to resorb bone. Three-dimensional images of the distal region of the femur showed robust trabecular bone loss (Fig.4A), and microCT analysis revealed a marked reduction in bone volume, thickness and the trabecular number, and an increase in trabecular separation in the RANKL-injected mice (Fig.4B). The treatment with CQ protected against trabecular bone loss in these RANKL-injected mice (Fig.4A). Consistent with this, the bone volume, the trabecular thickness and the trabecular number were increased, and the trabecular separation was decreased (Fig.4B). Since osteoblastic bone formation is not affected in this model[29], the protective effect of CQ on bone loss is due to the suppression of osteoclast differentiation and function. In addition, osteoblast differentiation and function *in vitro* was not affected by the CQ at the concentration used in this study (M.S., unpublished data). Taken together, our results suggest that CQ can protect against the bone loss induced by the enhanced bone resorption of osteoclasts.

Discussion

The lysosomal secretory vesicles in osteoclasts contain protons for decalcifying the mineralized matrix and enzymes for digesting organic matrix. Since the lysosomal enzymes such as cathepsins and MMPs are activated at a low pH, acidification of the lysosome is required for the degrada-

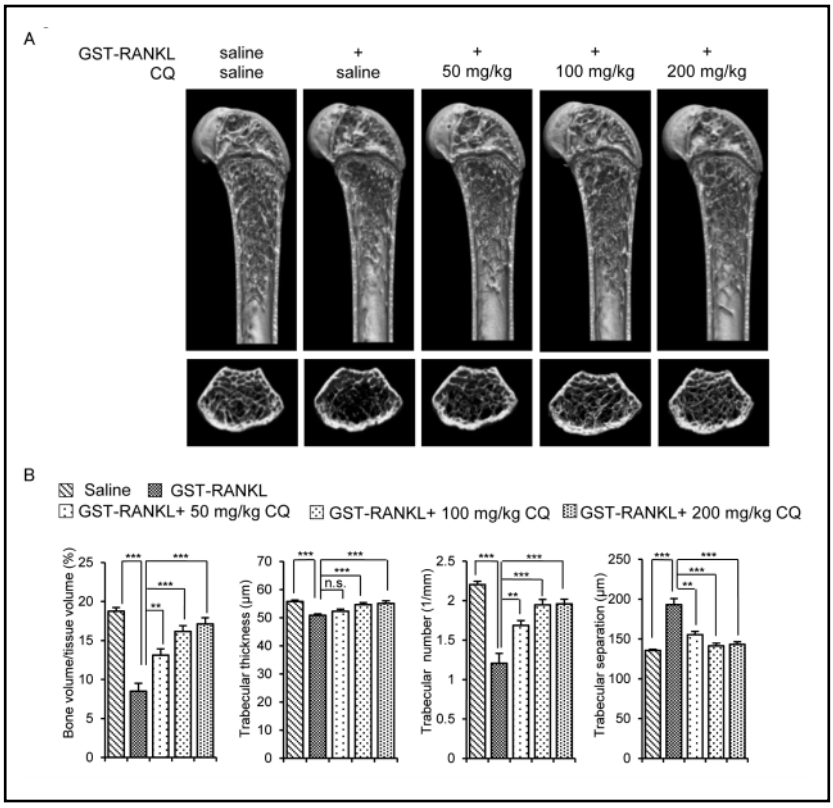

Fig.4 Protection against RANKL-induced bone loss by CQ treatment

(A)Microcomputed tomography (microCT) analysis of the femurs in mice treated with saline, GST-RANKL or GST-RANKL with 50, 100, 200 mg/kg CQ (upper photograph: longitudinal view; lower photograph: axial view of the metaphyseal region).

(B)The parameters of the metaphyseal region on microCT analysis. Data are represented as the mean ± SEM of 9 mice.

tion of bone matrix proteins. Previous reports have suggested that a selective inhibitor of the vacuolar-type H^+-ATPases BafA1, which suppresses acidification in lysosomes, inhibits the bone resorbing activity of osteoclasts[31, 32]. Furthermore, mutations in the *TCIRG1* gene, which encodes the a3 subunit of H^+-ATPase, results in severe osteopetrosis in humans and mice due to the impaired bone resorbing activity of osteoclasts[27, 33]. These previous reports demonstrated the importance of acidification in osteoclastic bone resorption. The lysosomotropic agent CQ is a weak base, and the unprotonated form of CQ passively diffuses into the lysosomal compartment. In the lysosome it is protonated, and the protonated form of CQ is unable to diffuse out of the lysosome. As a result, intralysosomal proton consumption is carried out by the uncharged CQ and the intralysosomal pH accordingly rises from 4.5 to 7.0, which in turn leads to alterations in lysosomal function[34]. AO staining revealed that the intralysosomal pH is increased in CQ-treated osteoclasts. Thus, it is conceivable that CQ inhibits the bone resorbing activity by a suppression of protons and hence the enzymatic activities of the bone-degrading proteinases in osteoclasts.

CQ also induces apoptosis in mature osteoclasts. CQ can increase lysosomal volume and enlarge the plasma membrane surface[35], which is a common event in apoptosis[36]. It was previously reported that CQ induces cell apoptosis in A549 lung cancer cells[37], neurons[38, 39], HeLa cells[40] and glioma cells[41] at high concentrations (more than 20 ～ 30 μM), but not at low concentrations. However, our data indicate that CQ induces apoptosis in mature osteoclasts at the concentration of 7.5 μM, suggesting that mature osteoclasts are highly sensitive to CQ compared to other cell types. In contrast, CQ does not induce apoptosis in osteoclast precursor cells, even at 10 μM. This result indicates that the CQ sensitivity changes during the course of osteoclast differentiation. Although the detailed mechanism remains to be elucidated, it may be caused by a change in the expression level of genes related to lysosome-dependent apoptosis during osteoclast differentiation.

We also found that CQ and BafA1 have inhibitory effects on osteoclast differentiation. While M-CSF is necessary for the proliferation and survival of osteoclast precursor cells, RANKL and Ig-receptor signals are essential for differentiation. Since CQ inhibits osteoclast differentiation with minimum effects on the proliferation or survival of pre-

cursor cells, CQ probably affects RANKL and/or Ig-receptor signaling. To explore the molecular mechanism by which CQ inhibits osteoclast differentiation, we examined the activation of the downstream pathways induced by RANKL, such as the Erk, p38, JNK and IKK pathways, and found that CQ did not affect the activation of these pathways (unpublished observations). Although the molecular mechanism is unclear at present, the data suggest that lysosomal function is also important for osteoclast differentiation. A recent study showed that lysosomal function modulates glucocorticoid receptor signaling in macrophages[42]. This finding, together with our results, indicates the importance of the lysosome in signal transduction.

Inflammation perturbs normal bone homeostasis and is known to induce bone loss, as it promotes local cartilage degradation as well as both local and systemic bone destruction by osteoclasts, along with inhibiting bone formation by osteoblasts[2]. Antirheumatic drugs such as methotrexate (MTX), leflunomide (Lef), bucillamine (Buc) and salazosulfapyridine (SASP), all of which are known to be immunomodulators (SASP/Buc) or immunosuppressants (MTX/Lef)[19], inhibit osteoclast differentiation and function[43, 44] under certain conditions. Previous reports suggested that the production of inflammatory cytokines such as TNFα and IL-6 are suppressed by CQ treatment in RA[15] and SLE patients[45] through the effects on macrophages and T-cells. Thus, CQ has been thought to ameliorate the course of arthritis by its inhibitory effect on these immune cells. The improved BMD in SLE patients treated with CQ[25, 26] has been attributed to the suppression of inflammation. However, in this study we found that CQ has a direct effect on osteoclast differentiation and function. Using a bone loss model in which exogenous RANKL stimulates osteoclast differentiation and function, we demonstrated that CQ treatment protects against bone loss *in vivo*. Our results clearly indicate that CQ plays a dual role in that it is involved in both the suppression of inflammation and bone destruction in RA.

CQ and HCQ have the same effects on the function of lysosome. However, it has been suggested that there are differences in efficacy and toxicity between CQ and HCQ[47]. In RA therapy, it was reported that HCQ was one half to two thirds as effective as CQ but one half in the toxicity[48, 49]. Thus, HCQ is thought to be less toxic and commonly prescribed than CQ in Canada, Australia and the USA. In Japan, it has been suggested that HCQ has a therapeutic efficacy in the treatment of SLE and lupus-related skin disease[49, 50], and a phase III clinical trial of HCQ for these diseases has just started.

Previous studies have demonstrated that CQ and HCQ cause retinal cell damage. The retinal cellular component, retinal pigment epithelium (RPE) is the primary target of CQ toxicity. The RPE is a single layer of cells adjacent to the photoreceptor outer segment (POS) of the retina, and the RPE plays a critical role in the maintenance of the POS by removing shed rod and cone debris[51]. This debris is endocytosed and then degraded in the lysosomes in the PRE[52]. Thus, the failure in the removal of the debris results in dysfunction of the POS cells, leading to retinopathy. CQ is also known to be an inhibitor of autophagy because it inhibits the function of autolysosomes, which is generated by the fusion of autophagosomes and lysosomes. Therefore, it is likely that CQ induces retinopathy by inhibiting autophagy. However, there is no direct evidence that autophagy is necessary for the function of the PRE in the maintenance of the POS, and the pathogenesis of retinopathy can be explained by the inhibition of the lysosomes.

Since retinopathy induced by CQ or HCQ is related to high daily dose (more than 750 mg per day) rather than long-term treatment[47, 48], an effort to reduce the dose of CQ or HCQ in the treatment is essentially required to reduce the side effect. It has been shown that the treatment of RA and SLE with a lower dose of HCQ in combination with MTX and/or SASP has a better therapeutic efficacy than a single drug treatment[53, 54]. We need to evaluate the effect of combined treatment with these drugs on the inflammation as well as osteoclastic bone resorption in the future. Considering that the treatment in a combination of antirheumatic drugs exerts a marked inhibitory effect on osteoclasts even at a low dose[55], the treatment by CQ or HCQ together with MTX and/or SASP would be more effective than a single treatment in the inhibition of osteoclasts. Moreover, since osteoclasts also mediate the bone loss in osteoporosis, CQ in combination with bisphosphonate may be useful for the treatment of osteoporosis.

Acknowledgements

We thank T. Ando, Y. Ogiwara, A. Yamamura, T. Koga, T. Nakashima, M. Oh-hora, K Okamoto, M. Guerrini, A. Yamaguchi and S. Kasugai for discussion and technical assistance. This work was supported in part by Ministry of Education, Culture, Sports, Science and Technology (MEXT) Scholarship (B.A.); a grant for ERATO,

Takayanagi Osteonetwork Project from the Japan Science and Technology Agency (H.T.); Grant-in-Aid for Young Scientist A and Challenging Exploratory Research from the Japan Society for the Promotion of Science (M.S.); grants for Global Center of Excellence Program from MEXT (H.T.); and a grant from Kowa Life Science Foundation (M.S.).

Conflict of interests

None

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