Targeting senescent osteal macrophages ameliorate excessive bone loss in postmenopausal osteoporosis: a new approach for prevention of osteoporotic fractures

Yoshio Nishida[1], M Alaa Terkawi[1], Keita Kitahara[1], Junki Shiota[1], Taiki Tokuhiro[1], Tomohiro Shimizu[1]
Hend Alhasan[1], Tsutomu Endo[1], Daisuke Takahashi[1], Norimasa Iwasaki[1]
[1]Department of Orthopedic Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan
Email of Presenting Author: nsd.yso@gmail.com

Disclosures: Y Nishida: None. MA Terkawi: None. K Kitahara: None. J Shiota: None. T Tokuhiro: None. T Shimizu: None. H Alhasan: None. T Endo: None. D Takahashi: None. N Iwasaki: None.

INTRODUCTION: Osteoporosis is the most common bone metabolic disease characterized by compromised bone volume and decreased mineral density resulting in an increased risk of fractures. It is estimated that over 370 million people worldwide were affected by osteoporosis in 2021, and 40% of women and 13% of men in ages of over than 50 years are at risk of experiencing one or more osteoporotic fractures in their lifetime [1]. Menopause is a major risk factor for skewing bone homeostasis toward excessive levels of bone resorption. Given the position of osteal macrophages in the bone microenvironment and their ability to secret osteoactive factors, understanding molecular and functional alterations in osteal macrophages in the post-menopause state should provide a clue for discovery of therapeutics for prevention of osteoporosis. Our earlier study demonstrated Osteal macrophages were heterogeneous subpopulations and had 6 subsets, and menopause made osteal macrophages to shift cell senescence and local inflammatory state in bone microenvironment [2]. These findings revealed that eliminating senescent osteal macrophages might be beneficial to ameliorate bone loss in the post-menopausal osteoporosis. Therefore, the aim of this study was to explore the pathological function of senescent osteal macrophages in osteoporosis and evaluate the therapeutic effects of targeting these cells in osteoporosis model.

METHODS: The procedures for the animal experiments were approved by the Institute of Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine (no. 22-0135). For in vitro oxidative stress model of macrophages, peritoneal macrophages that were isolated from SFF-BALB/c mice (CLEA, Tokyo, Japan) were seeded onto 24-well or 96-well plates and stimulated by treatment with hydrogen peroxide (Wako, Osaka, Japan) with or without 100nM β-estradiol (MedChemExpress, NJ, USA) and 20μM Glutaminase inhibitor compound (GIC) 968 (Cayman Chemical, MI, USA). Cells on 96-well plate were evaluated for the SA–β-Gal activity with a Senescence β-Galactosidase staining kit (Cell Signaling Technology, CST, USA). Cells on 24-well plate were collected to confirm the expression of target molecules using Western Blotting. For murine calvarial osteolysis model, SFF-BALB/c male mice were anesthetized then sagittal incision was made over the calvarial anterior site for implantation of oxidative stress-exposed macrophages (1.0×10^6). Pathological bone erosions were evaluated on day 5 using micro-CT (R_mCT2; Rigaku, Tokyo, Japan) and histopathology. For murine osteoporosis model, ovariectomized SFF-BALB/c female mice were randomly divided into three groups (PBS vehicle, GIC 968, CB-839). Mice were intraperitoneally injected with either 10 mg/ kg of GIC 968 or CB-839 (Cayman Chemical, MI, USA) in 500 μl 5% DMSO or the same volume of vehicle 3 times per week for 3 weeks starting at day 8 after the surgery. The mice were then anesthetized and euthanized by cervical dislocation (day 30 post surgery). Femurs and vertebrae were isolated and fixed in 10% formalin (Wako, Japan) for 24h and then scanned by micro-CT. For bone histomorphometric analyses, fixed femurs were decalcified in EDTA for 3 weeks and embedded in paraffin, and 5 μm-thick sections were prepared and stained with Tartrate-Resistant Acid Phosphatase (TRAP). Phospho-p53 (GeneTex) was applied as primary antibody to sectionized bone tissue for immunohistochemistry. Statistical analyses were performed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison procedure was used for comparison the differences among groups (GraphPad Software, La Jolla, CA, USA). The results were considered statistically significant when $p < 0.05$.

RESULTS: A remarkable increase in the expression of senescence markers in macrophages from OVX mice was found, as compared to these in the control mice (Fig. 1A, B). Moreover, supplementation of estrogen to cultured macrophages that had been treated with H_2O_2 decreased the harmful effects of oxidative stress on cells and suppressed the expression of genes involved in SASP (Fig. 1C). We next verified the beneficial effects of the GIC 968 after exposure to oxidative stress in vitro. It is noteworthy that the GIC968 treatment was effective in eliminating senescent macrophages and reduced the expression of inflammation markers in a manner that was comparable to the estradiol treatment (Fig. 1D). In consistent with these in vitro results, implantation of oxidative stress-exposed macrophages onto calvarial bones resulted in severe pathological bone loss in calvarial osteolysis model (Fig 2). These results revealed that presence of senescent macrophages in bone microenvironment may promote local inflammatory response accompanied with pathological bone loss leading to bone loss. Such findings suggest that senescent osteal macrophages can be a potential target for treatment of osteoporosis. Importantly, GIC968 treatment appeared to be effective in reducing the number of p-p53 positive lining cells but not osteocytes in femoral bone tissue (Fig. 3A), and alleviating bone loss in femurs compared to the vehicle-treated (control) in OVX mouse model (Fig. 3B). Consistent with these observations, the histological analysis revealed that the GIC968-treated OVX-mice exhibited a significant reduction in the numbers of TRAP-positive osteoclasts in the secondary spongiosa of femurs (Fig. 3C). These collective findings propose that targeting senescent osteal macrophages represents a promising therapeutic approach for the prevention of postmenopausal osteoporosis.

DISCUSSION: Our findings suggest that the decreased production of estrogen due to post-menopause state alters the osteal macrophages subsets, resulting in a shift toward cell senescence and inflammatory conditions in the bone microenvironment. These appear to be consistent with earlier findings highlighting that the accumulation of senescent myeloid cells in a bone microenvironment contributes to the development of inflammatory bone loss in the elderly [3]. Likewise, the increase in expression of senescence markers in macrophages skews these cells toward the inflammatory M1 phenotype that impairs bone metabolism and mediates skeletal fragility [4]. Importantly, the administration of GIC968 causes the elimination of senescent myeloid cells via inhibiting glutaminase alleviated excessive bone loss in OVX mice. In a support of our finding, the elimination of senescent cells by the inhibition of glutaminase 1 has been shown to ameliorate tissue microinflammation and prevent age-associated disorders [5]. Our data provide a new insight into the pathogenesis of osteoporosis and sheds light on a new therapeutic approach for the treatment of postmenopausal osteoporosis.

SIGNIFICANCE/CLINICAL RELEVANCE: The current study demonstrated targeting osteal macrophage senescence in the early stage of postmenopausal osteoporosis appears to be beneficial in terms of alleviating excessive bone loss and prevention of osteoporotic fractures.

REFERENCES: [1] Wade et al. Arch Osteoporos. 2014 [2] Yokota et al. ORS. 2023 [3] Farr et al. Bone and Mineral Research. 2016 [4] Kale et al. Immun Ageing. 2020 [5] Johmura et al. Science. 2021

ACKNOWLEDGEMENTS: The study was supported by research grants from Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research B; 23H03025), Hirose Foundation, and Kobayashi Foundation.

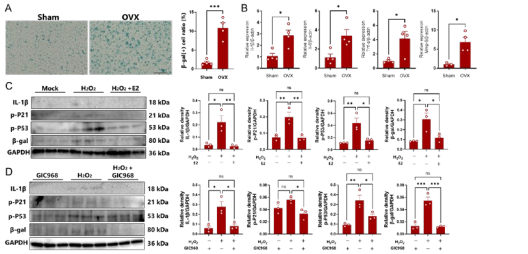

Figure 1. Role of estrogen in development of macrophage senescence and effect of estrogen or glutaminase inhibitor supplementation. A) Representative SA–β-Gal staining of macrophages and its quantification. Scale bar is 100 μm. B) Expression of molecules involved in SASP using qRT-PCR. C,D) Expression of molecules related to cellular senescence using western blotting. C; Results of estradiol treatment. D; Results of glutaminase inhibitor treatment.

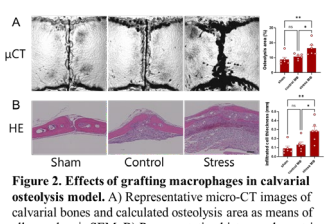

Figure 2. Effects of grafting macrophages in calvarial osteolysis model. A) Representative micro-CT images of calvarial bones and calculated osteolysis area as means of all samples ± SEM. B) Representative histomorphometric analysis of calvaral bone. Images are representative to HE-stained sections. Scale bar is 100 μm.

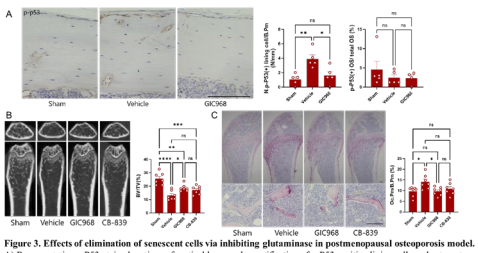

Figure 3. Effects of elimination of senescent cells via inhibiting glutaminase in postmenopausal osteoporosis model. A) Representative p-P53-stained sections of cortical bone and quantification of p-P53 positive lining cells and osteocyte. B) Representative micro-CT images of femoral bones and BV/TV values as means of all samples ± SEM. C) Representative histomorphometric analysis of femoral bone. Images are representative to TRAP-stained sections of secondary spongiosa. Scale bars are 100 μm.