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研究課題名（和文）Pathophysiological investigation of bone cell communications regulated by chemokine network

研究課題名（英文）Pathophysiological investigation of bone cell communications regulated by chemokine network

研究代表者

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研究成果の概要（和文）：ケモカイン受容体CCR5の骨代謝における役割を検討した。CCR5欠損マウスはRANKL誘導による骨粗鬆症モデルに耐性を示したことから、骨粗鬆症の発症に重要な分子であることが明らかとなった。さらなる解析から、CCR5を介した細胞内シグナルは、小分子GTPaseやインテグリン発現を調節し、破骨細胞の機能調節をしていることが明らかとなった。CCR5はHIVの共受容体でありAIDS薬物治療の標的分子であることから、本研究の臨床的意義も高いと思われた。

研究成果の概要（英文）：- Physiological roles of CCR5 in bone metabolism; Ccr5-deficient mice had significantly increased number and size of osteoclasts, although they did not show significant difference in BMD compared to their wild-type littermates. In keeping with osteoclast dysfuction, Ccr5-deficient mice were less susceptible to RANKL-induced bone loss, reflecting functional impairment of osteoclasts. - Elucidation of signaling pathway in Ccr5-/- mice; Ccr5-deficient osteoclasts showed decreased bone resorption activity accompanied with impaired and disorganized adhesive structures, demonstrated using confocal and super-resolution microscopy. Our molecular results suggested that CCR5-mediated signal was involved in integrin-mediated small GTPase activation that was required for proper osteoclasts function. Our findings unveil unique and essential roles of CCR5 in bone metabolism and bone destruction diseases, and have implications concerning bone physiopathology for the HIV therapy targeting CCR5.

研究分野：　Bone biology

キーワード：　osteoclast　chemokine　CCR5　RANKL

１．研究開始当初の背景

It is well known that HIV patients who receiving anti-HIV drugs, eventually leads to the emergence of drug resistance and sever side effects such as a high incidence of ostseoporosis. Furthermore, several epidemiclogic studies on human CCR5 polymorphism demonstrated that a 32-basepair deletion allele in the CCR5 gene affects not only transmitson of HIV-1 but also the reduced frequency of rheumatoid arthritis development. Therefore, it is possible that CCR5 regulates the osteolytic activity during normal bone turnover.

２．研究の目的

The G-protein-coupled receptor CCR5 is a co-receptor of HIV cell entry. Of note, epidemiological and pathological findings have reported that functional changes in CCR5 correlate with HIV transmission and bone destruction diseases and therefore a therapeutic target in. However, the roles of CCR5 in bone pathophysiology have not been well documented.

３．研究の方法

Ethical guidelines for animal and human studies All animal experiments were performed according to the Instiitutional Guidelines for the Care and Use of Laboratory Animals in Research and with the approval of the local ethics committees of both the Ehime Univiersity.

Mice Standard C57BL/6 mice (6 weeks-old, male) were obtained from CLEA Japan. *Ccr5*-deficient mice (*Ccr5*$^{-/-}$) were generated as previously described (32). All mice were backcrossed for 8 to 10 generations on the C57BL/6 background mice. Mice were all bred and maintained under pathogen-free conditions at the animal facilities of the Ehime University.

Osteoclast culture Mouse bone marrow cells isolated from 4-6 week-old mice cultured in α-MEM (Gibco BRL, Gaithersburg, MD) were used as sources of osteoclasts. The non-adherent cells were collected for bone marrow-derived macrophage and pre-osteoclast induction. Bone marrow-derived macrophages were induced with 50 ng/mL M-CSF and 100 ng/mL RANKL for additional 5-6 days. TRAP activity in the osteoclasts was determined by staining using TRAP staining kit (Wako). The non-adherent cells were collected for bone marrow-derived macrophage and pre-osteoclast induction. Bone marrow-derived macrophages were induced with M-CSF and RANKL for additional 5-6 days. The culture media were replaced every three days. TRAP activity in the osteoclasts was determined by staining using TRAP staining kit (Wako).

Immunocytochemical staining and fluorescence microscopy imaging osteoclasts were cultured into cover glass chamber fixed with 4% paraformaldehyde, permeabilized, and stained with the indicated specific Abs or Alexa488-labeled phalloidin (Molecular Probes), followed by Alexa594-conjugated Abs. The images were captured using an ECLISE Ni-E wide-field fluorescence microscope (Nikon, Japan) and analyzed by NISE Elements software (Nikon).

Mouse experiment To established bone loss model, sRANKL was administered to mice (rosmarinic acid, Ref17). The littermate of *Ccr5*+/+ and *Ccr5*-/- (6 week-old, male) mice were injected intraperitoneally with sRANKL (2 mg/kg) or PBS (vehicle). After 2 days, the mice were euthanized and blood samples were collected for serum isolation. Femora were fixed with 4% paraformaldehyde for 24 hr and reserved in 70% ethanol for next procedure.

Microcomputed tomography and Bone histomorphometry Micro-computed tomography (μCT) scanning was performed on proximal tibiae by μCT-40 (SCANCO Medical AG, Bruittesellen, Switzerland) with a resolution of 12 μm, and the microstructure parameters were three-dimensionally calculated as previously described (33). For the assessment of dynamic histomorphometric indices, calcein (at a dose of 20 mg/kg body weight) was injected twice (3 days interval) to wild-type and *Ccr5*$^{-/-}$ mice, respectively. The sections were stained with Villanueva, TRAP and toluidine blue and were analyzed using a semi-automated system (OsteoMeasure, Decatur, GA). The Nomenclature, symbols, and units used in the present study are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (34).

Inhibitory effect of human CCR5 for osteoclastogenesis and osteoblastogenesis Normal human natural osteoclast precursors cells and human mesenchymal stem cells were purchased from Lonza Walkersville, Inc.(Walkersville, MD), and maintained with Osteoclast Precursor Cell Basal Medium (Lonza) in the absence of supplemented growth factors. Multinuclear osteoclasts were cultured according to manufacturer’s instructions. Osteocalsts were supplemented with anti-CCR5 antibody, and the culture media was replaced every three days. Human osteoblastic cells were induced from human mesenchymal stem cells in osteogenic basal medium (Lonza) supplemented with ascorbic acid, β-glycerophosphate and dexamethasone for until harvest on day 14, with replacement once every three days in the presence of anti-CCR5 antibody.

４．研究成果

CCR5 deficiency impairs osteoclast adhesion

We first observed the dynamic movement of osteoclasts on culture dishes using time-lapse micrscopy can be used to visualize their migration and fusion. Osteoclasts isolated from *Ccr5*$^{+/+}$ and *Ccr5*$^{-/-}$ mice were infected adenovirus vector encoding GFP fusion, incubated 36 hr and imaged locomotion at intervals of 10 min (Fig.1A). A peripheral locomotion analysis showed a significant increase in contraction and CDI (cell deformation index) from osteoclasts of *Ccr5*$^{-/-}$ mice, indicating loosing the stability of cell attachment to matrix (Fig. 1B). We found the diminished expression levels of cell adhesion markers such as *integrin-αv* and *integrin-β3*, and markedly downregulated osteocytes enzymes such as *Mmp3* and *Mmp13* in *Ccr5*$^{-/-}$ cells. Thus Ccr5-deficiency affects cells locomotion, via disrupting the cell-attachment machineries including integrins.

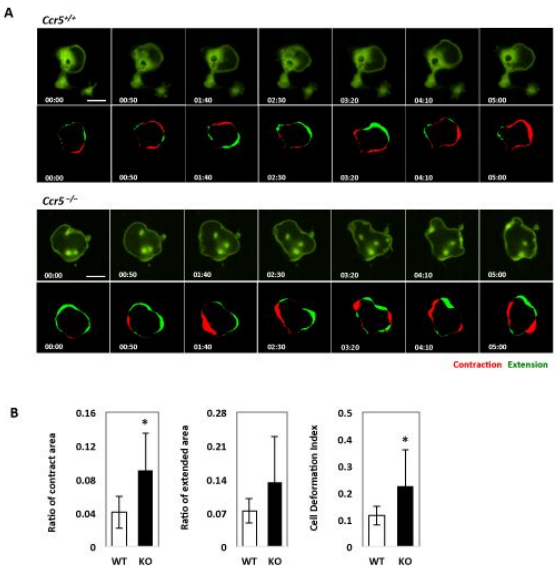

Fig.1. Osteoclastic locomotion impairment occurred by Ccr5-deficiency

Deletion of CCR5 in osteoclasts affect osteoclast function We further investigated the activity of bone resorption in *Ccr5*$^{-/-}$ osteoclasts. The actin rings formation (sealing zone) in *Ccr5*$^{-/-}$ was disrupted and the size was smaller than wild-type. A large numbers of resorption pits were observed in both *Ccr5*$^{+/+}$ and *Ccr5*$^{-/-}$ osteoclasts on dentin slices. Of note, *Ccr5*$^{-/-}$ osteoclasts ineffectively resorbed the dentin matrix and showed poor pit quality, as seen when we analyzed the quality of the resorptive pits by intensity (Fig. 2). The sealing zone maintain to local acidification and accumulation of matrix-degrading enzymes between the cell and bone surface. Therefore, these findings demonstrated that the absence of CCR5 resulted in abnormality morphology caused from fail of cytoskeletal arrangement and translate into functional decreased osteoclast.

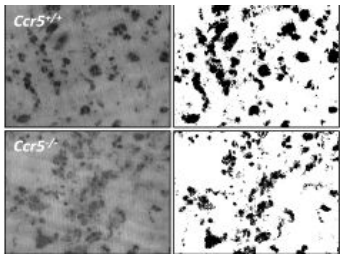

Fig. 2. CCR5-deficiency osteoclasts exhibit defects in bone resorbing activity

Increased bone mass due to less-susceptible RANKL-accelerated bone loss in Ccr5*$^{-/-}$ *mice Osteoporotic condition was induced by *in vivo* sRANKL administration (i.p., 2 mg/kg) in male *Ccr5*-/- and their litter-mate wild-type mice at 7 weeks of age. Vehicle (PBS) injection was also conducted as control experiments. In wild type mice, sRANKL administration induced significant reduction in pQCT-based parameters such as BMD, BV/TV, trabecular connective density (Conn. Dens.), trabecular number (Tb. N.) and Tb. Th., and increase in Tb.Sp., compared to those in the vehicle-injected group (Fig. 3B). Consistently, serum level of TRAP was significantly augmented in sRANKL-injected mice, though endogenous levels of RANKL and osteocalcin were not different between sRANKL and vehicle groups, thus validating a bone loss model. In contrast, sRANKL administration in *Ccr5*-/- mice did not alter these pQCT-based parameters and serum level of TRAP, indicating that *Ccr5*-/- mice are protected from sRANKL-induced osteoporosis. In histomorphometric analyses, osteoblastic parameters did not alter by sRANKL administration in either genotypes. In wild-type mice, osteoclastic parameters such as number of osteoclasts (N.Oc.), Oc.S./BS. and N. Oc./Oc.Pm. were siginificantly augmented (Fig. 3A). These data confirmed this protection from sRANKL-induced bone loss in *Ccr5*$^{-/-}$ mice, and suggested that CCR5 plays essential role in osteoclast function.

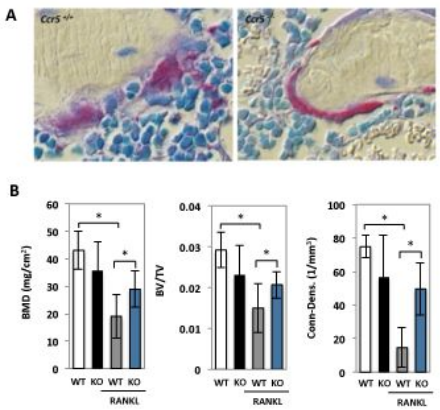

Fig. 3. Ccr5-/- male mice are less susceptible to RANKL-induced bone loss

CCR5-mediated signals involved in focal adhesion complex and its downstream signals We applied super-resolution microscopy imaging to allow clear independent actin core spots and cloud localization. Vinculin expression, as detect by immunetochemistry, and F-actin core, was defected association and integration of podosomal units in Ccr5-/- osteoclasts (Fig. 4A). Actin core size was also smaller than that of wild-type. Immunoblots data of FAK, Src and Akt signals, which promoted with RANKL showed that markedly reduced in Ccr5-/- ostseoclasts (Fig. 4B). To confirm whether CCR5 invovle in FAK-Src-Small GTPase axis, Rho GTPase family, Rac1, Rac2, RhoA and Cdc42 mRNA expression levels were examined during the course of osteoclastogenesis. Small GTPase signals except Cdc42, were showed signifynt augmentation specifically onpOC. Consistent with these results, G-LISA assay revealed that active form of Rac and Rho signals were significantly inhibited in pOC of Ccr5-/- mice (Fig. 4C). Thus, the mechanism by which CCR5 disrupts the cytoskeleton rearrangement is medicated from integrins and focal adhesion complex, via small GTPase signal.

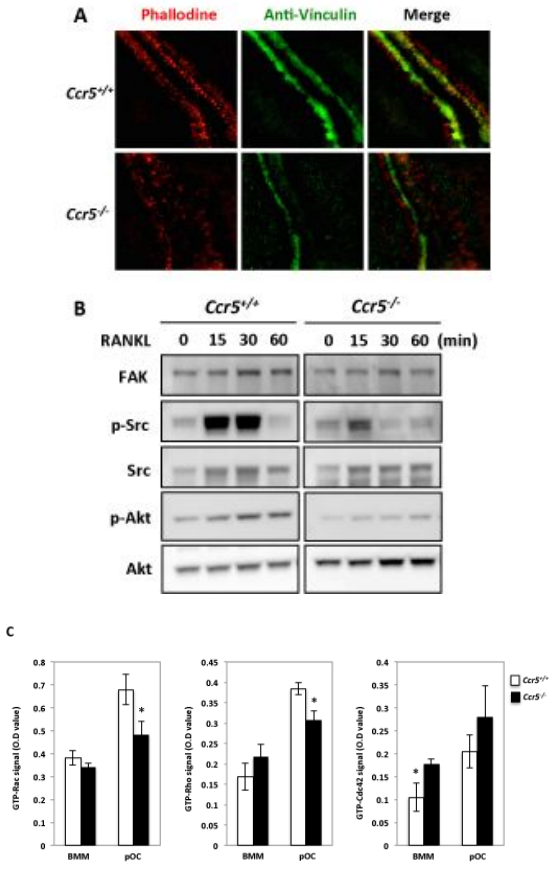

Fig. 4. Focal adhesion complex and small GTPase signals were defected by Ccr5-/- mice

Inhibitory effect of human CCR5-specific antagonists for osteoclastogenesis, not osteoblastogenesis We investigated the effects of the blockades of hCCR5 in osteoclastogenesis in vitro, by administrating its neutralizing antibody. Blockades of hCCR5 by anti-hCCR5 neuab obviously inhibited osteoclastogenesis in dose-dependent manners specifically inhibited the late stage of differentiation (Fig. 5A, B). In contrast, the blockade of hCCR5 was not showed significant in differentiation and mineralization of osteoblasts.

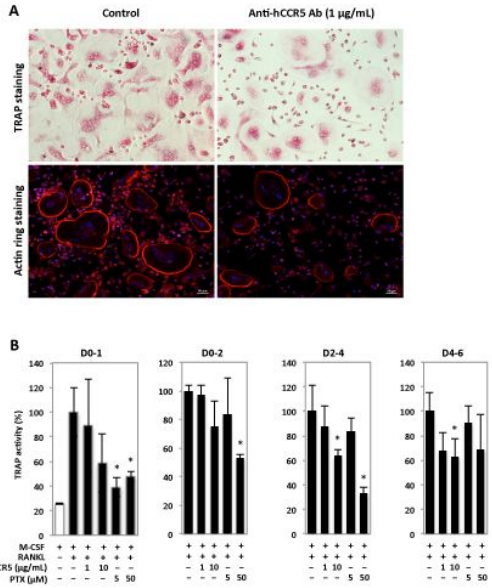

Fig. 5. Anti-CCR5 neutralizing antibody inhibited osteoclastogenesis in human cultured osteoclast precursors

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　4　件）

① Lee JW, Iimura T, Quantitative in situ fluorescence imaging to unveil the morphological and functional heterogeneity of osteocytes, Journal of Oral Bioscience, 57(2), 2015, 76-79
DOI:10.1016/j.job.2015.02.00, Referee Reading

②Lee JW, Asai M, Jeon SK, Iimura T, Yonezawa T, Cha BY, Woo JT, Yamaguchi A, Rosmarinic acid exerts an anti-osteoporotic effect in the RANKL-induced mouse model of bone loss by promotion of osteoblastic differentiation and inhibition of osteoclastic differentiation, Molecular Nutrition and Food Research, 59(3), 2015, 386-400
DOI:10.1002/mnfr.201400164, Referee Reading

③ Nishiyama Y, Matsumoto T, Lee JW, Saitou T, Imamura T, Moriyama K, Yamaguchi A, Iimura T, Changes in the spatial distribution of sclerostin in the osteocytic lacuno-canalicular system in alveolar bone due to orthodontic forces, as detected on multimodal confocal fluorescence

imaging analyses, Archives Oral Biology, 60(1), 2015, 45-54
DOI:10.1016/j.archoralbio.2014.08.013, Referee Reading

④ Lee JW, Iimura T, Functional heterogeneity of osteocytes in FGF23 production: the possible involvement of DMP1 as a direct negative regulator, Bonekey Reports, 543, 2014
DOI:10.1038/bonekey.2014.38, Referee Reading

〔学会発表〕（計 9 件）

① Lee JW, Hoshino A et al., C-C chemokine receptor 5, a co-receptor of HIV, -mediated signal regulates bone resorption via locomotion of osteoclasts, American Society of Bone Mineral Research 2015. 10. 11. Poster Presentation, Washington State Convention Center in Seattle, Washington in US

② Lee JW, **蛍光細胞イメージングと骨形態計測で見る骨代謝におけるケモカインシグナルの役割、歯科基礎医学会、**2015. 09. 11. Oral Presentation **新潟県新潟市新潟コンベンションセンター朱鷺メッセ**

③ Lee JW, Roles of C-C chemokine receptor 5, a co-receptor of HIV, -mediated signal in locomotion of osteoclasts and bone resorption, Japanese Society of Bone Mineral Research, 2015. 07. 25. Oral Presentation **東京都新宿京王プラザホテル**

④ Lee JW, Hoshino A et al., Roles of CCR5, a co-receptor of HIV, in regulation of functional motility of osteoclasts, Osteoimmunology, 2015. 07. 1. Poster Presentation **沖縄県宮古島市ホテルブリーズベイマリーナ**

⑤ Lee JW, Quantitative illumination on bone histology and cell biology by fluorescence imaging, International Symposium on Bio-Imaging and Gene Targeting Sciences in Okayama, 2015. 02. 15. Oral Presentation (Invitation) **岡山県岡山市岡山大学創立五十周年記念館金光ホール**

⑥ Lee JW, Imamura T, Iimura T, Cell cycle phase-dependent phosphorylation of Smads in skeletal cells, BMP conference 2014. 09. 18. Poster Presentation, Seminaris Hotels and Meeting Resorts, Berlin in German

⑦ Lee JW, Yamaguchi A, Iimura T, A possible role of DMP1 as a negative regulator of FGF23 production in functional heterogeneity osteocytes: Three dimensional morphological approaches, American Society of Bone Mineral Research 2014. 09. 13. Poster Presentation, George R. Brown Convention Center, Houston, Texas in US

⑧ Lee JW, Yamaguchi A, Iimura T, DMP1 **は骨細胞における** FGF23 **産生の抑制因子である－蛍光３次元形態計測と細胞生物学的アプローチ**, **日本骨代謝学会**, 2014. 07. 26. Poster Presentation **大阪県大阪市大阪国際会議場**

⑨ Lee JW, Yamaguchi A, Iimura T, Three-dimensional fluorescence morphometry reveals functional heterogeneity of osteocytes in FGF23 production regulated by DMP1, **骨形態計測学会**, 2014. 06. 13. Oral Presentation **北海道県札幌市さっぽろ芸文館**

〔図書〕（計 0 件）

〔産業財産権〕
○出願状況（計 0 件）

名称：
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発明者：
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〔その他〕
ホームページ等
なし

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