様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　元 年　 ６ 月 ２０ 日現在

機関番号：１６３０１
研究種目：若手研究(B)
研究期間：2016～2018
課題番号：１６Ｋ２０４１２

研究課題名（和文）Morphometrical and quantitative transcriptome analyses of C-C chemokine receptor 5 in functional structure of osteoclasts

研究課題名（英文）Morphometrical and quantitative transcriptome analyses of C-C chemokine receptor 5 in functional structure of osteoclasts

研究代表者
李　智媛（LEE, JIWON）

愛媛大学・プロテオサイエンスセンター・助教（特定教員）

研究者番号：７０７１１２７４

交付決定額（研究期間全体）：（直接経費）　　3,000,000円

研究成果の概要（和文）：以前の研究成果として、ヒト培養破骨細胞分化実験系において、抗CCR5中和抗体は破骨細胞の機能分化、特にアクチンリング形成を阻害した。そのメカニズムはCCR5がRANKLやインテグリンを介したシグナルと共役して、破骨細胞の細胞骨格と運動性を調節すること、その際、small GTPasesであるRacおよびRhoの活性化が重要かつ必要十分であることが明らかとなった。また、CCL5の中和抗体投与マウスでは破骨細胞形成が抑制され骨量が増加した。これらの観察結果は、上記の疫学研究を裏付ける結果であり、CCR5を標的としたHIV治療が骨吸収性疾患に対してもメリットをもたらす可能性を示した。

研究成果の学術的意義や社会的意義
HIV感染治療薬の標的分子であるCCR5が骨の代謝も調節していることを解明した。抗CCR5治療薬はHIV感染患者の延命に大きく貢献しますが、高齢化に伴う運動器疾患への影響が懸念されていた。今回の研究でCCR5が骨を吸収する破骨細胞にも存在し、CCR5の機能抑制は破骨細胞の骨吸収能を抑えることを解明した。さらに、動物実験では、CCR5の機能抑制が骨粗鬆症を抑えることを発見された。これらの実験観察は、抗CCR5治療薬はHIV感染症のみならず、骨粗鬆症を始めとする骨吸収性疾患に対してもメリットをもたらす可能性を明らかにした。

研究成果の概要（英文）：This study demonstrated that the blockade of CCR5 using its specific antibodies impaired in vitro human osteoclastogenesis with disorganized actin rings, but not osteoblastogenesis. Ccr5-deficient mice with dysfunctional osteoclasts were resistant to osteoporotic stimulation via the administration of receptor activator of nuclear factor kappa-B ligand (RANKL), which induces osteoporosis independently of the inflammatory and immunomodulatory responses. Furthermore, CCL5, a ligand for CCR5, enhanced the integrin- and chemokine-mediated pathways in osteoclast. The present study experimentally provides further evidence that CCR5 plays an essential role in bone destructive diseases through the functional regulation of osteoclasts, thus suggesting a skeletal benefit of the CCR5-targeting therapy.

研究分野：骨代謝・細胞生物学

キーワード：osteoclast　osteoblast　chemokine receptor 5　bone metabolism

１．研究開始当初の背景

Chemokine receptor (belong to the family of G-protein linked transmembrane receptors, GPCRs) is well known for their diverse signaling and distinct signaling pathways that mediated cell migration, actin cytoskeleton, cell adhesion and differentiation. Live imaging and super-resolution microscopy analyses revealed that Ccr5-deficient osteoclast showed larger in size and disorganized motility, cytoskeletal rearrangement and cell-attachment machineries including integrins, thus leading decreased bone resorption activity. In further our morphometrical analysis supporting that CCR5 deficiency mice showed loss of their function, consequently the bone mass had affected even abundant osteoclasts were exist in bone marrow.

２．研究の目的

In previous study, we found that changes in gene expression of chemokine receptor 5 (CCR5) during osteoclastogenesis are associated with osteoclast differentiation and distinct function. To validate this research results, we will investigate the chemokine-mediated osteoclast regulatory factor from omics profile by using microRNA microarray analysis system and elucidate the signal pathway how putative factors control the biological activities of osteoclast for drug development of bond disease.

３．研究の方法

3.1. Ethical guidelines for animal

All animal experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and with the approval of the local ethics committees of both the Ehime University.

3.2. Mice

Standard C57BL/6 mice (6 weeks-old, male) were obtained from CLEA Japan. Ccr5-deficient mice (Ccr5-/-) were generated. 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.

3.3. Osteoclast culture

Mouse bone marrow cells isolated from 4-6-week-old mice cultured in alpha-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 were determined by staining using TRAP staining kit (Wako).

3.4 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).

3.5. Cytomorphometrial analyses by fluorescence bio-imaging

To evaluate three-dimensional architecture of cultured osteoclasts obtained from Ccr5+/+ and Ccr5-/- mice, we will take three-dimensional fluorescence images by confocal-based super resolution microscopy (Nikon A1 with C-ER) equipped in Division of Analytical Bio-Medicine in Ehime University. Before imaging, intra-cellular filaments such as actin and tubulin are stained with phalloidin-Alexa 488 and anti-tubulin antibody, respectively. Super-resolution microscopy (Nikon N-SIM) is also applied to observe subcellular organelle dynamics, and cell adhesion machinery. Image analyses perform using IMARIS and ImageJ software.

3.6. Transcriptome analysis of CCL5 on osteoclasts

To find potent molecules that regulate osteoclast morphology and function under the control of CCR5-mediated pathway, we apply RNA-seq (RNA sequencing), also called whole transcriptome shotgun sequencing by a Next generation sequencer (Illumina MiSeq) equipped in Division of Analytical Bio-Medicine in Ehime University. RNA-seq can reveal presence and quantity of total RNA from a genome at a given moment in time. We will analyze transcriptomes of mRNA and micro RNA. Generally, micro RNAs play important roles in physiology and disease, and present tremendous therapeutic potential. Nevertheless, how micro RNAs regulate skeletal biology in still under-investigated.

Total RNA extracts are prepared from the cultured osteoclasts obtained from Ccr5+/+ and Ccr5-/- mice using RNeasy kit (Qiagen) according to the manufacture's instruction. Total RNA further separates into large and small RNA enriched fractions using the miRNeasy kit (Qiagen) columns and reagents. Quantified RNA samples will be sequenced and analyzed by Illumina MiSeq.

Data of RNA-seq will be analyzed using specific software (web-based tool, Database for annotation, visualization and integrated discovery etc.) according to the manufacturer's default

parameters and advices special expert from our common facility. To validate the results of microarray analysis, we investigate transcriptional expression of selected molecules in Ccr5+/+ and Ccr5-/- osteoclasts by using qRT-PCR.

3.7. Validation of candidate factor and signaling pathway from profiling

For functional cellular validation, we will construct RNA interference (RNAi) plasmid to block expression of candidate molecules using pSUPER basic vector (Oligoengine). These plasmids are electroporated to cultured osteoclasts (Neon, Invitrogen). These cells are analyzed by osteoclastic marker expression by qRT-PCR, pit formation assay and cytomorphometrical analyses as described above.

４．研究成果

4.1. CCL5 enhances integrin- and chemokine-mediated signaling.

Our previous study also reported that CCL5 and CCL9 (ligands for CCR1 and CCR5) were endogenously expressed by osteoclasts and were required for osteoclast differentiation in vitro. Ccl5 was highly detectable throughout the cultured period, whereas Ccl9 always remained at a basal level (data not shown). This finding suggested that CCL5 was more functional ligand than CCL9 in osteoclast differentiation at least in vivo. Treatment with recombinant mouse CCL5 (rmCCL5) significantly augmented the number of wild-type derived osteoclasts but not Ccr5-/- cells (Fig.1.), indicating that CCL5 enhanced RANKL-induced osteoclastogenesis through CCR5. The incubation with rmCCL5 also increased the numbers of actin rings and resorption pits of cultured wild-type osteoclasts on dentine slices in a dose-dependent manner (data not shown).

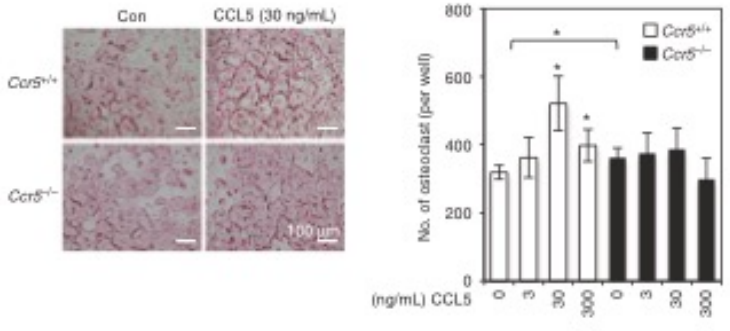

Fig.1. The effect of CCL5 on osteoclastogenesis was revealed by TRAP staining. Bone marrow cells that were isolated from Ccr5+/+ and Ccr5-/- mice were cultured with M-CSF and RANKL with or without recombinant CCL5. The number of multi-nucleated osteoclasts following exogenous CCL5 treatment was quantified. $P<0.05$ by Student's t-test.

4.2. Effects of CCL5 on focal adhesion signaling

We further investigated the roles of the CCL5 in the crosstalk with the RANKL-induced downstream signals. The rmCCL5 stimulation induced the phosphorylation of FAK, even at 0 min (prior to RANKL treatment) (Fig.2.). This did not occur after RANKL stimulation without rmCCL5. This elevated level of the phosphorylated FAK by the pre-incubation with rmCCL5 in 30 min after RANKL stimulation. This pre-incubation with rmCCL5 also enhanced the phosphorylation of Pyk2 prior to RANKL treatment. RANKL stimulation induced the phosphorylation of Src at 15-30 min and Pyk2 at 15-60 min after the stimulation. To confirm the induced phosphorylation of Src by RANKL or the combination of rmCCL5 and RANKL, we conducted immunoprecipitation with phosphor-tyrosine antibodies. The level of phosphorylated Src following the combination treatment with RANKL and CCL5 was markedly increased in comparison to that with RANKL alone.

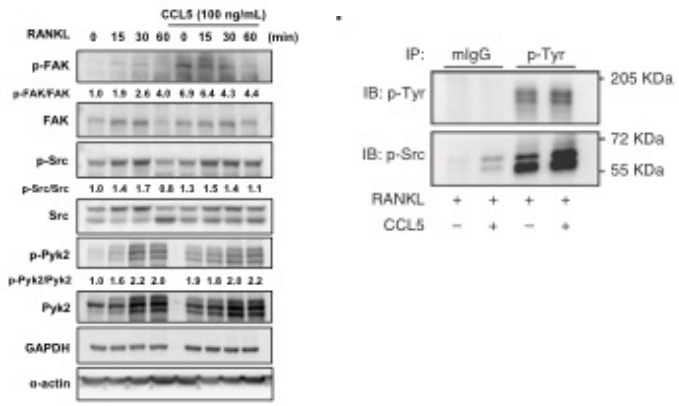

Fig.2. The effects of CCL5 on focal adhesion signaling. The effects of CCL5 on focal adhesion signal were investigated by immunoblotting. BMCs isolated from wild-type mice were cultured with M-CSF and RANKL for 3days and preincubated with rmCCL5 for 30 min prior to sRANKL stimulation for the indicated time. The phosphorylation levels of FAK, Src and Pyk2 were analyzed (left panel). BMCs were treated with RANKL alone or the combination of RANKL and CCL5 (100 ng/mL). Total cell lysates immunoprecipitated with phopspho-tyrosine kinase and immunoblotted with phosphor-Src (right panel).

4.3. RNA-sequencing expression profiling data

To investigate the changes in the transcriptional signatures induced by CCL5 in osteoclatogenesis, cultured osteoclasts at pOC stage were incubated with or without rmCCL5 for 2 days, and then subjected for RNA-sequencing. Genes with a log2 (fold-change) value of >0.5 and an adjusted *P*-value of <0.05 were tested for enrichment using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. A gene ontology analysis demonstrated that the greatest number of genes that were upregulated by CCL5 in osteoclatogenesis were found in pathways related to lysosomes, ECM-receptor interaction, focal adhesion, cell adhesion, osteoclast differentiation, and the chemokine signaling pathway. Interestingly, the pathway analysis demonstrated that transcriptionally upregulated molecules were involved in the integrin- and chemokine-mediated pathways (Fig.3).

Up-regulated pathway by CCL5 (vs. Control)	*P* value
Lysosome	1.5×10^{-15}
ECM-receptor interaction	5.0×10^{-9}
Focal adhesion	5.6×10^{-9}
Cell adhesion molecules	6.1×10^{-8}
Osteoclast differentiation	1.2×10^{-7}
Chemokine signaling pathway	3.8×10^{-4}

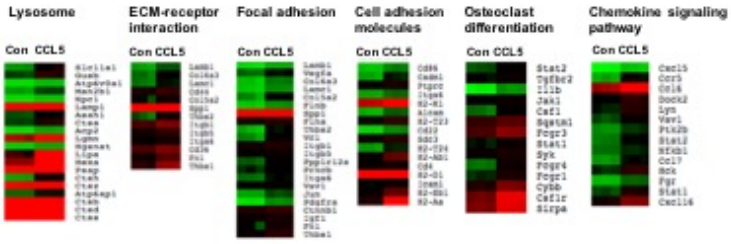

Fig.3. Gene ontology analysis of the genes that showed significantly altered expression levels in RNA-sequencing using cells incubated with RANKL alone and the combination of RANKL and CCL5 (100 ng/mL) (three analyses per condition). The significantly upregulated pathways and heat maps of the obtained results are shown. Red, high expression; green, low expression.

4.4. Critical roles of CCL5 in bone mass regulation.

Our in vitro analyses suggested that CCL5 is a major functional endogenous ligand for CCR5 and CCR1. CCL3 has been reported to be involved in models of pathological bone-destructive disease. Thus, we investigated the serum levels of CCL3 and CCL5 in physiological conditions. The serum concentration of CCL5 was ~60 ng mL-1, whereas the level of CCL3 was undetectable by ELISA-based method (Fig. 4a). The immunofluorescence of CCL5 in the bone marrow of the 8-week-old mouse tibia exhibited relatively intense signals in osteoblasts and osteoclasts covering the trabeculae, endothelial cells, and stromal cells (data not shown). To ascertain whether CCL5 exerts a functional role in the bone in vivo, the antibodies specific to mouse CCL5 (CCL5 neuAb) were intraperitoneally injected (once a week for 2 weeks) into 6-week-old C57BL/6J male mice (Fig. 4b). The μCT images of CCL5 neuAb-injected mice showed thickened cortical and trabecular bones in comparison to control mice (Fig. 4c). In fact, μCT-based parameters such as BMD, BV/TV, Conn-Dens, and Tb.N showed a significant increase in the bone architecture of CCL5 neuAb-injected mice (Fig. 4d). The histological analysis showed that the numbers of TRAP-positive osteoclasts in CCL5 neuAb-injected mice were markedly reduced in comparison to control mice (Fig. 4e). In CCL5 neuAb-injected bones, these cells were obviously smaller in size and occasionally exhibited a flattened shape. The histomorphometric parameters of osteoclasts, such as N.Oc, N.Oc/T.Ar, and No.Oc/B.Pm, were consistently reduced (to a significant extent) in CCL5 neuAb-injected bones (Fig. 4f). The parameters of osteoblasts, such as N.Ob, N.Ob/T.Ar, and N.Ob/B.Pm, were also severely reduced by the injection of CCL5 neuAb. These findings demonstrated that the blockade of CCL5 had an impact on the bone metabolism through the inhibition of bone osteoclasts and osteoblasts.

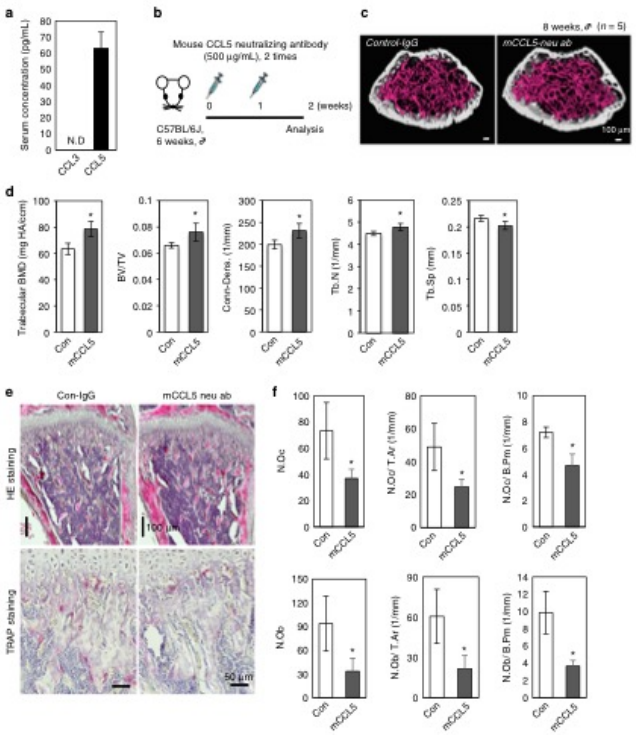

Fig.4. The blockade of CCL5 in vivo. Mouse anti-CCL5 neutralizing antibodies (rmCCL5 neuAb, 500 μg per mouse) were injected (once per week for 2 weeks) into 6-week-old male C57BL/6J mice (n=5). IgG was administered to the control group. μCT images (scale bars, 100 μm) and parameters (n=5 mice per group) are shown. Representatibe images of the distal femurs from the control IgG and mCCL5 ab groups. HE- (scale bar, 100 μm) and TRAP-stained sections (scale bars, 50 μm) are shown in the upper and lower panels, respectively. Quantitative bone histomorphometric analyses were conducted of the trabecular bones in the distal femurs of control IgG and mouse CCL5 neutralizing antibody-injected mice. P<0.05 (by Student's t-test) in comparison to control and mCCL5-neuAb mice. All values are shown as the mean ± SD, n=5.

５．主な発表論文等

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2. 2016. 09. 16 Invited speaker, The 14th Matsuyama International Symposium on Proteo-Sciences, “Unveiling novel regulatory mechanisms of osteoclasts by integrative approaches of bio-imaging and molecular omics” Ehime University, Ehime, Japan
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4. 2016. 07. 22 Invited speaker, Young Researcher Symposium - The 34th Annual meeting of Japanese Society for Bone and Mineral Research, “Roles of chemokine-mediated signaling in architectural function of osteoclasts” Osaka Convention Center, Osaka, Japan
5. 2016. 06. 04 Invited speaker, The 7th meeting of Bone Bio-Science, “Essential role of C-C chemokine-mediated signaling in mature osteoclast function” Okayama Convention Center, Okayama, Japan

〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年：
国内外の別：

〔その他〕
ホームページ等

６．研究組織
(1)研究分担者
研究分担者氏名：

ローマ字氏名：

所属研究機関名：

部局名：

職名：
研究者番号（8 桁）：

(2)研究協力者
研究協力者氏名：飯村　忠浩
ローマ字氏名：Iimura Tadahiro