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Form 3

Abstract of Thesis

Name （ Chen Siyu ）	
Title	Study of the role of Rab32/Rab38 in autophagy （ オートファジーにおけるRab32とRab38の役割に関する研究 ）

Abstract of Thesis

【Introduction】

Autophagy is intracellular degradation system. There exist two types of autophagy; macroautophagy and microautophagy. Macroautophagy progresses through formation of membrane structure, autophagosome, that delivers substrates to lysosomes. In microautophagy, lysosome directly entraps the substrate and degrades them. In sharp contrast to the situation that macroautophagy have been extensively studied in these decades, there are quite limited studies regarding microautophagy. In another line of classification, there exist two types of autophagy; non-selective autophagy and selective autophagy. Non-selective autophagy entraps non-selective cellular components represented by soluble cytosolic proteins. Selective autophagy entraps specific target, like organelle and bacteria. Each selective autophagy accompanies with specific names according to the target, and in the case of mitochondria, it is called mitophagy. Clearance of mitochondria is crucial for healthy cellular biological homeostasis. Abnormality in mitochondrial clearance occur in many diseases, which could be one of the pathogenesis mechanism, especially in neurological diseases.

Bone remodeling is a lifelong process where degradation and formation of bone tissue are required for bone homeostasis. Rab32 and Rab38 belong to a small GTPase protein family, which play regulatory role in intracellular vesicular trafficking, and close paralogues each other. Previous research in our laboratory had shown that the expression levels of Rab38 were significantly induced during the differentiation from bone marrow-derived macrophage (BMDM) to osteoclast. Both Rab32 and Rab38 are localized on the lysosome-like organelle membrane in macrophage and osteoclast. Interestingly, early endosome and mitochondria are entrapped inside those lysosome-like organelle, indicating that they are delivered by autophagy. Hence, I set to start the analysis which kind of autophagy is involved in these processes.

【Materials and Methods】

1. Cell culture

The Raw 264.7 cell line and HeLa cells stably expressing myc-Parkin were cultured in DMEM containing 10% FBS and 200 μg/mL penicillin/streptomycin. The culture condition was maintained at 37°C in 5% CO_2. BMDM collected from WT and Rab32 and Rab38 double knockout mouse bone were cultured in medium MEMα containing 10% FBS, 60 μg/mL kanamycin and 10% M-CSF. Cells were transfected with pMRX-puro-EGFP-Rab32, pMRX-puro-EGFP-Rab38 and pMRX-puro-Lamp1-EGFP plasmids to express GFP-Rab32, GFP-Rab38 and Lamp1-GFP. ATG7 KO Raw 264.7 cell line, a gift from Dr. Chao-Yuan Tsai (iFReC of Osaka University) was generated by using CRISPR-Cas9 gene editing technology.

2. Immunofluorescence

Cells were cultured on 12 mm cover glass and fixed with 4% PFA. Then cells were permeabilized and incubated with the primary antibody at room temperature for 1 hour or 4°C overnight. After washing 3 times with blocking buffer, it was incubated with the secondary antibody for 1 hour at room temperature. mounted on a slide glass for microscope obverse.

3. Dye staining and live cell imaging

Mitophagy Detection Kit called Mitophagy dye were used for observe mitophagy. Lysotracker and Mitotracker were used as lysosome and mitochondrial marker. The reagents were added to cells before CCCP treatment inducing mitochondrial damage. The live cells were directly observed under a confocal laser scanning TSC SP8 as 10

second interval for 5 minutes under an incubation temperature at 37°C.

4. BMDMs isolated from mouse bone and the differentiation of osteoclasts

Femur and tibia were collected from mouse and collected the epiphyses of both femur and tibia by flushing the bone marrow. BMDMs were cultured in MEMα medium supplemented with 10% FBS, 60 μg/mL kanamycin and 10% growth factor M-CSF condition medium. Osteoclasts differentiation were induced by stimulating macrophages with 400 ng/mL RANKL in MEMα completed medium for 6 days.

【Results and Discussion】

1. Rab32 and Rab38 are important for mitophagy in macrophage

Based on previous finding that mitochondria are entrapped in lysosome-like organelle, I first established the mitophagy analysis system in macrophage. First mitophagy assay kit was assessed about its feasibility detecting mitophagy using well established HeLa cells. Fluorescence signal was significantly increased depending on Parkin expression and CCCP treatment, both are required for mitophagy. Next BMDM were treated with CCCP, and mitophagy assay kit was subjected. As a result, fluorescent signal was increased even without extra Parkin expression. The fluorescent signals were mostly found within GFP-Rab32 and GFP-Rab38 positive ring-like structure representing lysosome-like organelle. By contrast, Rab32/Rab38 DKO BMDM cells showed a low efficiency of mitophagy. These results suggest that Rab32 and Rab38 are important for mitophagy.

2. Microautophagy mediated mitophagy occurs in BMDMs

LC3 is a protein that can be used as an autophagosome marker in macroautophagy process. I observed the locations of LC3 (autophagosome), LAMP-1 (lysosome), and Mitotracker (mitochondria) in BMDM and HeLa cells during mitophagy. LC3/LAMP-1/Mitotracker triple-positive structure were the main population in Hela cells, indicating that macroautophagy dependent mitophagy is the main process. By contrast LAMP-1/Mitotracker double-positive but LC3 negative structure was the dominant in BMDMs. Furthermore, the ratio of LAMP-1/Mitotracker double-positive vesicles was decreased in DKO BMDMs, compared to WT BMDMs. These results suggested that microautophagy could be the main pathway for mitophagy in BMDMs.

3. Rab32 and Rab38 dependent microautophagy mediated mitophagy is dominant in macrophages

Autophagy-related gene 7 (ATG7) is an essential regulator of autophagosome formation and ATG7 KO RAW 264.7 cell is deficient in macroautophagy. ATG7 KO showed a similar level of mitophagy after CCCP treatment compared with WT. Mitochondria were trapped in the GFP-Rab32 or GFP-Rab38 positive lysosome-like organelle even in ATG7 KO macrophages. I further observed live cell imaging that mitochondria are directly engulfed by lysosome-like organelle. This confirmed that and Rab32/Rab38 dependent microautophagy is dominant in macrophage.

4. Rab32 and Rab38 are involved in acidification of lysosome by affecting V-ATPase localization

Next to examine whether Rab32/Rab38 affect lysosome and lysosome-like organelle in BMDMs, immunofluorescence was performed using an anti-lamp-1 antibody. Lamp1 positive large vacuoles are significantly increased in DKO BMDMs compared to WT BMDMs. Next BMDMs were treated with lysotracker that stain acidified organelle. As a result, acidified organelles were much decreased in DKO BMDMs. These indicate that RAB32 and Rab38 affect the establishment of lysosome and or lysosome-like organelle.

5. Bone abnormalities appear in Rab32 and Rab38 double knockout mice

In the mouse model, the bones of fibula are thickened, hyperostosis, in DKO mice. The function of bone resorption should be affected in DKO mice. V-ATPase is a proton pump. Among three subunits, a1, a2, and a3 of V-ATPase, which maintains lysosome acidification, a3 subunit positive vesicles (lysosome) are specifically increased in osteoclasts and accumulate on plasma membrane providing the function of bone resorption. I found that a3 positive lysosome and the accumulation of a3 on plasma membrane were absent in Rab32 and Rab38 DKO osteoclasts. These results indicated that Rab32/Rab38 is involved in osteoclast functions.

様式７

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

Chen Siyu 氏はマクロファージにおけるオートファジー現象の解析を行った。マウス骨髄由来マクロファージやマクロファージ様細胞株 Raw264.7 細胞において、CCCP 処理によりミトコンドリアに障害を与えたとき、ミトコンドリアが Rab32 陽性のリソソーム様オルガネラに輸送されるオートファジー現象を見出した。

Rab32 およびそのパラログである Rab38 の二重欠損マウスに由来するマクロファージではこのオートファジー現象は著明に抑制されることを示した。またマクロオートファジーに必須なタンパク質 ATG7 を欠失した Raw264.7 細胞においても、ほぼ同じ効率でこのオートファジー現象は見られた。ライブイメージング観察により、実際にリソソーム様オルガネラが直接ミトコンドリアを取り込む画像が得られたことより、このオートファジー現象はミクロオートファジーによるものであることが明らかになった。

Rab32　Rab38 の二重欠損マウス由来の破骨細胞では、細胞膜への V 型 ATPase のサブユニット a3 の局在化に欠損を示し、骨吸収に十分な機能を発揮できない可能性が示唆される。実際 Rab32Rab38 の二重欠損老齢マウスにおいて、骨の形成に異常をきたしていた。

これらの発見はマクロファージおよび破骨細胞において Rab32 と Rab38 が重要な役割を演じることを示しており、特筆に値する。
よって、博士（歯学）の学位論文として価値のあるものと認める。