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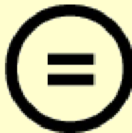

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이학석사학위논문

The Role of Adseverin in RANKL-Induced Osteoclastogenesis

RANKL에 의해 유도되는 파골세포 형성에서의 adseverin의 역할

2015 년 2 월

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이 논문을 이학 석사 학위 논문으로 제출함

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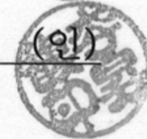

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ABSTRACT

The Role of Adseverin in RANKL-Induced Osteoclast Differentiation

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(Directed by Prof. Hong-Hee Kim, Ph.D)

Adseverin, a member of the gelsolin superfamily of actin binding proteins, is a Ca^{2+}-dependent actin filament-severing protein. The role of adseverin has been reported about exocytosis regulation through actin cytoskeleton rearrangement in secretory cells. However, the function of adsevrin in bone still remains unclear, and it has not yet been examined in osteoclastogenesis.

Here, I investigated the role of adseverin in osteoclastogenesis using bone marrow-derived macrophages (BMMs). I found that expression of the adseverin gene, *scinderin*, was up-regulated during RANKL-induced osteoclast differentiation. Knock-down of adseverin significantly blocked the increase of nuclear factor of activated T cell c1 (NFATc1), which is a key regulator of

osteoclastogenesis and also decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (MNCs). In addition, adseverin deficiency impaired the resorption activity and the secretion of bone degrading enzymes in osteoclast. These phenomenons were caused by decreased NFATc1 expression through NF-κB signaling.

Collectively, these findings indicate that adseverin plays an important role in osteoclastogenesis via regulation of NFATc1 expression.

Keyword : adseverin, osteoclast differentiation, NFATc1

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I. Introduction

Bone is a complex tissue remodeled and maintained by repetition of bone formation and resorption throughout life. This dynamic phenomenon is mediated by the two types of cells; osteoblast and osteoclast. Osteoblast forms bone matrix by depositing organic and inorganic components and osteoclast resorbs bone by secretion of degrading enzymes and ions. Osteoclasts are multinuclear cells differentiated from the monocyte/macrophage lineage cells derived from hematopoietic stem cells (Hayashi et al., 1998). There are two main factors needed for osteoclast differentiation; macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF is important for commitment of monocyte/macrophage lineage and osteoclast survival (Valledor et al., 1998) and RANKL, a tumor necrosis factor (TNF) family cytokine, induces the differentiation of osteoclasts in the presence of M-CSF. When RANKL binds to its receptor RANK (receptor activator of NF-κB), TNF receptor associated factor 6 (TRAF6) is recruited and NFκB and MAPKs signaling pathways are activated. At the same time, c-fos, AP-1 signal cascade and calcium signaling is also activated. All these signaling finally induces nuclear factor of activated T cells (NFAT) c1, the master transcription factor for osteoclastogenesis, and NFATc1 is autoamplificated and results in the osteoclast specific gene expression (Takayanagi, 2005). With expression of osteoclast specific genes including TRAP, cathepsin K and DC-

STAMP etc. osteoclasts become matured and functionally activated. In terms of function, osteoclast is included in secretory cells. Mature osteoclast secretes bone degrading enzymes into the resorption lacunae by exocytosis of vesicles containing enzymes. And for making resorption lacunae, osteoclast forms actin belt and sealing zone.

Adseverin, which is also called scinderin, is a member of the gelsolin super family of actin binding proteins and Ca^{2+}-activated actin filament severing protein. It has the highest degree of homology with gelsolin about 60% at the amino acid level but it has a more restricted expression (Kwiatkowski, 1999). Adseverin was first discovered in platelets, megakaryocytes and chromaffin cells and now it is known to be expressed in all secretory cells with involvement in actin cytoskeleton remodeling during exocytosis (Silacci et al., 2004). Like gelsolin, adseverin is composed of 6 gelsolin-like (G) domains. It contains three actin binding sites in the N-terminus and two calcium binding sites in the C-terminus. Actin binding of adseverin is inhibited by phosphatidylinositol-4,5-bisphosphate (PIP_2) and it is most likely owing to competition between actin and PIP_2 (Zhang et al., 1996). Upon stimulations, adseverin interacts with F-actin in the presence of Ca^{2+} and decreases the viscosity of actin gels as a result of actin filaments severing. Through immunofluorescence experiments, it was shown that adseverin is mainly localized in the subplasmalemma region with a mesh of actin filaments in chromaffin cell (Rodriguez Del Castillo et al., 1990). The localization of

adseverin has been confirmed in osteoclasts (Qi et al., 2014). Adseverin in chromaffin cells has been well characterized but the role of adseverin in osteoclasts has not been investigated. It has been reported that gelsolin$^{-/-}$ mice showed increased bone thickness, high bone mass, and defects in podosome assembly and bone resorption (Chellaiah et al., 2000). It gives the possibility of involvement of other gelsolin superfamily proteins in bone remodeling.

In this study, I found the adseverin expression is upregulated during differentiation of bone marrow-derived macrophages(BMMs) into osteoclast by RANKL stimulation. Using siRNA knock-down system, I discovered that adseverin promotes osteoclast differentiation through NF-κB and NFATc1 and it has an important role in osteoclastogenesis.

II. Materials and methods

Reagents and antibodies

Recombinant human M-CSF and human soluble RANKL were purchased from PeproTech (Rocky Hill, NJ). Antibodies against ERK, JNK, p38, p65 and Akt and phosphospecific antibodies for ERK (Thr202/Tyr204), JNK (Thr182/Tyr185), p38 (Thr180/Tyr182), p65 (Ser536) and pAkt (Ser473) were from Cell Signaling Technology (Cambridge, MA). Monoclonal antibodies against β-actin (AC-74) and secondary antibodies were from Sigma Aldrich (St.Louis, MO). Antibodies of adseverin (N17), NFATc1 (7A6), lamin B (M-20), α-tubulin (TU-02) and adseverin siRNA and negative control scrambled siRNA were purchased from Santa Cruz Biotechnology (SantaCruz, CA). A leukocyte acid phosphatase (TRAP) assay kit and all other reagents were obtained from Sigma Aldrich.

Culture of osteoclast

To obtain mouse bone marrow derived osteoclasts, 5 week old female ICR mice were sacrificed and the bone marrow was flushed from tibiae and femora. After eliminating erythrocytes with hypotonic buffer, cells were incubated overnight in alpha modified Eagle medium (α-MEM) with 10% fetal bovine serum (FBS) on culture dishes. Adherent cells were discarded and non-adherent

cells were incubated with 30 ng/ml M-CSF on petridishes further. After 3 days bone marrow-derived macrophages (BMMs) became adherent. These cells were collected by scraping and used as osteoclast precursor cells. BMMs were differentiated into osteoclasts by culturing with 30 ng/mL M-CSF and 120 ng/mL RANKL.

TRAP staining and counting of osteoclast

Cells were fixed with 3.7% formaldehyde for 15 min and permeablized with 0.1% TritonX-100 for 2 min. Then, by using a leukocyte acid phosphatase (TRAP) assay kit, staining was performed in the dark for 5-15 min, following the manufacturer's instruction. After staining, cells were washed with distilled water and observed by using a light microscope. For differentiation into pre-fusion osteoclasts (pOCs), it usually takes 2 days and 3-4 days are required to obtain mature osteoclasts in my experimental conditions. However, time for differentiation can be various between experiments. For counting of the mature osteoclasts, Osteomeasure software (OsteoMetrics,Inc., Dec-atur, GA) was used and the TRAP positive cells with 3 or more nuclei were regarded as multinuclear osteoclasts.

Gene knock-down by small interfering RNAs (siRNAs)

For testing the role of adseverin on RANKL-induced osteoclastogenesis, BMMs were transfected with 30 nM adseverin specific siRNAs (SantaCruz).

siRNAs and Hiperfect transfection reagent (Qiagen, Hilden, Germany) were mixed with serum- and antibiotics-free α-MEM. The mixtures were incubated for 20 min at room temperature and added to the cells. The cells were incubated overnight and then culture medium was changed to serum-containing alpha MEM.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells by using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and 3 μg RNAs were reverse-transcripted by Superscript II reverse-transcriptase (Invitrogen). The PCR reaction was performed in a thermal cycler (Bio-Rad, Hercules, CA). The initial denaturation step was 95℃ for 5 min and the following reaction cycle was denaturation at 95℃ for 30s, annealing at 57℃ for 30 s and extension at 72℃ for 30 s. These cycles were repeated for 27 cycles. The oligonucleotide sequences of primers used are: adseverin, 5'-GAG GAA CAG ACC CAG CAA AT-3'(sense) and 5'-GCC ACA GAG GCC TGT ATC TT-3'(antisense); HPRT, 5'-CCT AAG ATG AGC GCA AGT TGA A-3'(sense) and 5'-CCA CAG GGA CTA GAA CAC CTG CTA A-3'(antisense). PCR products were electrophoresed in a 1.5% agarose gel and visualized with a UV illuminator after staining with ethidium bromide.

Quantitative real-time PCR

Real-time PCR was performed with ABI7300 real-time system (Applied Biosystems,Warrington, UK) using KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc. Wilmington, MA). The PCR conditions were the initial enzyme activation step at 95℃ for 3 min and following cycles of denaturation at 95℃ for 3 s and amplification at 60℃ for 33 s. These cycles were repeated for 40 times. All reactions were run in triplicates and the housekeeping gene HPRT was used for normalization. Gene expression level was determined as fold change of comparative cycle threshold. The following primer sets were used: adseverin, 5'-TCC AGA GCA GAG AGC TTC AA-3'(sense) and 5'-TTC GCA GTC AGA TCA TTG GT-3'(antisense); NFATc1, 5'-CCA GTA TAC CAG CTC TGC CA-3'(sense) and 5'-GTG GGA AGT CAG AAG TGG GT-3'(antisense); TRAP, 5'-CGA CCA TTG TTA GCC ACA TAC G-3'(sense) and 5'-TCG TCC TGA AGA TAC TGC AGG TT-3'(antisense); cathepsin K, 5'-ATA TGT GGG CCA CCA TGA AAG TT-3'(sense) and 5'-TCG TTC CCC ACA GGA ATC TCT-3'(antisense); DC-STAMP, 5'-GGG TGC TGT TTG CCG CTG-3'(sense) and 5'-CGA CTC CTT GGG TTC CTT GCT-3'(antisense); v-ATPase(Atp6v0d2), 5'-GGG AGA CCC TCT TCC CCA CC-3'(sense) and 5'-CCA CCG ACA GCG TCA AAC AAA-3'(antisense); and HPRT, 5'-CCT AAG ATG AGC GCA AGT TGA A-3'(sense) and 5'-CCA CAG GGA CTA GAA CAC CTG CTA A-3'(antisense).

Nuclear fractionation and preparation of protein lysate

Nuclear and cytoplasmic protein lysate were prepared with the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's protocols. In brief, cells were washed with ice-cold PBS and treated with cell lysis buffer. After incubation on ice, the sample was spin-downed and the supernatant was used as cytoplasmic extract. The nuclear pellet was washed with PBS twice and lysed with pre-chilled nuclear lysis buffer. After centrifugation, the supernatant was used as the nuclear extract. Protein concentration was measured using a detergent-compatible colorimetric assay kit (Bio-Rad). Nuclear and cytoplasmic protein extracts were subjected to SDS-PAGE and Western blotting.

Resorption assay

BMMs were seeded on 48 well plate coated with calcium phosphate and cultured with 30 ng/ml M-CSF and 120 ng/ml RANKL. After 7 days, the plate was rinsed with distilled water and stained according to the von Kossa staining method. Photographs were taken under a light microscope and the resorption area was quantified using image J.

Immunocytochemistry

BMMs were cultured on 15 mm glass cover slips in 24 well plate and then fixed with 3.7% formaldehyde. After permeabilization with 0.1% triton X-100, cells were blocked with PBS containing 1% BSA for 1 hr 30 min and incubated

with an anti-adseverin antibody in PBS containing 1% BSA overnight at 4℃. Subsequently, cells were washed and stained with rhodamine-phalloidin (actin; red) and a FITC-conjugated secondary antibody for 2 hrs. After washing with PBS, the cells were mounted by mounting solution with DAPI (nucleus; blue) and images were obtained using a ZEISS LSM700 confocal microscope (Carl Zeiss Microimaging GmbH, Goettingen, Germany).

Cell proliferation assay (CCK assay)

Control and adseverin knock-downed BMMs were treated with 120 ng/ml RANKL in the presence of 30 ng/ml M-CSF for upto 3 days. Cells were incubated with 10% CCK solution in cell culture medium for 30 min at 37°C. After incubation, optical density was measured with an ELISA reader at 450 nm.

Western blot

Cells were lysed with a lysis buffer containing 120 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 0.5% NP40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors (Roche, Mannheim, Germany). The protein concentration of the cell lysates was measured by Dc protein assay kit (Bio-Rad) and the equal amount of protein was loaded onto 8% or 10% SDS-polyacrylamide gel. After transfer onto nitrocellulose membranes (Amersham Pharmacia, Uppsala, Sweden), the membrane was blocked with 5% non-fat skim milk for 1 hr and

then incubated with primary antibodies overnight at 4℃. The membrane was washed with TBST several times and incubated with HRP-conjugated secondary antibodies in 2% skim milk for 1 hr. The immunoreactivity was detected with ECL reagents in a dark room.

MMP assay

For checking a secretion of activated MMPs, BMMs were cultured with 30 ng/ml M–CSF and 120 ng/ml RANKL for 2 days and the conditioned media was collected. The activity of MMPs secreted from cells was measured by Abcam MMP Activity Assay Kit according to the manufacturer's instructions. In brief, the pro-MMPs in conditioned media were activated by APMA at 37℃ for 2 hrs and then MMP green substrate was added. After incubation for 30 min at room temperature the fluorescence intensity was measured with a Fluostar optima fluorescence plate reader (BMG Labtech, Offenburg, Germany) at 490/525 nm.

Statistical analysis

Statistical differences between results were tested using the Student's t test. P values less than 0.05 were regarded as significant.

III. Results

Adseverin expression is upregulated in RANKL-induced osteoclastogenesis

Osteoclastogenesis is a complex process that is related with numerous genes expression and function. To find what genes regulate osteoclast differentiation, I searched genes of which expression are changed during osteoclastogenesis. Through analyses of microarray and GEO data (http://www.ncbi.nlm. nih.gov/geo/, GSE17563), I found adseverin was increased 40 folds in 48 hrs after RANKL stimulation. In my experiments for osteoclast differentiation using bone-marrow derived macrophage, adseverin was up-regulated up to 50-80 folds by RANKL in a time and dose dependent manner (Figure 1A, 1C). However, at high dose RANKL (240 ng/ml), adseverin expression was not changed or rather decreased. These results were confirmed by western blot (Figure 1B). In addition, to find whether adseverin is also expressed in osteoblasts, I checked the mRNA expression level in calvarial osteoblasts by qRT-PCR. However, adseverin expression was very low in osteoblasts (data not shown). These observations suggest a possibility that adseverin may have some roles in RANKL-induced osteoclastogenesis.

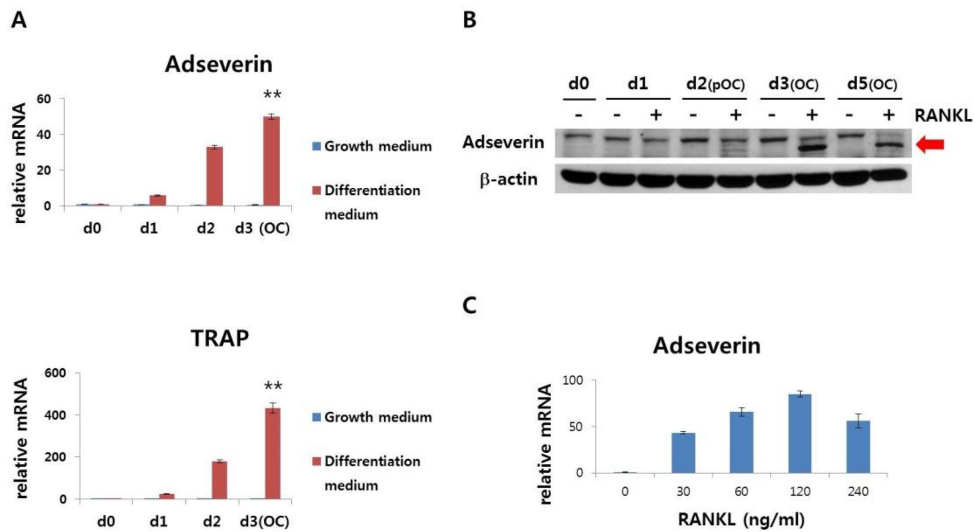


Figure 1. Adseverin expression in RANKL-induced osteoclastogenesis.

(A) BMMs were cultured with 30 ng/ml M-CSF and 120 ng/ml RANKL for 0, 1, 2 and 3 days. Total RNA was isolated from cell lysates and analyzed by quantitative real-time RT-PCR (qRT-PCR). The mRNA expression level was normalized to HPRT. The mRNA level of TRAP, a marker gene for osteoclast differentiation, was also analyzed. (B) Western blot was performed with adseverin and β-actin antibodies. Protein lysates were prepared for 0, 1, 2, 3 and 5 days from M-CSF/RANKL-treated BMMs as described in A. (C) BMMs were stimulated with RANKL for the indicated doses in the presence of 30 ng/ml M-CSF. Cells were cultured for 3 days and analyzed by qRT-PCR.

Osteoclast differentiation in adseverin knock-downed BMMs

To observe an effect of adseverin on osteoclastogenesis, I used small interfering RNAs (siRNA) knock-down system. Before evaluating the functional role of adseverin, its knock-down was confirmed by RT-PCR. Because adseverin expression is increased by RANKL, cell lysate was prepared at the second day after RANKL treatment. I found adseverin mRNA was prominently decreased in cells transfected with adseverin siRNAs compared to control siRNA transfected cells (Figure 2A). siRNAs transfected BMMs were cultured in the presences of M-CSF and soluble RANKL for 4 days and then TRAP staining was performed. TRAP is an enzyme highly expressed in mature osteoclasts and it is used as an osteoclast marker. In adseverin knock-downed cells, the number of TRAP positive multi-nucleated osteoclasts was significantly decreased and its size also smaller than control cells (Figure 2B). So, it suggests that adseverin plays an important role in RANKL-induced osteoclast differentiation.

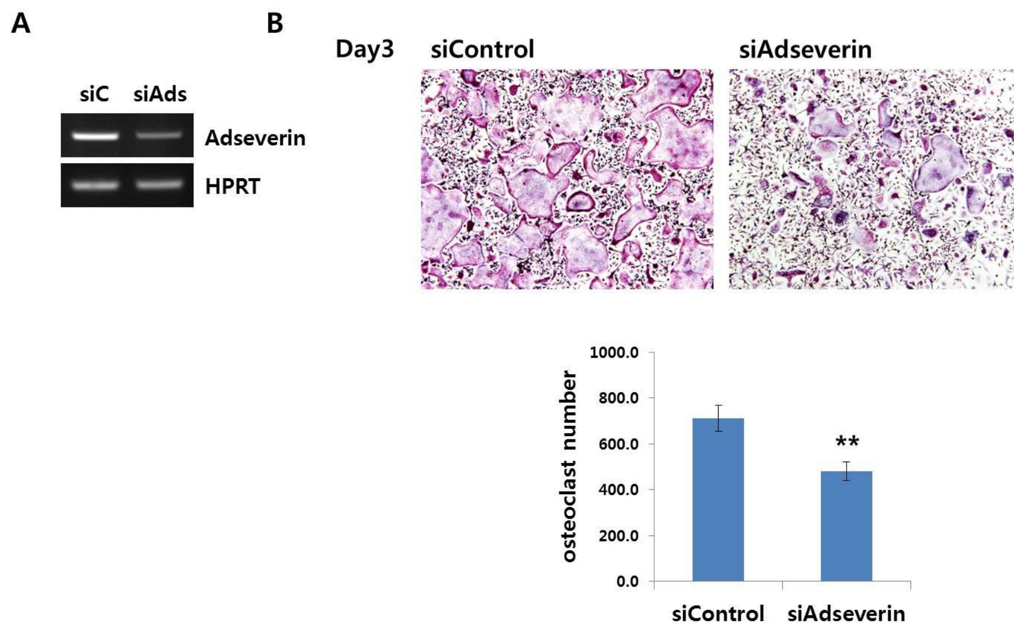


Figure 2. Osteoclast differentiation in adseverin knock-downed BMMs.

(A) BMMs were transfected with 30 nM adseverin specific siRNAs and further cultured with 120 ng/ml RANKL in the presence of 30 ng/ml M-CSF for 2 days. Adseverin mRNA expression was analyzed by RT-PCR. (B) siRNAs transfected BMMs were cultured with M-CSF and RANKL for 4 days. Cells were fixed and stained with TRAP solution. TRAP-positive MNCs were photographed under a light microscope (magnification x100) and counted. The number of MNCs is mean value of 4 wells of 48 well plate.

Adseverin affects resorption activity of osteoclasts

The main physiological function of osteoclast is resorbing bone. The majority of bone is made of bone matrix which is composed primarily of organic collagen and inorganic hydroxyapatite. With forming the actin ring along the cell boundary on the bone surface, osteoclast makes "sealing zone" and secretes many enzymes and ions through ruffled membrane called "ruffled border". Secreted enzymes like a collagenase or MMPs degrade organic part of bone and ions including proton acidify resorption compartment and solubilize inorganic minerals. Because I found the morphological defect in adseverin knock-downed osteoclasts, next I wondered whether the function of osteoclast is also affected. To examine resorption activities of adseverin-depleted osteoclasts, I cultured BMMs with RANKL and M-CSF on calcium phosphate coated dishes for 7 days. Before action of proteolytic enzymes, tightly packed hydroxyapatite crystals must be dissolved and it is achieved by HCl secretion. I used a calcium phosphate coated plate for mimicking inorganic part of the bone. After 3 days, mature osteoclasts started to be formed and at day 7, calcium phosphate was stained by the von Kossa method. Expectedly, compared with the control cells, adseverin-depleted osteoclasts could not resorb the calcium phosphate. About 80% of resorption areas were decreased in knock-downed cells (Figure 3). These results indicate that adseverin affects the function of osteoclasts in terms of resorption.

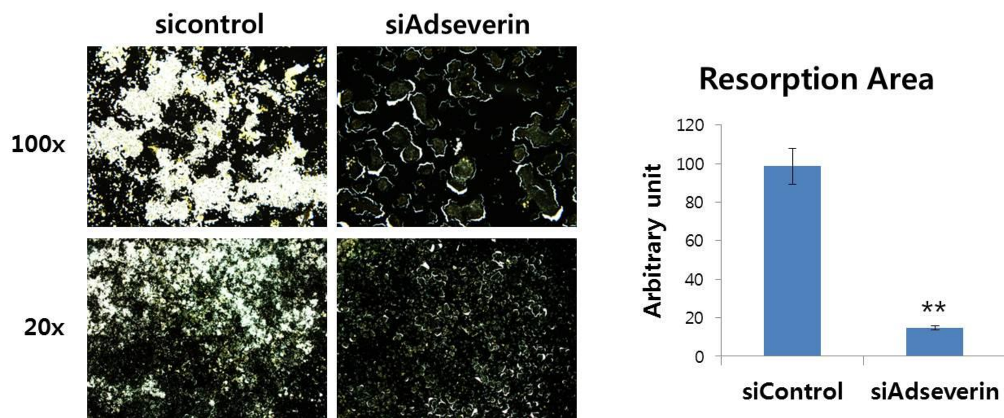


Figure 3. Impaired resorption activity of adseverin-depleted osteoclasts. BMMs were seeded on the calcium phosphate-coated 48 well plate. Cells were transfected with 30 nM adseverin siRNAs and further cultured with M-CSF and RANKL for 7 days. After washing of cells, von Kossa staining was performed. Resorption area was analyzed by Image J.

Effect on actin ring formation and localization of adseverin

After migration to the resorption area, osteoclasts attach to the bone surface and seal the resorption site from its surroundings. This structure, called sealing zone, produces a separated space for resorption where can be acidified and degrading enzymes can be secreted. It is also known that attachment of osteoclast to the bone surface at sealing zone is mediated by $\alpha v\beta 3$ integrin (Holt and Marshall, 1998; Nakamura et al., 1996). Actin ring is very important structure for resorption as well as sealing zone. It is one of the main morphological characters of mature osteoclasts. To test whether the defect of resorption activity in adseverin knock-downed osteoclasts is caused by actin ring formation defect, immunostaining experiments was performed. In control cells, most of adseverin were located in subplasmalemma region nearby actin ring but in adseverin knock-downed cell adseverin proteins were diffused and expressed at low level. Osteoclasts were formed with low number and had only one or two nuclei with small size in adseverin-deficient cells. Furthermore, with this diminished differentiation, the actin ring belt was not observed in adseverin-depleted osteoclasts (Figure 4). The defects in adseverin knock-downed osteoclasts including actin ring formation may be caused by down-regulation of osteoclast differentiation.

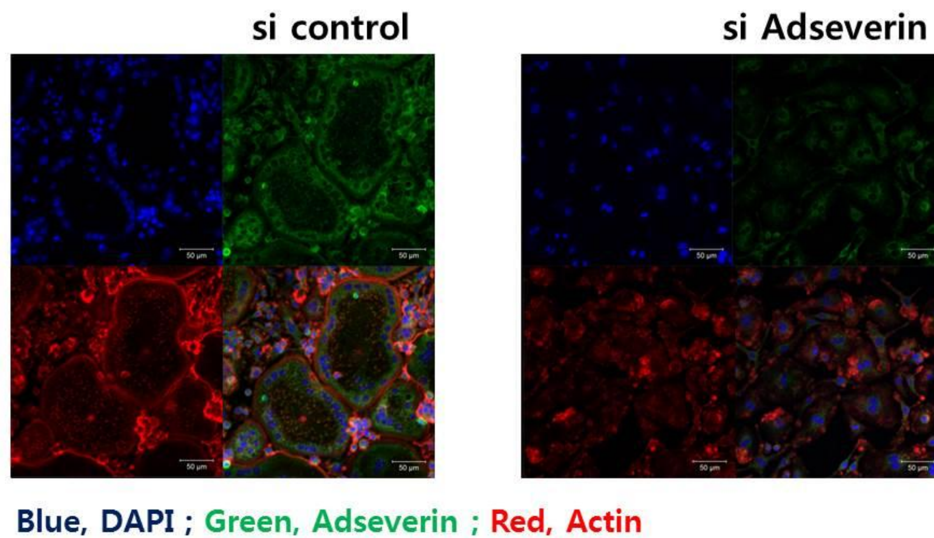


Figure 4. Effect on actin ring formation and localization of adseverin.

BMMs were cultured with 30 ng/ml M-CSF and 120 ng/ml RANKL for 4 days. Immunostaining of actin ring with rhodamine-phalloidin (actin; red) was performed and adseverin was co-stained using an adseverin-FITC antibody. Nuclei were also stained with DAPI.

Adseverin does not affect the proliferation of BMMs

I next inquired whether adseverin controls the osteoclast differentiation by regulation of osteoclast precursors proliferation. Because fusion between cells can occur more easily in high cell density, I thought that if adseverin increases cell proliferation, osteoclast formation will also be increased. To test the effect of adseverin in proliferation, I performed a CCK cell proliferation assay. However, there was no significant difference between adseverin-deficient cells and control cells (Figure 5). Although it was reported that adseverin has anti-cancer effect by inhibiting cell proliferation (Wang et al., 2014), it does not appear to have any role in proliferation rate of osteoclast precursors.

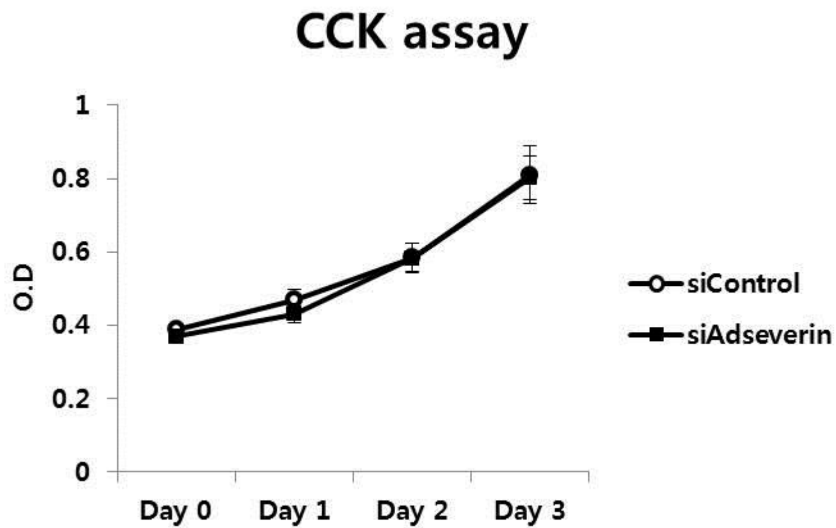


Figure 5. Effect of adseverin on cell proliferation.

BMMs were seeded on 96 well plate and differentiated into osteoclasts by stimulation with 120 ng/ml RANKL in the presence of 30 ng/ml M-CSF for upto 3 days. Then, CCK assay was performed.

Adseverin controls expression of NFATc1 which is the key regulator of osteoclastogenesis

NFATc1 is the transcription factor gene strongly induced after RANKL stimulation (Takayanagi et al., 2002) and it is regarded as a master transcription factor for osteoclastogenesis. NFATc1 is auto-amplified and induces osteoclast-specific genes efficiently (Asagiri et al., 2005). To investigate whether adseverin regulates NFATc1 in RANKL-induced osteoclastogenesis I checked the expression level of NFATc1 in adseverin knock-downed BMMs. At day 3 after RANKL stimulation, mRNA level of NFATc1 was significantly decreased in knock-downed cells compared to the control cells (Figure 6A) and protein expression was also reduced dramatically (Figure 6B). Because NFATc1 auto-regulates its own expression as a transcription factor, decreased expression of NFATc1 accompanies the decreased nuclear translocation. Therefore, I assessed the nuclear translocation of NFATc1 by western blot with the nuclear fraction lysate. As expected, the amount of NFATc1 is decreased in adseverin knock-downed cells in both nucleus and cytoplasm (Figure 6C).

NFATc1 directly induces several genes important for osteoclastogenesis such as TRAP, cathepsin K, DC-STAMP and v-ATPase (Kim et al., 2008; Reddy et al., 1995). TRAP is responsible for bone resorption activity (Takayanagi et al., 2002) and cathepsin K, a lysosomal cysteine protease, is also a key enzyme degrading the bone matrix (Georgess et al., 2014). Dendritic cell-specific transmembrane protein (DC-STAMP) (Kukita et al., 2004; Yagi et al., 2005)

and v-ATPase subunit d2 (ATP6v0d2) (Lee et al., 2006) are also well characterized as essential molecules for mononuclear osteoclast fusion. To further confirm whether NFATc1 expression regulated by adseverin affects the expression of osteoclast specific genes, I checked mRNA level of TRAP, cathepsin K, DC-STAMP and v-ATPase. All these genes were decreased upto 56% at day 3 after RANKL stimulation in adseverin-deficient cells compared with control cells (Figure 7). These results indicated that adseverin regulates osteoclast differentiation via NFATc1 and its downstream genes expression.

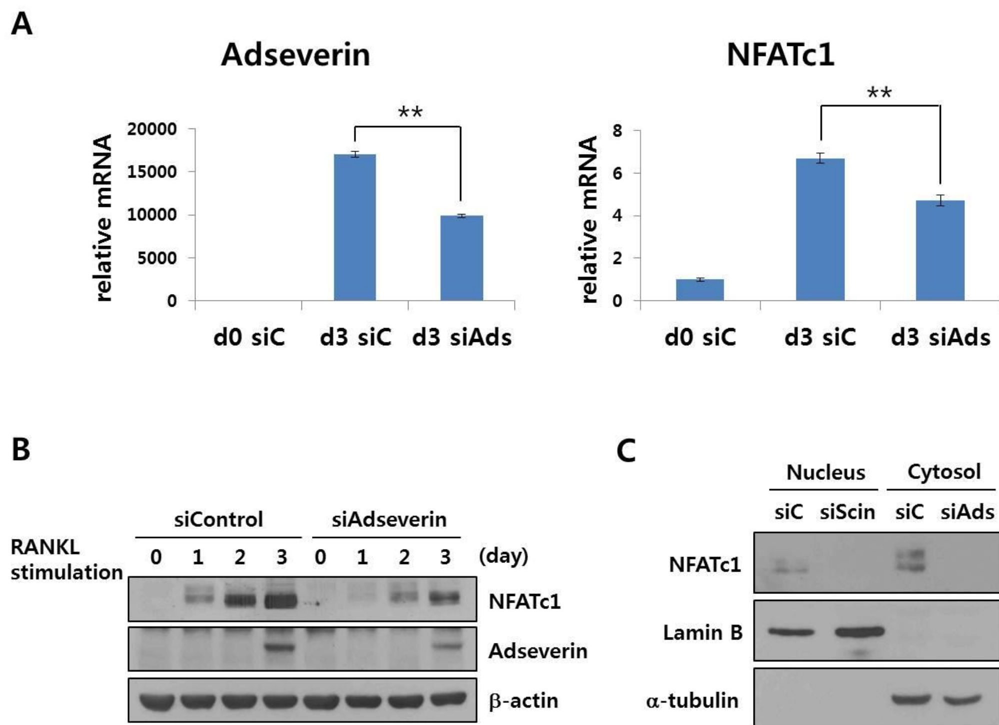


Figure 6. Adseverin regulates NFATc1 expression in osteoclasts.

(A) BMMs were transfected with 30 nM adseverin siRNAs and further cultured with M-CSF and RANKL for 3 days. Adseverin and NFATc1 mRNA expressions were analyzed by qRT-PCR. (B) Western blot was performed with adseverin and NFATc1 antibodies. Protein lysates were prepared at 0, 1, 2 and 3 days from M-CSF/RANKL-treated BMMs after siRNA transfection. (C) After siRNA transfection BMMs were cultured for 2 days in the presence of M-CSF and RANKL. Then, nuclear and cytoplasmic protein lysates were prepared by

nuclear fractionation and used for western blot. Lamin B and α-tubulin were used for controls of nucleus and cytoplasm proteins, respectively.

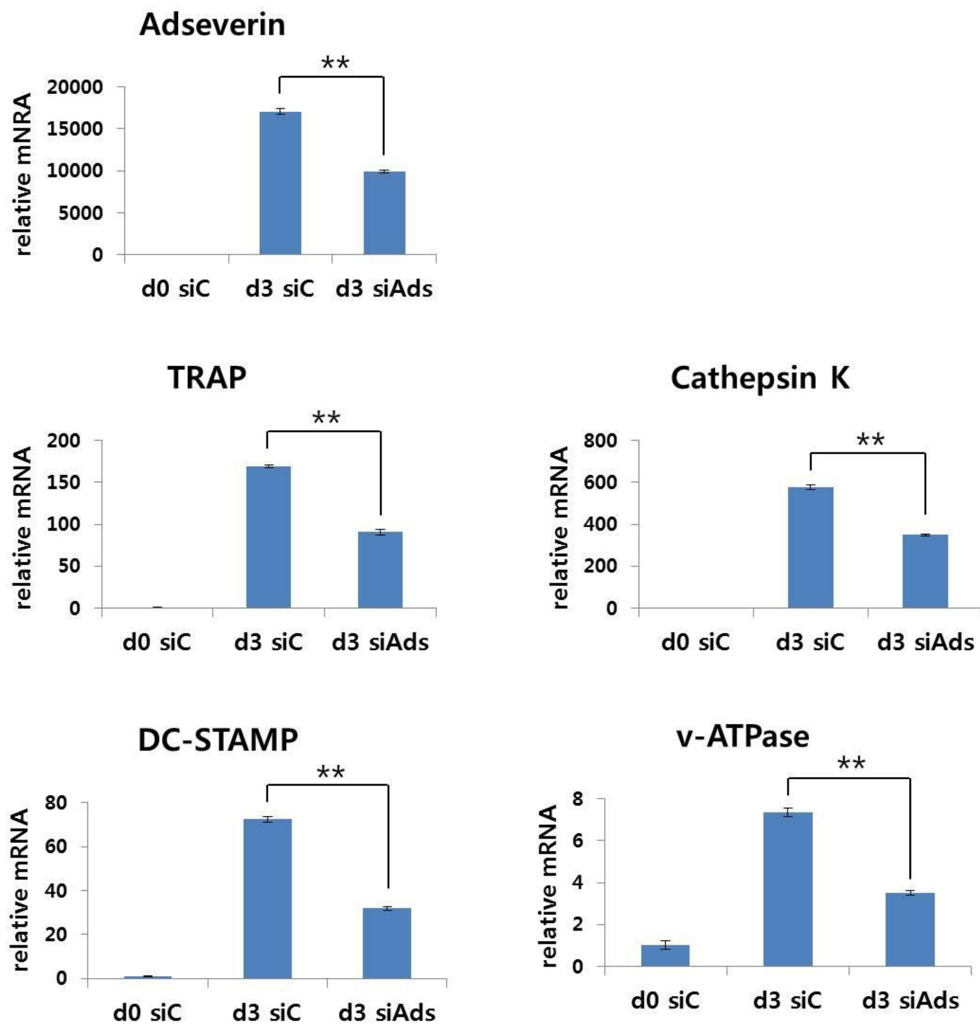


Figure 7. Expression of NFATc1 downstream gene in adseverin-depleted osteoclasts.

BMMs were transfected with 30 nM adseverin siRNAs and further cultured with M-CSF and RANKL for 3 days. Adseverin, TRAP, cathepsin K, DC-STAMP and v-ATPase expressions were analyzed by qRT-PCR

Adseverin regulates osteoclastogenesis via NFκB signaling, not MAPKs.

Next, I wondered how adseverin regulates NFATc1 expression in RANKL-induced osteoclastogenesis. The mechanism of RANKL signaling has been studied broadly. The binding of RANKL to its receptor RANK on BMMs results in the recruitment of TRAF6 which activates the NFκB, Akt and mitogen-activated protein kinase (MAPK) pathways including Erk, Jun N-terminal kinase (JNK) and p38 (Kobayashi et al., 2001; Wong et al., 1998). I first assessed MAPKs signaling pathway. However, there were no significant differences between knock-downed cells and control cells (Figure 8A). Subsequently, I tested phosphorylation of Akt and p65. Contrary to MAPKs phosphorylation, p65 phosphorylation was decreased in adseverin-depleted cells compared to control cells (Figure 8B), but phospho Akt level was not changed (Figure 8C). These data demonstrate that adseverin plays a role in RANKL-induced osteoclastogenesis via regulation of the NFκB signaling, not MAPKs pathway.

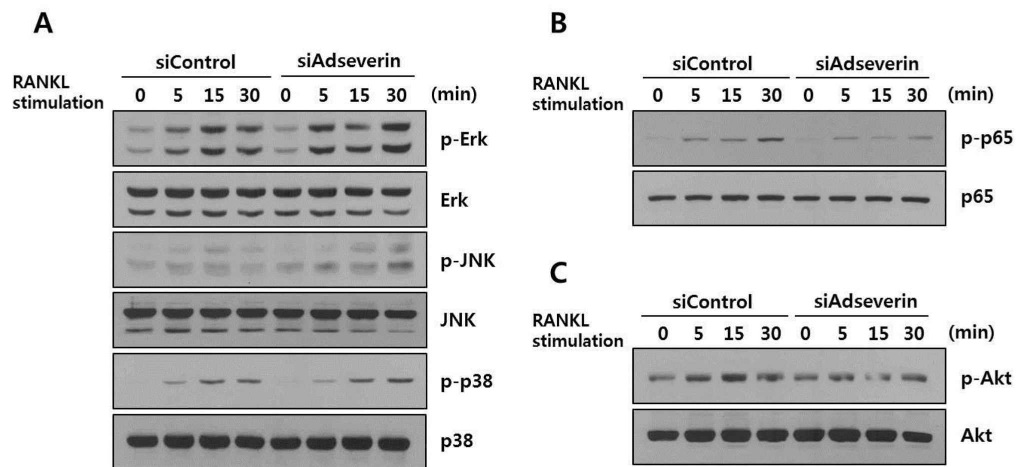


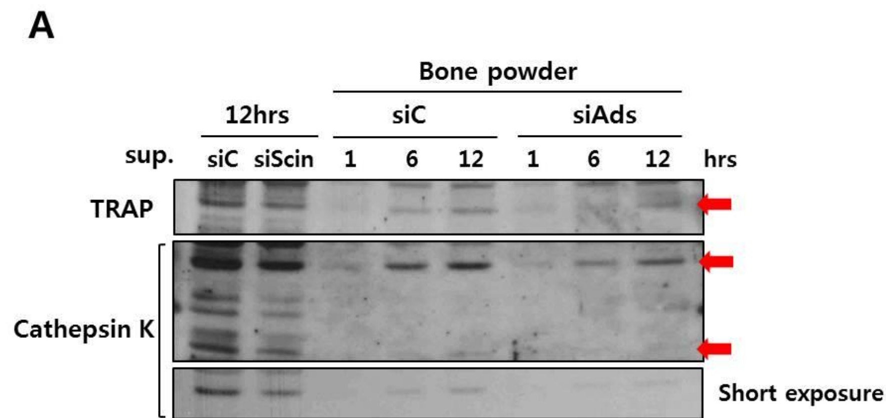
Figure 8. Adseverin regulates osteoclastogenesis via NFκB signaling, not MAPKs

After siRNA transfection, BMMs were cultured with M-CSF and RANKL for 2 days. The cells were starved for 4 hrs in alpha MEM without serum and then stimulated with 600 ng/ml RANKL. Stimulated cells were harvested at 0, 5, 15, 30 min and protein lysates were prepared for western blot. (A) Western blot performed with antibodies for phospho- or total form of MAPKs (Erk, JNK, p38). (B) and (C) Western blot performed with antibodies for phospho- or total form of p65 and Akt.

Enzyme secretion in adseverin-depleted osteoclasts

For resorbing bone, many vesicles are extensively transported to the ruffled border and delivers hydrochloric acid and proteases to resorption lacuna, which is the space between ruffled border and bone surface (Vaananen et al., 2000). Therefore, exocytosis is important for resorption activities of osteoclasts. Because it is known that adseverin plays a role in exocytosis in several cells including chromaffin cells (Trifaro, 1999; Zhang et al., 1996), I inquired whether adseverin affects the secretion of enzymes in osteoclasts. TRAP, cathepsin K and matrix metalloproteinase (MMP) are major enzymes secreted from the osteoclast and these enzymes degrade the bone matrix. I first examined the secretion of TRAP and cathepsin K by western blot. Bone particles were used for mimicking the resorption environment and there was a report that bone particles increase the cathepsin K secretion (Cremasco et al., 2012). Mature osteoclasts formed by 3 day culture with RANKL were further incubated with serum free α-MEM containing bone particles. Western blot was performed using the supernatant of culture. As shown in Figure 9A, the secreted TRAP and cathepsin K level were increased in a time-dependent manner. However, adseverin knock-downed osteoclasts showed lower level than control cells (Figure 9A). Next, I further examined whether MMPs secretion is also decreased in adseverin-deficient osteoclasts. Among the MMP family members, MMP-9 (gelatinase B) has the major role in osteoclasts. Conditioned medium collected from mature osteoclasts for 12 hrs were examined using a MMP

activity assay kit. As expected, amounts of the activated MMPs were decreased about 63% in conditioned medium from adseverin knock-downed cells (Figure 9B). These data suggest that adseverin regulate the enzyme secretion of osteoclasts.



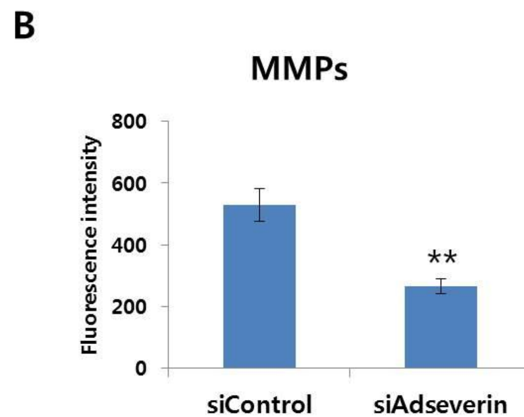


Figure 9. Enzyme secretion is diminished in adseverin knock-downed osteoclasts.

(A) After siRNAs transfection, BMMs were cultured with M-CSF and RANKL for 2 days. The cells were further incubated in serum-free α-MEM with or without bone particles. Bone particles were obtained from dentin slice by grinding. Supernatant was collected at 1, 6 and 12 hrs after incubation and

western blot was performed with TRAP and cathepsin K antibodies. (B) As described in A, supernatant collected from cell culture without bone particle was used. This sample was collected for 12 hrs. Total MMPs activated was estimated using a MMP assay kit according to the manufacturer's instructions.

IV. Discussion

Osteoclast is closely associated with calcium regulation and Ca^{2+} signaling is one of the main signaling involved in osteoclast differentiation. In an initial step of osteoclast differentiation, cytoplasmic calcium level is increased and after that, calcium oscillations are followed. Adseverin is a Ca^{2+}-regulated protein. It has actin severing activity only in the presence of Ca^{2+}. Based on these backgrounds, the relevance of adseverin to osteoclast differentiation is assumable. And in fact, adseverin expression was increased during osteoclast differentiation. Even though it is not investigated whether calcium plays a role between adseverin and osteoclast differentiation, through my study, it is proved that adseverin controls osteoclast differention via regulation of NFATc1 expression by NF-κB signaling.

In osteoclasts, extensive vesicles containing various molecules are transported to the ruffled border and release the molecules to the resorption lacunae (Vaananen et al., 2000) and these molecules including matrix degrading enzyme act on the bone effectively. In this course, exocytosis occurred and accompanied by focal and transient disruption of the cortical actin network (Vitale et al., 1991). Therefore, it brings second possibility of adseverin's role in osteoclast resorption. In chromaffin cells, disruption of F-actin and adseverin cortical fluorescent ring by stimualtaion is reported (Qi et al., 2014; Trifaro et al., 2008) and recently, it is also reported that osteoclast has a similar effect by RANKL stimulation (Qi et al., 2014). However, in my study, clear

subplasmalemma localization of adseverin with actin ring in osteoclast stimulated by RANKL was detected. Therefore, It is required to confirm whether RANKL stimulation induces redistribution of adseverin and actin severing effect by this process regulates osteoclast differentation and function.

Adseverin and gelsolin shares the function in terms of actin severing and have a highest homology in their family. However there are some differences between the two proteins. For example, they have different molecular weight, isoelectric points, chromatographic behavior, and peptides map by proteolytic digestion and have no cross-reactivity of the antibodies. In addition, adseverin has different affinity of Ca^{2+} binding sites from gelsolin (Rodriguez Del Castillo et al., 1990) and it may be the reason adseverin is more specialized on exocytosis and expressed in limited tissues, especially secretory cells. It is reported that gelsolin knock-out mice has defect in osteoclast and bone in vivo and in vitro (Chellaiah et al., 2000). In this study I investigated the existence and roles of adseverin in osteoclasts by in vitro study. However, in vivo, adseverin has been not investigated. Further studies in vivo will help to understand the biological and physiological roles of adseverin.

Recently, existence of actin in the nucleus as well as in the cytoplasm has become evident. Furthermore, nuclear function, which includes nuclear transport, gene transcription, DNA replication or structural maintenance, of several actin-binding proteins also have been proposed. The members of gelsolin family including flighlessI, supervillin and gelsolin are sometimes

observed in the nucleus and play roles as coactivators for nuclear receptors. Supervillin associates with the androgen receptor (AR) and facilitates transactivation of AR (Sampson et al., 2001; Ting et al., 2002). Gelsolin also has been identified to promotes nuclear translocation as a coregulator for the AR. (Nishimura et al., 2003). In addition, much evidences propose gelsolin as further upstream protein in signaling cascade. For example, gelsolin cleaves caspase 3 and its products act as effectors of apoptosis (Geng et al., 1998) and gelsolin regulates collagen phagocytosis via Rac-dependent cytoskeletal control (Arora et al., 2004). Although, adseverin has not been investigated about its roles as a signal transducer or transcriptional regulator, these evidences of other members of gelsolin family open up the possibilities of adseverin playing such functions.

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ABSTRACT IN KOREAN

RANKL에 의해 유도되는 파골세포 형성에서의 adseverin의 역할

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송 민 경

1. 연구목적

뼈는 조골세포에 의한 뼈 형성과 파골세포에 의한 뼈 흡수에 의해서 끊임없이 재구성되어지며 파골세포의 불균형한 활성은 골다공증, 골석화증 그리고 류마티스성 관절염 등의 다양한 질병에 연관된다. Adseverin은 칼슘에 의해 활성화 되어 액틴 필라멘트를 절단하는 단백질로서 분비세포의 세포 외 유출에 중요한 역할을 하는 것으로 알려져 있으나 파골세포에서의 역할은 알려진 바가 없다. 본 연구는 파골세포의 형성 및 분화에 있어서 adseverin의 역할을 밝히고자 하였

다.

2. 연구방법

마이크로어레이를 통해 파골세포 분화 시 발현에 변화가 있는 유전자를 선별하였고 그 중에서 adseverin이 파골세포가 분화함에 따라 크게 증가하는 것을 발견하였다. 골수유래 대식세포에서 파골세포의 분화를 유도하는 싸이토카인인 RANKL의 처리농도 및 처리 후 경과시간에 따른 adseverin의 발현을 qRT-PCR과 western blot 기법을 통해 확인하였고 siRNA knock-down system을 이용하여 adseverin이 결핍된 상황에서의 파골세포 분화를 평가하였다. 형성된 파골세포는 tartate-resistant acid phosphatase(TRAP) 염색을 통하여 확인 하였고 immunocytochemistry 기법을 이용하여 파골세포 내의 adseverin의 위치와 액틴링 형성을 확인하였다. 또한 calcium phosphate가 코팅된 세포배양판 위에서의 세포 배양 및 골 흡수에 작용하는 효소인 MMP의 activity assay를 통하여 파골세포의 골 흡수 능력을 평가하였다. Adseverin이 파골세포 전구체의 세포증식을 조절하는지 확인하기 위하여 CCK assay를 시행하였으며, adseverin의 파골세포 분화 조절 메커니즘을 규명하기 위하여 nuclear

fraction, western blot 및 qRT-PCR기법을 이용하였다.

3. 연구결과

RANKL의 처리 후 경과 시간 및 처리량에 의존하여 adseverin의 발현이 증가하였다. adseverin을 결핍 시켰을 때에, 형성되는 파골세포의 수와 크기가 확연히 감소되었고 파골세포의 골 흡수 기능에 중요한 구조인 액틴링 형성에 결손이 나타났으며 골 흡수율이 저하됨을 확인할 수 있었다. 하지만 이는 세포 증식 감소로 인한 결과는 아니며 파골세포의 중요 조절자인 NFATc1의 발현 감소에 의한 것으로 보인다. Adseverin이 knock-down되었을 때 NFATc1의 mRNA 및 단백질 발현량이 감소해 있었으며 전사인자인 NFATc1의 핵으로의 이동률 또한 떨어져 있는 것이 확인되었다. 이에 더해 파골세포의 분화와 기능에 중요한 역할을 하는 NFATc1의 하위 유전자인 TRAP, cathepsin K, DC-STAMP 및 v-ATPase의 발현이 감소되는 현상을 관측했다. 이러한 adseverin에 의한 NFATc1의 조절은 RANKL에 의한 NF-κB의 활성화에 영향을 주기 때문인 것으로 생각된다. 이에 덧붙여 adseverin이 골 흡수에 중요한 효소들의 분비에 영향을 주는지 알아보기 위해 파골세포의 배양 상청액을 분석한 결과 분비되

는 효소들의 양이 adseverin이 knock-down된 군에서 감소되어 있는 것을 확인할 수 있었다.

4. 결 론

Adseverin은 RANKL에 의해 유도되는 파골세포 형성 시에 발현이 증가하여 파골세포의 분화 및 골 흡수 능력을 향상시킨다. 이는 NF-κB를 통하여 파골세포 분화에 중요한 전사인자인 NFATc1의 발현을 증가시키고 이에 따른 파골세포 특이적인 여러 단백질들의 발현을 증가시킴으로서 나타난다.

주요어 : adseverin, 파골세포 분화, NFATc1

학번 : 2013-21810