Aster saponin A2 inhibits osteoclastogenesis through mitogen-activated protein kinase-c-Fos-NFATc1 signaling pathway

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ABSTRACT

Background: In lipopolysaccharide-induced RAW264.7 cells, Aster tataricus (AT) inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells and MAPKs pathways and critical pathways of osteoclast development and bone resorption.

Objectives: This study examined how aster saponin A2 (AS-A2) isolated from AT affects the processes and function of osteoclastogenesis induced by receptor activator of nuclear factor kappa-B ligand (RANKL) in RAW264.7 cells and bone marrow macrophages (BMMs).

Methods: The cell viability, tartrate-resistant acid phosphatase staining, pit formation assay, polymerase chain reaction, and western blot were carried out to determine the effects of AS-A2 on osteoclastogenesis.

Results: In RAW264.7 and BMMs, AS-A2 decreased RANKL-initiated osteoclast differentiation in a concentration-dependent manner. In AS-A2-treated cells, the phosphorylation of ERK1/2, JNK, and p38 protein expression were reduced considerably compared to the control cells. In RAW264.7 cells, AS-A2 suppressed the RANKL-induced activation of osteoclast-related genes. During osteoclast differentiation, AS-A2 suppressed the transcriptional and translational expression of NFATc1 and c-Fos. AS-A2 inhibited osteoclast development, reducing the size of the bone resorption pit area.

Conclusion: AS-A2 isolated from AT appears to be a viable therapeutic therapy for osteolytic illnesses, such as osteoporosis, Paget’s disease, and osteogenesis imperfecta.

Keywords: Saponins; osteoclasts; tartrate-resistant acid phosphatase; macrophage colony-stimulating factor; RANK ligand

INTRODUCTION

Osteoporosis is caused by a discrepancy between bone resorption and bone formation. Overactive osteoclasts cause uncontrolled bone resorption, leading to metabolic bone illnesses, such as osteoporosis and rheumatoid arthritis [1]. Receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) drive monocyte-macrophages to develop into osteoclasts [2-4]. After pairing RANKL to the
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Conflict of Interest
The authors declare no conflicts of interest.

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RANK, it interacts with tumor necrosis factor receptor-associated factors, stimulating several downward signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [5]. In osteoclast differentiation, activated T-cell cytoplasmic 1 (NFATc1) nuclear factor has proven crucial transcriptional components. This process is required for the production of genes related to osteoclastogenesis, such as tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, cathepsin K, and matrix metalloproteinase (MMP)-9 [6]. Therefore, targeting RANKL-mediated signaling to reduce NFATc1 expression could be an option for managing osteoclastogenesis-related diseases, including osteoporosis [7,8].

*Aster tataricus* (AT) is a member of the Asteraceae family. In China, Japan, and Korea, AT rhizomes and roots are used to treat cough, asthma, pharyngitis, and dysuria [9]. Scopoletin, kaempferol, caffeoylquinic acids, aster saponins (ASs), and shionon are compounds isolated from the root of AT that have high antioxidant, anti-cancer, and anti-inflammatory properties [10,11]. Su et al. [12,13] reported that AT has anti-inflammatory characteristics in lipopolysaccharide-induced RAW264.7 cells by blocking the MAPKs and NF-κB pathways, which are essential for osteoclast development and bone resorption. On the other hand, the effects of AT on osteoclast differentiation and the route that underpins are unknown.

This study analyzed the effects of AS-A2 (3-O-α-L-arabinopyranosyl-(6)-β-D-glucopyanosyl-2β, 3β-dihydroxyolean-12-en-28-oic-acid-28-O-β-D-apiofuranosyl-(3)-α-L-rhamnopranosyl-(2)-β-D-xylopyranoside) isolated from AT, on the potential mechanisms and function of RANKL-induced osteoclastogenesis in bone marrow macrophage (BMM) cells and RAW264.7 cells.

MATERIAL AND METHODS

Materials
AS-A2 was isolated by Drs. Xiang Dong Su and Seo Young Yang (Chungnam University, Korea) as reported elsewhere [12,13]. AS-A2 was solubilized in dimethyl sulfoxide (Daejung Chemicals and Metals, Korea) and normalized to the required concentration. The following items were obtained from Gibco: Eagle’s Minimal Essential Medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin (Gibco, USA). Dojindo Molecular Technologies provided the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). Sigma-Aldrich supplied the TRAP (leukocyte acid phosphatase) kits (Sigma-Aldrich, USA). The M-CSF and recombinant soluble mouse RANKL were obtained from R&D (R&D Systems, USA). Cell Signaling Technology (USA) and Santa Cruz Biotechnology (USA) provided the primary and secondary antibodies.

Cell culture
The American Type Culture Collection (ATCC, USA) provided the mouse leukemic monocyte/macrophage cells and the RAW264.7 cells cultivated in DMEM high glucose supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin antibiotic at 37°C in a humidified incubator under 5% CO₂.

Cytotoxicity assays for cell proliferation and viability
A cytotoxicity test was conducted using the CCK-8 to inspect the impact of AS-A2 on the viability of RAW264.7 cells. Briefly, RAW264.7 cells (5 × 10³ cells /well) were cultured for 16
h in 96-well plates. The cells in each well were then treated with varying doses of AS-A2 for 24 h. The cells were incubated for 2 h after adding the CCK-8 solution. A microplate reader (Power wave HT; BioTek, USA) measured the optical density at 540 nm. The outcomes are reported as the mean ± SD from three wells, and the cell viability is reported as a percentage of the control.

**Osteoclast formation and TRAP staining assay**

For osteoclast formation, $1 \times 10^3$ RAW264.7 cells/well were seeded in 96 well plates in the presence of 50 ng/mL of RANKL with AS-A2 at concentrations of 0.05, 0.5, and 5 µM. The cell culture medium was switched on alternate days for six days. Before being stained using a TRAP staining kit, the cells were treated for 10 min with 4% paraformaldehyde for fixation. The TRAP-positive multinucleated cells (TRAP + MNCs) were counted as osteoclast-like cells using an optical microscope (IX71; Olympus, Japan).

**Preparation of mouse BMMs and bone resorption pit assays**

Osteoclast progenitor cells differentiated from BMMs were extracted from the tibia and femur of C57BL/6 mice using α-MEM [14]. Briefly, a threefold volume of Gey's solution was added for at least 15 min to separate the blood cells. The BMMs were then incubated in α-MEM containing 10% FBS for one day. After proliferation, suspended cells were assembled and kept for three days in α-MEM having 10% FBS and 30 ng/mL M-CSF. BMMs adhering to the cell culture plate base were identified and differentiated into osteoclasts. BMMs ($3 \times 10^3$ cells/well) were seeded in a medium holding 30 ng/mL M-CSF and 50 ng/mL RANKL were used to co-culture the BMMs for six days with or without AS-A2 at a dose of 0.05, 0.5, and 5 µM to generate osteoclastogenesis.

In the presence of 30 ng/mL M-CSF and 50 ng/mL RANKL, $5 \times 10^3$ BMMs were treated with various concentrations of AS-A2 (0.005 to 5 µM) in 96-well plates for one day to determine the cytotoxicity to the BMMs.

A bone resorption assay kit (#CSF-BRA-48 KIT; Cosmo Bio, Japan) covered with a calcium phosphate plate was used to analyze the bone resorption pit tests. For six days, BMMs cells were cultivated in the α-MEM medium with 30 ng/mL M-CSF, 50 ng/mL RANKL, and AS-A2. Every other day, the culture media were replaced. A TRAP staining kit was used to stain the cells for osteoclast staining. Image J software (National Institutes of Health, USA) was used for the measurement of the pit area.

**RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)**

RAW264.7 cells ($1 \times 10^5$) were cultured in six-well plates and treated with AS-A2 (1, 5, and 10 µM) or vehicle in the presence of RANKL. For six days, the culture medium was switched every other day. A TRizol reagent (Invitrogen, USA) was used to extract the total RNA, and reverse transcription into cDNA was performed using a ReverTra Ace qPCR RT kit (Toyobo Biotechnology, Japan). HiPi DNA polymerase premix (ElpisBio, Korea) or i-MaxTMII DNA polymerase (iNtRON Biotechnology, Korea) were used for the RT-PCR steps. Bioneer (Korea) provided the PCR primers for RT-PCR, and all experiments were performed in triplicate. Table 1 lists the specific primer sequences. A small amount of Redsafe (iNtRON) was added to 2% agarose gel to make the nucleic acid staining solution, which was used to separate the PCR products. Specific quantities of genes were visualized under a UV trans-illuminator (Gel-Doc; Bio-Rad, USA) and were analyzed using a standard size 100 bp DNA loading ladder (iNtRON).
Western blot analysis

RAW264.7 (5 × 10^5) were cultured in a 60 mm cell culture dish in an α-MEM medium. The cells were treated with RANKL (50 ng/mL) and AS-A2 (1, 5, and 10 µM). After 24 h, the cells were lysed using a protein extraction reagent (iNtRON), as reported previously [15]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to isolate equal quantities of proteins, which were then transferred to polyvinylidene fluoride nitrocellulose membranes (Bio-Rad). For 1 h, 5% non-fat skim milk blocked the membrane. The specific primary antibodies were then used for probing at 4°C. After overnight incubation, horseradish peroxidase-conjugated secondary antibodies were used to probe the primary antibodies for 1 h. A Clarity Western ECL Substrate kit (Bio-Rad) was used to observe the immunoreactive bands, and Image J software was used for quantitative analysis.

Statistically analysis

A triplicate test was conducted for all experiments, and the data are provided as mean ± SD. A one-way analysis of variance and t-test were used to examine the statistical significance. A p-value less than 0.05 was considered significant.

RESULTS

AS-A2 inhibits RANKL-induced osteoclast differentiation in RAW264.7 cells

Fig. 1A presents the molecular structure of AS-A2. RAW264.7 cells were used to determine how AS-A2 affected cell survivability. The cell viability was examined using a CCK-8 (Dojindo) after the cells were grown for 24 h with increasing AS-A2 concentrations. The viability of the RAW264.7 cells at 0.01, 0.1, 1, and 10 µM of AS-A2 for 24 h was unaffected, as shown in Fig. 1B. As a result, up to 10 µM AS-A2 was used in the experiment. RAW264.7 cells were used to determine if AS-A2 is involved in osteoclast differentiation, where RANKL (50 ng/mL) was used to stimulate the cells for osteoclast differentiation. The presence of multinuclear TRAP-positive cells (MNC) identified the osteoclasts. The TRAP staining results indicated the significant osteoclast differentiation in RAW264.7 cells induced by the RANKL. On the other hand, at the AS-A2 doses in Fig. 1C, AS-A2 suppressed RANKL-induced osteoclast differentiation. The number of TRAP-positive MNCs was also reduced dramatically by 0.01 (p < 0.05), 0.1 (p < 0.005), 1 (p < 0.005) and 10 (p < 0.005) µM concentrations of AS-A2 (Fig. 1D).

| Target genes (accession number) | Primer (forward, reverse) | Annealing Tm (°C) | PCR cycles |
|----------------------------------|---------------------------|-------------------|------------|
| TRAP (NM_007388)                 | 5′-ctggctggccctacaagatc-3′ | 54                | 30         |
|                                  | 5′-gatagggctggaggg-3′      |                   |            |
| MMP9 (NM_013599)                 | 5′-ctgtagccccacttact-3′    | 57.5              | 36         |
|                                  | 5′-agtagctctgctccaggg-3′   |                   |            |
| Cathepsin K (NM_007802)          | 5′-agggggtatagctagc-3′     | 57.5              | 26         |
|                                  | 5′-ccagcagagagcatc-3′      |                   |            |
| c-Fos (NM_010234)                | 5′-atgggctctcctgcaacac-3′  | 57.5              | 30         |
|                                  | 5′-gctgccaataataactc-3′    |                   |            |
| NFATc1 (NM_198429)              | 5′-aggtatgtaagggctgg-3′    | 55                | 35         |
|                                  | 5′-aggtatgtaagggctgg-3′    |                   |            |
| RANK (MGI:1314891)               | 5′-aagcctggagcataagc-3′    | 54                | 30         |
|                                  | 5′-acccctctctcctcag-3′     |                   |            |
| β-actin (NM_007393)              | 5′-tcctacaaggtggctgg-3′    | 50                | 26         |
|                                  | 5′-tcctacagctcctcaggg-3′   |                   |            |

RT-PCR, reverse-transcription polymerase chain reaction; PCR, polymerase chain reaction.
AS-A2 reduces osteoclast differentiation in BMMs

This study examined how AS-A2 affected the RANKL-induced osteoclast development in BMMs. The CCK-8 kit determined the cell viability. Compared to the control group, AS-A2 does not have cytotoxic effects on BMMs at the concentrations utilized (Fig. 2A). For the RANKL-induced osteoclast differentiation assay, varying concentrations (0.01, 0.1, 1, and 5 µM) of AS-A2 were treated in BMMs for six days in the RANKL-induced osteoclast differentiation assay. RANKL increased osteoclast development significantly as evaluated by MNC formation from the TRAP staining data. In the BMMs, the quantity of MNCs in the TRAP-positive stained cells in AS-A2-treated groups was reduced dramatically by a treatment of AS-A2 with 0.1, 1, and 10 µM doses (Fig. 2C). AS-A2 could prevent RANKL-induced osteoclast development from BMMs in vitro.

AS-A2 inhibits RANKL-induced osteoclast differentiation signaling pathways

MAPKs, an essential signaling pathway during osteoclast differentiation [16], were examined to learn more about the molecular mechanism of AS-A2 on RANKL-induced differentiation in RAW264.7 cells. In RANKL-induced osteoclasts, the phosphorylation of ERK1/2, JNK, and p38 protein expression was increased within 15 min compared to the vehicle group. The phosphorylation of ERK1/2, JNK, and p38 protein in the AS-A2-treated cells was reduced considerably (Fig. 3). These results confirm that AS-A2 downregulates the MAPKs pathway during the inhibition of RANKL-induced differentiation of osteoclast in RAW264.7 cells.
AS-A2 inhibits the expression of RANKL-induced osteoclast related gene expression

Next, this study examined whether AS-A2 inhibited the production of TRAP, cathepsin K, MMP-9, and RANK, which are all osteoclast-specific genes [17]. RANKL increased the expression of osteoclast differentiation marker genes dramatically compared to the vehicle group, as demonstrated by RT-PCR. On the other hand, at 5 or 10 µM, AS-A2 reduced TRAP, MMP-9, and RANK mRNA expression dramatically, but AS-A2 did not affect cathepsin K mRNA expression (Fig. 4). These results suggest that AS-A2 can inhibit the RANKL-induced activation of osteoclast-related genes in RAW264.7 cells.
AS-A2 suppresses RANKL-induced expression of NFATc1 and c-Fos in RAW264.7 cells

This study examined the effect of AS-A2 on the mRNA and protein expression of NFATc1, a vital transcriptional factor of osteoclast differentiation [15]. c-Fos expression, which has been linked to osteoclast development via NFATc1 downregulation, was also studied [18]. RT-PCR showed that the mRNA expression of NFATc1 and c-Fos were increased by a treatment with RANKL at a concentration of 10 µM, whereas AS-A2 diminished the level of NFATc1 and c-Fos transcription dramatically (Fig. 4A). Furthermore, a western blotting study showed that RANKL induced protein expressions of NFATc1 and c-Fos, whereas treatment with 10 µM AS-A2 decreased the NFATc1 and c-Fos significantly for six days (Fig. 5A). These findings showed that AS-A2 suppresses NFATc1 and c-Fos transcription during osteoclast differentiation.

AS-A2 suppresses bone resorption in BMMs

M-CSF and RANKL favored the formation of resorption pits when BMMs were cultivated in calcium-coated plates for six days during the pit formation assay. The formation of resorption pits with a RANKL treatment had a large number and area compared to the vehicle cells, as shown in Fig. 6A, but AS-A2 decreased the RANKL-induced size of the bone resorption pits at 5 and 10 µM of AS-A2. These findings suggest that AS-A2 inhibits osteoclast development, resulting in a smaller pit region (Fig. 6B).
DISCUSSION

Increased RANKL activity causes osteoclast differentiation, leading to bone resorption and illnesses, such as osteoporosis, Paget’s disease, rheumatoid arthritis, and bone metastasis [19]. As a result, inhibiting osteoclast differentiation and function may aid in preventing or treating osteoclast-related disorders [20].

Bisphosphonates and denosumab are well known as antiresorptive agents. On the other hand, long-term use of these agents has serious side effects. Excessive inhibition of bone resorption may suppress bone formation and cause necrosis [21]. Hence, new drugs should
be introduced for bone diseases. AT is used to treat various illnesses throughout Eastern Asia, including Korea and China [9]. Cheng et al. [22] found polyphenols, triterpenes, and saponins in the roots of AT. Different compounds comprised of caffeoylquinic acids, ASs, and aster peptides from the root of AT have expectorant, antitussive, and anti-inflammatory properties [10]. AT has anti-inflammatory properties by blocking the MAPK and NF-κB signaling pathways [12,13]. This study examined the effects of AS-A2 on RANKL-induced differentiation in RAW264.7 cells and BMMs. In vitro, AS-A2 blocked RANKL-induced osteoclast development without causing cytotoxicity. This study examined how AS-A2 inhibitory action affected the BMMs viability and bone resorption. At the stated doses of AS-A2, AS-A2 decreased the RANKL-induced bone pit area dramatically. Further studies in post-ovariectomy osteoporosis will be needed to understand AS-A2 regulation better. RANKL, a critical factor regulating mature osteoclast function and viability, activated osteoclast differentiation from macrophages [23].

The MAPK pathway was activated by the pairing of RANKL to its receptor RANK [24]. Furthermore, RANK/RANKL interaction enhances the specific intracellular signaling transduction pathways of MAPKs, such as ERK, JNK, and p38, which are important in osteoclastogenesis [25]. The cell survivability and development of the ruffle border, as well as cell polarity maintenance, are connected to the ERK activity [26]. The development of osteoclast precursors into osteoclasts, and hence to bone resorption, is dependent on the p38-mediated signals [27]. JNK is involved in the generation of osteoclast precursors and the survival of osteoclasts [28]. This RANKL stimulated ERK and JNK phosphorylations were suppressed by AS-A2 in RAW264.7 cells. Overall, AS-A2 plays a significant role in suppressing the MAPK pathways, including ERK and JNK, while inhibiting osteoclast differentiation and function.

NFATc1 and c-Fos are vital transcription factors for osteoclastogenesis and the differentiation of osteoclasts [29,30]. NFATc1 promotes RANKL signaling in the development of osteoclasts as a down regulator of c-Fos, NF-κB, and MAPKs [18]. By interacting with c-Fos, NFATc1 induces the auto-amplification of NFATc1 and the transcription of osteoclast-specific genes, such TRAP, MMP-9, and cathepsin K, [28,31,32]. AS-A2 suppressed the expression of RANKL-induced NFATc1 and c-Fos, which is consistent with earlier studies, indicating that the RANKL/RANK axis activation leads to NFATc1 expression downstream. Cathepsin K is a cysteine proteinase found primarily in osteoclasts that cleaves important bone matrix proteins and plays a crucial role in bone resorption by degrading the organic phase of bone [18,33-35]. Furthermore, AS-A2 also suppressed c-Fos, NFATc1, RANK, MMP-9, TRAP, and cathepsin K, which are highly active genes implicated in RANKL-induced osteoclastogenesis.

In conclusion, AS-A2 inhibited RANKL-induced osteoclast differentiation in RAW264.7 cells and BMMs. In addition, AS-A2 inhibited the MAPKs pathway expression and downregulated the transcription factors, such as c-Fos and NFATc1. Therefore, AS-A2 isolated from AT appears to be a viable therapeutic therapy for osteolytic illnesses, such as osteoporosis, Paget’s disease, and osteogenesis imperfecta.

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