Inhibition of andrographolide in RAW 264.7 murine macrophage osteoclastogenesis by downregulating the nuclear factor-kappaB signaling pathway

Y.Q. Ren[1] and Y.B. Zhou[2]

[1]Department of Orthopedics 1, Yuyao People's Hospital, Yuyao, ZheJiang Province, China
[2]Trauma Ward, Department of Emergency Medicine, Yuyao People's Hospital, Yuyao, Zhejiang Province, China

Corresponding author: Y.B. Zhou
Email: zhouyunbin001@163.com

Genet. Mol. Res. 14 (4): 15955-15961 (2015)
Received August 10 , 2015
Accepted October 13, 2015
Published December 7, 2015
DOI http://dx.doi.org/10.4238/2015.December.7.7

ABSTRACT. This study aims to investigate the effects of andrographolide (AGP) on osteoclast formation in RAW 264.7 murine macrophage cells. The effects of AGP on cell viability were determined in RAW 264.7 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of AGP on osteoclast formation were tested by osteoclast staining with tartrate-resistant acid phosphatase (TRAP). The effects of AGP on receptor activator of nuclear factor-kappaB (NF-kappaB) ligand (RANKL)-induced, NF-kappaB-dependent transcription in RAW 264.7 cells were assessed using luciferase reporter assays. The results demonstrated that the viability of osteoclast precursor RAW 264.7 cells was not affected by AGP treatment at a concentration of 0.4 to 10 μM. Additionally, the number of TRAP-positive osteoclasts was significantly reduced by the same concentrations of AGP treatment. AGP also inhibited RANKL-induced NF-kappaB activation in a dose-dependent

fashion as evidenced by luciferase reporter assays. In summary, this study demonstrates that AGP inhibits osteoclastogenesis in RAW 264.7 murine macrophage cells through downregulation of the NF-kappaB signaling pathway.

Key words: Andrographolide; Osteoclastogenesis; Nuclear factor-kappaB; RAW264.7; Macrophages

INTRODUCTION

Osteoporosis, also known as low bone mineral density, is an important risk factor for fracture in elderly women (Gallagher and Levine, 2011). Osteoclasts, originate from hematopoietic progenitors, are essential for bone homeostasis, and play a vital role in both the development of osteoporosis and the metastasis of tumors to bone. The formation of osteoclasts involves several factors such as receptor activator of nuclear factor-kappaB (NF-kappaB) ligand (RANKL) and macrophage colony-stimulating factor (Novack, 2011). Many researchers have shown that RANKL is directly involved in the differentiation of macrophages into osteoclasts (Wang et al., 2003; Kukita and Kukita, 2013). RANKL is able to activate mature osteoclasts (Burgess et al., 1999), and induce osteoclast polarization and hypercalcemia (Xu et al., 2000). During stimulation with RANKL, the receptor RANK binds to signaling adaptor molecules such as tumor necrosis factor receptor-associated factor adapter proteins (Keating et al., 2007), which leads to the activation of the NF-kappaB and c-Jun N-terminal kinase signaling pathways (Darnay et al., 1998).

Andrographolide (AGP) is a diterpenoid lactone component found in the herbal medicine *Andrographis paniculata*, which is widely used in Asia (Zhao et al., 2002). Several studies have demonstrated that AGP exhibits various biological activities, including antitumor (Yang et al., 2014), anti-inflammatory (Ku and Lin, 2013), antiviral (Wen et al., 2014), and hepatoprotective effects (Lee et al., 2014). It has been demonstrated that AGP can attenuate inflammation by inhibiting NF-kappaB activation through covalent modification of the reduced cys62 residue of p50 (Xia et al., 2004). Furthermore, AGP was also shown to suppress tumor growth by inhibiting NF-kappaB signaling activation (Zhang et al., 2014). We examined the effects of AGP on osteoclast differentiation and on RANKL-induced NF-kappaB-dependent transcription in RAW 264.7 murine macrophages.

MATERIAL AND METHODS

Cell culture and cell viability assay

RAW 264.7 murine monocytes/macrophage cells were purchased from the Cell Storehouse of the Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Life Technologies, USA) containing 10% fetal bovine serum (FBS; Life Technologies), penicillin (100 U/mL, Life Technologies) and streptomycin (100 µg/ml, Life Technologies) in a humidified incubator with 5% CO_2 at 37°C. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 5 days of treatment with various concentrations of AGP. RAW 264.7 cells were seeded on 96-well plates at a density of 5 x 10^2 cells/well. Then, the cells were treated with AGP at concentrations of 0 to 10 µM for 5 days. The relative amount of viable cells was calculated by measuring the absorbance at 570 nm.

Osteoclastic differentiation activity

Cells were treated with different concentrations of AGP (0 to 10 µM). The differentiation of osteoclasts from RAW 264.7 cells was induced by adding 50 ng/mL recombinant murine RANKL in alpha-minimal essential medium (Life Technologies) with 10% FBS for 5 days. After treatments, the cells were fixed with 4% paraformaldehyde and stained for the osteoclast enzyme marker, tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase kit (Life Technologies). In our study, TRAP-positive, multinucleated cells were counted as osteoclasts only if the cells showed more than three nuclei.

Quantitative real time-polymerase chain reaction (qPCR) to evaluate TRAP mRNA expression

After treatment with RANKL and AGP for 5 days, total RNA was extracted with RNeasy mini kit (Qiagen, USA). The reverse transcription reaction for cDNA was synthesized from about 1 µg total RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for each sample. The specific primers used in our study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): mouse GAPDH: forward, 5'-AACGGGAAGCTTGTCATCAATGGAAA-3'; reverse, 5'-GCATCAGCAGAGGGGGCAGAG-3'; mouse TRAP: forward, 5'-CTGGAGTGCACGATGCCAGCGACA-3'; reverse, 5'-TCCGTGCTCGG CGATGGACCAGA-3'. qPCR experiments were performed in triplicate using the SYBR Green PCR Master Mix using a 7900HT qPCR system thermal cycler (Applied Biosystems, USA). The Ct values of samples were normalized to GAPDH mRNA.

NF-kappaB luciferase reporter gene activity assay

RAW 264.7 cells were seeded on 48-well plates and cultured for 24 h. Cells were then pretreated for 1 h with AGP or vehicle as a control, followed by addition of RANKL (50 ng/mL) for 8 h. Luciferase activity was measured using the Promega Luciferase Assay System (Promega, USA). The data were normalized to that of the vehicle control.

Statistical analysis

The differences between the treatment and control groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. All statistical analyses were performed using the GraphPad PRISM software (GraphPad PRISM version 5.0, USA). $P < 0.05$ was considered to be significant. Data are reported as means ± standard error of the mean (SE).

RESULTS

AGP inhibits RANKL-induced osteoclast differentiation of RAW 264.7 cells

In order to test the cytotoxic effect of AGP on murine RAW 264.7 cells, the MTT assay was employed. The results demonstrated that the viability of RAW 264.7 osteoclast precursor cells was not affected by AGP treatment at concentrations up to 10 µM (Figure 1). At the same

concentrations, the number of TRAP-positive osteoclasts was significantly decreased by AGP treatment in a dose-dependent manner (Figure 2).

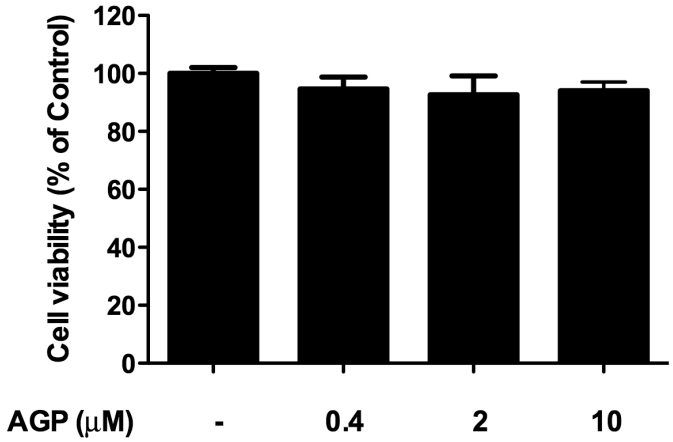

Figure 1. Effect of andrographolide on cell viability of RAW 264.7 murine macrophage cells. Data are reported as means ± SE from three independent experiments.

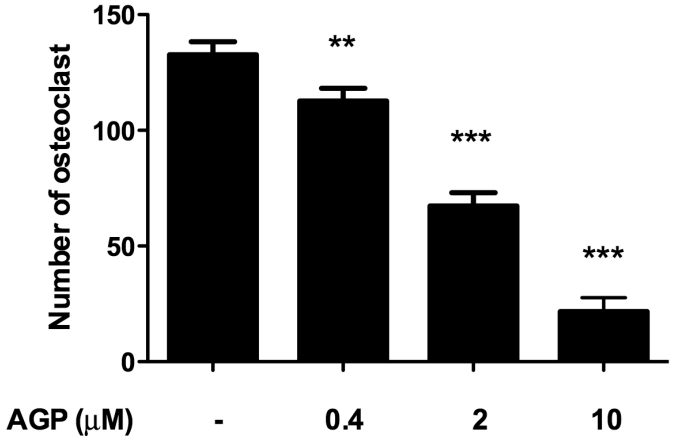

Figure 2. Andrographolide inhibition of receptor activator of nuclear factor kappa B ligand-induced osteoclastogenesis from RAW 264.7 murine macrophage cells after 5 days. Data are reported as means ± standard errors of the mean (error bars) from three independent experiments. **P < 0.01, ***P < 0.001 versus the dimethyl sulfoxide treatment control.

AGP inhibited TRAP mRNA expression in RANKL-induced osteoclast differentiation of RAW 264.7 cells

The data from qPCR analysis indicated that AGP could significantly downregulate TRAP mRNA expression in a dose-dependent manner at concentrations of 0.4 to 10 μM after 5 days of treatment (Figure 3).

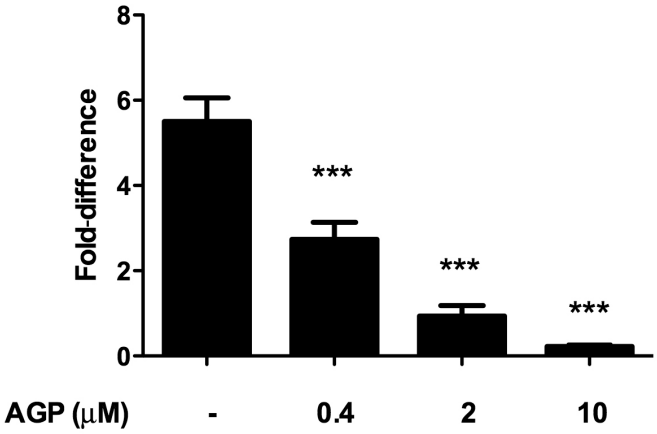

Figure 3. Effects of andrographolide on tartrate-resistant acid phosphatase mRNA expression in receptor activator of nuclear factor kappa B ligand-treated RAW 264.7 murine macrophage cells. Data are reported as means ± standard errors of the mean (error bars) from three independent experiments. ***P < 0.001 versus the dimethyl sulfoxide treatment control.

AGP inhibited NF-kappaB activity in RANKL-induced osteoclast differentiation of RAW 264.7 cells

The results showed that the transcriptional activity of NF-kappaB was dramatically increased by RANKL treatment. However, NF-kappaB activity was significantly inhibited by AGP treatment in a dose-dependent manner (Figure 4).

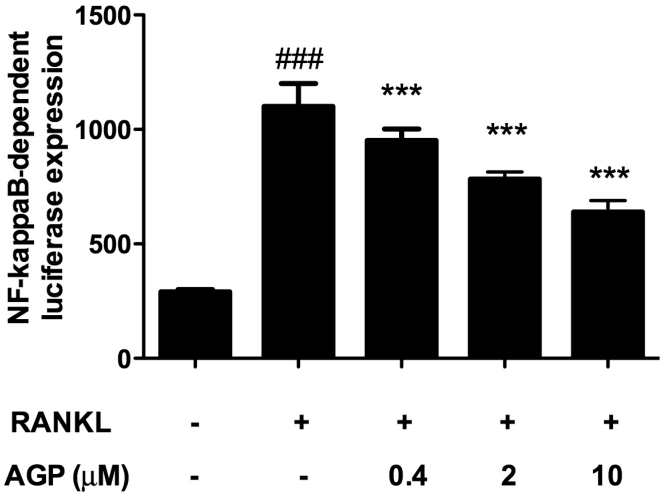

Figure 4. Effects of AGP on NF-kappaB activity in receptor activator of nuclear factor kappa B ligand (RANKL)-treated RAW 264.7 murine macrophage cells. Data are reported as means ± standard errors of the mean (error bars) from three independent experiments. ###P < 0.001 versus no RANKL treatment control; ***P < 0.001 versus the dimethyl sulfoxide treatment control.

DISCUSSION

AGP, the major active ingredient of the herbal medicine *A. paniculata*, has been shown to exert various biological activities, including antitumor (Yang et al., 2014), anti-inflammatory (Ku and Lin, 2013), antiviral (Wen et al., 2014), and hepatoprotective effects (Lee et al., 2014). In this study, we showed that AGP significantly inhibited RANKL-induced osteoclast differentiation from RAW 264.7 murine macrophage cells and decreased the number of osteoclasts and the mRNA expression of TRAP. These observations were similar to those that have recently been reported in the context of bone metabolism (Zhai et al., 2014).

In addition, AGP also blocked RANKL signaling by significantly reducing RANKL-induced NF-kappaB transcriptional activity. The interaction of RANKL with RANK results in the activation of NF-kappaB and other proteins, and this process plays an important role in osteoclastogenesis (Boyce et al., 1999). NF-kappaB is activated by RANKL in RAW 264.7 cells and is required for osteoclast formation (Franzoso et al., 1997; Wang et al., 2003). Several studies have suggested that p50 and p52 expression are crucial for RANK-expressing osteoclast precursors to differentiate into osteoclasts in response to osteoclastogenic molecules such as RANKL (Xing et al., 2002; Wang et al., 2003). Therefore, suppression of NF-kappaB activation would be an effective approach to inhibit osteoclast formation. Interestingly, in our current study, AGP was shown to be able to reduce RANKL-induced NF-kappaB activity and inhibit RANKL-induced differentiation of RAW 264.7 cells into osteoclasts. These observations indicate that AGP is a potent herbal ingredient that is able to regulate bone metabolism.

In summary, our study has provided important evidence for the effects of AGP on bone metabolism as evidenced by the inhibition of osteoclast formation. Our observations also highlight that AGP can effectively inhibit osteoclastogenesis and that the mechanism of action of AGP is related to the NF-kappaB pathway. However, more studies are required to explore the detailed signaling pathways involved in *in vitro* systems and to establish the effect of AGP on bone metabolism *in vivo*.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Research Fund of Yuyao People's Hospital with the reference #YJQ02A.

REFERENCES

Boyce BF, Xing L, Franzoso G and Siebenlist U (1999). Required and nonessential functions of nuclear factor-kappa B in bone cells. *Bone* 25: 137-139.

Burgess TL, Qian Y, Kaufman S, Ring BD, et al. (1999). The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J. Cell Biol.* 145: 527-538.

Darnay BG, Haridas V, Ni J, Moore PA, et al. (1998). Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappab and c-Jun N-terminal kinase. *J. Biol. Chem.* 273: 20551-20555.

Franzoso G, Carlson L, Xing L, Poljak L, et al. (1997). Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev.* 11: 3482-3496.

Gallagher JC and Levine JP (2011). Preventing osteoporosis in symptomatic postmenopausal women. *Menopause* 18: 109-118.

Keating SE, Maloney GM, Moran EM and Bowie AG (2007). IRAK-2 participates in multiple Toll-like receptor signaling pathways to NFkappaB via activation of TRAF6 ubiquitination. *J. Biol. Chem.* 282: 33435-33443.

Ku CM and Lin JY (2013). Anti-inflammatory effects of 27 selected terpenoid compounds tested through modulating Th1/Th2 cytokine secretion profiles using murine primary splenocytes. *Food Chem.* 141: 1104-1113.

Kukita A and Kukita T (2013). Multifunctional properties of RANKL/RANK in cell differentiation, proliferation and metastasis. *Future Oncol.* 9: 1609-1622.

Lee JC, Tseng CK, Young KC, Sun HY, et al. (2014). Andrographolide exerts anti-hepatitis C virus activity by up-regulating haeme oxygenase-1 via the p38 MAPK/Nrf2 pathway in human hepatoma cells. *Br. J. Pharmacol.* 171: 237-252.

Novack DV (2011). Role of NF-kappaB in the skeleton. *Cell Res.* 21: 169-182.

Wang C, Steer JH, Joyce DA, Yip KH, et al. (2003). 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits osteoclastogenesis by suppressing RANKL-induced NF-kappaB activation. *J. Bone Miner. Res.* 18: 2159-2168.

Wen L, Xia N, Chen X, Li Y, et al. (2014). Activity of antibacterial, antiviral, anti-inflammatory in compounds andrographolide salt. *Eur. J. Pharmacol.* 740: 421-427.

Xia YF, Ye BQ, Li YD, Wang JG, et al. (2004). Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. *J. Immunol.* 173: 4207-4217.

Xing L, Bushnell TP, Carlson L, Tai Z, et al. (2002). NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J. Bone Miner. Res.* 17: 1200-1210.

Xu J, Tan JW, Huang L, Gao XH, et al. (2000). Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-kappaB ligand. *J. Bone Miner. Res.* 15: 2178-2186.

Yang SH, Wang SM, Syu JP, Chen Y, et al. (2014). Andrographolide Induces Apoptosis of C6 Glioma Cells via the ERK-p53-Caspase 7-PARP Pathway. *Biomed. Res. Int.* 2014: 312847.

Zhai ZJ, Li HW, Liu GW, Qu XH, et al. (2014). Andrographolide suppresses RANKL-induced osteoclastogenesis *in vitro* and prevents inflammatory bone loss *in vivo*. *Br. J. Pharmacol.* 171: 663-675.

Zhang QQ, Ding Y, Lei Y, Qi CL, et al. (2014). Andrographolide suppress tumor growth by inhibiting TLR4/NF-kappaB signaling activation in insulinoma. *Int. J. Biol. Sci.* 10: 404-414.

Zhao J, Yang G, Liu H, Wang D, et al. (2002). Determination of andrographolide, deoxyandrographolide and neoandrographolide in the Chinese herb *Andrographis paniculata* by micellar electrokinetic capillary chromatography. *Phytochem. Anal.* 13: 222-227.