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Original Article

The Regulation of $p27^{Kip-1}$ and Bcl2 Expression Is Involved in the Decrease of Osteoclast Proliferation by A2B Adenosine Receptor Stimulation

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A2B adenosine receptor (A2BAR) is known to be a regulator of bone homeostasis, but the regulatory mechanism of A2BAR on the osteoclast proliferation are poorly explored. Recently, we have shown that stimulation with BAY 60-6583, a specific agonist of A2BAR, significantly reduced macrophage-colony stimulating factor (M-CSF)-induced osteoclast proliferation by inducing cell cycle arrest at G1 phase and increasing the apoptosis of osteoclasts. The objective of this study was to investigate the regulatory mechanisms of cell cycle and apoptosis by A2BAR stimulation. The expression of A2BAR and M-CSF receptor, c-Fms, was not changed by A2BAR stimulation whereas M-CSF effectively induced c-Fms expression during osteoclast proliferation. Interestingly, A2BAR stimulation remarkably increased the expression of $p27^{Kip-1}$, a cell cycle inhibitor, but the expression of Cyclin D1 and cdk4 was not affected. In addition, while BAY 60-6583 treatment reduced the expression of Bcl2, an anti-apoptotic oncogene, it failed to regulate the expression of Bax, a pro-apoptotic marker. Taken together, these results imply that the increase of $p27^{Kip-1}$ inducing cell cycle arrest at G1 phase and the decrease of Bcl2 inducing anti-apoptotic response by A2BAR stimulation contribute to the down-regulation of osteoclast proliferation.

Key Words: A2B adenosine receptor; Osteoclast proliferation; M-CSF; Bone homeostasis

INTRODUCTION

Bone is a dynamic tissue that regulated by a constant remodeling process requiring a considerable amount of energy. Bone-forming osteoblasts and bone-resorbing osteoclasts are critical for bone homeostasis (Teitelbaum, 2000; Harada and Rodan, 2003). The proliferation of osteoclasts derived from hematopoietic progenitors of the bone marrow-derived monocyte/macrophage (BMM) is mainly regulated by M-CSF. Binding of M-CSF to M-CSF receptor, c-Fms, stimulates proliferation, survival and differentiation of osteoclasts (Teitelbaum and Ross, 2003; Ross, 2006). The importance of M-CSF in osteoclast biology is demonstrated by *op/op* mice lacking the gene (*Csf1r*) encoding M-CSF receptor, which developed severe osteopetrosis due to a decrease in tissue macrophages and osteoclasts (Wiktor-Jedrzejczak et al., 1990; Kodama et al., 1991; Dai et al., 2002).

The increase of proliferation and/or activity of osteoclasts results in a variety of bone diseases including osteoporosis

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and osteoarthritis (Rodan and Martin, 2000), and, when bones are fractured or repair, the production of adenosine is enhanced from increased extracellular nucleotide molecules such as ATP (Ham and Evans, 2012; Mediero and Cronstein, 2013; Kim et al., 2017). There are four adenosine receptors (A1R, A2AR, A2BAR and A3R) that are members of the G protein-coupled receptor family. Although all of adenosine receptors are expressed in osteoblasts and osteoclasts, they show the different biological actions (Orriss et al., 2010; Ham and Evans, 2012; Mediero and Cronstein, 2013). Among them, whereas A2BAR stimulation with its specific agonist BAY 60-6583 decreases osteoclast differentiation, it increases osteoblast differentiation (Carroll et al., 2012; Trincavelli et al., 2014; Corciulo et al., 2016; Kim et al., 2017). A2BAR seems to stimulate apoptosis as a p53-induced cell death priming receptor (Long et al., 2013). Moreover, our recent study represents that A2BAR stimulation increases apoptotic response of osteoclasts and induces cell cycle arrest in the G1 (Oh and Lee, 2017). However, there have been little studies on the regulatory mechanism of A2BAR stimulation on osteoclast proliferation.

Cyclin D1 and Cdk4 are important regulators that link mito genic signals to the cell cycle machinery, whereas $p27^{Kip-1}$ inhibits G1 progression by binding to cyclin/cdk complexes and inhibiting their activity (Baldin et al., 1993; Toyoshima et al., 1994; Ekholm and Reed, 2000). Bcl2, an anti-apoptotic oncogene, promotes cell proliferation, and Bax, a pro-apoptotic marker, activates caspase3 and causes apoptosis (Hockenbery et al., 1993; Yamashita et al., 2008). Here, we show that the induction of $p27^{Kip-1}$ expression and the decrease of Bcl2 expression by A2BAR stimulation contributes to the inhibition of osteoclast proliferation.

MATERIALS AND METHODS

Isolation of bone marrow osteoclast precursors

Isolation of bone marrow osteoclast precursors was performed as described in previous research (Oh et al., 2017). In brief, bone marrow cells from femur of 4-6-week-old C57BL/6 mice were flushed out with a sterile 21-gauge syringe and incubated in alpha-MEM media containing 10% FBS and 10 ng/ml M-CSF (R&D Systems) for 24 h. Non-adherent cells were harvested and cultured in the media with 20 ng/ml of M-CSF for 3 days. Adherent cells were used as BMMs after non-adherent cells were removed.

Real-time PCR

The total RNA was isolated from BMMs treated with M-CSF (20 ng/ml) and/or BAY 60-6583 (5 μM) and reverse transcribed using SuperScript III reverse transcriptase (RT) (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed with the Brilliant UltraFast SYBR Green QPCR Master Mix (Agilent Technologies) and specific primers (QIAGEN) for the indicated genes in triplicate using an Mx3000P instrument (Agilent Technologies). *Hprt* was used for endogenous control. The thermal cycling conditions were as follows: 3 min at 95℃, followed by 40 cycles of 95℃ for 10 s, 60℃ for 20 s, and 1 cycle of 95℃ for 1 min, 55℃ for 30 s and 95℃ for 30 s. All quantitation was normalized to *hprt* (Kim et al., 2017).

Western blot analysis

BMMs were stimulated with M-CSF (20 ng/ml) and/or BAY 60-6583 (5 μM) as indicated and then lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl-fluoride). Whole cell extracts were prepared by centrifugation and then the supernatants were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were blotted onto a polyvinylidene difluoride membrane and immunoblot detection was performed with polyclonal antibodies specific to c-Fms, A2BAR, β-actin (as a loading control), Bcl2, Bax (Santa Cruz Biotechnology Inc) and monoclonal anti-mouse $p27^{Kip-1}$ antibody (PharMingen), followed by horseradish peroxidase-conjugated secondary antibodies and enhanced using an ECL detection kit (Amersham Biosciences) (Kim et al., 2017).

Statistical analysis

Results are presented as means ± standard deviations (SD) from at least 3 independent experiments and statistical analyses were determined using Student's *t* test, if not, in-

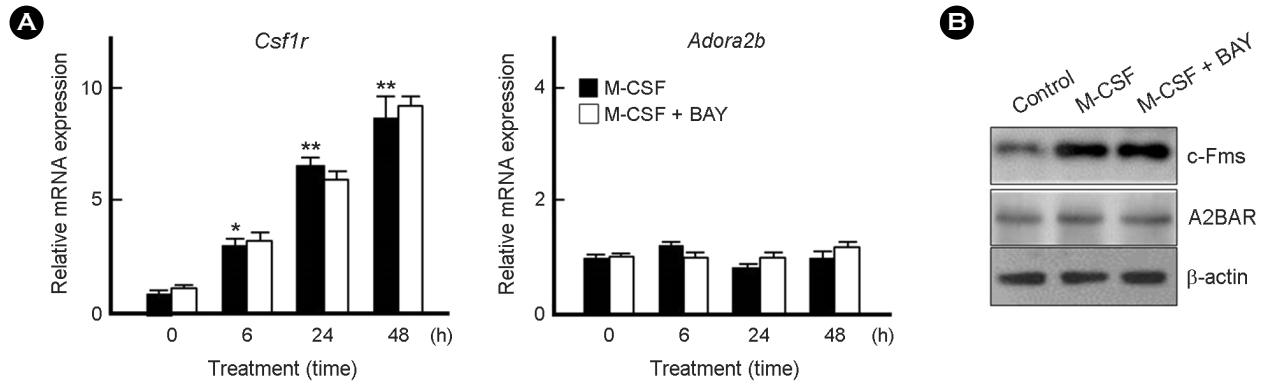

Fig. 1. Effect of A2BAR stimulation on the expression of c-Fms and A2BAR. (A) Primary osteoclast precursors were incubated with or without BAY 60-6583 (BAY, 5 μM) in the presence of M-CSF (20 ng/ml) for the indicated times. Total RNAs were isolated from the cells and then subjected to real-time PCR using *Adora2b*- and *Csf1r*-specific primers. All quantitation was normalized to *hprt*. Results are representative of at least three independent experiments. *$P<0.05$ and **$P<0.005$ vs. non-treated cells. (B) Primary osteoclast precursors were treated with M-CSF (20 ng/ml) or M-CSF (20 ng/ml) and BAY 60-6583 (BAY, 5 μM) for 72 h and then the cell lysates were subjected to Western blot analysis.

dicated. $P<0.05$ was considered to be statistically significant.

RESULTS

A2BAR stimulation does not affect the expression of c-Fms or A2BAR during osteoclast proliferation

To investigate how A2BAR stimulation regulates osteoclast proliferation, we first examined whether the expression of A2BAR is affected by BAY 60-6583 treatment during osteoclast proliferation. Whereas M-CSF treatment induced the expression of *Csf1r*, a gene encoding M-CSF receptor, in a time-dependent manner, the expression of *Adora2b* (a gene encoding A2BAR) was not changed by it (Fig. 1A). Moreover, the addition of BAY 60-6583 in the presence of M-CSF failed to regulate the expression of *Adora2b* as well as *Csf1r* (Fig. 1A). The same was true when the protein levels of c-Fms (M-CSF receptor) or A2BAR were measured (Fig. 1B). These results suggest that A2BAR stimulation decreases osteoclast proliferation without affecting the expression of c-Fms or A2BAR.

A2BAR stimulation induces $p27^{Kip-1}$ expression

In the previous study, we found that BAY 60-6583 treatment increased cell accumulation at the G1 phase with a decrease at the G2/M phase (Oh and Lee, 2017). To identify regulatory mechanism of cell cycle by A2BAR stimulation, the expression of cyclin D1, Cdk4 and $p27^{Kip-1}$ after BAY 60-6583 treatment was determined. Interestingly, BAY 60-6583 treatment remarkably induced $p27^{Kip-1}$ expression in the presence of M-CSF (Fig. 2A). However, the expression of cyclin D1 and Cdk4 was not affected by it (Fig. 2A). When protein levels of $p27^{Kip-1}$ were analyzed, we found that M-CSF treatment showed the inhibitory effect on the expression of $p27^{Kip-1}$ and the addition of BAY 60-6583 reversed the effect of M-CSF (Fig. 2B). These results strongly suggest that the cell cycle arrest at the G1 phase through the induction of $p27^{Kip-1}$ expression by A2BAR stimulation may be responsible for the decrease of osteoclast proliferation.

A2BAR stimulation suppresses Bcl2 expression

The results that A2BAR stimulation increased apoptosis of osteoclast precursors in the previous study (Oh and Lee, 2017) led us to investigate the change of apoptosis-related genes by BAY 60-6583 treatment. Whereas the expression of Bcl2, an anti-apoptotic oncogene, was significantly suppressed by the A2BAR stimulation, Bax, an apoptotic marker, was not affected by it (Fig. 3A and 3B). In addition, since the balance between Bcl2 and Bax determines cell fate, Bcl2/Bax ratio was verified by immunoblotting. The Bcl2/Bax ratio was remarkably decreased by BAY 60-6583 treatment (Fig. 3C), which demonstrates that the decrease of

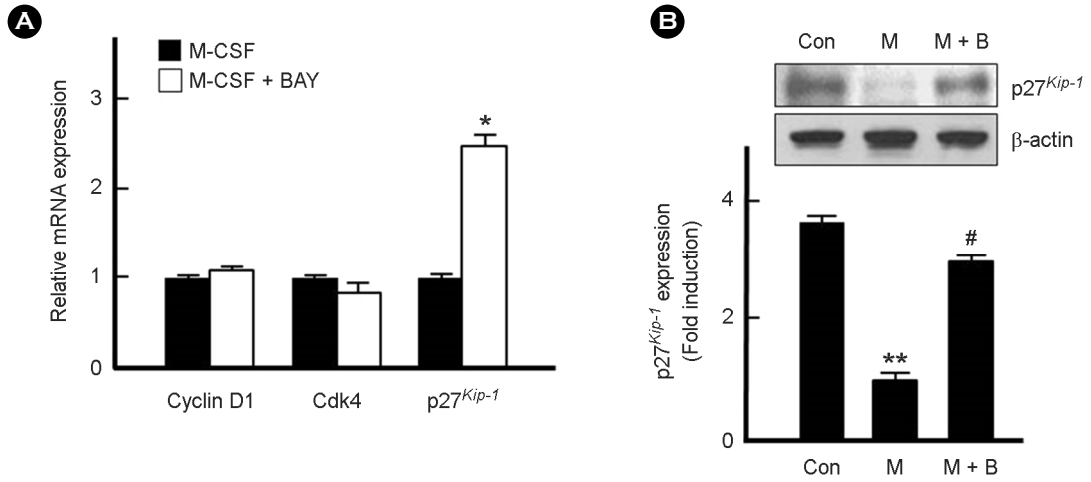

Fig. 2. The induction of $p27^{Kip-1}$ expression by A2BAR stimulation. (A) Primary osteoclast precursors were incubated with or without BAY 60-6583 (BAY, 5 μM) in the presence of M-CSF (20 ng/ml) for 24 h. Total RNAs were isolated from the cells and then subjected to real-time PCR. All quantitation was normalized to *hprt*. Results are representative of at least three independent experiments. *P<0.05 vs. M-CSF-treated cells. (B) Primary osteoclast precursors were M-CSF-starved for 6 h and then treated with or without BAY 60-6583 (BAY, 5 μM) in the presence of M-CSF (20 ng/ml) for 48 h. Total proteins were isolated and then subjected to Western blot analyses (upper panel). Protein bands were quantified by densitometry, and the level of $p27^{Kip-1}$ was normalized to that of β-actin (lower panel). **P<0.005 vs. control cells. #P<0.05 vs. M-CSF-treated cells.

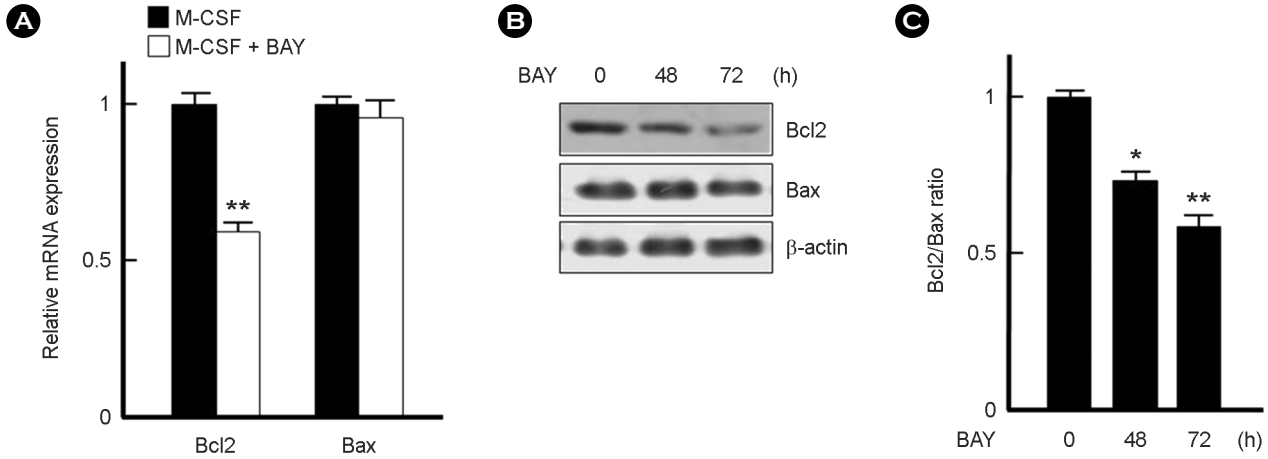

Fig. 3. The suppression of Bcl2 expression by A2BAR stimulation. (A) Primary osteoclast precursors were incubated with or without BAY 60-6583 (BAY, 5 μM) in the presence of M-CSF (20 ng/ml) for 24 h. Total RNAs were isolated from the cells and then subjected to. All quantitation was normalized to *hprt*. Results are representative of at least three independent experiments. **P<0.005 vs. M-CSF-treated cells. (B and C) Primary osteoclast precursors were treated with BAY 60-6583 (BAY, 5 μM) in the presence of M-CSF (20 ng/ml) for indicated times. Total proteins were isolated and then subjected to Western blot analyses (B). Protein bands were quantified by densitometry, and Bcl2/Bax ratio was calculated (C). *P<0.05 and **P<0.005 vs. non-treated cells.

Bcl2 expression by A2BAR stimulation contributes to the apoptosis of osteoclast precursors.

DISCUSSION

Bone remodeling is a finely regulated mechanism and

adenosine and its four receptors are well known regulators of bone homeostasis (Mediero and Cronstein, 2013). A2BAR KO mice display a delay in normal fracture healing (Carroll et al., 2012) and a decrease of bone mineral density by an impaired osteoblast differentiation and an increase of osteoclast differentiation (Trincavelli et al., 2014; Corciulo et al., 2016). It has been reported that p53, a master cell death regulator, upregulated A2BAR expression and stimulation of A2BAR enhanced p53-mediated cell death (Long et al., 2013). Our prior study demonstrated that the stimulation of A2BAR with BAY 60-6583 increases apoptotic response of osteoclasts and induces cell cycle arrest in the G1 phase (Oh and Lee, 2017). In the present study, we provide the molecular regulatory mechanism of A2BAR stimulation on osteoclast proliferation.

In contrast to the roles of RANKL in recent study (Kim et al., 2017), M-CSF did not affect A2BAR expression. However, it remarkably increased the expression of c-Fms during osteoclast proliferation. The addition of BAY 60-6583 failed to regulate the expression of A2BAR and c-Fms. These results imply that A2BAR stimulation regulates M-CSF-mediated osteoclast proliferation without altering A2BAR and c-Fms expression.

$p27^{Kip-1}$, a G1 cyclin-dependent kinase inhibitor, inhibits cell cycle progression by binding to cyclin/cdk complexes and inhibiting their activity (Toyoshima et al., 1994). Whereas the expression of cyclin D1 and Cdk4 was not changed by A2BAR stimulation, it increased $p27^{Kip-1}$ expression, which explaining the decrease of osteoclast proliferation through the cell cycle arrest in the G1 phase by A2BAR stimulation. Moreover, the involvement of $p27^{Kip-1}$ in the adenosine inhibition of macrophage proliferation (Xaus et al., 1999) supports our results.

Bcl2 seems to play critical anti-apoptotic role in osteoclasts but not in osteoblasts since $Bcl2^{-/-}$ mice showed increased bone mass by the reduced numbers of osteoclasts whereas proliferation and differentiation of osteoblasts were normal (Bowler et al., 2001). In this study, A2BAR stimulation remarkably suppressed the Bcl2 expression, but, Bax, a pro-apoptotic oncogene, was not affected by it. Thus, the decrease of Bcl2/Bax ratio by BAY 60-6583 treatment may be responsible for the decrease of osteoclast proliferation.

Given the critical roles of Akt on the osteoclast proliferation, it is intriguing to investigate whether $p27^{Kip-1}$ or Bcl2 are in a downstream of Akt since the activation of Akt by M-CSF promotes osteoclast proliferation and survival by transducing anti-apoptotic signals (Ross, 2006; Pixley et al., 2004; Bradley et al., 2008). Moreover, BAY 60-6583 treatment effectively blocked the activation of Akt by M-CSF (Oh and Lee, 2017).

Further studies of reciprocal regulation between adenosine receptor signaling pathway and c-Fms signaling pathway in osteoclasts could give a hint for understanding pathophysiology of bone-related diseases.

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CONFLICT OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.

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