

## The roles of adenosine and adenosine receptors in bone remodeling

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## 1. ABSTRACT

Adenosine regulates a wide variety of physiological processes including heart rate, vasodilation and inflammation through the activation of specific cell surface adenosine receptors. In addition to these well-established roles of adenosine, recent genetic and pharmacological research has implicated adenosine as an important regulator in bone remodeling. The secretion of adenosine and the presence of its four receptors in bone cells have been well documented. More recently, we provided the first evidence that adenosine regulates osteoclast formation and function through A<sub>1</sub> receptor (A<sub>1</sub>R), and showed that A<sub>1</sub>R-knockout mice have significantly increased bone volume as a result of impaired osteoclast-mediated bone resorption. Moreover, adenosine A<sub>1</sub>R-knockout mice are protective from bone loss following ovariectomy further supporting the involvement of adenosine in osteoclast formation and function. This short review summarizes current knowledge related to the roles of adenosine and adenosine receptors in bone formation and remodeling. A deeper insight into the regulation of bone metabolism by adenosine receptors should assist in developing new therapies for osteoporosis.

## 2. INTRODUCTION

Bone remodeling is a process that takes place throughout life and is required not only for skeletal growth but also to maintain normal bone structure. Bone remodeling consists of coupled bone resorption by osteoclasts and bone formation by osteoblasts. If anything goes awry in this balance, as with advancing age or during disease conditions such as rheumatoid arthritis (RA), skeletal abnormalities will develop such as osteoporosis. Great progress has been made over the last decade in understanding the intercellular and intracellular processes that mediate and regulate osteoclastogenesis and osteoclast activity (1-11). Three molecules play central roles in the regulation of osteoclastogenesis: the receptor activator of NF- $\kappa$ B ligand (RANKL), a constitutive transmembrane protein on osteoblasts which is also present as a soluble mediator (1-5); RANK, the receptor for RANKL and (2, 6-8); the soluble natural decoy receptor for RANKL, osteoprotegerin (OPG) which is secreted from osteoblasts (9-11). The balance of RANKL and OPG regulates the extent and degree of osteoclastogenesis (12-13). Moreover, the dissection of these pathways indicates that the osteoblast communicates with osteoclast

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progenitors and regulates their differentiation into osteoclasts via both secretion of signaling molecules and by direct cell-cell interactions.

There is a growing body of evidence to suggest that bone resorption during inflammatory bone destruction is regulated by the immune system giving rise to a new field of research named osteoimmunology (14-17). The key player in the RANK-RANKL-OPG axis, RANKL, was originally identified as an activator of dendritic cells expressed by activated T cells (1-2). Another molecule that is secreted by T cells and has proven to be a regulator of osteoclastogenesis is interferon-gamma (IFN-gamma) (18). IFN-gamma promotes degradation of TNF receptor-associated factor 6 (TRAF6), a critical adaptor protein involved in RANKL signaling, via the ubiquitin-proteasome pathway (19). The signaling receptor for RANKL, RANK, expressed on osteoclast progenitors, is also abundant in dendritic cells and detectable in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (20). Clearly, the identification of these molecules and the cells expressing them within the bone microenvironment will be critical to furthering our understanding of the regulation of osteoclastogenesis.

Because mature osteoclasts require high adenosine 5'-triphosphate (ATP) levels to support bone resorption and biosynthetic intermediates to supply many cellular constituents, the high turnover rate of ATP and its breakdown products, including adenosine, in osteoclasts also seems to play a role in regulating osteoclast differentiation (21-23). Based on our recent studies we propose that adenosine alterations are also important regulators of RANKL-stimulated osteoclast formation and differentiation. This review will summarize prior knowledge and recent developments in the area of adenosine regulation of bone remodeling.

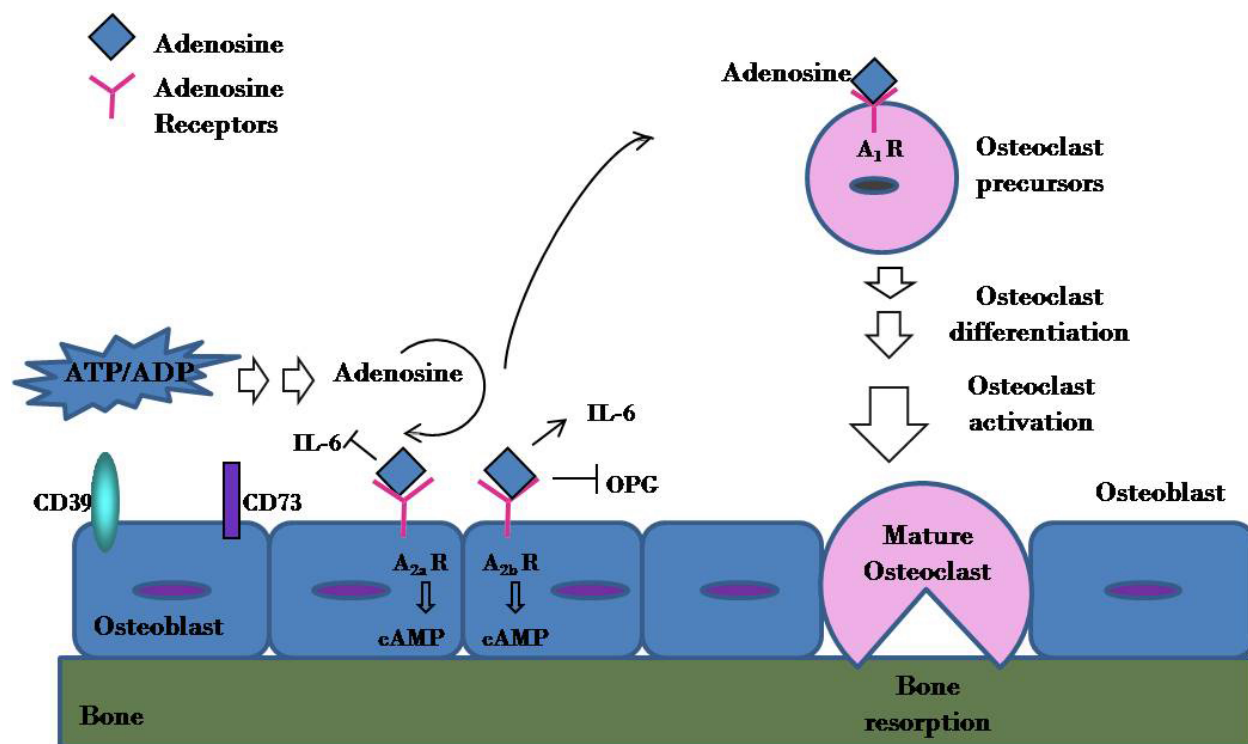
### 3. ADENOSINE PRODUCTION AND EXPRESSION OF ADENOSINE RECEPTORS IN BONE

Adenosine is generally released from cells as a result of ATP catabolism. In stress situations like ischemia or hypoxia extracellular adenosine concentrations may be measured at micromolar concentrations due to a massive ATP degradation with minimal ATP regeneration leading to the release of adenosine through bidirectional cell surface nucleoside transporters. The other major pathway that contributes to high extracellular adenosine concentrations is the extracellular degradation of adenine nucleotides (ATP, ADP and AMP) to adenosine by a cascade of ectonucleotidases, including CD39 (nucleoside triphosphate phosphohydrolase) and CD73 (5'-ectonucleotidase). Adenosine exerts its action via the four types of receptors located on cell membranes and its accumulation is limited by its rapid catabolism to inosine by adenosine deaminase (ADA) outside of the cell or rephosphorylation to nucleotides within the cell.

When there is bone fracture or mechanical microdamage extracellular ATP in a lesion site can reach high concentrations (24-26). Since this purine nucleotide has a very short half-life and is known to be rapidly

hydrolyzed to adenosine, it is reasonable to speculate an increase in levels of adenosine within the bone microenvironment. Accordingly, it has been described by Evans and colleagues in a recent study that human osteoblast precursor cell line, HCC1 and human primary bone marrow stromal cells (BMS) produce adenosine (27). We and others have clearly demonstrated that CD73 is expressed by both human and mouse mesenchymal progenitors that are capable of differentiating into osteoblasts (27-30). The study performed by Evans and colleagues confirmed that the CD73 expressed on HCC1 cells is a functional ectonucleotidase by comparing the degradation rates of exogenously added 1, N<sup>6</sup>-ethenoAMP in the presence and absence of the CD73 inhibitor, AOPCP (27). The same authors went on to suggest that adenosine acts in an autocrine fashion to modulate the activity of osteoblast precursors via A<sub>2b</sub> receptor which is activated only when the extracellular adenosine reaches high nanomolar to low micromolar levels. These observations indicate that osteoblast progenitors produce relative high levels of adenosine that likely acts on surrounding cells in bone and bone marrow, including osteoclast progenitors, in a paracrine manner (Figure 1).

Adenosine receptors are widely distributed on cells including inflammatory and immune cells and the cells of bone. Both the undifferentiated osteoblast precursor cells (HCC1 and BMS) and differentiated osteoblasts express all four receptors (A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub> and A<sub>3</sub>) (27-28). A mature osteoblast cell line, MG-63 expresses A<sub>1</sub>, A<sub>2a</sub>, and A<sub>2b</sub> receptors, but not the A<sub>3</sub> receptor (31). Monocytes and macrophages also express adenosine receptors; for example, the mouse macrophage cell line RAW 264.7 which can be induced to osteoclastic differentiation *in vitro* by culturing with recombinant RANKL (32). Splenocytes and marrow-derived osteoclast progenitor cells also express adenosine receptors (32). All four adenosine receptors have been detected in these cells (32). Recent work from our laboratory has clearly revealed that mouse bone marrow-derived monocyte/macrophage precursor cells (BMMs) express all four receptors and A<sub>1</sub> receptor is functionally predominant, as shown by the increased bone density in A<sub>1</sub>R-knockout mice and defective osteoclast differentiation *in vitro* by osteoclast progenitor cells from A<sub>1</sub>R-knockout mice (32-33). It is interesting to note here A<sub>1</sub> receptor expression in BMMs is up-regulated during osteoclast differentiation (32). Adenosine receptors have also been described on a number of different mesenchymal cell types that are important in the pathophysiology of inflammatory knee osteoarthritis, including equine, human and bovine chondrocytes and fibroblast-like synoviocytes (34-39). Recent work has demonstrated that inflammatory cytokines, including TNF and IL-1, stimulate increased expression and function of adenosine receptors (40-42), a finding confirmed by recent studies that demonstrate that adenosine A<sub>2a</sub> and A<sub>3</sub> receptors are up-regulated in both rheumatoid arthritis patients and that anti-TNF therapy leads to reduction of A<sub>2a</sub> receptor expression (43). Similarly, there is increased adenosine receptor expression on inflammatory cells in monosodium iodoacetate-induced osteoarthritic animal models (44). In these experiments, Bar-Yehuda *et al.* found CF101, a selective A<sub>3</sub> agonist,



**Figure 1.** Schematic diagram illustrating the potential roles played by extracellular adenosine and adenosine receptors in modulating bone cell function. Adenosine 5'-triphosphate (ATP) released from osteoblasts can be degraded to adenosine by a cascade of ectonucleotidases, including CD39 and CD73. Adenosine may regulate osteoclast differentiation from hematopoietic stem cells in a paracrine manner through A<sub>1</sub> receptor on osteoclast precursors. Adenosine at higher concentration might regulate osteoblast function in an autocrine manner through A<sub>2a</sub> and A<sub>2b</sub> receptor on osteoblast precursors.

prevented apoptosis of chondrocytes, which resulted in cartilage protection (44). Although it may be related to the anti-inflammatory properties of A<sub>3</sub> receptors these studies highlight the potential significance of the adenosine receptors in osteoarthritis.

#### 4. ADENOSINE AND OSTEOBLASTS

Early studies conducted by Shimegi and colleagues suggest that adenosine stimulates proliferation of osteoblast-like cells, the MC3T3-E1 osteoblastic cell line, although it is not as mitogenic as ATP and the receptor type involved was not addressed in these studies (45-46). Subsequent work has provided evidence for the importance of adenosine and adenosine receptors in osteoblast activity, although controversy exists regarding their roles (27, 31). Employing adenosine receptor analogues Evans and colleagues reported that adenosine modulates endogenous secretion of cytokines including interleukin 6 (IL-6) and OPG from HCC1 cells predominantly through A<sub>2b</sub> receptors (27). Russell *et al.* also describe adenosine receptor-dependence of cytokine expression from MG-63 cells, however, in contrast to Evans *et al.*, they reported adenosine does not change the basal secretion of IL-6 from MG-63 cells. Instead adenosine attenuates lipopolysaccharide (LPS)-induced IL-6 release from osteoblast through A<sub>2a</sub> receptors, at least in part (31). The reasons for these discrepant results remain unclear,

although they may reflect the peculiarities of these cell lines. It is interesting to note both studies suggest the involvement of cAMP/PKA signaling in the action of adenosine, a downstream signaling molecule for both A<sub>2a</sub> and A<sub>2b</sub> receptors (27, 31).

Although the effects of adenosine and its receptors on osteoblast remain to be clearly established, older studies in patients lacking adenosine deaminase (ADA) and a recent study with genetic mouse models strongly underlines the importance of adenosine regulation of osteoblast function (47-48). It was widely recognized that in children lacking adenosine deaminase there was bone dysmorphismogenesis in addition to the clear immunodeficiency. By examining two immunodeficient mouse models, ADA<sup>-/-</sup> and Pag2 gamma c<sup>-/-</sup> which lack T, B and NK cells, Sauer and colleagues reported that a specific bone phenotype characterized by lower bone mass and impaired bone mechanical competence in ADA<sup>-/-</sup> mice is a consequence of alterations in purine metabolism (47). Their work further revealed the skeletal defects in ADA<sup>-/-</sup> mice are associated with markedly impaired osteoblast activity indicated by lower cell viability and reduced osteoblast-specific markers such as osteocalcin and N-terminal propeptide of type I procollagen (PINP) (47). It is noteworthy that the message level of Runx2, a key factor in osteogenesis, is normal in these ADA<sup>-/-</sup> mice, suggesting intact osteogenesis. Another intriguing finding is that ADA

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activity is 3-fold higher in mature wild type osteoblasts than in mesenchymal progenitor cells, further implicating the importance of adenosine in osteoblast function (47). In a recent study our group documented that osteoblast number and bone formation was unchanged in the bone of adenosine A<sub>1</sub> receptor-KO mice, indicating that adenosine receptors other than A<sub>1</sub>R in regulate osteoblast formation and function in mice (33).

### 5. ADENOSINE AND OSTEOCLASTS

Osteoclasts are highly specialized bone-resorbing cells derived from myeloid precursors. They share several important features with immune cells. For example, nuclear factor of activated T cells (NFATc1), the key transcription factor leading to terminal differentiation of osteoclasts, was first identified in activated T cells (49-50). Indeed, activated T cells stimulate osteoclast formation *in vitro* (51-52). Recent work has discovered that osteoclasts and immune cells share a requirement for regulatory signals that are mediated by immunoreceptor tyrosine-based activation motif (ITAMs) (53-54). Two ITAM adaptor proteins, DNAX-activating protein 12 (DAP12) and Fc receptor common gamma subunit (FcR-gamma), are required for RANKL-induced osteoclast differentiation and bone resorption (53-54). Given the intertwined connection and similarity between osteoclasts and immune cells it is plausible to speculate that osteoclast might use similar mechanisms to regulate cellular development and function.

The hypothesis that adenosine receptors regulate osteoclasts has been supported by our recent finding that adenosine receptors regulate osteoclast formation and function, as the presence and function of adenosine receptors in monocyte and macrophage has been well established. Our studies utilizing A<sub>1</sub>R-knockout mice and pharmacological approaches clearly demonstrated that A<sub>1</sub>R contributes to osteoclast formation and function (32-33). We had previously observed that adenosine regulates the phorbol myristate acetate (PMA)-stimulated formation of multinucleated giant cells by cultured human peripheral blood monocytes through A<sub>1</sub> receptors. 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), a specific inhibitor of the A<sub>1</sub> receptor, almost completely abrogated phorbol ester-stimulated macrophage fusion into giant cells (55). Consistent with this observation, our more recent studies showed significantly increased trabecular and cortical bone density in 6-month-old A<sub>1</sub>KO mice compared with wild type (WT) mice (33). Morphologically, there were a normal number of osteoclasts in the bone but there was a marked loss of ruffled borders of the osteoclasts in A<sub>1</sub>R-knockout mice as compared with those from WT mice (33). In *in vitro* studies osteoclast formation was markedly impaired in BMMs isolated from A<sub>1</sub>KO animals when compared to WT controls (32). In addition, DPCPX potently inhibited the differentiation of osteoclast from WT BMMs and both blockade and deletion of adenosine A<sub>1</sub> receptors diminished osteoclast bone resorption *in vitro* (32-33).

Regarding the regulatory mechanism of adenosine on osteoclast function, observation of adenosine production from osteoblast raises a question: does

adenosine regulate the osteoclast formation and function in an autocrine and/or paracrine fashion? As a cue for resolving this question, our recently identified role of A<sub>1</sub>R in osteoclastogenesis suggests that adenosine may exert its action on osteoclast precursors in both manners. On one hand it can regulate the proliferation of osteoblast and secretion of cytokines from osteoblast, which in turn modifies osteoclastogenesis in a paracrine pathway. For example, IL-6 and OPG are both important regulators of osteoclastogenesis secreted by osteoblast in response to adenosine (10, 27, 56). On the other hand, adenosine generates a concentration gradient from osteoblast to osteoclast precursors in the bone microenvironment and regulates the osteoclast formation and function in an autocrine fashion. This pathway is mediated through binding to specific surface receptors (Figure 1).

### 6. ADENOSINE AND BONE HOMEOSTASIS

Since all four adenosine receptors may be expressed on the same cell or different cells in a tissue it is likely that the affinity of the various adenosine receptors for the ligand dictates which receptor's effects will predominate under specific conditions. In general the A<sub>1</sub> receptor has the highest affinity for adenosine, followed by the A<sub>2a</sub> receptors. A<sub>2b</sub> and A<sub>3</sub> receptors are lower affinity receptors. Thus, under basal, non-stressed conditions when adenosine concentrations are low the functional effects of A<sub>1</sub> receptors will dominate. Modest stress or pharmacologic induction of adenosine will lead to A<sub>2a</sub> receptor predominance and severe stresses will lead to predominance of A<sub>2b</sub> or A<sub>3</sub> receptor functions. Furthermore, the finding that adenosine receptors regulate both osteoclast function and inflammatory process reveals a potential network of signaling cross-talk between osteoclast resorption and immune system. These findings suggest that osteoclasts are indeed highly regulated by multiple factors simultaneously, which can partially explain the inability of some early experiments to demonstrate an effect of adenosine on osteoclasts or the apparent normality of osteoclast formation *in vitro* with macrophage colony-stimulating factor (M-CSF)/RANKL-treated precursors ADA deficient mice (47, 57). To understand the precise roles of adenosine and adenosine receptors further studies detailing the analysis of bone phenotypes of knockout mice deficient in individual adenosine receptors will be required.

### 7. THERAPEUTIC IMPLICATIONS

Our recent studies in models of osteoporosis indicate that adenosine A<sub>1</sub> receptors may be useful targets for the treatment or prevention of osteoporosis since both deletion and pharmacologic blockade of A<sub>1</sub> receptors prevented osteoporosis following ovariectomy (32-33). Pharmacological blockade of the A<sub>1</sub>R may also be a useful target in treating diseases characterized by excessive bone turnover such as osteoporosis, prosthetic joint loosening and other conditions in which osteoclasts play a pathogenic role (e.g. Paget's disease).

The studies discussed here clearly suggest that agents that promote, directly or indirectly, activation of

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adenosine receptors on cells of the bone and joint might be useful in the treatment of inflammatory joint and bone diseases such as rheumatoid arthritis. Indeed, evidence suggests that we are already targeting adenosine receptors in the treatment of inflammatory bone and joint treatments. Thus, our laboratory and others have shown that the antiinflammatory effects of low-dose methotrexate, the anchor drug in the treatment of rheumatoid arthritis and other forms of inflammatory arthritis, are mediated by adenosine (58-59). Methotrexate inhibits inflammatory bone destruction both in animal models of inflammatory arthritis and in patients with inflammatory arthritis and it is likely that adenosine, acting at its receptors, plays a role in suppression of bone injury in this setting although it is not clear at present whether this effect is direct or indirect via reduction in inflammation (60-62).

Thus it is likely that adenosine receptors may be targeted for the treatment of bone diseases and the bone manifestations of inflammatory diseases. The utility of adenosine receptors as targets for the treatment of bone disease will require a great deal more studies.

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**Abbreviations:** A<sub>1</sub>R: A<sub>1</sub> receptor, RANKL: the receptor activator of NF- $\kappa$ B ligand, OPG: osteoprotegerin, RA: rheumatoid arthritis, TRAF6: TNF receptor-associated factor 6, IFN- $\gamma$ : interferon- $\gamma$ , ATP: adenosine 5'-triphosphate, CD39: nucleoside triphosphate phosphohydrolase, CD73: 5'-ectonucleotidase, ADA: adenosine deaminase, BMS: marrow stromal cells, BMMS: bone marrow-derived monocyte/macrophage precursor cells, LPS: lipopolysaccharide, IL-6: interleukin 6, NFATc1: the nuclear factor of activated T cells, ITAMs: immunoreceptor tyrosine-based activation motif, DAP12: DNAX-activating protein 12, FcR- $\gamma$ : Fc receptor common  $\gamma$  subunit, PMA: phorbol myristate acetate, DPCPX: 8-cyclopentyl-1, 3-dipropylxanthine, PINP: N-terminal propeptide of type I procollagen, M-CSF: macrophage colony-stimulating factor, RA: rheumatoid arthritis

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