Published in final edited form as:

Sci Transl Med. 2012 May 23; 4(135): 135ra65. doi:10.1126/scitranslmed.3003393.

Adenosine A_{2A} Receptor Activation Prevents Wear Particle-Induced Osteolysis

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Abstract

Prosthesis loosening, associated with wear-particle-induced inflammation and osteoclast-mediated bone destruction, is a common cause for joint implant failure, leading to revision surgery. Adenosine A_{2A} receptors (A_{2A}R) mediate potent anti-inflammatory effects in many tissues and prevent osteoclast differentiation. We tested the hypothesis that an A_{2A}R agonist could reduce osteoclast-mediated bone resorption in a murine calvaria model of wear-particle-induced bone resorption. C57Bl/6 and A_{2A} knockout (A_{2A}RKO) mice received ultrahigh-molecular weight polyethylene particles (UHMWPE) and were treated daily with either saline or the A_{2A}R agonist CGS21680. After 2 weeks, micro-computed tomography of calvaria demonstrated that CGS21680 reduced particle-induced bone pitting and porosity in a dose-dependent manner, increasing cortical bone and bone volume compared to control mice. Histological examination demonstrated diminished inflammation after treatment with CGS21680. In A_{2A}RKO mice, CGS21680 did not affect osteoclast-mediated bone resorption or inflammation. Levels of bone-resorption markers receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), cathepsin K, CD163, and osteopontin were reduced following CGS21680 treatment, together with a reduction in osteoclasts. Secretion of interleukin 1β (IL-1β) and TNFα was significantly decreased, whereas IL-10 was markedly increased in bone by CGS21680. These results in mice suggest that site-specific delivery of an adenosine A_{2A}R agonist could enhance implant survival, delaying or eliminating the need for revision arthroplastic surgery.

INTRODUCTION

One of the most clinically successful and cost-effective interventions in health care is total hip arthroplasty, in terms of reducing pain, improving function, and enhancing quality of life

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Author contributions: B.N.C. designed most of the experiments. A. Mediero was the primary person responsible for carrying out all experimental procedures. T.W. bred the A_{2A}R KO animals and helped in surgery. S.F. helped design the experiments. W.H. and A. Mazumder. carried out the human cell experiments.

Competing interests: B.N.C. holds patents numbers 5,932,558; 6,020,321; 6,555,545; 7,795,427; adenosine A₁R and A_{2B}R antagonists to treat fatty liver (pending); adenosine A_{2A}R agonists to prevent prosthesis loosening (pending). B.N.C. is a consultant for Bristol-Myers Squibb, Novartis, CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, and Medivector.

Data and materials availability: A_{2A}RKO mice are available from J. F. Chen (Harvard University) and from B.N.C.

in patients with debilitating hip disease (1). However, despite continual changes in surgical technique and implant design, the percentage of revision total hip arthroplasty cases—where the hip implant is removed or replaced—in the United States has not decreased over time (2). More than 25% of revision procedures involve arthrotomy and removal of the prosthesis. Understanding the causes of total hip arthroplasty failure and the types of revision total hip arthroplasty procedures being performed is essential for improving long-term patient outcomes (3). One of the most important factors for long-term survival of joint components is bone growth onto the surface of the implant and one of the major causes of prosthesis loosening is inflammation and osteoclast-mediated bone resorption in response to wear particles near the prostheses (4). Wear particles are debris from joint replacements, whether polymeric, metallic or ceramic, that can stimulate the recruitment of inflammatory-mediating cells and osteoclasts (bone-resorbing cells) to the local site.

The small molecule adenosine is present in all cells and extracellular fluids and is generated from the catabolism of adenine nucleotides in response to oxidative stress, ischemia, and hypoxia. Adenosine can modulate a variety of physiological processes by interacting with specific cell-surface receptors and is necessary for normal cell metabolism and growth. The adenosine receptors (or P1 receptors) are a class of G protein-coupled receptors, which are thought to play a protective role in inflammation, ischemia, and other pathologies upon activation. There are four subtypes of adenosine receptors—A₁, A_{2A}, A_{2B}, and A₃—each encoded by a separate gene and each possessing a unique pharmacological profile and function (5). Classically, adenosine receptor signaling occurs through inhibition or stimulation of adenylate cyclase, although other pathways, such as phospholipase C, calcium ions, and mitogen-activated protein kinases, are also relevant (6). Interestingly, adenosine A₁ and A₂ receptors (A₁R and A₂R) were first defined on the basis of their antagonistic effects on cyclic AMP (cAMP): A₁R inhibits cAMP accumulation, whereas A_{2A}R stimulates cAMP. In general, these receptors have opposing functional effects (7). For example, in inflammation, adenosine A₁R appears to promote multinucleated giant cell formation from human peripheral blood monocytes contrary to A_{2A}R, which inhibits multinucleated giant cell formation (8).

Adenosine receptors are direct and indirect targets for several existing drugs, as well as a number of drugs in development. Potent and selective agonists at the adenosine A₁R are in development to control heart rate in tachyarrhythmias, whereas antagonists of the receptor may be useful for the treatment of dementia and anxiety disorders (6). Adenosine and short-lived selective A_{2A}R agonists have been approved or are in development for use as a coronary vasodilator for pharmacologic stress testing (9). Antagonists of the receptor are currently in testing for the treatment of Parkinson's Disease (6). Adenosine A_{2A}R possess a wide variety of anti-inflammatory effects (10) and adenosine, by targeting either A_{2A}R or A₃R, mediates the anti-inflammatory effects of low-dose methotrexate, the most widely used drug for rheumatoid arthritis (11).

Previous results from our laboratory demonstrated that osteoclasts express all four adenosine receptor subtypes and, moreover, that deletion or blockade of adenosine A₁R diminishes osteoclast differentiation and function *in vitro* (12). *In vivo*, adenosine A₁R blockade or deletion diminished bone resorption, leading to osteopetrosis (increased bone density) in knockout mice and inhibition of post-ovariectomy-induced bone loss (12, 13). More recently, we have observed that adenosine A_{2A}R activation suppresses osteoclast formation *in vitro* (14). Mice lacking adenosine A_{2A}R have increased numbers of osteoclasts in their bones and diminished bone density (14). In general, as noted above, it is possible that since both adenosine A₁ and A_{2A} receptors (as well as A_{2B} receptors) are expressed on osteoclast precursors and have opposing effects on osteoclast development, wherein blockade or loss of one receptor leads to an apparent increase in baseline function of the other in response to

ambient adenosine concentrations. Thus, the increase in osteoclast number and function observed in A_{2A}R knockout (A_{2A}R KO) mice could be from unopposed A₁R functions; where, conversely, the diminished osteoclast function observed in A₁KO mice could result from enhanced A_{2A}R activity. Neither of these receptors is thought to play a role in regulating osteoblast function, which appears to be under control of adenosine A_{2B}R (15, 16).

Thus, we hypothesized that adenosine A_{2A}R agonists might be useful for the prevention of osteoclast-mediated bone resorption and inflammation at the site of prosthesis wear. If this hypothesis is correct, then site-specific delivery of an adenosine A_{2A}R agonist could enhance implant survival, delaying or eliminating the need for revision arthroplastic surgery. To test this, we examined the effects of application of an adenosine A_{2A}R agonist in a well-described model of wear particle-induced bone resorption (17) in both wild-type and A_{2A}RKO mice.

RESULTS

A_{2A}R agonist prevents calvaria osteolysis in mice

In our initial studies we formed an air pouch over the calvaria in adult C57Bl/6 (wild-type, WT) mice and injected either ultrahigh-molecular weight polyethylene (UHMWPE) particles or vehicle (saline) daily for 2 weeks, at which time the mice were sacrificed and the calvaria harvested. Micro-computed tomography (μCT) examination of the affected calvaria in these mice demonstrated pitting and increased porosity of the calvaria of the mice administered particles, as compared to particle-free vehicle (Fig. 1A). Injection of 1 μM CGS21680, a selective adenosine A_{2A}R agonist, into the pouch at the same time as the particles significantly reduced the area of particle-induced bone pitting in a dose-dependent manner (IC₅₀=8.9 nM) by as much as 41.4% (Fig. 1B).

In CGS21680-treated WT mice that had been exposed to the UHMWPE particles, the reduction in bone was significantly less than in the saline-treated, particle-exposed mice ($P<0.01$) (Table 1). Bone volume/total volume (BV/TV) was significantly greater in sham (untreated) animals than in either particulate group (Table 1A). Thus, an adenosine A_{2A}R agonist markedly diminished wear particle-induced bone loss. In vitro, the concentration of agonist that has maximal inhibitory effect on bone resorption is 1 μM (14), which is reflected in in vivo studies as well (Fig. 1B). To investigate the role of adenosine A_{2A}R in wear particle-induced bone resorption, we studied the effects of these particles on bone resorption in A_{2A}R KO (C57Bl/6 background) mice in vivo in the presence of wear particles alone or wear particles with 1 μM CGS21680 administered simultaneously. The adenosine A_{2A}R agonist did not diminish the area of particle-induced bone pitting and porosity (Fig. 1C, D). Bone morphometry confirmed that CGS21680 did not diminish wear particle-induced bone resorption in the knockout mice (Table 1B), further establishing the role of the A_{2A}R in wear particle-induced osteolysis.

In order to determine the translational potential for an A_{2A}R agonist in wear particle induced-bone resorption we determined whether administration of CGS21680 injected every other day for two weeks affected bone resorption. μCT on day 14 revealed that administering CGS21680 every other day exerted a similar effect as daily injections on wear particle-induced bone loss (-Fig. 1E).

A_{2A}R agonist inhibits osteoclast differentiation in human bone marrow

Because only mouse models were used, we next examined whether A_{2A}R stimulation offers similar protection from wear particle-induced bone resorption in human osteoclast precursors derived from bone marrow of healthy patients or patients with multiple myeloma

(Fig. 2). We observed that the A_{2A}R agonist CGS21680 inhibited osteoclast differentiation in vitro by human bone marrow cells in a dose-dependent fashion (maximal decrease of 85±4% compared to control) in both myeloma (Fig. 2B) and healthy patients (Fig. 2C). Pre-treatment with 1 μM ZM241385, an A_{2A}R antagonist, reversed the effect of CGS21680 on osteoclast formation in the myeloma patient cells (Fig. 2B). In vitro, human cells appeared to be more sensitive to A_{2A}R activation than mouse cells (14), requiring only nanomolar concentrations of CGS21680 to potently inhibit osteoclast differentiation (14, 16). The decreased inhibition at higher concentrations of CGS21680 in the human cells may be related to effects of other adenosine receptors, such as A_{2B}R, which are stimulated by CGS21680 at high concentrations.

Adenosine A_{2A}R activation decreases wear particle-induced inflammation

Histological examination of particle-exposed WT mouse calvarias demonstrated an inflammatory cell infiltration on the outside bone surface as seen with the H&E staining (Fig. 3A). We observed a reduction in inflammatory infiltrate on the bone surface in a dose-dependent fashion by treatment with CGS21680 (Fig. 3B; fig. S1), with an IC₅₀=1 nM. As expected, CGS21680 (1 μM) did not diminish the inflammatory infiltrate induced by wear particles in A_{2A}RKO mice (Fig. 3C, D). Interestingly, there appeared to be more inflammatory infiltrate in A_{2A}R KO mice exposed to wear particles as compared to the WT animals (Fig. 3C), as evidenced by comparison to saline-treated calvaria in both WT and A_{2A}RKO mice (Fig. 3E).

Osteoclasts are diminished by adenosine A_{2A}R activation

Exposure to polymeric wear particles increased the number of TRAP-positive osteoclasts—which cause bone resorption—in the WT mice calvaria (Fig. 4A, B). Conversely, treatment of UHMWPE particle-exposed animals with CGS21680 reduced the number of TRAP-positive osteoclasts in a dose-dependent manner in calvaria, with an IC₅₀=2.69 nM (Fig. 4A, B; Fig. S2). These results were consistent with the μCT analyses showing reduced bone resorption upon treatment with the receptor agonist (Fig. 1).

Moreover, there was a 26% increase in the number of osteoclasts in the calvaria of A_{2A}R KO mice exposed to polymeric wear particles compared to WT particle-treated calvarias (Fig. 4). Treatment with CGS21680 (1 μM) did not reduce the number of osteoclasts after wear particle exposure in A_{2A}R KO mice (Fig. 4C, D), a finding consistent with the phenotype previously demonstrated in these animals (14). Interestingly, although wear particle-exposed A_{2A}R KO mice showed a significant increase in the inflammatory infiltrate surrounding the bone (Fig. 3) and in the number of osteoclasts associated with the bone (Fig. 4), basal bone resorption was not altered when compared to the wild-type animals in the untreated (sham) A_{2A}RKO mice.

Localization of osteoclasts, macrophages, inflammation, and bone remodeling markers

To delineate the specific effects of wear particles on bone metabolism and the capacity of the adenosine A_{2A}R agonist to block or reverse these effects we examined several immunohistochemical markers in treated calvaria. In all mice, cathepsin K—a lysosomal cysteine protease involved in bone remodeling and resorption and used as a marker for osteoclasts—was present in trabecular bone where osteoclasts were located (Fig. 5A). There was an increase in the number of cathepsin K-positive cells in the wear particle-exposed animals, which was reduced by treatment with CGS21680 (Fig. 5B). Similar changes were noted in cells staining positive for receptor activator of nuclear factor-κB (RANK) and receptor activator of nuclear factor-κB ligand (RANKL) (Fig. 5A, B), which are key factors for osteoclast differentiation and activation. These findings confirm that wear particle

exposure increases the number of osteoclasts, as demonstrated also by TRAP staining (Fig. 4A, B; fig S2).

In the untreated mice, there were relatively few CD163+ macrophages, which are localized in the periosteal area and in the tissue overlying the bone, as compared to the wear particle-exposed tissues (Fig. 5). In the particle-exposed animals that were also treated with CGS21680, there was a marked reduction in expression of all of these markers (Fig. 5), nearing the levels present in the saline-only (sham) control. Tumor necrosis factor α (TNF α) expression was localized in the same areas as CD163 in the particle-exposed tissues (Fig. 5A, B), as expected because TNF α is secreted by macrophages. The mesenchymal precursor cells that expressed α -smooth muscle actin (α SMA) were generally colocalized with the osteoclasts (Fig. 5), which correlates with the osteogenic potential of this cell population.

Osteopontin—an extracellular matrix protein that initiates the development of osteoclasts' ruffled borders—colocalizes with osteoclasts on the surface of bone. This marker was overexpressed in particle-exposed cells as compared to sham, and was reduced in the CGS21680-treated mice (Fig. 5). Osteocalcin, a protein secreted by osteoblasts, was localized to cells adherent to bone in all three treatment groups of mice (Fig. 5) consistent with the hypothesis that wear particles did not reduce bone formation. Finally, osteoprotegerin—a protein that is secreted as a decoy receptor for RANKL—was expressed in a similar distribution to osteocalcin and was reduced in the wear-particle-exposed, saline-treated mice (Fig. 5). Treatment with CGS21680 increased the levels of osteoprotegerin in particle-exposed animals. CGS21680 treatment altered the expression of many of these markers in a dose-dependent manner (Fig. 5B;).

As with the other effects described in previous sections, CGS21680 did not affect osteoclast or osteoblast marker expression in A_{2A}R KO mice (fig. S3A, B), providing further evidence that the beneficial effects of CGS21680 in wild-type mice were mediated via adenosine A_{2A} receptors.

CGS21680 regulates secretion of cytokines, chemokines, and bone metabolism markers in calvaria

The interaction between the immune system and bone resorption has been well established (18). Osteoblasts and T cells play an important role in osteoclast differentiation by secreting cytokines and chemokines (19). Therefore, we examined the effect of wear particle exposure and CGS21680 treatment on secretion of cytokines, chemokines, and bone metabolism markers by the inflammatory and bone cells in the calvaria cultured *ex vivo* for 72 hours. Calvaria exposed to wear particles secreted more macrophage colony-stimulating factor (M-CSF), interleukin 1 β (IL-1 β), and TNF α than untreated animals (Fig. 6). When treated with CGS21680, levels of M-CSF, IL-1 β , and TNF α decreased to normal or below-normal levels, indicating that activation of A_{2A}R inhibits the release of pro-inflammatory cytokines from macrophages and osteoclasts. In addition, CGS21680 treatment reduced the concentration of secreted RANKL to levels lower than those found in the supernatants of calvaria that were not exposed to wear particles (Fig. 6), an observation consistent with the decrease in osteoclast differentiation that we have previously observed *in vivo* (14) and with the increase in osteoclast number observed in A_{2A}RKO mice (Fig. 4). IL-6 and granulocyte M-CSF (GM-CSF) levels were not changed significantly in the supernatants of any treatment group, whereas interferon γ -induced protein 10 (IP-10) and monocyte chemoattractant protein 1 (MCP1) levels were reduced in particle-exposed groups (Fig. 6). Treatment with CGS21680 did not rescue the levels of IP-10 and MCP1 (chemoattractants for monocytes/macrophages).

The osteocalcin level was significantly reduced in the supernatants of wear particle-treated calvaria—an effect that was reversed by treatment with CGS21680. Interestingly, IL-10 levels were markedly increased in the supernatants of the particle-exposed, CGS21680-treated calvaria (Fig. 6), a finding that is consistent with prior demonstrations that adenosine A_{2A}R stimulation increases IL-10 production by murine and human macrophages (20, 21), and correlates with prior studies that link a potent osteoclastogenesis inhibitory effect for this cytokine (22).

DISCUSSION

We used the well-established calvarium model with direct exposure to UHMWPE particles to model aseptic loosening *in vivo* and study the effect of an adenosine receptor agonist on this process (17). We report here that wear particles induced increased pitting, porosity, and bone loss in the calvaria of mice. There was a marked increase in the number of osteoclasts in the calvaria of mice exposed to wear particles, in addition to inflammation, which is associated with bone resorption, as indicated by inflammatory mediators (IL-1 β , TNF α) and soluble stimuli for osteoclast formation (RANKL, M-CSF). Adenosine A_{2A}R stimulation reduced bone pitting and loss, abated inflammation, and lowered the number of osteoclasts present in a dose-dependent fashion. Moreover, adenosine A_{2A}R activation led to an increase in the secretion of the potent anti-inflammatory cytokine IL-10—a cytokine that also diminishes osteoclast formation. These effects were mediated via activation of the adenosine A_{2A}R, which was confirmed using adenosine A_{2A}R KO mice unresponsive to treatment with the receptor agonist. Interestingly, although basal bone resorption was not altered in A_{2A}R KO animals, the wear-particles induced greater osteoclast differentiation in these mice, likely due to a greater effect of other adenosine receptors, such as A₁R, which potentiate osteoclast formation.

Our results suggest the therapeutic potential of adenosine A_{2A}R agonists for the prevention and treatment of prosthesis loosening. In the current study, we provide evidence that A_{2A}R activation directly inhibits both murine and human osteoclast differentiation suggesting the translational potential of this approach to prevention of prosthesis loosening. Because A_{2A}R must be activated at the outset of osteoclast differentiation to exert its inhibitory effect (14), the best approach would be to include adenosine A_{2A}R agonists into the cement used at the time of joint replacement surgery. Alternatively, once the wear particle-induced bone damage has occurred, local treatment with A_{2A}R agonists may halt or delay prosthesis loosening.

There are several potential explanations for the effects of adenosine A_{2A}R activation on bone loss, including inhibition of inflammation, with diminished secretion of stimuli for osteoclast formation; and stimulation of secretion of soluble factors from osteoblasts, mesenchymal stem cells, bone marrow stem cells, and T cells that inhibit osteoclast formation and function; and direct inhibition of osteoclast formation and function as we observed *in vitro* with osteoclast precursors derived either from C57Bl/6 mice or adenosine A_{2A}R KO mice (14). In those experiments, CGS21680 directly inhibited M-CSF/RANKL-stimulated osteoclast formation in wild-type cells, but not A_{2A}KO cells, and a selective adenosine A_{2A}R antagonist completely blocked that effect (14).

Several studies have described the effect of purinergic signaling on bone metabolism, although most focus on the role of P2 nucleotide receptors (23), both in humans (24) and in animals (25). In contrast, there are few reports on the role of adenosine receptors in regulating bone metabolism. Recently, evidence for the presence of all four adenosine receptors has been found in human primary bone marrow stromal cells during proliferation and differentiation of osteoblast cells from postmenopausal women cultured under

osteogenic conditions (26). In contrast to our findings in bone marrow-derived osteoclasts Pellegatti and colleagues reported that A_{2A}R stimulation reverses ATP-mediated inhibition of osteoclast formation (via the P₂X₇ receptor) by stimulated peripheral blood monocytes (27). The difference between their results and those reported here is most likely due to differences in receptor expression/function in peripheral blood monocytes as compared to actual bone marrow derived osteoclast precursors.

Another contributing factor to CGS21680-mediated suppression of wear particle-induced bone loss could be suppression of inflammation, leading to diminished secretion of cytokines or other molecules that stimulate osteoclast formation. RANKL is both secreted by osteoblasts and expressed on the surface of these cells, where it can ligate RANK—a member of the TNF receptor super family—on the surface of osteoclasts (28). Prior work has demonstrated that RANKL/RANK interactions are critical for wear particle-mediated stimulation of bone resorption. Ren and colleagues (29) reported that wear particles induced strong pouch tissue inflammation in RANK^{-/-} mice, as manifested by inflammatory cell infiltration, pouch tissue proliferation, and increased gene expression of IL-1 β , TNF α , and RANKL, but was not associated with osteoclastic bone resorption. This suggests that RANKL provides an osteoclast commitment signal that overrides subsequent particle effects that normally result in granuloma formation (fig. S4).

Although we observed no wear particle-induced change in RANKL secretion, we found that CGS21680 suppressed RANKL secretion by wear particle-treated calvaria; such diminished RANKL secretion could lead to less osteoclast differentiation and bone resorption activity. Moreover, adenosine A_{2A}R activation directly suppressed the secretion of M-CSF, a cytokine required for differentiation of osteoclast precursor cells, by wear particle-stimulated calvaria. Thus, it is likely that adenosine A_{2A}R stimulation both suppressed the secretion of osteoclast differentiation factors and inhibited osteoclast response to these factors (fig. S4).

Wear particles induce a strong inflammatory response and adenosine A_{2A}R activation is known to suppress pro-inflammatory factors, such as TNF α and IL-1 β and diminishes inflammatory cell responses to these ligands (10), most likely via direct inhibition of NF- κ B (30). In addition, both of these factors stimulate increased expression of adenosine A_{2A}R (31) and increase the function of these receptors by preventing adenosine A_{2A}R desensitization (32). Our observations are consistent with the hypothesis that adenosine A_{2A}R ligation diminishes osteoclastogenesis and bone resorption as a result of diminished proinflammatory molecule secretion (fig. S4).

Anti-inflammatory factors produced by activated immune cells are able to suppress osteoclastogenesis (33) by two complementary mechanisms: direct inhibition of osteoclast differentiation, as discussed above, and by regulation of IL-10 and osteoprotegerin (33). IL-10 is a key factor in bone resorption in periodontitis (34), and it directly inhibits osteoclast differentiation by blocking RANKL-induced NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) and c-Fos/c-Jun proto-oncogene expression (35). Osteoprotegerin binds RANKL, diminishing its availability to bind RANK and, thus, to stimulate osteoclastogenesis. We found that adenosine A_{2A}R stimulation in wear particle-exposed calvaria markedly increased IL-10 secretion and partially reversed the particle-mediated suppression of osteoprotegerin—effects that are consistent with suppression of osteoclast differentiation and function.

In this study, we also found that treatment with the adenosine A_{2A}R agonist diminished osteopontin and increased osteocalcin levels in bone in the presence of UHMWPE particles. Changes in these proteins, induced by wear particles, have been described recently in the

periprosthetic tissue of patients with aseptically loosened prostheses (36). Osteopontin has also been shown to be required for the progression of titanium particle-induced osteolysis in murine calvaria (37). Moreover, osteopontin may regulate TNF α secretion in the murine calvaria model of wear particle-induced bone resorption (37).

The observations we report here, together with the extensive prior documentation that adenosine A_{2A}R agonists are potent pharmacologic regulators of inflammation (38), indicate that delivery of an adenosine A_{2A}R agonist in bone cement may enhance orthopedic implant survival, delaying or eliminating the need for revision arthroplasty surgery by diminishing bone resorption by osteoclasts, inflammation, and, in turn, prosthesis-loosening. The observation that both daily and alternate day injection of CGS21680 exerted similar inhibition of osteoclast differentiation suggests that inclusion of an A_{2A}R agonist in bone cement or as a prosthesis coating might prevent the early inflammatory reaction that results from early degeneration of the prosthesis, cement, or other components and thereby increase the survival of the prosthesis.

Nonetheless, a number of challenges remain in translating this observation into a therapeutic adjuvant for orthopedic or dental implants. Developing potent A_{2A} agonists that are stable, soluble in biologic solutions and which are able to survive thermal degradation during hardening of cement will need to be considered. Other considerations in developing an agonist include investigating agents that do not interfere with hardening or durability of the cement and that permit both survival of the adenosine receptor agonist in the cement and release upon breakdown of the cement. We have not tested these characteristics for CGS21680 in the current study because other agents with more favorable characteristics (e.g. half-life in biologic solutions, stability, etc.) remain under investigation.

MATERIAL AND METHODS

Animals

C57Bl/6 (wild-type, WT) mice and adenosine A_{2A}R knockout (KO) mice age 6–8 weeks were used. A_{2A}R KO mice were a gift of J. F. Chen (Boston University School of Medicine). Female A_{2A}R KO mice were bred onto a C57BL/6 background (10 backcrosses) in the New York University School of Medicine (NYU SoM) Animal Facility. A_{2A}R KO animals were derived from four original heterozygous breeding pairs for each mouse strain. Mice described as WT were all maintained on the C57Bl/6 background by the breeder (Taconic Laboratories). Genotyping was performed by PCR, as reported previously (39). All protocols were approved by the NYU SoM Institutional Animal Care and Use Committee (IACUC).

Human primary bone marrow stromal cell culture

Bone marrow-derived mononuclear cells were isolated from four multiple myeloma or healthy patients (use was approved by the Institutional Review Board of NYU School of Medicine by Ficoll density gradient using Histopaque-1077 (Sigma-Aldrich) according to the manufacturer's instructions. Subsequently, cells were washed with PBS and seeded as 1×10^5 cells/cm² in α MEM supplemented with human M-CSF (30 ng/ml) for two days. Cells at this stage were considered M-CSF-dependent bone marrow macrophages (BMMs) and used as osteoclast precursors. Induction of differentiation to osteoclasts was achieved by culturing the BMM cells with α MEM containing M-CSF (30 ng/ml) and recombinant human RANKL (30 ng/ml) in the presence or absence of CGS21680 (- Tocris) (10 nM to 10 μ M). ZM241385, an A_{2A}R antagonist, as applied in one study at 1 μ M (Tocris). TRAP staining was performed as previously described (14).

Wear particle preparation

UHMWPE particles, a gift of P. H. Wooley (Via Christi Regional Medical Center), had a mean particle size (equivalent circle diameter) of $1.74 \pm 1.43 \mu\text{m}$ (range 0.05–11.06), with more than 34% of the particles smaller than $1 \mu\text{m}$. For decontamination from endotoxins, the particles were washed twice in 70% ethanol for 24 h at room temperature. The particles were washed in phosphate-buffered saline (PBS) and dried in a desiccator.

Surgical procedure

WT ($n=84$) and A_{2A}R KO ($n=18$) mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine, and a 1-cm midline sagittal incision was made over the calvarium anterior to the line connecting both ears. WT ($n=19$) and A_{2A}R KO ($n=6$) mice received no particles (sham, untreated mice) and the incision was closed without any further intervention. The remaining animals received 3mg of dried UHMWPE particles. Of the 83 mice receiving particles (71 WT, 12 KO), 19 WT and 6 KO mice received 20 μl of 0.9% saline (control) at the site of surgery prior to particle application, and the rest received 20 μl of CGS21680 at concentrations ranging from 1 nM to 1 μM (9 WT mice for each concentration and 6 A_{2A}R KO mice for CGS21680 1 μM), beginning immediately after incision closure and continuing every day until sacrifice (2 weeks later). Water and food were given *ad libitum* until sacrifice. Animals were sacrificed after 14 days in a CO₂ chamber and the calvaria were removed, fixed, and prepared for μCT , histological staining, and cytokine measurements.

Micro-computed tomography (μCT)

After sacrifice, WT ($n=6$) and A_{2A}R KO ($n=4$) calvaria per treatment group were fixed in 70% ethanol and prepared for high-resolution μCT . This 3D imaging technology was used to perform qualitative and quantitative analyses of resorbing areas in murine calvarial bone. Analyses were performed in the μCT core at the Hospital for Special Surgery using the Scanco Medical MicroCT 35 Scanner with 25- μm resolution (KVp: 5T $\mu\text{A}/45$). Every field of view chosen for analysis was scanned by increments using a CCD detector, with an integration time of 400 ms. For qualitative analysis, 3D images of the mice heads were then reconstructed from the cross-sectional slices using the software provided by Scanco Medical MicroCT 35 and processing was done to get direct morphometric measurements in 3D.

For quantitative analysis of UHMWPE particle-induced osteolysis, a square-shaped region of interest across the parietal bone of approximately 4 mm right and left of the midline suture of the skull was placed in one of the 2D-reconstructed slices, as described previously (40). A Matlab software application for analysis of the calvaria bone resorption was utilized. The method used is based on a simple segmentation of the original image in order to extract the resorbed areas. We used a basic thresholding coupled to a morphological close operator to obtain accurate segmentation results and a region of interest (ROI) of total area. Using a counter, the number of white pixels (resorbed area) and the number of black pixels (non resorbed area) was calculated and a percentage of the total area resorbed was estimated by the ration resorbed area/total area.

Histological studies

WT ($n=3$) and A_{2A}R KO ($n=3$) calvaria per group were removed and fixed in 4% paraformaldehyde for 48 hours, followed by decalcifying in 10% EDTA for four weeks and paraffin embedding. Sections (5 μm) were cut and H&E staining was performed. Photomicrographs were taken at an original magnification of 100X or 400X. Inflammatory infiltration in midsagittal suture areas was quantified from 5 images per animal using Sigma Scan Pro Image analysis version 5.0.0 software.

TRAP staining was carried out with the Acid Phosphatase Leukocyte TRAP kit (Sigma-Aldrich) following the manufacturer's protocol after deparaffinized and acetate buffer washing processes.

Immunohistochemistry analysis of markers for osteoclasts [cathepsin K, RANK (Santa Cruz Biotechnology) and RANKL (Abcam)], macrophages [CD163 (Abcam)], inflammation [TNF α (Abcam)], and bone remodeling [α SMA, osteopontin, osteocalcin, and osteoprotegerin (Abcam)] were carried out in WT and A_{2A}R KO calvaria in paraffin-embedded sections. Briefly, deparaffinized and hydrated sections were incubated with Proteinase K Solution (20 μ g/ml in TE Buffer, pH 8.0) for 15 minutes in water bath at 37°C. After cooling, the sections were rinsed with PBS, the internal peroxidase was removed with 3% H₂O₂ in methanol by incubating sections for 15 minutes at room temperature. After rinsing sections in a BSA solution (3 wt% in PBS), 1 hour blocking at room temperature (BSA solution containing 1% Triton and 5% FBS) was performed. Primary antibody diluted in PBS with 3 wt% BSA (cathepsin K 1:25, RANK 1:100, RANKL 1:200, CD163 1:200; TNF α 1:200, α SMA 1:400, osteopontin 1:100, osteocalcin 1:200 and osteoprotegerin 0.5 μ g/ml) was incubated overnight at 4°C in a humidifying chamber. After three washes in PBS-BSA solution, secondary goat anti rabbit HRP antibody (Santa Cruz Biotechnology) was incubated with the sections for 1 hour at room temperature. Sections were developed Fast 3'3'-Diaminobenzidine (Sigma-Aldrich), prepared following the manufactures protocol and, after washing with PBS, counterstained with hematoxylin (Sigma-Aldrich) for 1 minute. Slides were mounted using Permount mounting media (Fisher Scientific).

Images were observed under light microscope (Nikon) equipped with Nis Elements F3.0 SP7 software and under a Leica microscope equipped with SlidePath Digital Image Hub Version 3.0 software.

Measurement of cytokines, chemokines, and bone metabolism markers in calvaria supernatants

Sham animals ($n=10$), saline-treated particle-exposed control animals ($n=10$), and CGS21680-treated, particle-exposed animals ($n=10$) were sacrificed 14 days after surgery and calvarias were harvested. The harvested calvarias were placed in 2 ml of α MEM medium containing 10% FBS and 1% penicillin-streptomycin (Invitrogen, Gibco) and incubated at 37°C in 5% CO₂ for 72 hours as described previously (41). The medium was then centrifuged to remove any debris. Concentrations of, GM-CSF, M-CSF, IP-10, MCP-1, IL-1 β , IL-6, TNF α , IL-10, RANK, and osteocalcin were simultaneously determined in calvaria tissue supernatant samples using multi-analyte profiling performed on the Luminex-200 system and the XMap Platform (Luminex Corporation). Calibration microspheres for classification and reporter readings as well as sheath fluid were also purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the Beadview software. All analyses were performed according to the manufacturers' protocols.

Statistical analysis

Statistical significance for differences between groups was determined by use of ANOVA or Student's *t* test. All statistics were calculated using GraphPad software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank S.R. Goldring for critical reading of the manuscript, M. Hawly and E. Sellam for developing the Matlab compiler software, M. Quirno, I. Immerman, S. Hadley, and R.D. Howell for help with surgical procedures, Y.J. Lee (NYU Hospital for Joint Diseases) for help in sectioning the samples, L. Lukashova for technical assistance in the microCT core (Hospital for Special Surgery), and L. Chiriboga for advice in the histology.

Funding: The National Institutes of Health (AR56672, AR56672S1, AR54897, AR046121) and the NYU-HHC Clinical and Translational Science Institute (UL1RR029893). NYUCI Center Support Grant, NIH/NCI 5 P30CA16087-31.

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Figure 1.

Bone pitting in mouse calvaria after exposure to wear particles. Mice were administered UHMWPE particles and either treated with saline or the adenosine $A_{2A}R$ agonist CGS21680. Control mice (sham) were not exposed to particles ($n=6$). Mice were injected daily and after 2 weeks animals were sacrificed. **(A)** Representative μ CT images of calvaria from wild-type animals. **(B)** Morphometric quantitation of bone pitting in the presence of varying doses of CGS21680 (1 nM to 1 μ M). Data were calculated as the percentage of the area of bone pitting in the saline-treated, particle-exposed mice. Each point represents the mean $\pm$ SEM of determinations in 6 individual mice per group. *** $P<0.001$ compared to control (100% pitting), ANOVA. **(C)** Representative μ CT images of calvaria from $A_{2A}R$ KO animals. **(D)** Morphometric quantitation of bone pitting in the presence of CGS21680 (1 μ M). Data were calculated as the percentage of the area of bone resorption in the saline-treated, particle-exposed mice. Each point represents the mean $\pm$ SEM of determinations in 4 individual mice per group. NS compared to control, ANOVA **(E)** Morphometric quantitation of bone resorption in the presence of varying doses of CGS21680 injected every other day in wild-type mice. Data were calculated as the percentage of the area of bone pitting in the saline-treated mice. Data are means $\pm$ SEM ($n=5$). *** $P<0.001$ compared to saline, ANOVA.

Figure 2.

Effect of A_{2A} R stimulation on osteoclast formation by human bone marrow cells. **(A)** Human primary bone marrow monocytes were fixed and stained for TRAP after culture for 7 days in the presence of M-CSF and RANKL, with or without CGS21680 (10 μ M to 10 nM). TRAP-positive cells containing three or more nuclei were counted as osteoclasts. **(B)** Number of TRAP+ osteoclast [% relative to control (RANKL+)] expressed as the means of 4 cultures in duplicate in myeloma bone marrow-derived osteoclasts. **(C)** Number of TRAP+ osteoclast [% relative to control (RANKL+)] expressed as the means of 4 cultures in duplicate in healthy bone marrow-derived osteoclasts. * P <0.05, *** P <0.001, compared to RANKL, # P <0.05 compared to CGS21680 1 μ M, ANOVA.

Figure 3.

Inflammation in mice calvaria after exposure to wear particles in the absence or presence of CGS21680. Calvaria were stained with hematoxylin & eosin to determine the presence of inflammation in the outer bone surface. (A) Calvaria were exposed to saline only (sham) or to UHMWPE particles, the latter in the absence or presence of the adenosine A_{2A}R agonist CGS21680 (1 μM). (B) The area of inflammatory infiltrate was quantified from (A) and expressed as a percentage of the area of the saline-treated, particle-exposed mice. Data are means±SEM (*n*=3 per group). ****P*<0.001, ANOVA. (C) Calvaria from A_{2A}R KO mice after exposure to saline only (sham) or UHMWPE particles without or with CGS21680 (1 μM). (D) The area of inflammatory infiltrate was quantified from (C) and expressed as a percentage of the area of the saline-treated, particle-exposed mice. Data are means±SEM (*n*=3 per group). (E) Comparison between WT and A_{2A}R KO in saline-treated, particle-exposed mice was quantified expressed as a percentage of the area of the sham mice. Data are means±SEM (*n*=3 per treatment group). ****P*<0.001, ANOVA.

Figure 4.

TRAP staining for osteoclasts in mice calvaria. (A) Representative images of saline only (sham) or UHMWPE particle-exposed calvaria with or without CGS21680 (1 μM) treatment. Histology for 10 to 100 nM CGS21680 is shown in fig. S2. Red cells are positive for TRAP staining (arrow). (B) Quantification of the number of osteoclast/hpf (high power field) in (A). Osteoclasts were counted in 5 different images for each of 3 mice. Data are means $\pm$ SEM ($n=3$ per group). *** $P<0.001$ related to saline, ANOVA. (C) Calvaria from A_{2A}ARKO mice after exposure to saline alone (sham) or wear particles in the absence or presence of CGS21680 (1 μM). Red cells are positive for TRAP staining (arrow). (D) Quantification of the number of osteoclast/hpf in (C). Osteoclasts were counted in 5 different images for each of 3 mice. Data are means $\pm$ SEM ($n=3$ per group). *** $P<0.001$ related to saline (ANOVA).

Figure 5.

Immunohistochemistry for markers of osteoclasts, macrophages, inflammation, and bone remodeling in wild-type mice. **(A)** Calvaria were processed and immunohistologic staining carried out on calvaria from sham or UHMWPE-exposed mice, the latter treated with either saline or CGS21680 (1 μ M). Shown are representative sections of calvaria (from $n=3$ mice) stained for cathepsin K, RANK, RANKL, CD163, TNF α , α SMA, osteopontin, osteocalcin, and osteoprotegerin. **(B)** Immunohistochemistry quantifications of cells/hpf (high power field) for (A). Data are means $\pm$ SEM for 5 different slides per untreated mouse ($n=3$) and each mouse treated with either particulate and saline ($n=3$) or particulate and 1 μ M CGS21680 ($n=15$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared to untreated, ANOVA. Scale bar indicates 50 μ m. All images are taken with the same magnification.

Figure 6.

Cytokine, chemokine, and bone metabolism markers in mouse calvaria supernatants. Calvaria from untreated (sham) animals and animals exposed to wear particles in the absence or presence of CGS21680 (1 μ M) were cultured in medium for 4 hours before collection of supernatants and quantification of cytokines/chemokines and bone metabolism markers. Data are expressed as mean percentages of sham $\pm$ SEM from $n = 10$ mice per treatment group assayed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus particle-exposed, saline-treated controls; ANOVA.

Table 1
μCT analysis of mouse calvarias after treatment

(A, B) Digital morphometric analysis of μCT images from wild-type (A) and A_{2A}R knockout (B) mice.

Treatment	<i>n</i>	Bone volume (BV) (mm ³)	BV/Total volume (TV)	Bone mineral density (BMD) (mg)
(A) Wild-type				
Sham	6	2.05 ± 0.09 *	0.7964 ± 0.0002 *	821.11 ± 138.79 **
Particulate + saline	6	2.46 ± 0.22	0.7436 ± 0.0005	810.28 ± 153.07
Particulate + 1 μM CGS21680	6	2.52 ± 0.19 *	0.7765 ± 0.0003 *	809.14 ± 161.64 *
(B) A _{2A} RKO				
Sham	4	2.50 ± 0.13	0.7644 ± 0.011 ***	832.79 ± 15.65
Particulate + saline	4	2.54 ± 0.11	0.7324 ± 0.012	822.84 ± 4.78
Particulate + 1 μM CGS21680	4	2.43 ± 0.24	0.7455 ± 0.020	816.41 ± 12.92

Data are means ± SEM.

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.005$, compared to saline (Student's *t* test).