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Actin and ERK1/2-CEBP β signaling mediates phagocytosis-induced innate immune response of osteoprogenitor cells

Heon Goo Lee^{a,b}, Hiroshi Minematsu^a, Kyung Ok Kim^a, Ayse Aydemir^a, Mike J. Shin^a, Saqib Nizami^a, Kook Jin Chung^{a,c}, Anny C. Hsu^a, Christopher R. Jacobs^b, and Francis Y. Lee^{a,*}

^aDepartment of Orthopaedic Surgery, Columbia University College of Physicians and Surgeons, New York, New York, USA

^bDepartment of Biomedical Engineering, Columbia University, New York, New York

^cDepartment of Orthopaedic Surgery, Kangnam Sacred Heart Hospital, College of Medicine, Hallym University

Abstract

Wear particles at the host bone-implant interface are a major challenge for successful bone implant arthroplasties. Current understanding of aseptic loosening consists of macrophage-mediated inflammatory responses and increasing osteoclastogenesis, which lead to an imbalance between bone formation and resorption. Despite its significant role in bone regeneration and implant osteointegration, the osteoprogenitor response to wear particles has been examined recent years. More specifically, the intracellular mechanism of osteoprogenitor mediated inflammation has not been fully elucidated. In this study, we examined the role of osteoprogenitors and the cellular mechanism by which metal wear particles elicit an inflammatory cascade. Through both *in vivo* and *in vitro* experiments, we have demonstrated that [1] osteoprogenitor cells are capable of initiating inflammatory responses by phagocytosing wear particles, which lead to subsequent accumulation of macrophages and osteoclastogenesis, and [2] the ERK_CEBP β intracellular signaling is a key inflammatory pathway that links phagocytosis of wear particles to inflammatory gene expression in osteoprogenitors. AZD6244 treatment, a potent inhibitor of the ERK pathway, attenuated particle mediated inflammatory osteolysis both *in vivo* and *in vitro*. This study advances our understanding of the mechanisms of osteoprogenitor-mediated inflammation, and provides further evidence that the ERK_CEBP β pathway may be a suitable therapeutic target in the treatment of inflammatory osteolysis.

Keywords

ERK; Actin; IL6; Cox2; Cytochalasin D; AZD6244; particle; osteolysis

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*Address correspondence to: Francis Y. Lee, Department of Orthopaedic Surgery, Columbia University Medical Center, 630 W 168th Street, Black Building 14-1412, New York, NY 10032, Phone: 212 305 7965, Fax: 212 305 2741, fl127@columbia.edu.

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Introduction

Advances in biomaterials have led to an increased usage of bone and dental implants in clinical settings. Despite this popularity of the implants, clinical studies have shown that wear debris mediated inflammation leads to operative site pain, limitation of motion, osteolysis-induced pseudarthrosis, and, consequently, bone implant failure.[1-3] Exposure to particulate wear debris influences host cellular responses including cell proliferation, apoptosis, osteogenic differentiation, and possible combinations of these factors.[4-6] Due to the clinical significance, inflammation at host bone-implant interface has become an important scientific issue.

The culprit behind aseptic implant loosening is believed to be the wear particles such as titanium (Ti), polyethylene, and cobalt chromium. These particles initiate phagocytosis through macrophages and monocytes, leading to a proinflammatory response. [7-9] Macrophages and monocytes have efficient phagocytic receptors including Fc γ receptor and complement receptor 3, which are activated upon particle binding. These activated receptors induce cellular signals which lead to remodeling of cytoskeletal elements, mainly actin, at the host-implant interface site of internalization. [10-12] Macrophages and monocytes accumulate at the site of damage and secrete several inflammatory cytokines such as IL1 β and TNF α , which significantly affect cellular behavior.[13] These cytokines induce an imbalance between bone formation and resorption by enhancing osteoclast formation. [14-16] In addition to IL1 β and TNF α , considerably altered IL6 and PGE2 levels are frequently detected in several pathological conditions such as osteoporosis and osteolysis around the bone implant. [8, 17, 18]

The primary function of osteoprogenitor cells, as a precursor of osteoblasts, is to form bone tissue that constitutes the skeletal system.[19] Recent studies have highlighted additional roles of osteoprogenitor cells in the regulation of osteoclastogenesis by elaborating the function of RANKL and MCSF.[20, 21] Since osteoprogenitors are resident cells at the host bone-implant interface, the fundamental questions arise as to the role of osteoprogenitor cells at the host bone-implant interface and their ability to initiate a cascade of inflammatory response. Although many studies have examined the interaction between biomaterial debris and macrophages[7-9], the role of osteoprogenitor cells in biomaterials-related inflammation has not been extensively studied.

The objectives of this study were to elucidate (1) the role of osteoprogenitor cells on metal particle-mediated osteolysis and (2) its phagocytic machinery in inflammatory cascade. To achieve the objectives, the responses of osteoprogenitors to Ti metal particles are explored through *in vivo* mouse calvaria model and *in vitro* experiments for elucidating inflammatory intracellular pathway.

Materials and Methods

Study Design

We utilized the mouse calvaria resorption model to study the role of osteoprogenitor cells in metal particle-mediated osteolysis. In this model, Ti particles were implanted on the mouse pericranium, inducing an inflammatory response with osteolysis, which closely mimic the inflammatory response observed in clinical aseptic loosening.[22] Outcomes were evaluated by immunohistochemical analyses. Next, the intracellular mechanism of osteoprogenitor cells in the inflammatory cascade was further investigated. We elucidated phagocytic capacity of osteoprogenitor cells by analyzing actin remodeling and investigated a key intracellular signal transduction process through Real time RT-PCR, ELISA, Western blot, and Immunostaining.

Titanium Particles

Commercially pure titanium particles (Ti) with an average diameter of 1-3µm were purchased from Johnson Matthey Chemicals (Ward Hill, MA) as previously described by Ragab *et al.*[23] For removal of lipopolysaccharide (LPS), ten cycles of the following two steps were performed. In the first step, Ti particles were incubated in 25% nitric acid at room temperature for 20 hours followed by washing three times with phosphate-buffered saline (PBS). In the second step, the Ti particles were treated with a mixture of 0.1 N NaOH and 95% ethanol at 30°C for 18-20 hours, followed by three times of washes with PBS between cycles. The endotoxin level was determined by a high-sensitivity version of the Limulus Amoebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA) as previously described (i.e.<0.01EU/ml). [27] Prior to use, particles were sonicated for 15 minutes.

Mouse Calvaria model

For the *in vivo* study, Dr. Schwarz's mouse calvaria resorption model was utilized with modifications.[22] Five 6-7weeks old male C57BL/6J mice were used in each group. They were anesthetized with 80 mg/kg of ketamine and 7 mg/kg of xylazine by intraperitoneal injection. 30 mg of Ti particles with/without 50mg/kg of AZD6244 (ARRY-142886) in 0.5 ml of sterile phosphate buffered saline were spread on pericranium with subcutaneous injection. As a major component of the pericranium, osteoprogenitor cell is one of the first cell lines to be exposed to metal particles in this model.[24-26] Mice in the sham group underwent surgery without particle injection. Calvarial bones with pericranium were harvested after 1, 3 and 7 days. They were fixed with 4% paraformaldehyde at 4°C for 6 h, and were decalcified with 10% EDTA for 2 days. 5 µm thick paraffin embedded calvarial bones were prepared for immunohistochemistry. All procedures are approved by Institutional Animal Care and Use Committee (IACUC).

Immunohistochemistry

The primary antibodies used were *phosphor*-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA), ERK1/2 antibody (Cell Signaling Technology, Danvers, MA), Cox2 antibody (Cell Signaling Technology, Danvers, MA), IL6 antibody (Abcam, Cambridge, MA), and CD11b antibody (Abcam, Cambridge, MA) at a 1:50 dilution. Immuno-staining was performed using the HRP-ABC and HRP-DAB Cell & Tissue Staining Kits (R&D Systems, Minneapolis, MN). The calvarial sagittal suture line was stained using the Acid Phosphatase Leukocyte (TRAP) Kit (Sigma-Aldrich), and TRAP positive multinucleated osteoclast cells were counted under 10× magnification.[22]

Cell Culture & Reagents

To understand the inflammatory mechanism of osteoprogenitors, we investigated two different types of osteoprogenitors: human mesenchymal stem cells (hMSC) that had been induced into osteogenic differentiation and the murine pre-osteoblastic cell line MC3T3-E1. Human mesenchymal stem cells (hMSC) (Cambrex Inc., Walkersville, MD) were cultured in Mesenchymal Stem Cell Basal Media (MSCBM) containing mesenchymal cell growth supplement (MCGS), L-Glutamine, and penicillin-streptomycin (Lonza). For osteoprogenitor differentiation, the medium was changed from M7SCBM to hMSC Differentiation Basal Medium-Osteogenic containing dexamethasone, L-Glutamine, Ascorbate penicillin-streptomycin, MCGS, and β-Glycerophosphate. The murine pre-osteoblastic cell line MC3T3-E1 cells were purchased from American Type Culture Collection (ATCC; Manassas, MD, USA) and maintained in Minimum Essential Medium α (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini BIO) and 1% antibiotic/antimycotic (Gemini BIO) at 37°C and 5% CO₂. 1×10⁶ differentiated hMSC or MC3T3-E1 cells were seeded on 10cm dishes two days before the Ti particle treatment. In

order to examine the role of actin in phagocytosis, actin polymerization was blocked by cytochalasin D (CYD). Cells were treated with CYD for 30min, prior to the particle treatment. AZD6244 (ARRY-142886), a potent, selective, and ATP-uncompetitive inhibitor of MAPK/ERK kinase1/2, was purchased from Selleck Chemicals (Houston, TX) and was used to treat the cells for 1 hour prior to the particle treatment. Cells were directly stimulated with Ti particles for 3 hours. Unattached particles were flushed by rinsing the plates 10 times with PBS followed by inverting the plates for 10 minutes to allow any residual unattached particles to be removed by gravity.

Quantification of Attachment of Ti Particles

For quantification of particle attachment, we increased the contrast between metal particles and entire cell surface by transfecting cells with GFP cDNA. Cells were seeded on 6-well tissue culture plates and transfected with 2 µg of cDNA using the FuGene HD transfection reagent (Roche) according to the manufacturer's instructions. The transfection efficiency was higher than 95%. A GFP fluorescence image obtained from fluorescence microscope (ZEISS) was used to portray the complete cell surface in green. An image from bright field microscopy view showed the Ti particles as dark hole. Using the GFP image as the threshold for the cell surface, a customized Java program analyzed the percentage of cell surface which occupied by Ti particles (dark spot). The area was calculated by summation of the total pixel area of the cell surface image and the Ti particle image. Twenty cells were tested.

Quantification of Internalization of Ti Particles

To quantify particle internalization, actin of osteoprogenitor cells was stained with 0.5 µM Alexa Fluor 488-conjugated phalloidin. (Molecular Probes, Eugene, OR) 3D images of GFP(+) actin stained cells were obtained with a confocal microscope with an axial slice thickness 0.22µm and reformatted in X, Y, and Z dimensions. To distinguish completely phagocytosed (internalized) particles and membrane-bound particles, actin filaments in a selected area were magnified and reformatted into X-Y (Axial), X-Z (Coronal) and Y-Z (Sagittal) planes. While the internalized particles are fully covered by actin filaments in axial, sagittal, and coronal view (Ring pattern: O), membrane-bound particles show a hemisphere of actin filaments in sagittal and coronal view (Cup pattern: U). For quantitative analysis of internalization, we selected three-hundred cells that exhibited Ti attachment and counted the number of cells with internalized particles.

RNA Isolation and Real-Time RT-PCR

Immediately after the stimulation, total RNA was isolated from cells using RNeasy Mini Kit (Qiagen). The 260/280 absorbance ratio was measured for verification of the RNA purity. Single stranded cDNA was synthesized from total RNA with the SuperScript III system (Invitrogen). Realtime RT-PCR for each target was performed with LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) using the Realplex system (Eppendorf). Primers sets used were: for mouse-GAPDH, (5'-AGAACATCATCCCTGCATCC-3') and (5'-AGTTGCTGTTGAAGTCGC-3'); for mouse-IL6, (5'-AAAGCCAGAGTCCTTCAGAG-3') and (5'-CTAGGTTTGCCGAGTAGATC-3'); for mouse-Cox2, (5'-ACATCGATGTCATGGAAGT-3') and (5'-GGACACCCCTTCACATTATT-3'); for human-GAPDH, (5'-GAAGGTGAAGGTCTGGAGTC-3') and (5'-GAAGATGGTGATGGGATTTC-3'); for human-Cox2, (5'-GAGTACCGCAAACGCTTTATGCT-3') and (5'-ACTGAATGAAGTAAAGGGACAGCC-3'). Human-IL6 primer was purchased. (santa cruz biotechnology, inc, Santa Cruz, CA). The thermal cycling conditions consisted of preheating (10 min at 95°C) and 40 cycles of denaturation (15 sec at 95°C), annealing (15 sec at 60°C) and elongation (20 sec at 72°C). For each sample, mRNA levels of each gene were normalized to GAPDH levels.

Protein Isolation & Western Blot Analysis

The Nuclear Extract Kit (Active Motif, Carlsbad, CA) was used to isolate nuclear and cytoplasmic extracts. These extracts were further homogenized by sonication for 15 min at a 10% duty cycle and 4 °C (Sonifier 250; Branson, Danbury, CT). The samples were centrifuged at 10,000×g for 10 min, and the supernatant was collected for analysis. Protein quantification was performed using the BCA Protein Assay reagents (Pierce, Rockford, IL) and 20 µg of protein extract was used for Western blotting. Samples were run in 4-20% SDS-polyacrylamide gels (Invitrogen) and electrotransferred to PVDF membranes which were blocked and probed with primary antibodies; *phospho*-ERK (pERK), ERK (tERK), and *phospho*-CEBP-β (pCREB β) (Cell signaling, Cambridge, MA) overnight at 4 °C. Blots were washed of excess antibodies in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and with 10% FBS, the blots were probed with a HRP-conjugated secondary antibody (Cell Signaling Technologies, Beverly, MA), and the protein were detected using the enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were stripped with Restore® Western blot stripping buffer (Pierce) and reprobed with Lamin A/C (Cell Signaling) and GAPDH (Chemicon, Temecula, CA) antibodies as loading controls.

Immunocytochemistry

After AZD pretreatment and Ti stimulation, cells on 4-chamber slide glasses (BD) were fixed with 4% paraformaldehyde and ethanol, rinsed with PBS, and air-dried overnight for immunocytochemistry. The slides were incubated in a blocking solution containing 5% serum, washed, and incubated with *Phospho*-ERK antibody (Cell signaling, Cambridge, MA) overnight at 4 °C. The slides were then washed and incubated with a goat anti-rabbit IgG secondary antibody conjugated with Alexa 488 (Invitrogen) for 2 hours and visualized using the Axiovert imaging system (Zeiss, Thornwood, NY). Images were analyzed with an NIH Image J program.

ELISA

With or without the CYD and AZD pretreatment, the cells were incubated with 0.05% v/v Ti for 3, 6, and 24 hours. Then the medium was collected for ELISA. Mouse IL6 and PGE2 ELISA kit (R&D system, Minneapolis, MN, USA) was used for a quantitative measurement.

Statistical Analysis

An one-way ANOVA was used to test for the effect of Ti, CYD, AZD, and combination effects on phagocytic activity and inflammatory gene expression. Student-Newman-Kuels multiple comparisons tests were performed to check for significance between levels for each of the factors. Statistical significance was taken as $p < 0.05$.

Results

In vivo Metal Particle Mediated Osteolysis in Mouse Calvaria model

The effects of osteoprogenitor cells on inflammatory osteolysis were assessed through time-course experiment. Ti particles (Black Arrow) were implanted on pericranium (white arrow) and harvested on days 1, 3 and 7 after the operation (Fig 1.A). Immunostaining of CD11b was performed to confirm the existence of macrophages in pericranium. Macrophages were not observed in pericranium in sham and Day 1 groups (Fig 1.B). In contrast, significant increase of macrophage accumulation was detected at the site of Ti particle implanted in day 7. As shown in Fig 1.C&D, the number of TRAP positive osteoclasts (red arrow) on calvaria also markedly increased from day 3 to day 7. All Sham groups of different days have an average of four TRAP positive osteoclasts around the suture line. pERK, Cox2, and IL6

immunostainings were conducted to evaluate the inflammation of pericranium. In the Ti treated group, significant elevation of pERK, Cox2, and IL6 was observed in the pericranium from day 1. Steady increase of pERK (Brown) was detected until day 7. (Fig 2.A) This elevation corresponds with the increasing level of Cox2 and IL6 expression between day 1 and day 7 (Fig 2.B&C).

Capacity of Phagocytosis in Osteoprogenitor Cell

To study the capacity of phagocytosis in osteoprogenitor cells, quantification of particle attachment on cell surface and internalization by actin remodeling were performed. Schematic diagram of cell surface occupied by particles when treated with Ti particles is shown in Fig 3.A. The custom-written JAVA program showed the Ti occupied area, which is compromised of attachment and internalization of Ti, increased in a dose-dependent manner (Fig 3.B). In the presence of CYD, the occupied area significantly decreased (Fig 3.B). Under fluorescence, actin staining revealed a significantly pronounced occurrence of Ti particle attachment on actin fibers (Fig 3.C). In the CYD treated group, a marked decrease of Ti particle attachment was detected. (Fig 3.C).

Actin internalization of Ti particles was investigated via analysis of confocal images. The no treatment group showed actin stress fibers in alignment whereas the Ti treated group showed disorganized actin fibers with actin cup and ring formation. (Fig 4.A) The completely internalized particles were identified through actin ring formation (**O** in Fig 4B), and membrane bounded particles were identified through actin cup formation. (**U** in Fig 4.B) In the presence of CYD, actin filaments depolymerized and became disorganized (Fig 4.A). The axial view of the Ti+CYD group showed a similar sized single, dark hole. Most particles, however, were membrane bounded particles. (Fig 4.A) Among the cells that exhibited Ti attachment, approximately 50% of the cells internalized Ti particles in the Ti-only treated group, while only 10% of the cells in the Ti+CYD group internalized the particles. (Fig 4.C)

Induction of IL6 and Cox2 Gene Expression by Ti Particle Mediated-Actin Remodeling

To investigate whether particle-mediated actin remodeling is involved in inflammatory gene expression, we assessed the relative mRNA expression of TNF α , IL1 β , IL6, and Cox2 using real time RT-PCR. Unlike macrophages, TNF α and IL1 β mRNA levels were not affected by Ti and CYD treatment. (Fig 5.A) The IL6 and Cox2 mRNA levels, however, significantly increased with dynamic actin remodeling after particle stimulation in both MC3T3-E1 and hMSC-derived osteoprogenitors (hMSCs). (Fig 5.C&D) As both internalization and attachment of Ti particles significantly decreased by CYD, IL6 and Cox2 gene expression decreased to a basal level even in the presence of Ti particles. (Fig 5.C) Correspondingly, the phosphorylation of ERK was elevated by Ti particles but inhibited by CYD in a dose dependent manner. (Fig 5.B)

Key Inflammatory Signaling Pathway in Osteoprogenitor Cells

We further clarified whether a loss of ERK has a distinct role in the inflammatory response in osteoprogenitor cells. The phosphorylation of ERK in MC3T3-E1 and hMSCs was inhibited by AZD. Cells treated with AZD exhibited a selective and drastic inhibition of the ERK phosphorylation (pERK) in both nucleus and cytoplasm. (Fig 6. B) Furthermore, AZD treatment decreased pERK level, which is induced by Ti particles in both MC3T3-E1 and hMSCs (Fig 6.A). The level of pERK expression correlated well with Cox2 and IL6 expression in MC3T3-E1 and hMSCs. Real time RT-PCR verified that Ti particles elevate the IL6 and Cox2 mRNA, while AZD pretreatment dramatically decreased the IL6 and Cox2 mRNA levels in the both MC3T3-E1 and hMSCs. (Fig 6.C&D).

Direct Effects of Cytochalasin D on the ERK Pathway Mediated-Inflammatory Responses

To clarify that particle-mediated actin remodeling is the main cause of the pERK, the direct effects of CYD on ERK and IL6 & Cox2 mRNA expression were examined. LPS is an endotoxin which elicits strong immune responses in animal cells. Since LPS induces phosphorylation of ERK without actin dynamics, we assessed the unexpected effects of CYD on ERK, IL6, and Cox2 expression using LPS. MC3T3-E1 cells were exposed to LPS for 30, 60, and 180 minutes with and without CYD pretreatment. CYD neither decreased nor increased the phosphorylation of ERK, which was elevated by LPS. (Supplement 1.A). Under the same condition, IL6 and Cox2 mRNA levels are also unaffected by CYD, but are increased by LPS. (Supplement 1.B&C)

The Role of ERK1/2-CEBP/β Pathway in IL6 and Cox2 Gene Expression

To investigate the ERK mediated inflammatory pathway in depth, we targeted C/EBP-β as a primary inflammatory transcription factor. Since immunocytochemistry analysis has shown that the phosphorylated-ERK (pERK) is located in both the nucleus and cytoplasm (Fig 6.B), we assessed the level of pERK and phosphorylation of C/EBP-β in the cytoplasm and nucleus, separately. Notably, when treated with Ti particle alone, MC3T3-E1 cells were found to up-regulate production of phosphorylated C/EBP-β (pC/EBP-β) and ERK in nucleus. This particle-mediated phosphorylated C/EBP-β was blocked by CYD and AZD, correlating with pERK expression (Fig 7.A).

Next, the levels of IL6 and PGE2 were measured by ELISA to assess the antiinflammatory effects of CYD and AZD. MC3T3-E1 cells were treated with Ti particles for three different time points (3hr, 6hr and 24hr) in the presence or absence of AZD and CYD pretreatment. Compared to the no treatment, the Ti treatment resulted in significant increase in IL6 and PGE2 production within 24 hours. The addition of AZD or CYD reduced the production of both IL6 and PGE2 at the 24hr point. (Fig 7.B&C)

Therapeutic potential of ERK inhibitor for inflammatory osteolysis

We have shown that activation of ERK signaling pathway is required for inflammatory response in osteoprogenitor cells. Thus, therapeutic effects of ERK inhibitor on preventing Ti particles mediated osteolysis were investigated through *in vivo model*. Expression of inflammatory mediators (Cox2, IL6, pERK) and osteoclastogenic gene (TRAP, CD11b) were evaluated. Four groups were tested in this experiments: Sham, Ti_Day7, AZD_Day7, and Ti+AZD_Day7. Consistent with our time course experiment, Ti treatment increased expression of pERK, Cox2, and IL6. AZD treatment significantly decreased the expression of these inflammatory responses. (Fig 8.A&B&C) Sham group showed similar level of inflammatory expression as the AZD only treated group. Similar trends to inflammatory responses were observed in TRAP and CD11b staining. Ti treated group showed increased number of TAP positive osteoclasts and macrophages. AZD treatment markedly decreased osteoclastogenesis induced by Ti treatment. (Fig 8.D&9)

Discussion

Osteolysis at the site of implant attachment is a multi-factorial problem. Particularly, imbalance of bone remodeling caused by wear particle around an implant is known to be a major cause of the osteolysis.[2, 27] Since osteoblasts derived from mesenchymal stem cells are responsible for both initial osseointegration of implants and ongoing bone regeneration[28, 29], it is important to elucidate the responses of osteoprogenitor cells to wear particles. While several studies identified that wear particles have adverse effects on viability, differentiation, and osteogenic function of osteoprogenitor cells[6, 28, 30], the participation of osteoprogenitor cells in the inflammatory process is not well studied. In this

study, we investigated innate immune functions of osteoprogenitor cells in inflammatory osteolysis and focused on the molecular mechanisms of osteoprogenitors in the presence of wear particles. Ti particles, which are commonly generated from numerous orthopaedic prostheses such as metal-on-metal bearing surfaces, spine pedicular screws, and dental implants, are widely used for inflammation studies. Since our study is focused on the in-depth mechanism of cellular inflammatory response to metal particles, we used Ti particles for precise analysis compared to other inflammatory studies.

Several studies have shown intense osteoclastogenesis and inflammation responses from mouse calvaria model.[22, 31, 32] Similar to these studies, our *in vivo* experiments also resulted in significant increase of TRAP positive osteoclasts and resorption pits on the calvaria suture line after Day7.(Fig 1.C&D) We did not quantify osteolysis by calculating the suture area due to a wide range of variable normal suture area. Due to significant geometrical differences of the calvaria, considerable error in calculating suture area was observed. Interestingly, our time course experiments of *in vivo* mice calvarial model revealed that particle contacted osteoprogenitor cells are able to initiate inflammatory cascade without the help of macrophages. High levels of pERK, Cox2, and IL6 expression were observed from Day 1 and steadily increased until Day7. The early increased levels of IL6 and Cox2 were involved predominantly in a late increase in number of osteoclasts and macrophages in Day7. (Fig1,8&9) IL6 and Cox2 driven-PGE2 are known to recruitment macrophages and to accelerate the induction of IL1 β and TNF α secretion in monocytes and macrophages, leading to osteoclast formation. [33-37] In summary, our results may imply that the increased IL6 and PGE2 levels from particle contacted-osteoprogenitor cells exaggerate the macrophage mediated-osteolysis by promoting osteoclast differentiation and assisting macrophage accumulation.

As other studies indicated, we also confirmed that osteoprogenitor cells engulf metal particles and induce pro-inflammatory cytokines.[6, 38, 39] Osteoprogenitor cells are capable of phagocytosing metal particles with dynamic actin remodeling. In this study, we identified that actin mediated phagocytosis results in activation of ERK pathway, leading to expression of inflammatory mediators. More specifically, IL6 and Cox2 expressions were found to be the end result of this ERK pathway. Similar to macrophages[40], inhibition of ERK pathway using AZD decreased inflammatory responses of osteoprogenitor cells. (Fig6) Interestingly, these phagocytosed particles increase IL6 and Cox2 gene expression in osteoprogenitor cell, while IL1 β and TNF α .gene expression was not affected by particles. Since C/EBP- β has downstream effects on the production of inflammatory gene expression such as IL6 and Cox2[41-43], we targeted C/EBP- β as a key transcription factor in metal particle mediated inflammatory response. Our immunoblot and immunocytochemical analyses demonstrate that pERK is located in the nucleus and cytoplasm leading to phosphorylation of C/EBP- β , which is a transcriptional factor of IL6 and Cox2. (Fig 6.B&8.A) These results indicate that ERK_ C/EBP- β pathway acts as a link between metal particle phagocytosis and inflammatory gene expression. Schematic illustration of the proposed intracellular signal pathway is given in Figure 10. The detailed receptor for metal particle await further investigation, but we can speculate that the osteoprogenitor cells might have an unrecognized innate immune receptors associated with actin-mediated phagocytosis and inflammatory intracellular signaling from this study.

Next, we extended our study to examine the effects of ERK inhibitor on osteoclastogenesis *in vivo*. AZD is a potent, selective, and ATP-uncompetitive inhibitor of MAPK/ERK kinase1/2.[44] It is an investigational anti-cancer agent and is still in phase II study under the U.S. Food and Drug Administration (FDA). Consistent with our *in vitro* experiments, AZD treatment significantly decreased pERK and inflammatory expression (IL6 and Cox2) in pericranium. (Fig 6, 8&9) The decreased inflammatory responses resulted in significant

decrease of osteoclastogenesis and macrophages accumulation on calvaria suture line in Day 7. (Fig 8&9) We have shown that ERK pathway is involved in inflammatory cascades in various cell lines including macrophages.[40] Thus, our data supports that ERK pathway could be an important therapeutic target for preventing inflammatory osteolysis.

Conclusion

In summary, this study supports our hypothesis that (1) osteoprogenitors are able to initiate inflammatory responses with well established phagocytic machinery and (2) phagocytosis-activated ERK-CEBP β signaling leads to downstream expression of Cox2 and IL6 expression. Although osteoprogenitors are able to phagocytose particles and initiate the inflammatory cascade, they may not be the main cause of osteolysis in clinical scenarios. Since a variety of cells are exposed to wear particles in human body, it is hard to define the main cause of the osteolysis. Our study, however, found that innate immune function of osteoprogenitors may have additional/synergistic effects on inflammatory osteolysis cooperating with macrophages. More specifically, we highlighted the molecular mechanisms of inflammatory cascades that can advance our understanding of osteoprogenitor-mediated inflammation. The study of the ERK-CEBP β pathway has revealed a potent therapeutic target for preventing osteolysis in future clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *In vivo* time course experiments of Ti particle induced osteolysis

Four different groups of mice calvarium were used for histology analyses; Sham, 1 day Ti treated, 3 days Ti treated, and 7 days treated groups. Ti particles (black arrow) were implanted on pericranium (white arrow). All sections of each group were stained with Tri-chrome (top row, A), CD11b (2nd row, B), and TRAP (3rd row, C). (A) Tri-chrome sections were photographed at 10X magnification. (B) CD11b positive cells in pericranium were observed at 40X magnification. (C) TRAP positive osteoclasts (red arrow) at suture line were detected at 40X and (D) quantified at 10X.

Figure 2. *In vivo* inflammatory responses of pericranium to Ti particle

To investigate the effects of inflammation on osteoclastogenesis, same four groups were used for histology analyses: Sham, 1 day Ti treated, 3 days Ti treated, and 7 days treated groups. All sections of each group were stained with (A) pERK, (B) Cox2, and (C) IL6. The expressions of these inflammation mediators at the pericranium were observed under 40X magnification.

Figure 3. Attachment of Ti particles on cell surface

(A) The schematic of a customized JAVA program for quantification of Titanium (Ti) particle-occupied area on the cell surface is shown. (B) Percentage of Ti-occupied area out of total cell surface is generated based on images from the JAVA program. Different dose of Ti particles were treated to MC3T3-E1 cells with/without 10µM of the Cytochalasin D (CYD) pretreatment. n=20. (C) Actin fluorescent image analysis of particle-occupied area illustrates that Ti particles (Bright View) are accumulated along with actin filaments (Fluorescent image) and 10µM of CYD are able to decrease particle attachment on actin filaments.

Figure 4. Actin remodeling for phagocytosing of Ti particles in osteoprogenitor cell
 (A) Actin filaments of selected area (yellow square box) were magnified and projected into X-Y, X-Z, and Y-Z planes by confocal microscopy to visualize actin remodeling. No treatment, Ti, CYD, and Ti+CYD group were compared. Scale bar in non-magnified images is 10 μ m. Scale bar in magnified images is 1 μ m. (B) After Ti treatment, vigorous dynamics including actin ring (O) and cup (U) formations were detected in axial, coronal, and sagittal planes. Scale bar represents 1 μ m. (C) The quantification analysis of cell associated with Ti particles is shown. Each group has n=300 and * denotes $P < 0.01$.

Figure 5. Ti particle mediated-actin remodeling activates the ERK pathway to induce inflammatory gene expression in MC3T3-E1 and hMSC-derived osteoprogenitor cells (A) Relative gene expressions of TNF α and IL1 β in MC3T3-E1 cells are assessed. No treatment (N), Titanium (Ti), Cytochalasin D (CYD), and Cytochalasin D + Titanium (Both) groups were compared. (B) Immunoblot of MC3T3-E1 cells show the level of phosphorylation of ERK 1/2 (phospho-p44, phospho-p42) after stimulation of Ti particles with/without CYD. (C&D) Relative gene expressions of IL6 and Cox2 in MC3T3-E1 and hMSC-derived osteoprogenitor cells are assessed. Every gene expression assay is normalized by GAPDH and n=6. Each symbol denotes significant differences (P<0.05).

Figure 6. ERK cascades is a key inflammatory pathway in osteoprogenitor cells

(A) Selective inhibition of pERK1/2 by AZD is assessed through immunoblot. (B) pERK fluorescent image analysis illustrates that (pERK) expression can be induced by titanium particle in MC3T3-E1 and hMSC-derived osteoprogenitor cells. These pERK expressions are also located in both the nucleus and cytoplasm. AZD treatment blocks phosphorylation of ERK expression in both the nucleus and cytoplasm. (C&D) Relative gene expressions of IL6 and Cox2 in MC3T3-E1 and hMSC-derived osteoprogenitor cells are assessed. No treatment (N), Titanium (Ti), AZD (AZD), and AZD + Titanium (Both) groups were compared. Every gene expression assay is normalized by GAPDH and n=6. Each symbol (*, #, \$) denotes significant differences ($P < 0.05$).

Figure 7. The ERK pathway mediates phosphorylation of CCAAT/enhancer-binding protein beta (CEBP-β) for inflammatory response to Ti particles

(A) Immunoblot of the cytoplasm and nucleus shows phosphorylated ERK1/2 (phospho-p44, phospho-p42), phosphorylated CEBP-β (phosphor-CEBP-β), GAPDH, and Lamin A/C after Ti particle stimulation with/without AZD or CYD in MC3T3-E1 cells. Effective separations of nuclei and cytoplasm have been confirmed by GAPDH and Lamin A/C detection. (B&C) Prostaglandin E2 (PGE2) and IL6 secretion is measured by ELISA. MC3T3-E1 cells were treated with Ti particles for three different time points (3hr, 6hr and 24hr) in the presence or absence of AZD and CYD pretreatment. Each symbol (*, #, \$) denotes significant differences ($P < 0.05$).

Figure 8. The effects of AZD treatment on inflammatory responses to Ti particles *in vivo*
 Four groups were examined to investigate the effects of AZD on inflammation: Sham, 7 day Ti treated, 7 days AZD treated, and 7 days AZD+Ti treated groups. Immunohistochemical staining of pericranium was performed with (A) pERK, (B) Cox2 (C) IL6 and (D) CD11b. Magnification, 40X.

Figure 9. AZD treatment reduces osteoclastogenesis induced by Ti particles *in vivo*

To investigate the effects of AZD treatment on osteoclastogenesis, quantification of TRAP positive osteoclasts at the suture line was performed at 10X magnification. Representative TRAP positive osteoclasts were photographed from four groups: Sham, 7 day Ti treated, 7 days AZD treated, and 7 days AZD+Ti treated groups. Magnification was set at 40X.

Figure 10.
Model for mechanism of inflammatory responses to Ti particles in progenitor cell.