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Intra-articular basic calcium phosphate and monosodium urate crystals inhibit anti-osteoclastogenic cytokine signalling

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SUMMARY

Objective: Basic calcium phosphate (BCP) and monosodium urate (MSU) crystals are particulates with potent pro-inflammatory effects, associated with osteoarthritis (OA) and gout, respectively. Bone erosion, due to increased osteoclastogenesis, is a hallmark of both arthropathies and results in severe joint destruction. The aim of this study was to investigate the effect of these endogenous particulates on anti-osteoclastogenic cytokine signalling.

Methods: Human osteoclast precursors (OcP) were treated with BCP and MSU crystals prior to stimulation with Interleukin (IL-6) or Interferon (IFN-γ) and the effect on Signal Transducer and Activator of Transcription (STAT)-3 and STAT-1 activation in addition to Mitogen Activated Protein Kinase (MAPK) activation was examined by immunoblotting. Crystal-induced suppressor of cytokine signalling (SOCS) protein and SH-2 containing tyrosine phosphatase (SHP) expression was assessed by real-time polymerase chain reaction (PCR) in the presence and absence of MAPK inhibitors.

Results: Pre-treatment with BCP or MSU crystals for 1 h inhibited IL-6-induced STAT-3 activation in human OcP, while pre-treatment for 3 h inhibited IFN-γ-induced STAT-1 activation. Both crystals activated p38 and extracellular signal–regulated (ERK) MAPKs with BCP crystals also activating c-Jun N-terminal kinase (JNK). Inhibition of p38 counteracted the inhibitory effect of BCP and MSU crystals and restored STAT-3 phosphorylation. In contrast, STAT-1 phosphorylation was not restored by MAPK inhibition. Finally, both crystals potently induced the expression of SOCS-3 in a MAPK dependent manner, while BCP crystals also induced expression of SHP-1 and SHP-2.

Conclusion: This study provides further insight into the pathogenic effects of endogenous particulates in joint arthropathies and demonstrates how they may contribute to bone erosion *via* the inhibition of anti-osteoclastogenic cytokine signalling. Potential targets to overcome these effects include p38 MAPK, SOCS-3 and SHP phosphatases.

Introduction

A number of studies have demonstrated that endogenous particulates such as gout-associated monosodium urate (MSU) crystals and osteoarthritis (OA)-associated basic calcium phosphate (BCP) crystals contribute to joint inflammation primarily through the production of pro-inflammatory cytokines and cartilage-degrading enzymes. MSU crystals, arising as a result of persistent hyperuricaemia, induce matrix metalloprotease (MMP) expression by chondrocytes[1] and Tumor Necrosis Factor (TNF)-α and IL-8 production by monocytes. However, the acute joint inflammation observed is primarily driven by macrophages, through the activation of a large multi-protein complex called the inflammasome which culminates in the activation of IL-1β, a master regulator of local and systemic inflammation[2–6]. Intra-articular BCP crystals activate and promote mitogenesis in osteoarthritic synovial fibroblasts and upregulate MMP expression by chondrocytes[7,8]. In terms of cytokine production, they have been shown to induce IL-6 by chondrocytes[9] as well as TNF-α and IL-1β production by macrophages[10–13]. Both BCP and MSU crystals induce the production of

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prostaglandin E2 (PGE2) by fibroblasts[14,15]. Importantly, PGE2 was shown to promote osteoclastogenesis in a co-culture of murine osteoblasts and osteoclast precursors cells (OcP)[16], while TNFα and IL-1β were reported to upregulate the expression of RANKL by osteoblasts and synovial fibroblasts, thus promoting osteoclastogenesis through the activation of RANK-expressing OcP[17]. We have recently demonstrated that, in addition to the inflammasome, BCP crystals activate membrane-proximal kinases leading to the upregulation of damage-associated molecules, such as S100 proteins, which may contribute further to joint degradation[13]. Therefore, components of the inflammasome, as well as tyrosine kinases, represent potential therapeutic targets for the treatment of crystal-related arthropathies.

Despite recent advances in OA research, the complex processes involved in OA pathogenesis have hindered the development of a successful disease-modifying drug. As the disease progresses, increased cartilage permeability allows for signals to be transmitted from the synovial fluid and cartilage to the underlying subchondral bone. This may account for the observed bone deterioration and sclerosis or the formation of osteophytes and bone cysts, hallmarks of OA progression resulting from altered osteoclast activity[18,19]. Bone erosion is also a common feature of tophaceous gout whereby granulomatous lesions, known as tophi, develop around a core of MSU crystals and tartrate-resistant acid phosphatase (TRAP) positive "osteoclast-like" cells have been identified within the tophus and at sites of bone erosion in gout patients[20,21].

Cytokines act directly on OcP and, depending on the stage of osteoclastogenesis, may promote or inhibit their differentiation. For example, TNF-α is pro-osteoclastogenic in the early stage of differentiation but anti-osteoclastogenic at the later stage, while IL-1β is pro-osteoclastogenic at both stages[22]. IL-6 and IFN-γ are also associated with OA and gout, however, due to their pleiotropic nature, the precise role of these cytokines in osteoclastogenesis has been difficult to elucidate[23–25]. IL-6 is reported to work synergistically with TNF-α to induce the formation of "osteoclast-like" cells from OcP both *in vitro* and *in vivo*[26]. On the other hand, IL-6-deficient mice were shown to exhibit advanced osteoarthritic changes upon aging[27] while administration of recombinant IL-6 resulted in reduced cartilage destruction[28]. Furthermore, IL-6 transgenic mice display decreased osteoclast numbers and decreased bone turnover, further supporting a role for IL-6 in the suppression of osteoclastogenesis[29]. IFN-γ is suggested to act on T cells to induce the secretion of RANKL and TNF-α which promote osteoclastogenesis[30]. Conversely, IFN-γ has been reported to inhibit RANKL signalling in OcP through the rapid degradation of TRAF6 and consequent inhibition of NF-κB and JNK[31]. Additionally, it has been demonstrated that IFN-γ can inhibit IL-1-induced MMP-13 expression and this effect is diminished in OA chondrocytes compared to healthy chondrocytes due to reduced IFN-γ receptor expression[32] thus providing further evidence that this cytokine may negatively regulate destructive processes in the joint.

In addition to endogenous particulates, it is well established that wear debris such as poly (methyl methacrylate) (PMMA) bone cement and titanium particles generated from orthopaedic implants can drive inflammation in the joint. This leads to increased osteoclastogenesis resulting in periprosthetic osteolysis and eventual implant failure[33–36]. Furthermore, it has been demonstrated that, in addition to driving inflammation, both particulates can inhibit anti-osteoclastogenic signalling by IL-6 *via* the induction of suppressor of cytokine signalling (SOCS)-3, which negatively regulates IL-6-induced STAT-3 signalling. In the same study, titanium particles also inhibited IFN-γ-induced STAT-1 activation but failed to significantly induce the expression of SOCS-1, a key negative regulator of STAT-1 activity. Thus, the inhibition of STAT-1 signalling was believed to be SOCS-1-independent[37].

Further insight into the mechanisms by which endogenous particulates exert their effects will contribute to our understanding of the pathological processes associated with crystal deposition. In this study, we sought to determine if BCP and MSU crystals have the capacity to inhibit anti-osteoclastogenic cytokine signalling. We report that, similar to PMMA bone cement, both particulates are capable of inhibiting IL-6/IFN-γ-dependent STAT activation in human OcP. Furthermore, we demonstrate that this occurs primarily *via* the p38 mitogen-activated protein kinase (MAPK) and the upregulation of SOCS proteins. Finally, we demonstrate that BCP crystals upregulate the protein tyrosine phosphatases, SH2-containing tyrosine phosphatase (SHP)-1 and SHP-2 which may represent an additional mechanism by which particulates can inhibit anti-osteoclastogenic JAK/STAT signalling.

Materials and methods

Reagents

The Syk inhibitor (R788) was obtained from AdooQ BioScience (Irvine, CA), p38 (SB203580), JNK (SP600125), MEK/ERK (PD98059) inhibitors and MSU crystals were from Invivogen (San Diego, CA). PMMA particles (diameter range 1–10 μm) were obtained from Polysciences, Inc. (Warrington, PA). BCP crystals in the form of hydroxyapatite (HA) were synthesized by alkaline hydrolysis of brushite as described[38]. Recombinant human IL-6 and IFN-γ were obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). Recombinant human M-CSF and RANKL were from PeproTech (Rocky Hill, NJ). Lymphoprep was obtained from Stemcell Technologies (Grenoble, France). All primary antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts). Secondary antibodies, cell culture reagents, Acid Phosphatase, Leukocyte (TRAP) Kit and all chemicals were from Sigma–Aldrich (St. Louis, Missouri).

Culture of OcP

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from leukocyte-enriched buffy coats from anonymous healthy donors, obtained with permission from the Irish Blood Transfusion Board, St. James's Hospital, Dublin. $CD14^+$ myeloid cells were positively selected from PBMC using anti-CD14 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and shown to be >90% pure, as determined by flow cytometry. $CD14^+$ cells were cultured overnight in sterile petri dishes (10×10^6 cells/dish) in RPMI 1640 medium supplemented with 1% penicillin–streptomycin and 10% Foetal Bovine Serum and containing M-CSF (10 ng/ml). Cells were harvested and recultured overnight in RPMI containing M-CSF (10 ng/ml) in 6-well plates (for immunoblotting assays) or 24-well plates (for real-time polymerase chain reaction (PCR)). Consistent with previous reports[37,39], these cells are considered to be OcP due to their ability to differentiate into osteoclasts in the presence of RANKL. Late OcP were obtained by further differentiation with M-CSF (25 ng/ml) and RANKL (100 ng/ml) for 48 h. Mature, multinucleated (three or more nuclei), TRAP-positive osteoclasts were obtained after 14 days of culture, with replacement of RPMI (containing M-CSF and RANKL) every 3 days. Cells were counted under a microscope at three predetermined sites of area 0.3 × 0.4 mm and a mean value (per donor) was calculated ($n = 3$ healthy donors).

Cell treatments

MAPK activation

OcP (1.5×10^6 cells/well) were stimulated with BCP crystals (100 μg/ml), PMMA particles (500 μg/ml) or MSU crystals (100 μg/