Titanium particles up-regulate the activity of matrix metalloproteinase-2 in human synovial cells

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Abstract

Purpose Wear debris particle-induced osteolysis and subsequent aseptic loosening is one of the major causes of failure of total joint replacement. The purpose of this study was to investigate the effect of titanium implant material and inflammatory cytokines on human synovial cells and the development to osteolysis and aseptic loosening.

Methods This study investigated the effect of titanium implant material on the ECM-degraded MMP-2 in human synovial cells and analyzed the contribution of synovial cells in osteolysis and aseptic loosening.

Results When human synovial cells are exposed to titanium materials, MMP-2 activity is induced by 1.72 ± 0.14 -fold with Ti disc and 3.95 ± 0.10 -fold with Ti particles, compared with that of the controls, respectively. Inflammatory cytokines TNF α and IL-1 β are also shown to induce MMP-2 activity by 3.65 ± 0.28 -fold and 6.76 ± 0.28 -fold, respectively. A combination of Ti particles and cytokines induces MMP-2 activities to a higher level (10.54 ± 0.45 -fold). Inhibitors of various signal pathways involved in MMP-2 reverse Ti particle-induced MMP-2 activities.

Conclusions Synovial cells surrounding the bone–prosthesis interface may contribute to production of MMP-2, and NF κ B

inhibitors may be explored as potential therapeutics to alleviate wear debris-induced osteolysis and aseptic loosening.

Keywords Prosthetic arthroplasty · Osteolysis · MMP-2 · Inflammatory cytokines · Synovial membrane

Introduction

Total joint replacement is the most common and successful treatment for severe degenerative or other arthritic joint diseases, such as end-stage osteoarthritis or rheumatoid arthritis [1–3]. However, aseptic loosening is a major factor resulting in the late failure of total joint replacement and limits the longevity of prosthetic arthroplasty [4–6]. The progressive peri-prosthetic osteolysis, which can be identified radiographically as the radiolucent zone at the bone-implant interface, may result in substantial bone loss, loss of fixation, implant loosening and even clinical failure [5, 7, 8]. Although the underlying mechanism of osteolysis is unclear, it is generally accepted that the osteolysis is initiated by inflammation caused by the particles generated from continuous wear, abrasion and/or corrosion of implant components [1–5, 9, 10].

One of the pathological characteristics of the osteolysis in the failed total joint arthroplasty is the formation of a membranous tissue (or interfacial membrane) at the interface of the bone-prostheses [11]. The interfacial membrane is a granulomatous tissue which is mainly composed of fibroblasts, macrophages and foreign body giant cells [9, 11, 12]. It has been postulated that these cells may phagocytize the particles at the interface of bone-prosthetic materials and cause inflammation. Inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), can activate the osteoclasts and induce a cascade of the osteolytic events [5, 10, 12–14]. It was shown that human macrophages incubated with the prosthetic materials (titanium particles) released high

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amounts of TNF- α and IL-6. A combination of TNF- α and Ti particles showed an additive effect on inhibition of osteoblast proliferation and alkaline phosphatase production, suggesting that this interaction may play an important role in the pathology of peri-prosthetic osteolysis [15].

Matrix metalloproteinase 2 (MMP-2) is one of the most important members of MMPs which play an essential role in the extracellular matrix (ECM) degradation [16]. MMP-2 contains three repeats of a type II fibronectin domain in the catalytic domain which bind to gelatin, collagens and laminin and degrade gelatin and most types of collagens [17, 18]. It has been reported that the expressions and activities of MMP-2 were modulated by Ti particles and some cytokines in human macrophages, nucleus pulposus tissue, and fibroblasts [19–21].

Metal-on-metal (MoM) articulations in THA have become an attractive option for young, active patients, and with low revision rates of long-term follow-up [22]. However, little is known about the effect of Ti particles on synovial cells. It was shown that synovial cells were present in or around the peri-prosthetic membrane and may play an important role in wear debris-induced osteolysis [23, 24]. Here, we hypothesize that Ti particle-induced inflammatory response in synovial cells participates in the ECM turnover at the interface of the bone-prostheses through MMP-2. We thus focus on the synovial membrane around the interface of the bone-prosthetic materials and investigate the effect of titanium implant material on the ECM-degraded MMP-2 in the synovial cells. We find that when human synovial cells are exposed to titanium materials, MMP-2 activity is significantly induced with Ti disc and Ti particles. Inflammatory cytokines TNF- α and IL-1 β have also been shown to induce MMP-2 activity. A combination of Ti particles and cytokines induces MMP-2 activities to higher levels. Inhibitors of various signal pathways reduce MMP-2 activities in synovial cells to basal level after incubation with Ti particles. Taken together, our results suggest that synovial cells surrounding the bone-prosthesis interface may contribute to the production of MMP-2 and that NF κ B inhibitors (e.g. Bay-11) may be explored as potential therapeutics to alleviate wear debris-induced osteolysis and aseptic loosening.

Materials and methods

Procurement of human synovial cells and cell culture

The use of postoperative human synovial tissues followed the ethical guidelines approved by the Institutional Ethics Committee of Chongqing Medical University. Human synovial membranes were obtained from four healthy donors (ages from 30 to 45, two males and two females) undergoing limb amputation after traumatic injuries at the First Affiliated Hospital of Chongqing Medical University. The donor tissues were immediately washed with phosphate buffered saline

(PBS) with penicillin and streptomycin (200 U/ml), and then cut into 2×2×2 mm³ pieces. The small tissue pieces were suspended in 10 % FBS-DMEM (fetal bovine serum, high-glucose DMEM, 0.1 mM nonessential amino acids, 4 mM L-glutamine, and antibiotics) and incubated at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. After the cells migrated out from the minced tissues and attached to the bottom of the flask, the attached cells were allowed to grow to confluence (95 %). These cells were then collected and frozen in liquid nitrogen until use. All experiments were carried out with synovial cells less than five passages. Cells from four different donor patients were used independently in the experiments. All assays were repeated at least three times.

Titanium disc treatment

Titanium disc (commercial titanium disc donated by K-L Paul Sung of University of California at San Diego, California, USA; diameter, 15.5 mm; height, 2.5 mm; Fig. 1a) was washed three times with nitric acid and sodium hydroxide solutions, sterile phosphate-buffered saline (PBS), and then placed onto 24-well plates (Corning, NY, USA), which were pre-coated with 5 pg/ml poly-D-lysine (Sigma, St. Louis, MO, USA) at 37 °C for 40 min and then type I collagen (Sigma) at 5 pg/ml for 30 minutes. The titanium disc was washed with sterile PBS before the synovial cells were seeded. Cells were cultured in complete medium for 24 hours, changed to 2 % FBS medium for additional 16 hours, and then replaced with 1 % FBS medium for sample collections at 24, 48, 72, 120 and 168 hours after 1 % FBS DMEM treatment. At each time point, we collected 200 μ l culture samples and immediately supplemented 200 μ l 1 % fresh FBS DMEM into the cultured cells.

Titanium particle treatment

The synovial cells were trypsinized and seeded onto the type I collagen-coated six-well plates (Corning). Cells were allowed 24 hours to attach and equilibrate in complete medium. Then, the culture medium was removed and replaced with 2 % FBS medium for 16 hours for starvation. Prior to titanium particle treatment, the culture medium was replaced with fresh 1 % FBS medium. The titanium particles (commercial titanium particles donated by K-L Paul Sung of University of California at San Diego, California, USA) ($\leq 20 \mu$ m, 0.1 wt %, Ward Hill, MA) were divided into three groups (group I: $\geq 10 \mu$ m & $\leq 20 \mu$ m; group II: $\geq 5 \mu$ m & $\leq 10 \mu$ m, group III: $\leq 5 \mu$ m), and added into the culture medium of the synovial cells [25]. We collected culture media samples at 12, 24, 48 and 72 hours after treatment.

TNF- α or/and IL-1 β treatment

Cells were seeded onto the type I collagen-coated six-well plates. Cells were allowed 24 hours to seed and equilibrate.

Fig. 1 The titanium (Ti) substrates promote MMP-2 activity in synovial cells. **a** The schematic diagram of synovial cell (SC) treatment. **b** Zymographic analysis of MMP-2 activity in a time-dependent increased fashion; higher activities of MMP-2 in SC on the titanium substrates. **c** Quantitative analysis of MMP-2 activities using Bio-Rad imaging software (Quantity One 4.6.3 software). **d** The morphology of synovial cell. Optical densities (OD) of the pro-MMP-2 and active-MMP-2 bands were added as the total value of activities for MMP-2. The OD values of 24 h, 48 h, 72 h, 120 h and 168 h were normalized to 24 h in control and treated groups, respectively. The data were the mean of four different experiments ($n=4$). *Significant difference with respect to control ($p<0.05$)

Then, the culture medium was removed and replaced with 2 % FBS medium for 16 hours for starvation, prior to TNF- α or/

and IL-1 β (Peprotech, New Jersey, USA) treatment at different concentrations (TNF- α at 1, 10 and 20 ng/ml, or IL-1 β at 1, 5 and 10 ng/ml) [26]. After being replaced with 1 % fresh FBS medium, samples of 600 μ l culture medium were collected at 12, 24, 48 and 72 hours after the treatment for zymographic assays.

The use of signal pathway inhibitors

Commercially available inhibitors were used to screen for their effects on MMP-2 production in synovial cells in response to Ti particles. The inhibitors included KT5720 (10 nM, Sigma) for the PKA pathway, Curcumin (50 mM, Sigma) for the AP-1 pathway, Bay 11-7082 (30 mM, Calbiochem, San Diego, CA) for the p65 subunit of NF- κ B pathway, Bay 11-7085 (20 mM, Calbiochem, San Diego, CA) for the p50 subunit, SP600125 (10 mM, Sigma) for the c Jun N-terminal kinase (JNK) pathway, PD98059 (50 mM, Sigma) for the ERK pathway, and SB203580 (3 mM, Sigma) for the p38 pathway according to the manufacturers' instructions. After the titanium particle treatment for 12 hours, the above inhibitors were added to the culture medium. The medium samples were collected at 12, 24, 48 and 72 hours post treatment for zymographic assays.

Zymographic analysis of MMP-2 activity

MMP-2 activity was assessed in the collected culture medium samples using 0.05 % gelatin zymography. Briefly, 10 μ l of each sample were mixed with an equal volume of 2x Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue, no β -mercaptoethanol) and separated on a 10 % SDS-PAGE gel that was copolymerized with 0.05 % gelatin. To regain enzymatic activity, gels were washed three times for 1.5 hours in 2.5 % Triton X-100 at room temperature after electrophoresis to remove SDS. Washed gels were then incubated in proteolysis buffer (50 mM CaCl₂, 0.5 M NaCl, 50 mM Tris, pH 7.8) at 37 $^{\circ}$ C for 12–16 hours. Following incubation, gels were rinsed in 2.5 % Triton X-100 solution and stained at room temperature with Coomassie blue (45 % methanol, 10 % acetic acid, 0.25 % Coomassie blue R-250) for 1 hours with gentle agitations. Gels were destained (40 % methanol, 7.5 % acetic acid, 52.5 % H₂O) until blue bands appeared clearly from Coomassie blue background. The gels were then scanned using a densitometer (Bio-Rad, Hercules, CA, USA), and quantitative analysis was performed using Quantity One 4.6.3 software (Bio-Rad).

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) to determine whether differences existed among groups. Post-hoc analysis utilized Fisher's protected

least significant differences (PLSD). A p -value < 0.05 was considered statistically significant.

Results

Human synovial cells exhibit high levels of MMP-2 activity after interaction with titanium disc or particles

MMP-2 plays an essential role in the ECM degradation [16–18]. We first seeded the synovial cells onto the Ti disc and collected culture medium samples at 24 hours, 48 hours, 72 hours, 120 hours and 168 hours for zymographic analysis. We found that MMP-2 activities increased in a time-dependent fashion in both control and Ti disc groups (Fig. 1b). However, the Ti disc group showed a much higher activity than that of the control group ($p < 0.05$), and the MMP-2 activities increased up to 172 % after titanium disc treatment when compared with the control within a week.

We were able to successfully isolate synovial membrane cells from the four healthy donors. The isolated cells could be passaged for up to four passages (Fig. 1d). When the cells were treated with exogenous inflammatory factor TNF- α and IL-1 β , no significant cytotoxicity was observed at the doses used in this study as assessed by Trypan blue staining (data not shown). The synovial cell proliferative activities were determined using MTT assay and not significantly affected by TNF- α and IL-1 β (up to 50 ng/ml) (data not shown) [26, 27].

We further analyzed MMP-2 activity in the synovial cells incubated with Ti particles ($\leq 20 \mu\text{m}$, 0.1 wt %) in three groups based on particle sizes. Our early study found that titanium particles can increase the gene expression of MMP-1,2,3, and the MMP-2 expression is more than MMP-1 and 2 (data not shown). We found that the activities of MMP-2 were increased in the synovial cells in all groups treated with Ti particles while smaller size particles induced higher MMP-2 activities (Fig. 2a). At 72 hours, the MMP-2 activities were up-regulated to 1.66 ± 0.07 folds in group I, 2.12 ± 0.12 folds in group II and 3.95 ± 0.10 folds in group III (Fig. 2b).

Inflammatory cytokines induce MMP-2 activities in human synovial cells

It was reported that after total joint replacement the chronic inflammation appeared in and/or around the interface of the bone-prostheses with the elevated inflammatory factors [28, 29]. We found that both TNF- α and IL-1 β up-regulated the production of MMP-2. Furthermore, the IL-1 β had a greater influence on MMP-2 activity than TNF- α , while the combination of the two factors induced a much higher amount of MMP-2 production than each factor alone (Fig. 3). At 72 hours, the combination induced MMP-2 up to 8.94 ± 0.3 folds compared to normal control; in comparison, IL-1 β

Fig. 2 The titanium (Ti) particles induce high levels of MMP-2 production in synovial cells. **a** Elevated MMP-2 activities induced by different sizes of Ti particles. The particle sizes were: group I ($\leq 20 \mu\text{m}$, $\geq 10 \mu\text{m}$), group II ($\leq 10 \mu\text{m}$, $\geq 5 \mu\text{m}$), group III ($\leq 5 \mu\text{m}$). **b** Quantitative analysis of zymography with Quantity One 4.6.3 software. All treatment groups were compared to the control group at different time points. The data presented were the mean of four different experiments ($n = 4$). * $p < 0.05$ vs untreated control; & $p < 0.05$ vs groups I and II

induced up to 4.28 ± 0.27 folds (5 ng/ml) and TNF- α up to 3.32 ± 0.28 folds (10 ng/ml) (Fig. 3b).

Titanium particles promote higher MMP-2 activity in synovial cells in the presence of inflammatory factors

At the interface of the bone-prostheses, the surrounded synovial membrane is exposed to the micro-environment where titanium particles and inflammatory factors both exist. To mimic the in vivo environment in the cultured synovial cells, we added the Ti particles and inflammatory factors to reveal the MMP-2 activity. We found a combination of Ti particles and TNF- α induced a higher activity than each factor alone at 72 hours (5.48 ± 0.38 folds vs. control) (Fig. 4b). The combination of Ti particles and IL-1 β had higher activity at 48 hours (up to 7.76 ± 0.40 folds vs. control) (Fig. 4d). Moreover, a lower concentration of IL-1 β induced the same level of MMP-2 activity compared with a higher TNF- α . The combination of Ti particles, TNF- α and IL-1 β induced both pro- and active-enzymes of MMP-2 to the highest level (9.08 ± 0.41 vs. control, 72 hours).

Fig. 3 Inflammatory factors enhance MMP-2 activities in synovial cells (SCs). **a** The cells were treated with varied concentrations of TNF- α (1 ng/ml, 10 ng/ml and 20 ng/ml) and IL-1 β (1 ng/ml, 5 ng/ml and 10 ng/ml). Zymography showed the inflammatory factor-induced MMP-2 activities in SCs. The gels shown were representative of four different experiments ($n=4$). **b** Quantification of MMP-2 activities showed increases of MMP-2 activities in SC induced by TNF- α and IL-1 β . Optical densities of the pro-MMP-2 and active-MMP-2 bands were added as the total value of activity for MMP-2. Then, the OD values of all treated groups were compared to the control group at indicated time points. The data were presented as the mean of four different experiments ($n=4$). Scale bars, SD. * $p<0.05$ vs. untreated control; & $p<0.05$ vs. other groups

Signal pathway inhibitors reverse the elevated MMP-2 activity induced by titanium particles

We next analyzed if inhibitors of several signaling pathways would antagonize the titanium particle-induced production of MMP2. We found that inhibition of the PKA pathway (with KT5720), the JNK pathway (with SP600125), the ERK pathway (with PD98059) and the P38 pathway (with SB203580) effectively reduced MMP-2 production, while inhibition of the NF- κ B pathway (inhibition of p65 subunit with bay11-7082, p50 subunit with bay11-7085) significantly reduced MMP-2 activity (Fig. 5). Inhibition of the AP-1 pathway with curcumin also significantly down-regulated MMP-2 activity. Although AP-1 is important for cell proliferation and migration, it may not be an appropriate target for therapy [30], whereas targeting the NF- κ B pathway may be explored as a potential therapeutic for chronic tissue degradation and osteolysis-mediated aseptic loosening.

Discussion

Aseptic loosening is one of the most frequent causes of the late failure of total joint arthroplasty. The severe loosening of the

prosthesis and/or osteolysis may lead to peri-prosthetic fractures [31]. It is increasingly accepted that the interaction between wear particles and cells in the interface of the prostheses plays a key role in the osteolysis [9–11]. The interaction of the cells around the bone-prostheses interface leads to inflammatory response. Inflammatory cytokines, such as TNF- α , IL-1 and IL-6, have been shown to be involved in prosthetic osteolysis [10, 32, 33]. Numerous studies have been carried out about the aseptic loosening in macrophages, fibroblasts and osteoclasts in or around the interface of bone-prostheses [5, 10, 12–14]. It has been suggested that the synovial cells presented in the peri-prosthetic membrane may be important targets of wear debris during osteolysis [23, 24]. We postulated that the synovial membrane around interface of bone-prostheses may react to particles and contribute to the imbalance of articular extracellular matrix (ECM).

MMP-2 is an important member of the MMP family and can degrade the ECM components such as gelatin, collagens, laminin and fibronectin [16–18]. MMP-2 protein exists in two forms, active-MMP-2 of 62 kDa and pro-MMP-2 of 72 kDa. While both forms carry out the same enzymatic reaction, the 72 kDa MMP-2 has approximately 10 % of the activity of 62 kDa MMP-2 [34]. MMP-2 activities have been shown to

Fig. 4 Ti particles up-regulate MMP-2 activity through inflammation. **a, b** 10 ng/ml TNF- α induced higher MMP-2 activity of synovial cells in the presence of Ti particles (size $\leq 5 \mu\text{m}$). **c, d** 5 ng/ml IL-1 β combined with 10 ng/ml TNF- α promoted much higher activities of MMP-2 in the presence of Ti particles (size $\leq 5 \mu\text{m}$). Quantitative analysis was done by using Quantity One 4.6.3 software (Bio-Rad). The data were the representative of four different experiments ($n=4$). * $p<0.05$ vs. untreated control; & $p<0.05$ vs. Ti particles group; # $p<0.05$ vs. Ti+TNF- α +IL-1 β group

Fig. 5 Signal pathway-specific inhibitors reverse Ti particle-induced MMP-2 activity in synovial cells. **a** The SCs were treated with Ti particles (size $\leq 5 \mu\text{m}$) and the medium samples were collected at 12, 24, 48 and 72 h after being treated with inhibitors. Zymography showed the MMP-2 activities. **b** Quantitative assays were done by using Quantity One 4.6.3 software (Bio-Rad). The data are presented as the mean of three independent experiments ($n=3$). Scale bars, SD. * $p<0.05$ vs. control

present in the interface tissue from failed total hip arthroplasty [35, 36]. Nakashima et al. described that macrophages exposed to titanium and polymethylmethacrylate (PMMA) particles increased expression and high production of MMP-2 and MMP-9 [19]. Fibroblasts isolated from the interface membranes are also able to enhance expression of collagenase (i.e. MMP-1) and stromelysin (i.e. MMP-12) in response to Ti particles [12]. We find that Ti disc and Ti particles up-regulate MMP-2 production in human synovial cells. These results are consistent with those findings in macrophages and fibroblasts, suggesting that the synovial membrane may be an important contributor of the secreted matrix-degraded protease in or around the bone-prostheses interface.

The relationship between inflammatory factors and MMP-2 has been studied in many cell types [12, 13, 16, 19–21]. In synovial cells, we find that TNF- α and IL-1 β can effectively enhance the activities of MMP-2. It was reported that Ti particles induced macrophages to secrete inflammatory cytokines, such as TNF- α and IL-1 β [28]. Our results in which Ti particles are shown to upregulate MMP-2 activities in synovial cells suggest that inflammatory cytokines may be important mediators of osteolysis-induced aseptic loosening. Accordingly, when the Ti particles combine with inflammatory cytokines, we find a synergetic effect on elevated MMP-2 activity. The increased MMP-2 production may activate other members of MMP proteins and thus increase the overall MMP activities [37, 38], leading to a significant degradation of the articular ECM and underlying one of the most important causes involved in aseptic loosening and periprosthetic osteolysis.

High activities of MMP-2 in the interface of bone–Ti prosthetic materials last a relatively long period of time after total joint replacement [38], leading to a chronic degradation of the articular ECM. Mulhall et al. reported the anti-inflammatory treatment with N-acetylcysteine and diclofenac suppressed particulate debris-induced TNF- α and IL-1 β expressions in monocyte-macrophage cells [39, 40]. Here we explore the possibility to block the signal pathways involved in MMP-2. It has been shown that multiple signal pathways may be involved in the activity of MMP-2, including PKA, JNK, ERK, p38, AP-1 and NF- κ B pathways [28, 41–43]. However, the signal pathways underlying the increased activities of MMP-2 in osteolysis in synovial cells are not clear. In this study, we found that while the inhibition of PKA, JNK, ERK, and P38 pathways can reduce the activity of MMP-2, the inhibition of AP-1 and NF- κ B pathways results in more pronounced reduction in MMP-2 activity. AP-1 is an important pathway responsible for cell proliferation and migration [33]. An inhibition of the AP-1 pathway may reduce the viability, proliferation, and migration of cells around the interface after total joint replacement. On the other hand, targeting the NF- κ B pathway may be explored as potential therapeutics for chronic tissue degradation.

NF- κ B exists in several dimeric forms, but the p50/p65 heterodimer is the predominant one [44]. As NF- κ B controls a large number of normal cellular functions, the appropriate concentration of inhibitors should be further investigated. In this study, we found that concentrations of Bay11 (Bay11-7082, 30 mM; Bay11-7085, 20 mM) can reduce MMP-2 almost to the basal level. Conversely, over inhibition of MMP-2 may break the normal remodeling balance of the articular ECM to remove old and damaged tissues.

In conclusion, our results indicate that smaller titanium particles can stimulate higher MMP-2 production. Moreover, the inflammatory conditions can potentiate Ti particle-induced MMP2 production in human synovial cells. By analysing a panel of pathway-specific inhibitors, we found that targeting the NF- κ B pathway may be explored as a potentially novel therapeutic to relieve and manage aseptic loosening and periprosthetic osteolysis.

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