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Co-Cr-Mo Alloy Particles Induce Tumor Necrosis Factor Alpha Production in MLO-Y4 Osteocytes: A Role for Osteocytes in Particle Induced Inflammation

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Abstract

Wear debris-induced osteolysis is purportedly the limiting problem affecting the long term results of joint arthroplasty. Pathogenic effects of wear debris in peri-implant cells such as macrophages, osteoblasts and osteoclasts have been well studied. In contrast, the affects of wear-debris on osteocytes, which make up over 90% of all bone cells, remains unknown. We hypothesized that metal implant debris can induce the proinflammatory response in osteocytes. This study demonstrated the effects of cobalt-chromium-molybdenum alloy (Co-Cr-Mo) particles on a well-characterized MLO-Y4 osteocyte cell line. Co-Cr-Mo alloy particle treatment significantly ($p < 0.05$) up-regulated tumor necrosis factor alpha (TNF α) gene expression after 3 and 6 hr and TNF α protein production after 24 hr, but down-regulated interleukin-6 (IL-6) gene expression after 6 hr. Co-Cr-Mo alloy particle treatment also induced osteocyte apoptosis after 24 hr. This apoptotic effect was partially (40%) dependent on TNF α . Therefore, our results suggest that osteocytes play a role in particle induced inflammation and bone resorption following total hip arthroplasty by inducing pro-inflammatory cytokines and inducing osteocyte apoptosis.

Keywords

Wear debris; Osteolysis; MLO-Y4 cell; Tumor necrosis factor α ; Osteocyte apoptosis

Introduction

The number of revision total hip arthroplasties and total knee arthroplasties performed in United States has been increasing [23] due to longer life expectancies, an aging population, and the use of joint replacements in younger, more active patients. Among the total number of total hip and total knee arthroplasties, approximately 17.5% and 8.2%, respectively, were revision arthroplasties between 1990 and 2002. [22] A recent study projected the growth of

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total hip and total knee revisions by 137% and 601%, respectively, between 2005 and 2030. [21] Late aseptic loosening of components is the most common reason for implant failure, causing approximately 75% of failures. [10] Implant failure causes significant morbidity and accounts for substantial health care expenditures.

Aseptic loosening, one of the major complications following total joint arthroplasty, is mostly induced by wear debris particles from prosthetic materials. [17] Wear particles generated by implants are phagocytosed by macrophages, leading to activation and release of pro-inflammatory mediators which accelerate bone resorption around the implant. [28] However, the precise etiology of aseptic loosening still remains unclear.

Osteocytes, which are the most abundant cells in bone, are terminally differentiated cells of the osteoblast lineage that have become embedded in mineralized matrix and may send signals that regulate bone modeling and remodeling. [2] Recent studies have demonstrated the contribution of osteoblasts to osteoclast generation and/or recruitment. For example, expression of pro-inflammatory mediators such as IL-6 by cultured osteoblasts can be induced by wear debris, [40] which can subsequently lead to osteoclast activation.

However, the interaction between osteocytes and implant debris has not been well characterized. Lohmann et al. showed that the addition of ultra-high molecular weight polyethylene to MLO-Y4 osteocytes in culture increased prostaglandin E2 and nitric oxide production suggesting that osteocytes may be involved in aseptic loosening by affecting monocytes and macrophages as well as osteoclasts that causes bone resorption. [24] Recently investigation elucidating the mechanisms responsible for aseptic loosening demonstrated that IL-6 and TNF α are major pro-inflammatory cytokines involved in aseptic loosening. [12,14,19,28,31,33,43] However, it remains unknown whether implant wear debris can elicit production of pro-inflammatory cytokines; IL-6 and TNF α , by osteocytes. We hypothesized that metal implant debris particles can induce the production of pro-inflammatory cytokines, IL-6 and TNF α in osteocytes. Thus, the purpose of this study was to determine the *in vitro* response of osteocytes to Co-Cr-Mo alloy particles with respect to the expression and production of pro-inflammatory cytokines, IL-6 and TNF α .

Despite the abundant evidence that the potent cytokine TNF α is not only produced by monocytes and macrophages in an autocrine response to wear particles but also acts in paracrine manner to increase osteoclast mediated bone resorption, [7,8,28] TNF α has been reported to induce osteocyte apoptosis [1,34] which involves activation of caspase 3 and 7. [34] Moreover, recent studies indicates link of osteocyte apoptosis to the control of local bone resorption. [5,35,36,39] Therefore, we further analyzed the role of TNF α in osteocyte apoptosis under Co-Cr-Mo alloy particle treatment.

Materials and methods

Co-Cr-Mo alloy particles

Co-Cr-Mo alloy particles (ASTM F-75; Bioengineering Solutions Inc, Chicago, IL, USA) were used in this study. The mean particle diameter was 1.2 μm (range 0.5–10 μm ; Fig. 1). Particles were endotoxin cleaned using PyrocleanTM, CitrisurfTM and ethanol and were determined to be endotoxin free by Kinetic QCL (i.e. < 0.1 EU per mg of particles; Lonza, Walkersville, MD, USA). Prior to use particles were incubated in fresh 70% ethanol with shaking overnight. The particles were then washed and suspended in sterilized phosphate buffered saline (PBS; Lonza, Walkersville, MD, USA) to a stock concentration of 1.0×10^7 particles/ ml.

Cell culture of MLO-Y4 osteocytes

MLO-Y4; murine long bone-derived osteocytic cell line, derived from 14 day-old transgenic mice containing the SV40 large T-antigen driven by the osteocalcin promoter [18] were kindly provided by Dr. Lynda F. Bonewald (University of Missouri-Kansas City, Kansas City, MO, USA) for use in this study. The MLO-Y4 cell line display an osteocyte-like phenotype. Cells were cultured at 37 °C, 5% CO₂, 95% air in α -MEM supplemented with 2.5% fetal bovine serum, 2.5% calf serum and antibiotics on tissue culture plastic dishes coated with rat tail collagen (BD Biosciences, San Jose, CA, USA) as previously described [18]. Cells were plated at a density of 1.0×10^5 cells/ well in 12-well culture plates. After 24 hrs, the MLO-Y4 cells were treated with the desired number of Co-Cr-Mo alloy particles (0, 1, 10 particles/ cell, Fig. 2A, 2B) for 3, 6 and 24 hr without subsequent media change.

To investigate the mechanism of induction of TNF α gene expression by Co-Cr-Mo alloy particles, actinomycin D (ActD; Sigma-Aldrich, St. Louis, MO, USA) or cycloheximide (CHX; Sigma-Aldrich, St. Louis, MO, USA) were added to the cultures to inhibit RNA synthesis and de novo protein synthesis, respectively, as described previously [30]. Cells underwent 3 hr incubation of ActD (5 μ g/ ml) or CHX (10 μ g/ ml) with or without addition of Co-Cr-Mo alloy particles. The ActD treated group underwent 1 hr of pre-treatment with ActD before Co-Cr-Mo alloy particle stimulation.

Real-time Quantitative PCR

Total RNA from the culture was extracted after the treatment of Co-Cr-Mo alloy particle (3 and 6 hr) by a standard protocol using TRIzol® (Invitrogen, Carlsbad, CA, USA) [9,29,30]. Reverse-transcription was performed to generate first-strand DNA (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA, USA) and used for the PCR template.

Quantitative gene expression analyses were carried out using real-time PCR by means of the SYBR® Green I nucleic acid gel stain (BioWittaker Molecular Applications, Rockland, ME, USA) and the Smart Cycler® system (Cepheid, Sunnyvale, CA, USA) as described previously [9,29,30]. Genes studied in this investigation were IL-6 and TNF α . All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific primer sets from a previous study were used [11].

Protein quantification / Luminex assays

MLO-Y4 culture supernatants were collected at 24 hr and frozen at -80°C. The levels of IL-6 and TNF α in cell culture supernatants were determined by mouse cytokine MILLIPLEX™ MAP kit (Millipore, Billerica, MA, USA), as described previously [4]. Briefly, cytokine capture antibody-coupled polystyrene microspheres were provided with distinct ratios of two dyes (red and infrared fluorophores) for each cytokine to be tested. Assay supernatants were incubated with the anti-cytokine bead sets, followed by incubation with a detection antibody coupled to microspheres. The microspheres and reporter molecule were read on a Luminex 100 (Luminex, Austin, TX, USA). Determination of cytokine concentrations from the fluorescence values obtained was calculated from standard curves. The sensitivity of the multiplex kit was 3.4 and 1.4 pg/ ml, for IL-6 and TNF α , respectively.

Determination of Apoptosis

To determine whether apoptosis is activated in Co-Cr-Mo alloy particle treated osteocytes, MLO-Y4 cells were treated with Co-Cr-Mo alloy particles. Caspase 3, 7 activity was measured using the Caspase-Glo™ 3, 7 assay (Promega, Madison, WI, USA). MLO-Y4 cells were plated in white-walled plates. After a 24 hr culture period, the MLO-Y4 cells were treated with the desired numbers of Co-Cr-Mo alloy particles (0 or 10 particles/cell) for 24 hr without

subsequent media change. Caspase 3, 7 activity was determined after 30 min incubation by measuring luminescence with a Victor plate reader (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. For studies blocking TNF α , MLO-Y4 cells were co-treated with anti-mouse TNF α neutralizing antibody (clone: MP6-XT22, R&D Systems, Minneapolis, MN, USA) at 10 μ g/ ml.

Statistical analysis

All values are presented as mean $\pm$ standard deviation of at least 4 replicates. These values were analyzed with two-way ANOVA and the Bonferroni/ Dunn test as a post hoc test for all groups using GraphPad Instat® software (GraphPad Software Inc., San Diego, CA, USA). Significance was defined as probability values less than 0.05 ($p < 0.05$).

Results

IL-6 gene expression

Co-Cr-Mo alloy particles did not change the expression level of the IL-6 gene at 3 hr ($p > 0.200$) (Fig. 3A). However, at 6 hr, the expression level in the particle treated group was significantly lower compared to control, at both 1 particle per cell ($p = 0.002$) and 10 particles per cell ($p = 0.015$) concentrations.

TNF α gene expression

The addition of Co-Cr-Mo alloy particles to the culture significantly affected gene expression of TNF α ($p < 0.001$) (Fig. 3B). The increase in TNF α gene expression in the high dose group was 671% at 3 hr after treatment ($p = 0.033$) and 761% at 6 hr after treatment ($p = 0.012$). Co-Cr-Mo alloy particles did not change the expression level of the TNF α gene in the low dose group at either 3hr or 6hr. Consequently, 10 particles per cell were used for all further experiments.

Effect of ActD and CHX on TNF α expression

In order to investigate the mechanism of induction of TNF α gene expression by Co-Cr-Mo alloy particles, cells underwent ActD and/or CHX treatment. Without the addition of ActD or CHX, Co-Cr-Mo alloy particles induced significantly higher expression of TNF α . ActD, an inhibitor of RNA synthesis, alone reduced the transcription of TNF α in the absence of Co-Cr-Mo alloy particles. The addition of Co-Cr-Mo alloy particles with ActD treatment did not change the expression level of TNF α . Inhibition of de novo protein synthesis by CHX alone led to elevated level of TNF α . Addition of Co-Cr-Mo alloy particles in the presence of CHX led to higher TNF α mRNA expression similar to that observed in the absence of CHX. (Fig. 4)

IL-6 production

There was no significant change in IL-6 production into the culture media by Co-Cr-Mo alloy particle treatment (Fig. 5A). IL-6 concentrations in the media of the control group and the Co-Cr-Mo particle treated group were 868.81 ± 605.28 pg/ ml and 1412.38 ± 325.72 pg/ ml, respectively.

TNF α production

Addition of Co-Cr-Mo alloy particles (10 particles per cell) to the culture significantly increased TNF α production, after 24 hr (Fig. 5B). TNF α concentration in the media of Co-Cr-Mo alloy particle group was 13.03 ± 1.61 pg/ ml while it was under the detection limit in the control group.

Caspase 3, 7 activity

MLO-Y4 cells treated with Co-Cr-Mo alloy particles at a ratio of 1:10 demonstrated nearly 90% increase in activated caspase 3, 7 after 24 hr, which was significantly higher than the level of activated caspase 3, 7 in the control ($p < 0.001$) (Fig. 6). In addition, exposure to anti-TNF α antibody, a specific neutralizing antibody for TNF α , significantly reduced the level of activated caspase 3, 7 by 40% (Fig. 6).

Discussion

The results of the present study support our hypothesis that metal implant debris can induce the production of pro-inflammatory cytokines, IL-6 and TNF α in osteocytes. In addition, we also report that Co-Cr-Mo alloy particles activate apoptosis in osteocytes/MLO-Y4 cells and this activation is, in part, regulated by TNF α . This is the first report to show that Co-Cr-Mo alloy particles have a direct effect on increasing TNF α gene expression and protein secretion in osteocytes/ MLO-Y4 cells, *in vitro*. The present study supports a new potential role of osteocytes in implant loosening since it has been reported that osteocytes residing near the implant surface are in direct contact with the implant via the canalicular system, [32] which links the osteocyte lacunae with the peri-implant surface.

Upregulation of the TNF α gene was observed at 3 and 6 hr after dosing with 10 Co-Cr-Mo alloy particles per cell. At a lower dose of 1 particle per cell the expression level for TNF α did not change. Thus Co-Cr-Mo alloy particles appear to stimulate osteocytes in a dose-dependent manner. This dose-dependent effect of Co-Cr-Mo alloy particles is well accepted for macrophages where 10:1 Co-Cr-Mo alloy particles per cell have been shown to maximally induce IL-1 β secretion while lower (5:1 particles per cell) and much higher (20:1 particles per cell) doses did not, in human monocytes (where the size of particles are approximately 1–5 microns in diameter. [4] The studies using RNA and protein inhibitors indicated that the up-regulation of TNF α by Co-Cr-Mo alloy particles is regulated by an intracellular transcriptional pathway and is independent of *de novo* protein synthesis. Our protein secretion data further supports the gene expression results by detecting TNF α only in the Co-Cr-Mo alloy particle treated group.

To date, substantial progress has been made to understand the mechanisms of aseptic loosening. [13,15,16,37] Pro-inflammatory cytokines induced in response to wear particles are responsible for the downstream processes leading to osteolysis. [15] TNF α , a central pro-inflammatory cytokines mediating osteolysis is up-regulated during aseptic loosening. [12, 14,31,43] Studies utilizing transgenic mice that lack TNF α signaling found that those mice were partially protected from particle-induced osteolysis in the calvaria model. [25,28] In addition, genetic or pharmaceutical blockade of TNF α inhibited both osteoclast differentiation and osteolysis induced by wear particles in the murine calvaria model. [7,8] Moreover, contribution of natural variance within the promoter region of the TNF α gene to the risk of osteolysis has been reported. [41]

IL-6 is also known as one of the important pro-inflammatory cytokines mediating osteolysis. Its level is also up-regulated in patients experiencing osteolysis [12,14,20,31]. IL-6 primarily stimulates bone resorption indirectly via RANKL production by osteoblasts, other mesenchymal cells, and lymphocytes. [27,38,42] In our study, expression of the IL-6 gene did not change following Co-Cr-Mo alloy particle treatment at 3 hr while the expression level was lower than the control at 6 hr. Moreover, IL-6 protein secretion did not change significantly compared to control group. These observations might be due to the constitutive high expression level of IL-6 in osteocytes and MLO-Y4 cells. Chenoufi et al. have shown that production of IL-6 increases during differentiation of human osteoblasts [6] and a recent report by Yang et

al. found MLO-Y4 cells express 3 times higher levels of IL-6 compared to 2T3 osteoblasts. [44]

The osteocytes, making up over 90% of all bone cells, which reside in bone matrix, [2] have been considered to be metabolically inactive cells [26] and little attention has been given to osteocytes in comparison to studies of osteoblasts and osteoclasts. However, there is now evidence that osteocytes regulate bone formation and resorption. [5,35,36,39] Several studies have shown that osteocytes undergo apoptosis during bone fatigue-induced microdamage and subsequently, the bone containing the apoptotic osteocytes is resorbed by osteoclasts. Cardoso et al. recently reported that rats treated with an apoptosis inhibitor completely blocked both fatigue-induced osteocyte apoptosis and the activation of osteoclastic resorption, suggesting that apoptotic events following fatigue-induced microdamage may play a substantial role in determining the subsequent course of tissue remodeling. [5] More recently, Tatsumi et al. reported significantly lower bone strength with increased intracortical porosity and microfractures, osteoblastic dysfunction, and trabecular bone loss with microstructural deterioration and adipose tissue proliferation in the marrow space in osteocyte-ablated transgenic mice. [35] These results support previous findings in which osteocyte apoptosis contributes to the control of local bone resorption [36] by sending signals to recruit osteoclasts. [39] Our data support the concept that factors other than microdamage may induce osteocytes to undergo apoptosis and activate bone resorption. TNF α -mediated apoptosis has been reported for a variety of cells, including MLO-Y4 cells and murine osteoblasts. [1] In addition, a previous report demonstrated that TNF α -induced apoptosis of osteocytes involves activation of caspase 3 and 7. [34] In the present study, we observed that Co-Cr-Mo alloy particle treatment induced TNF α production and apoptosis indicated by increase in caspase 3, 7 activity. In order to dissect the role of TNF α in Co-Cr-Mo alloy particle-induced apoptosis, we further utilized neutralizing antibody for TNF α and found that TNF α contributes to nearly 40% of the increased apoptosis activity induced by Co-Cr-Mo alloy particle.

Although, it has been reported that osteocytes exist near the implant surface [32] and the fact that wear particles exist in Haversian canals in pathological samples obtained from patients (Robert M. Urban, personal communication), the degree to which wear debris particles come into contact with osteocytes *in vivo* remains unknown. However, Busse et al. successfully detected considerable quantity of heavy metal cobalt in the peri-prosthetic mineralized bone tissue. [3] In addition, the mechanism for osteocytes to recognize/sense the Co-Cr-Mo alloy particles is unknown and needs further investigation.

In summary, we have demonstrated that Co-Cr-Mo alloy particles induce production of TNF α and apoptosis in osteocytes. This apoptotic effect of Co-Cr-Mo alloy particles is, at least in part, mediated by TNF α . These responses together with recent knowledge on the connection between osteocyte apoptosis and initiation of bone resorption makes it reasonable to suggest that osteocytes play an important role in regulating peri-prosthetic bone resorption following total hip arthroplasty by inducing pro-inflammatory cytokines and inducing osteocyte apoptosis.

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Fig. 1. Characterization of Co-Cr-Mo alloy particle used in the current study. The graph indicates particle size distribution measured by particle size analyzers. The mean particle diameter was 1.2 μm (range 0.5 – 10 μm). A cumulative data with % PASS is presented as a line graph (left y-axis). Bar graph indicates relative data with % CHAN (right y-axis).

Fig. 2. Experimental set-up for the Co-Cr-Mo alloy particles treatment *in vitro*. (A) MLO-Y4 cells were treated with desired numbers of Co-Cr-Mo alloy particles. Control; no particles, Low dose; 1 particle / cell, High dose; 10 particles / cell. (B) Distribution of Co-Cr-Mo alloy particle in MLO-Y4 culture visualized by phase contrast microscopy from high dose group. Black dots indicate Co-Cr-Mo alloy particles.

Fig. 3. Effects of Co-Cr-Mo alloy particles on expression level of pro-inflammatory cytokine genes; (A) IL-6 and (B) TNF α , in MLO-Y4 cells. After particle treatment, at low and high dose, cells were collected for real-time PCR. The gene expression level at each time point is demonstrated as a relative expression level to GAPDH from real-time PCR. Mean values $\pm$ SD (n = 4) are represented. The asterisk indicates statistically significant difference (*p < 0.05).

Fig. 4.

Upregulation of TNF α gene expression level at 3 hr depends on transcription. MLO-Y4 cells were co-treated with Co-Cr-Mo alloy particles or CHX (10 μ g/ml) or ActD (5 μ g/ml) as described in Materials and methods. After incubating for 3 hr, RNA samples were isolated for real-time PCR. Mean values $\pm$ SD (n = 4) are represented. The asterisk indicates statistically significant difference (*p < 0.05).

Fig. 5. Effects of Co-Cr-Mo alloy particles on protein production of pro-inflammatory cytokines; (A) IL-6 and (B) TNF α , in MLO-Y4 cells. After particle treatment, supernatants were collected for Luminex assays. Mean values $\pm$ SD (n = 6) are represented.

Fig. 6.
Effects of Co-Cr-Mo alloy particles on apoptosis in MLO-Y4 cells and the role of neutralizing antibody which targets TNFα. Apoptosis was measured by means of increase in caspase 3, 7 activity. Mean values ± SD (n = 4) are represented. The asterisk indicates statistically significant difference (**p < 0.001, *p < 0.05).