Original Article

UHMWPE wear particles and dendritic cells promote osteoclastogenesis of RAW264.7 cells through RANK-activated NF-κB/MAPK/AKT pathways

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Abstract: Dendritic cells (DCs) were shown to enhance UHMWPE particle promoted osteoclastogenesis in RAW264.7 macrophages. This study aimed to elucidate the signaling network mediating the osteoclastogenic effects of UHMWPE particles and DCs. RAW264.7 cells were induced with UHMWPE particles and/or DC2.4 cells. The expression of Rank was silenced by shRNA. The activation of p38MAPK, AKT, and NF-κB was inhibited specific inhibitors. The osteoclasts were assessed by TRAP staining. The expression of osteoclastogenic genes and activation of p38MAPK, AKT, and NF-κB were analyzed by qPCR and/or Western blot. UHMWPE particles and DC2.4 cells cooperatively induced RAW264.7 macrophages to differentiate into osteoclasts, activated p38MAPK, AKT, and NF-κB pathways, and upregulated Nfatc1 and osteoclast markers, which was abolished by silencing Rank or inhibited by inhibitors of p38MAPK, AKT, and NF-κB in RAW264.7 cells. These data demonstrated the complexity of signaling network and cell-cell interactions involved in UHMWPE particles-induced osteolysis.

Keywords: UHMWPE wear particle, dendritic cells, osteoclastogenesis, RANKL/RANK

Introduction

Ultra-high molecular weight polyethylene (UHMWPE) is the most used material for the load bearing of large joint replacements (e.g. the tibial plateau in total knee replacement and the acetabular cup in total hip prostheses) as it has excellent wear resistance and low coefficient of friction [1]. However, along with oxidative damage, wear is one of the main material-related causes of UHMWPE failure. Oxidative damage and wear are closely related as oxidative degradation leads to the decrease of wear resistance among other mechanical properties [1, 2].

Inflammation [3-6] and osteolysis [5-9] are the two major biological responses elicited by UHMWPE wear particles. Inflammation and osteolysis are two closely connected processes as UHMWPE-induced macrophage trafficking promotes inflammation and osteolysis [7, 10]. Wear particles initially promote the production and local release of pro-inflammatory cytokines and chemokines including MCP-1, TNF-α, different interleukins (IL) (IL1β, IL-6, IL-8, IL-10, IL-12p40, IL-11), and GM-CSF into the synovial fluid and interfacial tissues [6, 11]. These cytokines and chemokines can further amplify the polarization of macrophages and osteoclast precursor cells in perimplant tissues, leading to increased local osteoclastogenesis and inhibited osteoblast differentiation and function [12, 13] which results in the prosthesis loosening and eventually the UHMWPE replacement failure.

Osteoclasts are differentiated from the monocyte/macrophage hematopoietic lineage [14, 15]. The necessary and required cytokines for osteoclast differentiation and activation are macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [15]. RANKL binds to its cognate receptor RANK on osteoclast progenitors and activates downstream signaling cascades...
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and transcription factors including NF-κB, AP-1 (c-Fos/c-Jun), NFATc1, and MITF, which in turn activate the expression of osteoclastogenic genes and osteoclast markers [15, 16]. Among them, NFATc1 is the master regulator of osteoclast differentiation that regulates a host of osteoclast specific genes such as calcitonin receptor, integrin β3, osteoclast-associated receptor (OSCAR), tartrate resistant acid phosphatase (TRAP), and cathepsin K [17].

UHMWPE wear particles were shown to induce murine monocytic RAW264.7 cells to spontaneously differentiate into osteoclasts [18]. Moreover, our previous study showed that dendritic cells DC2.4 cells co-culture enhanced UHMWPE wear particles-induced osteoclastogenesis in RAW264.7 cells [19]. This study aimed to elucidate the signaling pathways mediating the induction of RAW264.7 macrophages differentiation into osteoclasts by UHMWPE and DC2.4 dendritic cells.

Materials and methods

Cell culture

RAW264.7 (ATCC, Manassas, VA) and DC 2.4 (Huixing Bio. Tech, Shanghai, China) cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher, Shanghai, China), 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Beyotime, Shanghai, China) at 37°C in humidified air with 5% CO₂. The cells were split when necessary. The stable cell lines RAW264.7-shControl and RAW264.7-shRank cells were selected with the G418 (ThermoFisher).

Materials

PF184 was purchased from R&D Systems (Minneapolis, MN) and SB203580 and Perifosine were purchased from Selleck Chemicals, (Houston, USA).

RANK silencing by shRNA

Three pairs of shRNA (CACCGGAGGATGCTTCCAAGATCTCTCACCTTGGAACCACCTTCC and AAAAGGAGAAGTGGTCCAGAAGATCTCTGGAACACTTCTCCTCC targeting 902-922; CACCGGAGGATGCTTCCAAGATCTCTCACCTTGGAACCACCTTCCG and AAAAGGAGAAGTGGTCCAGAAGATCTCTGGAACACTTCTCCTCC targeting 902-922; and CACCGGAGGATGCTTCCAAGATCTCTCACCTTGGAACCACCTTCC targeting 1603-1624) targeting mouse Rank gene (NM_009399) were designed using BLOCK-iT™ RNAi Designer (Invitrogen, Carlsbad, CA) and cloned into pENTR™/U6 vector (Invitrogen) according to manufacturer’s protocol. The resultants vectors were transfected into RAW264.7 cells to test their silencing efficiency by qPCR. The shRNA targeting mouse Rank gene from 972 to 992 was the most potent one in silencing Rank expression and sent for making lentiviral vector and packaging lentivirus (FulenGen, Guangzhou, China) for following silencing experiments.

TRAP staining

TRAP staining was performed according to method described previously [19]. Twenty thousand cells/well were plated in 4-well Lab-Tek TM chamber slides (Thermo Fisher, Waltham, MA) in the presence of specified treatment for 4 days. Cells were washed twice with PBS, fixed with 10% buffered formalin for 5 min and subjected to tartrate resistant acid phosphatase (TRAP) staining (Sigma, St Louis, MO) following the manufacturer’s instructions. Slides were mounted with Aqua-Mount and images were obtained using an upright microscope (Olympus IX71).

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA extraction, reverse transcription, and quantitative real-time PCR were performed as we described previously [19]. Briefly, total RNA (0.5 µg) was reverse-transcribed into cDNA using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR amplification was carried on an ABI 7500 Fast (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq™ kit (Takara, Dalian, China). The PCR primers were CTCGCTGTCCTGTAATCTGC and TCTGACCTGAACCATAAGCA for matrix metallopeptidase 9 (MMP9), CCTGTTAACGGAAGGA and GGGAGCAGGGCTAC-TACACA for calcitonin receptor (Calcr), CACCCTTAGTCTTCCGCA and CCCACATCCTGCT-ATTGTGA for cathepsin K (Cstk), CACCCGACCTGCAGCTGGTGG for TNF receptor superfamily member

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Figure 1. DC 2.4 cells enhanced UHMWPE particles induced osteoclastogenic responses of RAW264.7 cells. (A) TRAP staining of RAW264.7 cells treated with or with UHMWPE particles in the presence or absence of DC 2.4 cells (200×). The mRNA (B) and protein (C) levels of Nfatc1, Calcr, Acp5, Cstk, Itgb3, and Mmp9 were assessed by qPCR and western blot. (D) The phosphorylated and total IKKβ, NF-κB p65, p38MAPK, and AKT were assessed by western blot. (E) Quantitative analyses of the ratio between phosphorylated and total IKKβ, NF-κB p65, p38MAPK, and AKT proteins. Ctrl, vehicle control; U, UHMWPE particles (cell/particle ratio at 1:300); D, DC2.4 cells (1/10 of RAW264.7). Data expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared to Ctrl; #, P < 0.05 compared to U.
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For gene expression, RAW264.7 macrophages were culture in plates coated with or without UHMWPE particles [19] combined with specified treatments for 24 hr before collected for protein extraction whereas RAW264.7 cells were treated for 45 min for protein phosphorylation analyses. Western blot was performed as we described earlier [19]. The primary antibodies against MMP-9 (ab194314), TRAP (ab133238), cathepsin K (ab19027), calcitonin receptor (ab11042), Integrin β3 (ab38460), NFATc1 (ab3447), RANK (ab13918), and β-Actin (ab8226) were purchased from Abcam (Shanghai, China). Antibodies against NF-κB p65 (#6956), phospho-NF-κB p65 (#3036), p-IKKβ (#2694), IKKβ (#2684), p-p38MAPK (#9216), p38 (#9212), AKT (#4685), and p-AKT (#9271) were purchased from Cell Signaling (Danvers, MA).

Statistical analysis

Every experiment was independently carried out at least three times with more than three repeats each time. The data was expressed as mean ± standard deviation. Statistical analyses were done using Graphpad 6 and the differences between treated groups and control were assessed using student t-test. It was con-

Figure 2. Silencing Rank expression abolished UHMWPE particles and DC 2.4 cells induced osteoclastogenic responses of RAW264.7 macrophages. (A) TRAP staining showed that RAW264.7-shRank almost did not have osteoclast after UHMWPE particles and DC 2.4 cells treatment whereas RAW264.7-shCtrl had large amount of osteoclasts upon induction. The upregulation of mRNA (B) and protein (C) levels of Nfatc1, Calcr, Acp5, Cstk, Itgb3, and Mmp9 upon UHMWPE particles and DC 2.4 cells treatment was lost in RAW264.7-shRank cells. (D) Western blot showed that silencing Rank expression blocked UHMWPE particles and DC 2.4 cells induced activation of IKKβ, NF-κB p65, p38MAPK, and AKT in RAW264.7 macrophages. U, UHMWPE particles (cell/particle ratio at 1:300); D, DC2.4 cells (1/10 of RAW264.7). Data expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared to Ctrl; #, P < 0.05 compared to U.
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Considered statistically significant when a $p$ value was less than 0.05.

**Results**

**UHMWPE wear particles and dendritic cells cooperatively induced osteoclastogenesis in RAW264.7 macrophages by activating MAPK and AKT pathways**

TRAP staining showed that UHMWPE wear particles induced significant osteoclast differentiation from RAW264.7 macrophages, which was strongly enhanced by the coculturing of dendritic cells DC2.4 even though DC2.4 coculturing did not induce obvious osteoclast differentiation by itself (Figure 1A). Consistently, the mRNA (Figure 1B) and protein (Figure 1C) levels of master regulator Nfatc1 and osteoclast marker genes Calcr, Acp5, Cstk, Itgb3, and Mmp9 were substantially increased by UHMWPE particles and further elevated by dendritic cell coculturing in the presence of UHMWPE particles.

UHMWPE wear particles promoted the activation of p38MAPK, AKT, and IKKβ/NF-κB pathways in RAW264.7 cells (Figure 1D and 1E). After 45 min treatment with UHMWPE particles alone or with DC2.4 co-culturing, the phosphorylation of IKKβ, NF-κB p65, p38MAPK, and AKT were markedly increased compared to...
RAW264.7 treated with vehicle or co-cultured with DC2.4 alone (Figure 1D and 1E).

RANK/RANKL pathway was required for the osteoclastogenesis induced by UHMWPE alone and cooperatively with DC2.4

Next we tested whether RANK/RANKL signaling pathway was required for UHMWPE wear particles alone or cooperatively with dendritic cells induced osteoclastogenesis of RAW264.7 macrophages. Rank expression was silenced by shRNA targeting nucleotides 972 to 992 of mouse rank gene (NM_009399) compared to scrambled shRNA (shCtrl) (Figure 2B and 2C). Consequently, the expression levels of Nfatc1, Calcr, Acp5, Cstk, Itgb3, and Mmp9 were drastically reduced in RAW264.7-shRank cells compared to RAW264.7-shCtrl cells (Figure 2B and 2C). Moreover, RAW264.7-shRank macrophages greatly lost osteoclastogenic responses when treated with UHMWPE particles in conjunction with DC2.4 coculturing (Figure 2A). UHMWPE wear particles and DC2.4 induced phosphorylation of p38MAPK, AKT, IKKβ, and NF-κB was blocked in Rank-silenced RAW264.7 cells (Figure 2D).

Inhibition of NF-κB, p38MAPK, and AKT activation attenuated UHMWPE and DC2.4 induced osteoclastogenesis of RAW264.7 macrophages

As p38MAPK, AKT, and NF-κB were activated in RAW264.7 macrophages by UHMWPE particles and DC2.4 coculturing, we next examined the effects of the blockade of these signaling pathways on the osteoclastogenesis. The activation of IKKκ/NF-κB, p38MAPK, and AKT in RAW264.7 by UHMWPE particles and DC2.4 was inhibited by PF184, SB203580, and Perifosine, respectively (Figure 3A), which in turn led to drastic reduction of osteoclasts differentiated from RAW264.7 cells induced by UHMWPE wear particles and DC2.4 coculturing (Figure 3B). The combination of PF184, SB203580, and Perifosine almost completely blocked UHMWPE wear particles and DC2.4 coculturing induced osteoblast differentiation of RAW264.7 macrophages (Figure 3B). The expression levels of Nfatc1, Calcr, Acp5, Cstk, Itgb3, and Mmp9 were also significantly inhibited by blockade of p38MAPK, AKT and NF-κB activation (Figure 3C and 3D).

Discussion

UHMWPE wear particles and co-cultured dendritic cells cooperatively promoted osteoclastogenesis of RAW264.7 macrophages with activation of p38MAPK, AKT and IKKβ/NF-κB signaling pathways and upregulation of osteoclastogenic genes. Using shRNA silencing Rank expression stopped osteoclastogenic responses of RAW264.7 cells upon the induction of UHMWPE particles and DC2.4 cells. Moreover, inhibitors of IKKβ/NF-κB, p38MAPK, and AKT (PF184, SB203580, and Perifosine, respectively) substantially inhibited UHMWPE particles and DC2.4 cells induced osteoclast differentiation of RAW264.7 macrophages and upregulation of osteoclastogenic genes.

We and others have demonstrated that dendritic cells augmented UHMWPE wear particles-induced inflammatory and osteoclastogenic responses [19, 20]. The activation of NF-κB pathway was at the center of wear particle-induced inflammatory cytokines and chemokines production by macrophages and periprosthetic osteolysis [19-21]. UHMWPE wear particles induced activation of NF-κB was dependent on receptors expressed by macrophages [22, 23]. RANK was required for UHMWPE-induced osteoclastogenesis as UHMWPE particles failed to induce osteoclast differentiation and bone resorption in RANK-/- mice despite strong inflammatory response [23], which was consistent with the current data that silencing Rank expression in RAW264.7 cells led to drastic reduction of osteoclasts differentiated from RAW264.7 cells induced by UHMWPE wear particles and DC2.4 coculturing (Figure 3B). The combination of PF184, SB203580, and Perifosine almost completely blocked UHMWPE wear particles and DC2.4 coculturing induced osteoclast differentiation of RAW264.7 macrophages (Figure 3B). The expression levels of Nfatc1, Calcr, Acp5, Cstk, Itgb3, and Mmp9 were also significantly inhibited by blockade of p38MAPK, AKT and NF-κB activation (Figure 3C and 3D).
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Studies have shown that the RANK/RANKL/OPG system plays an indispensable role in bone resorption induced by UHMWPE particles and activation of inflammatory responses only was not enough to promote osteoclastogenesis [23, 24]. Simultaneously activation of a host of RANKL/RANK downstream signaling pathways was a prerequisite for UHMWPE particle-induced osteoclast differentiation and osteolysis, which could be inhibited by blocking any one of the signaling pathways [5, 6, 9, 11, 22]. Using shRNA targeting RANK and using specific inhibitors we demonstrated that among others, p38MAPK, AKT, and NF-κB were activated in RAW264.7 macrophages downstream of RANKL/RANK signaling by concurrent treatment of UHMWPE particles and DC2.4 coculture. NF-κB was the main player mediating RANKL/RANK regulation of osteoclastogenic gene expression besides its role in inflammatory responses [21] whereas PI3K/AKT [27] and p38MAPK [28] were participated in mediating RANKL/RANK signaling to promote osteoclast differentiation.

Taken together, the cooperative effects of UHMWPE particles and DC2.4 dendritic cells on osteoclastogenesis were through RANK receptor on RAW264.7 macrophages, which further activated downstream signaling network including p38MAPK, AKT, and NF-κB among other players. The osteoclastogenic effects of UHMWPE particles and DC2.4 were abolished by silencing Rank expression or inhibited by specific inhibitors against p38MAPK (SB-203580), AKT (Perifosine), and NF-κB (PF184) (Figure 4), indicating the complexity of UHMWPE-induced peri-prosthetic osteolysis.

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Disclosure of conflict of interest

None.

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