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Ubiquitin Proteasome System Regulates Biological Particles Interaction in Particle Disease (PD) via NF-κB Signaling

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Considering their outstanding mechanical character, it is inevitable to utilize titanium and titanium composite for biomedical engineering application [1-6]. However, the particles releasing from these bulks or composites of biomaterials after long term implanting in human body will cause cell apoptosis or cell death, inflammation, bone resorption and other tissues destruction, which we called “Particle disease” (PD) [7-9]. While researchers in biomaterials focus on the “Particle disease” and try to find the therapy for it, but little is reported about the biological interaction of particles, an important and complicated problem. Recently, we have found that nanosized alumina particle prevents PD induced by titanium particle and ubiquitin proteasome system (UPS) takes an important role in the biological particles interaction via NF-κB signaling pathway.

Titanium (Ti) is widely applied for implants biomaterials in different kinds of manner such as porous Ti, Ti metal, Ti composite, Ti bone augmentation material and so on, due to its outstanding properties of mechanics and bioactivity [1-6,10,11]. While Ti implants goes through a fast development period and becomes the most widely utilized implant material in the last 20 years, it still has inherent disadvantages [7,8]. The Ti composites take the advantage from the component of the composite and exhibit lots of outstanding characters in biological engineering [6,7,10,11]. However, like other researchers, we are also wondering these questions such as: when the particle release from the composite, then what will happen? Is there any way to better design the composite and prevent the PD caused by the particle releasing from implants in human body? The wear particles could be observed in the PD cases, which induced peri-implant ostolysis and is the main complication for implant fail [12-14].

Ubiquitination is a reversible post-translational modification, and UPS is not only the important system to control protein concentration but also conduct cell signaling, cell circle, tumorigenesis, immune response and so on [15-19]. During the ubiquitination, E3 ubiquitin ligases conjugate ubiquitin moieties to targeted substrates with mono-ubiquitination or different linkage-specific poly-ubiquitinated chains [15,16,20]. SCF (Skp1-Cullin1-F-box protein) E3 ligases are well characterized Cullin-based E3 ligases, and beta-transducin repeat-containing protein (β-TRCP) as F-box proteins confers substrate specificity to the $SCF^{\beta\text{-}TRCP}$ E_3 ligase complex [15-17,20]. Under stimulation, β-TRCP as a positive regulator of NF-κB signaling pathway will induce the degradation of IκBα, the endogenous inhibitor of NF-κB signaling, and

promote the activation of NF-κB signaling pathway [20-22]. Our preliminary study has showed that $SCF^{\beta\text{-}TRCP}$ E3 ligase complex bound CYLD and induced the degradation of CYLD, the negative regulator of NF-κB signaling pathway [20]. In osteoclast precursor cells, depletion of β-TRCP induced CYLD accumulation and TRAF6 deubiquitination and suppressed the RANKL-induced osteoclast differentiation. Bortezomib (BTZ) is the first proteasome inhibitor to be utilized in clinical treatment [23,24]. Our preliminary study had shown that at safe concentrations (≤ 1 nM) BTZ could block the activation of NF-κB signaling pathway by inhibiting degradation of IκBα, preventing nuclear translocation of NF-kB/p65 complex and suppressing the protein expression of proinflammatory cytokines in LPS-stimulated periodontal ligament cells [24]. It also could inhibit activator of NF-kB ligand/osteoprotegerin and prevent alveolar bone absorption in ameliorates experimental periodontitis in rats [24].

Aluminum is considered as a biomaterial with low acute cytotoxicity compared with other metal materials and has been utilized for diagnostic, therapeutic material, and artificial replacement for bone defect [25]. Recently, we found that nano-sized aluminum particles could prevent apoptosis and necrosis of MG-63 cells induced by micro-sized titanium (Al-NPs) particles. The Apoptosis and Necrosis Assay was performed by flow cytometry and showed that MG-63 cells were undergoing obviously apoptosis induced by Ti particles at 10 μg/ml and 50 μg/ml, and 30% of MG-63 cells were apoptosis after exposed to 50 μg/ml Ti paritcles. The most interesting discovering was that the Al-NPs remarkably prevented the apoptosis induced by Ti particles in MG-63 cells. Regularly, the toxicity of particle is in a concentration dependent manner. In order to minimize the effect of concentration, we performed the following *in vitro* experiments with the low concentration of 10 μg/ml Ti, 10 μg/ml Al-NPs, and 5 μg/ml Ti+ 5 μg/ml Al group with 50 μg/ml Ti and 50 μg/ml Al groups as positive controls.

Before we detected how the UPS and NF-κB signaling pathway regulated the biological interaction of particles, we had to figure out the effects of BTZ on the viability and cytotoxicity of MG-63 cells. Recently, the research of BTZ is far more than anticancer activity and low concentration of BTZ is applied in anti-inflammatory [24,26-28]. Our preliminary study had showed that there was no significant difference in cell proliferation after 24 hours of treatment with BTZ at concentrations <1 nM in periodontal ligament cells [24]. Next, we detect the toxicity of BTZ on MG-63 cells. MTT proliferation assay and Apoptosis assay with flow cytometry showed that there was no significantly different cell proliferation and pro-apoptotic effect on MG 63 under the concentration of 1nM, and 0.5 nM BTZ was considered as the non-cytotoxic concentration and utilized in subsequent experiments.

Next, Western blot assay showed that 10 μg/ml Ti particles induced the degradation of IκBα and the expression of IL-6 an P65, while the Al-NPs accumulated both the concentration of P-IκBα and IκBα in 10 μg/ml Al-NPs and 5 μg/ml Ti+ 5 μg/ml Al group, and BTZ has the same effects as Al-NPs. Compared with Ti particles, Al-NPs induced a stronger expression of LC3, an indicator of autophage, in the MG-63 cells in immunofluorescence staining assays. And the activated autophage would due to the suppressing of NF-κB signaling and prevent the apoptosis of MG 63 as shown above [29,30]. In RT-PCR assay, it again showed that the activation of NF-κB signaling pathway induced by Ti particles leading to the expression of inflammatory mediators, including IL-1β and TNF-α, could be decreased by Al-NPs and BTZ. And the expression of caspase-3, which was a downstream of noncranonial NF-κB and an indicator of cell apoptosis, was in the same pattern as IL-1β and TNF-α.

β-TRCP is the positive regulator of NF-κB signaling pathway [20-22]. And the next Western blot assay showed that Ti particles induced the activation of NF-κB signaling pathway by evoking the expression of β-TRCP, and this elevation of β-TRCP could be prevented by Al-NPs and BTZ. And the Enzyme-linked Immunosorbent Assay (ELISA) also showed that Ti particles-induced IL-1β expression could be prevented by by Al-NPs and BTZ. In conclusion, the Al-NPs and BTZ prevented the inflammation induced by Ti particle via the NF-κB signaling pathway *in vitro*.

In order to confirm the discovery *in vitro*, we performed particles-induced calvarial osteolysis mouse model *in vivo*. Histological assessment indicated that Ti particles promoted severely bone destruction, but the Al-NPs and BTZ treatment attenuated Ti particles-induced bone erosion. Immunohistochemistry assay had further confirmed the inflammatory and bone remodeling related cytokines. It showed that Ti particles increased the expression of inflammatory cytokines such as TNF-α, IL-1 and IL-6, which were decreased by the Al-NPs and BTZ treatment. And Al-NPs and BTZ treatment also evoked a higher level of OPG for bone reconstruction.

Senior patients, who are with a fail implant in body, especially hip replacement, are dangerous to suffer a secondary surgery. Our results will guide us to have a clearer understanding of the biological interaction of different type of particles (especially the Ti and Al particles) in PD and offer a bigger picture of PD for implant design to prevent PD and pharmacy design to treat PD. Considering the importance and urgency of

dramatic treatment for PD in such an aging society, our study about PD is undergoing and looking for more novel discoveries and transformable results.

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