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Vitamin E-stabilized UHMWPE: Biological response on human osteoblasts to wear debris

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A B S T R A C T

UHMWPE doped with vitamin E was introduced to provide oxidation resistance upon sterilization, without affecting UHMWPE's mechanical properties. Particle-induced macrophage activation leads to periprosthetic bone resorption, requiring total joint replacements. During osteolysis, osteoblasts produce osteoimmunological factors such as RANKL and OPG, and the inhibitors of the Wnt pathway DKK-1 and Sclerostin. This study investigated in vitro how vitamin *E*-blended-UHMWPE wear debris might affect osteoblast-mediated osteolysis and the production of RANKL, OPG, Sclerostin and DKK-1, compared to conventional UHMWPE wear debris.

Human osteoblastic SaOS2 cells were incubated with wear particles from Vitamin E doped and conventional UHMWPE and the gene expression and protein production of IL-6, RANKL, OPG, DKK-1, and Sclerostin was evaluated, RANKL, a bone erosion marker, was reduced, while OPG, a bone protective marker, were increased by the vitamin *E*-blended UHMWPE compared to conventional UHMWPE.

Vitamin E doped UHMWPE reduced Sclerostin level, and partially affected DKK-1 production, thereby protecting against bone erosion. In conclusion, Vitamin *E*-blended UHMWPE induced an osteoimmunological response in bone cells that had positive effects on the osteolysis induced by wear debris, reducing aseptic loosening of the implants.

In conclusion, this is the first study showing that Vitamin *E*-blended UHMWPE induced an osteoimmunological response in bone cells that positively affect the osteolysis induced by wear debris, thereby reducing the aseptic loosening of the implants.

1. Introduction

Ultra-high-molecular-weight polyethylene (UHMWPE) wear debris stimulates a chronic inflammatory response [1], mainly mediated by infiltrating macrophages which are responsible for the phagocytosis of wear particles [2–5]. This inflammatory response, in turn, induces the differentiation of osteoclast precursors into mature osteoclasts, stimulates osteoclast activity, and consequently promotes local bone erosion, ultimately leading to bone loss and implant failure [6–7].

To reduce wear rates and oxidative degradation of UHMWPE, different strategies have been developed, such as UHMWPE cross-linking and UHMWPE doped with vitamin E (α-tocopherol) [8,9]. UHMWPE cross-linking provides better shape memory properties and resistance, but it is obtained by radiation, which stimulates the formation of free radicals in the crystalline phase [10,11], responsible for oxidative degradation of the material [12]. Vitamin E is the most abundant and effective antioxidant in the body, and its main role is to react with free radicals in cell membranes and protect polyunsaturated fatty acids from degradation due to oxidation. Thus, UHMWPE doped with Vitamin E was introduced as a method to provide oxidation resistance, without modifying UHMWPE's mechanical properties [13–15].

Besides macrophages, osteoblasts too can phagocytose wear debris and produce inflammatory mediators such as IL-6, TNFα, IL-β in response to the debris and soluble factors that regulate osteoclastogenesis [16].

In the past decade, growing attention has been directed to

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osteoimmunological factors, which are soluble mediators linking bone tissue to the immune system. The main osteoimmunological markers are RANKL (receptor activator of NfKB ligand), its receptor RANK and the decoy receptor osteoprotegerin (OPG) [17]. Osteoblasts produce RANKL which binds to its receptor RANK on the surface of osteoclasts [18]. This regulates the osteoclast differentiation and activation associated with increased bone resorption. OPG is also secreted by osteoblasts and protects the skeleton from excessive bone resorption by binding to RANKL and preventing it interacting with RANK.

In addition to the RNK-RANKL-OPG system, the Wnt signaling pathway is vital for bone remodeling in both physiological and pathological conditions [19]. Wnts are a large family of small secreted morphogenic proteins heavily involved in bone development, and they contribute to maintaining the homeostasis of bone mass [20,21]. Wnts suppress bone resorption by up-regulation of OPG expression and down-regulation of RANKL expression in osteoblasts. [22]. Recent osteoimmunological markers expressed by osteoblasts are the two inhibitors of the Wnt pathway, Sclerostin and DKK-1. DKK-1 blocks the interaction of Wnt with β-catenin, leading to β-catenin degradation [23], while Sclerostin (also known as SOST) is an osteocyte-soluble factor that negatively regulates Wnt signaling. Both are markers of bone resorption.

Several studies have described the role of osteoblasts in wear particle-induced osteolysis [7,16,24–27], but there is little evidence about the effect of UHMWPE particles on the osteoblast-mediated osteoimmunological response. In particular, we found no evidence of how the addition of vitamin E to UHMWPE influences the osteoblast production of osteoimmunological soluble factors affecting osteoclast activity and ultimately bone erosion.

This study investigated in vitro how vitamin *E*-blended UHMWPE wear debris might affect osteoblast-mediated osteolysis, focusing in particular on the production of the markers RANKL, OPG, Sclerostin and DKK-1 compared to conventional UHMWPE wear debris.

2. Methods

2.1. Wear particles generation

UHMWPE wear particles were generated by four different UHMWPE articular inserts (raw material GUR 1020): Material A) A moderately cross-linked vitamin *E*-blended UHMWPE (60 kGy electron-beam irradiated) (vitamin E concentration 0.1 wt%), EtO sterilized (*Vital-XE®, Permedica S.p.A.*); Material B) standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized *(Permedica S.p.A);* Material C) vitamin *E*-blended UHMWPE (vitamin E concentration 0.1 wt%) not cross-linked, EtO sterilized (*Vital-E®, Permedica S.p.A.*); Material D) standard UHMWPE (without vitamin E and not cross-linked), electron-beam sterilized (25 kGy) (*Permedica S.p.A*).

All UHMWPE wear particles were generated by rubbing the articular inserts slowly (230 rpm) for 10 days against ceramic ball heads using a combined drilling and tapping machine (IM company, Italy). A load of 1000 N was applied to the machine to boost wear rates. UHMWPE particles were produced and released directly into a closed sterile recipient containing 500 mL of ultrapure water with 0.2% sodium azide (antibacterial additive).

2.2. Particle size and size distribution

We investigated particle size and the size distribution of nanometric debris by photon correlation spectroscopy (range: 1 nm – 10 μm), and used the Single Particle Optical Sensing technique for particles up to 0.5 μm (dimensional range: 0,5 μm – 500 μm).

The former enabled us to calculate the mean hydrodynamic diameter (D_H) and the size distribution of nanometric debris particles stabilized with a 5% poly(vinyl alcohol) solution. We used a dynamic light scatter (DLS) Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK), equipped with a backscattered light detector, operating at 173°. All analyses were carried out in disposable polystyrene cuvettes at a constant temperature of 25 °C. The results were calculated using the Dispersion Technology Software (DTS, Malvern Instruments Ltd., Worcestershire, UK) and are reported as intensity distribution.

The zeta potential of the nanoparticles was assessed by M3-PALS (Phase Analysis Light Scattering), using the same equipment as for the size measurement. The analyses were done in a capillary cuvette at 25 °C, with polystyrene latex as reference material. Three measurements were taken on all samples.

An Accusizer 770 (PSS Inc., USA) with the Single Particle Optical Sensing technique was used to determine the size distribution of the micrometric particles in the samples, previously stabilized with a poly (vinyl alcohol) solution. Particle size was expressed as the cumulative percentages of the distribution, where d_{10}, d_{50}, d_{90} and d_{99} are the diameters at respectively 10%, 50%, 90% and 99% of the population.

2.3. Cell culture

SAoS2 cells, a permanent line of human osteoblast-like cells, were obtained from a partner institute and grown in RPMI1640 (Invitrogen, Germany) with l-glutamine, 10% fetal bovine serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO, USA). Cells were cultured in 5% CO_2 at 37 °C in 12-well culture plates (Corning, USA).

2.4. Addition of particles to the cultures

Before use, particles were sterilized by UV irradiation overnight. Cells were seeded in growth medium at the concentration of 21,000/mL. After 72 h, when the cells had reached confluence, media were removed, wells were washed with PBS 1× without calcium and magnesium (Sigma Aldrich, Italy) and the experimental media containing the four types of wear particle (described above) diluted in growth media at concentrations of 1:1, 1:10, 1:100, 1:1000, or pure media (as control) were added. After 24 h, 48 h and 72 h incubation, the supernatants of each well were collected and stocked at −20 °C for ELISA, while cells were processed for total RNA extraction, as described below.

2.5. Cell viability

Cell viability was measured by Trypan blue (Sigma Aldrich, Italy) exclusion, which is based on the principle that live cells have intact cell membranes that exclude Trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye, then visually examined to determine whether cells take up or exclude the dye. After incubation with Trypan blue viable cells are unstained while nonviable cells have blue cytoplasm. The total and number of living cells were counted in a Burker chamber. Three replicates were run for each count.

2.6. RNA extraction using TRI Reagent

Total RNA was extracted from SaOS2 cells, which were then treated with trypsin to detach them from the plate, washed in PBS 1× and centrifuged at 3000 rpm (1008 rcf) for 5 min at 4 °C. After removing the supernatant, the TRI Reagent solution (Sigma Aldrich, Italy) (1 mL) was added to the pellet. To fragment the pellet, the samples were vortexed and passed through a 1-mL syringe, then centrifuged at 12,000 rpm (16,128 rcf) for 10 min at 4 °C. The supernatant phase containing RNA was transferred to a new tube and incubated for 5 min at room temperature (RT). Chloroform (200 μL) was added and the tube was incubated for 15 min at RT to ensure separation. Centrifugation was repeated at 12,000 rpm (16,128 rcf) for 15 min at 4 °C and the resulting supernatant containing RNA was transferred to a new micro-tube. Isopropanol (500 μL) was added and the solution was gently agitated, then maintained for 10 min at RT. After that the samples were

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