Supplementary Information

Evaluation of biological responses to micro-particles derived from a double network hydrogel

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MATERIALS AND METHODS

Ethics statement

Experimental procedures on the animal were approved by the Institute of Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine (17-0085).

Preparation of DN gel mico-particles

The PAMPS/PDMA DN gel was synthesized using two-step sequential polymerization method[1]. Briefly, PAMPS hydrogel was obtained by radical polymerization using N,N-Methylenebisacrylamide (MBAA) as a crosslinker and 2-oxoglutaric acid as an initiator. The concentrations were 1 mol/L for monomer, 4 mol% for the crosslinker, and 0.1 mol% for the initiator. The aqueous solution containing a monomer, crosslinker, and the initiator was injected into a cell consisting of a pair of glass plates separated by silicone rubber. The solution was irradiated with a UV lamp (wavelength 365 nm) for about 6 h in an argon atmosphere. The obtained PAMPS hydrogel (1st network) was immersed in an aqueous solution of 2 mol/L DMA, containing 0.1 mol % MBAA and 0.1 mol % 2-oxoglutaric acid for 1 day until reaching the equilibrium. The 2nd network (PDMA) was subsequently polymerized in the presence of the PAMPS hydrogel by irradiating UV for 6 h between two plates of glasses in an argon atmosphere. After polymerization, the PAMPS-PDMA DN gel was immersed in pure water for 1 week and the water was changed twice every day to remove any unreacted materials. For

grinding of DN gel, DN gel was pulverized in a nitrogen atmosphere using a pulverizer (NJ-50C, Aisin Nanotechnologies), with the conditions as follows: 1st cycle: processing amount 120 g/h, feed pressure 1.5 MPa, grinding pressure 0.6 MPa; 2nd cycle: processing amount 60 g/h, feed pressure 1.5 MPa, grinding pressure 1.0 MPa; 3rd cycle: processing amount 60 g/h, feed pressure 1.5 MPa, grinding pressure 1.0 MPa; 4th cycle: processing amount 60 g/h, feed pressure 2.0 MPa, grinding pressure 1.8 MPa. After two cycles of grinding, the samples were classified with a 10 μm mesh, and the permeation sample was set to a 10 μm group. The four-cycle grinding samples were set to a 4 μm group. Sizes of powder particles in a dry and wet condition were examined using ImageJ (NIH, USA) for the size distribution measurement. In addition, morphology of particles was examined by scanning electron microscope (JSM-6010LA, JEOL Ltd, Japan). Thereafter, particles were autoclaved, washed by PBS three times, and suspended in appropriate volume of PBS for further use. An endotoxin detection kit (Thermo Fisher Scientific, USA) was used to detect endotoxin in DN gel particles according to the procedure recommended by the manufacturer's instructions. Levels of endotoxin in all samples were below the detection limit of the kit (0.01 EU/mL).

Isolation and stimulation of murine peritoneal macrophages

Peritoneal macrophages were obtained from peritoneal cavity of 8-week-old male C57BL/6 mice (CLEA, Tokyo, Japan) 5 days after peritoneal injection with 1 ml of 4%

thioglycolate (Sigma-Aldrich, USA). The peritoneal macrophages were harvested in 10 ml sterile phosphate-buffered saline (PBS; Nacalai Tesque, Kyoto, Japan), washed twice with sterile PBS, suspended in growth medium containing minimum essential medium Eagle (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), a 5% penicillin/streptomycin solution, and 5% L-glutamine, and then seeded on a 96-well plate (1 x 10^4). Non-adherent cells were washed out and cells were stimulated with 10 ng/ml lipopolysaccharide (LPS; Sigma) as a positive control, 8 µM-sized latex beads polyester (Sigma) or DN gel particles for 48h. Beads and particles were suspended in a fresh growth medium at 0.1 mg/ml for stimulation of macrophages.

Stimulation of murine peritoneal macrophages in vivo

The 3 mg latex beads polyester (Sigma) and sterile DN gel particles were suspended in 300 µl PBS and then injected in the peritoneal cavity of 8-week-old male C57BL/6 mice (CLEA, Japan). Peritoneal macrophages were harvested after 48h, washed by PBS and 1×10^5 cells were placed in Eppendorf tubes for further analysis.

Osteoblast cultivation and stimulation

Murine osteoblasts were extracted from calvarial bones of 4-day-old C57BL/6 mice (CLEA, Japan) as earlier reported[2]. Pre-osteoblasts were cultured in a complete medium containing MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), a 1%

penicillin/streptomycin solution, and 50 µg/ml ascorbic acid for 14 days to obtain mature osteoblasts. The cultures were regularly replenished with fresh media every 3 days. Mature osteoblasts were stimulated with DN gel particles or latex beads at 0.1 mg/ml in a complete medium for 48h. Cells were lysed and stored at -80 °C for further gene and protein expression analysis.

Quantitative real-time polymerase chain reaction (qRT–PCR)

Cells were homogenized and lysed using TRIzol Reagent (Invitrogen), and RNA was extracted using NucleoSpin RNA (Takara, Shiga, Japan). The cDNAs were synthesized using the GoScriptTM reverse transcriptase kit (Promega, Madison, USA) and assayed using the SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan) and gene-specific primers as follows: Tnf-α (mouse: forward AGCCGATGGGTTGTACCTT, reverse ATAGCAAATCGGCTGACG), Il-6 (mouse: forward TAGTCCTTCCTACCCCAATTTCC, reverse TTGGTCCTTAGCCACTCCTTC), Col1a1 (mouse: forward, reverse), Vegfa (mouse: forward, reverse), Gapdh (mouse: forward TGCAGCGAACTTTATTGATG, reverse ACTTTGTCAAGCTCATTTCC). Gene expression was determined by the $2^{-\Delta\Delta Ct}$ method with amplification efficiencies ranging between 90 and 110%.

Western blot analysis

Cells were lysed using RIPA lysis buffer (ATTO corporation, Tokyo, Japan), and then mixed with samples buffer EzApply (ATTO, Osaka, Japan). After heating the samples at 100°C for 5 min, the extract proteins were subjected to SDS-PAGE gels and transferred electrophoretically onto polyvinylidene difluoride membrane (Immobilon-P Membrane; Merck, Darmstadt, Germany). The membranes were blocked in 5%-skimmed milk and then incubated with each target primary antibody at the optimal concentration recommended by the manufacturer's instructions. The secondary antibody conjugated with HRP (Cell signaling technology) was used for the detection of the bound antibody. Signals were detected by Ez WestLumi Plus (ATTO, Tokyo, Japan) and Quantity One v. 4.6.9 (Bio-Rad) software.

Particle-induced inflammatory osteolysis model

We used our procedure for the osteolysis model induced by particles to examine the effects of particles on bone[3]. Briefly, 8-week-old male C57BL/6 mice (CLEA, Tokyo, Japan) were anesthetized by an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Head hair was shaved in preparation for making a sagittal incision (< 1 cm) over the calvaria anterior site. The latex beads polyester, 4 μm, and 10 μm DN gel particles were suspended in 100μl PBS and then implanted onto the calvarial bone for 7 days. Control mice received the same surgical procedure (Sham) without further particles implantation, but PBS injection. The incision was closed using stainless steel clips. Mice were sacrificed, and their

calvariae were subjected to micro-CT Analyses and bone histomorphometry analysis. Their tissues on the calvariae were collected for gene expression analysis using qRT-PCR. For the analysis of bone pits, fixed calvariae were analyzed by high-resolution micro-computed tomography (micro-CT) scanning R_mCT2 scan (Rigaku, Tokyo, Japan) at a 10-mm isotropic resolution. Pits (%) on the calvariae were calculated by the ImageJ (NIH, USA). Furthermore, 10% formalin-fixed calvariae were decalcified in 10% EDTA (Wako, Osaka, Japan) for 3 days and then embedded in paraffin. Five-micron thickness sections were stained with hematoxylin and eosin (H&E) and tartrate resistance acid phosphatase staining (TRAP; Sigma-Aldrich, Missouri, USA). ImageJ (NIH, USA) was used to analyze images and to quantify cell infiltration into tissues.

Statistical analysis

Statistical analyses were performed using GraphPad Software (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison was used to compare gene expression, bone resorption area, inflammatory infiltrate, and TRAP-staining area. Results were presented as means ± standard errors of the means (SEM) and were considered statistically significant when $p < 0.05$.

References

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