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Effect of poly(vinyl alcohol) (PVA) wear particles generated in water lubricant on immune response of macrophage

S. Omata[a,*], Y. Sawae[a,b], T. Murakami[a]

[a]*Research Center for Advanced Biomechanics, Kyushu University, Fukuoka, Japan*
[b]*Department of Mechanical Engineering, Faculty of Engineering, Kyushu University, Fukuoka, Japan*

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Abstract

To develop a novel biotribological material for artificial cartilage implant with a lubricity supplement of the joint surface, we focused on two types of poly(vinyl alcohol) (PVA) hydrogel: repeated freeze–thawing (PVA-FT) and cast-drying (PVA-CD) gels. Here we observed the morphology of wear particles generated during a reciprocating wear test and assessed macrophage immune responses by applying hydrogel wear particles. As a result, PVA-CD had a significantly lower total amount of wear than did PVA-FT. The size distributions of PVA-FT and -CD wear particles were similar. Most of the particles were nanoparticles up to approximately 50 nm in diameter. Considering the particle volume distribution, there were very few micron- and submicron-sized wear particles around 1 μm in diameter. In SEM observations of dried PVA wear particles, both distributions of wear particles of PVA-FT and -CD were similar. Micron-sized wear particles were chiefly formed by close packing of 20- to 50-nm-sized particles. Biochemical and immunological evaluations revealed no cytotoxic effects of wear particles on macrophages. Cytokine synthesis of both wear particle-stimulated groups was significantly lower than that of the lipopolysaccharide-stimulated positive control. Therefore, it is suggested that PVA wear particles do not affect the macrophage immune response.

Keywords: Artificial cartilage implant; Poly(vinyl alcohol) (PVA) hydrogel; Macrophage immune response; Wear particles; Biotribology

1. Introduction

Healthy articular joints allow us to enjoy activities of daily life comfortably. However, the restorative ability of damaged articular cartilage to spontaneously recover is limited because the cartilage is aneural and avascular. To treat an end-stage symptomatic cartilage defect, or osteoarthritis (OA), surgical intervention such as total joint replacement is an effective means to expeditiously recover quality of life. In most total joint replacements, the combination of ultra-high molecular weight polyethylene (UHMWPE) and metal or ceramics has been used. The wear resistance of these rubbing materials has been improved, but significant wear occurs under severe frictional condition. In such cases, the endurance life of the artificial joints is limited to 10–20 years by aseptic loosening due to wear particle-induced osteolysis as the active resorption of bone matrix by osteoclasts associated with the wear particles [1–3].

In brief, the osteolysis is induced by synthesizing various proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and prostaglandin E2, from immunocytes, i.e. macrophages and leukocytes [2 and references therein]. The synthesized cytokines from macrophages stimulate the surrounding osteoclasts and activate bone resorption, which is induced because the activated osteoclast pumps a high concentration of proton between bone and the osteoclast. Thus, unbalance of a bone remodeling occurs and induces osteolysis and aseptic loosening. Although there are numerous reports regarding this process, influence of artificial cartilage wear particles on cytokine synthesis and the osteolysis is still unclear.

There are many reports on the various failure modes of artificial joints. By applying fairly large compression and shear load to the

*Corresponding author. Office address: 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan. Tel./fax: +81 92 802 3891.
E-mail address: s-omata@mech.kyushu-u.ac.jp (S. Omata).
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articular surface, failure modes, such as chipping and delamination, have been reported [4,5]. To prevent cytokine-induced osteoclast formation and osteolysis, it is important to control the morphology of wear particles and to inhibit to generate it. It is well known that phagocytosis of small particles induces cytokine synthesis [2,6]. Wu et al. determined the mechanical properties and three-dimensional topographic morphology of UHMWPE wear particles and showed an understanding of the wear mechanisms in an artificial joint [7]. They separated the polyethylene wear particles into three groups based on particle size and found that the nano- and submicron-sized particles had a higher modulus and smoother surface than those of micron-sized particles and the bulk UHMWPE sample. Currently, to reduce the wear of joint materials, the UHMWPE is applied by gamma radiation cross-linking and vitamin E is added to the polyethylene to inhibit oxidation by gamma radiation. Moreover, several researchers are actively developing surface modifications of rubbing materials using various coating methods such as diamond-like carbon (DLC) and poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) [8].

Since biocompatible hydrogels and sponges for artificial cartilage implant (ACI) have high lubricity and cushioning properties, several researchers have focused on modifying the surface of the artificial joint materials with soft tissues to reduce the friction and extend the endurance limit of the implant system [9–12]. Blum et al. examined the mechanical and tribological properties of a functionalized freeze–thawing poly(vinyl alcohol) (PVA) hydrogel with an organic fatty acid-derived boundary lubricant [13,14]. Conducting a uniaxial pin-on-plate wear test against a carbon steel flat-end pin and bovine articular cartilage, they demonstrated that the boundary lubricant functionalized for PVA hydrogel suppressed accretion of wear loss of the modified PVA gel. Studies by Gonzalez et al. modified a PVA hydrogel composite with hydroxyapatite (HA) to the physical and chemical characteristics and promoted its crosslinking and stability [15]. According to characteristics of PVA hydrogel wear particles, in wear testing a PVA hydrogel in distilled water, smaller particles were generated during the early testing period and numerous larger-sized particles had attached to the surface of the gel sheet [16]. Several stretched and elongated particles were generated by increasing the friction test time. Several remarkable hydrogels, such as a double-network hydrogel [17] and slide-ring hydrogel [10], have been developed for artificial cartilage implantation and/or other medical applications. Recently, several clinical investigations have reported that the PVA hydrogels were used as artificial cartilage and meniscus and remained unscratched for over 2 years [12]. However, it is necessary to keep attempting these clinical investigations for a long term in the future.

To develop a novel biotribological material for ACI, Murakami et al. focused on a PVA hydrogel as an artificial cartilage biomaterial with ultra-low wear and friction coefficient [18]. Here we observed the morphology of wear particles and assessed macrophage immune responses by applying hydrogel wear particles to confirm the validity and safety of the gel in human joints. We conducted the tribological wear test using two types of PVA hydrogels – repeated freeze–thawing (PVA-FT) and cast-drying (PVA-CD) gels [19] – and assessed the characteristics of the wear particle size distribution and nano- and micron-sized particle shape. We then confirmed the effect of PVA wear particles on the immune response of THP-1-derived macrophages by assaying two cytokines, IL-1β and TNF-α.

2. Materials and methods

2.1. Reagents

We used the following reagents: RPMI-1640 (31800-022), Alexa Fluor 568 goat anti-mouse IgG antibody (A11031), Alexa Fluor 568 goat anti-rabbit IgG antibody (A11011), Alexa Fluor 488 phalloidin (A12379), 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; D1306): Life Technologies; Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS(−); T900): Takara Bio; lipopolysaccharide (LPS; L3012), TritonX-100 (T9284), antibiotic antimycotic solution (A5955), which was premixed with penicillin, streptomycin and amphotericin B: Sigma-Aldrich; fetal bovine serum (FBS; S1560-500; lot: S06537S1560): Biowest; paraformaldehyde (PFA), phorbol 12-myristate 13-acetate (PMA; 162-23591), globulin free bovine serum albumin (BSA; 015-15103): Wako; CD14 antibody (Mouse-Mono(CC-G33); MCA2678GA): AbD Serotec; interleukin-1β (IL-1β) antibody (rabbit polyclonal, PBOIL1BI): Thermo Scientific; tumor necrosis factor-α (TNF-α) antibody (rabbit monoclonal, 8184S): Cell Signaling Technology.

2.2. Preparation of PVA hydrogels

Two types of PVA powder were obtained, one was purchased from Kishida Chemical Co., Ltd., Japan (polymerization degree: 2000, saponification degree: 98.4–99.8 mol%) and the other was obtained from Kuraray Co., Ltd., Japan (polymerization degree: 1700, saponification degree: 98–99 mol%). We prepared 20% w/w PVA solution using Kishida's powder for PVA-FT gel by the repeated freezing–thawing method with five freeze–thaw cycles. Freezing proceeded at −20 °C for 18 h and thawing at 4 °C for 6 h. We prepared 15% w/w PVA solution using Kuraray's powder for PVA-CD gel by the cast-drying method. The PVA powder was solved at 95 °C and was dried in an environment test chamber (Espec) to form a constant weight PVA solution [19,20]. We subsequently soaked the dried PVA gel into distilled water and stored it at room temperature for swell. The water used to soak the PVA-FT and -CD was used as a lubricant. When using saturated PVA-elution water as a lubricant, the elution of molecules from PVA wear particles could be suppressed during the wear test and subsequent storage, while it is easy to elute for uncrosslinked PVA molecules from gel to surrounding water solvent until it comes to equilibrium of the PVA molecule concentration between the gel and the solvent.

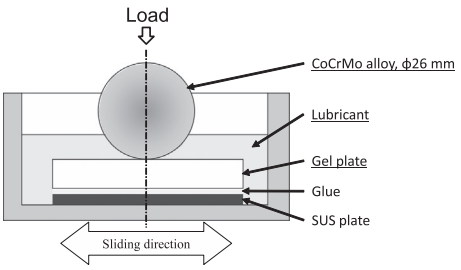

Fig. 1. Schematic drawing of a uniaxial reciprocating ball-on-plate tribometer for preparing PVA wear particles.

2.3. Tribological test and evaluations

2.3.1. Wear test and wear particle evaluations

To collect PVA hydrogel wear particles, we made a purpose-build wear test machine using a uniaxial reciprocating ball-on-plate tribometer as shown in Fig. 1. We tested a pair of specimens; the stationary upper specimen was a spherical 26-mm-diameter Co–Cr–Mo alloy and the reciprocating lower specimen was a 2-mm-thick PVA hydrogel plate. The applied load was 4.9 N and the maximum contact pressure was approximately 0.1 MPa [21]. Sliding speed was 50 mm/s. The reciprocating stroke was 50 mm, and total sliding distance was 3.0 km. The test was conducted in atmospheric air at 37 °C and 100% relative humidity. After the wear test, we collected the tested lubricant and dehydrated it at 2500*g* by ultrafiltration with an Amicon Ultra-15 centrifugal filter unit with Ultracel-100 membrane (UFC910008; Merck Millipore). We subsequently added 0.1% w/w tritonX-100 solution for a final volume of 15 mL for washing and left the solution for 1 h at room temperature. After centrifuging the solution at 2500*g* for dehydration, we added autoclaved distilled water for a final volume of 15 mL and recentrifuged five times to obtain a solution of washed wear particles. The solution of washed wear particles was stored in a refrigerator at 4–8 °C until use. Wear particles collected from PVA-FT and -CD were called WP-FT and WT-CD, respectively.

2.3.2. Determination of wear amount

The evaluation of total wear amount of hydrogel specimen by profilometry of rubbing surfaces or gravimetric method is very difficult. The viscoelastic and swelling behavior of hydrogels affects the surface profile, and the time-dependent water evaporation from hydrogel changes its weight in the gravimetric method using electric balance for weighing of specimen. Therefore, to estimate the total amount of PVA wear, we assessed the concentration of PVA molecules in lubricant by total carbon analysis [22]. We adjusted the washed wear particle solution up to 15 mL with the same lubricant and measured the total carbon content of the solution using a total organic carbon (TOC) analysis system (multi N/C 3100; Analytik Jena). A standard calibration curve was prepared in the concentration range 2–2000 μg/mL from each PVA solution by serial dilution of the 2000 μg/mL standard solution with ultra-pure water (Merck Millipore). After applying the calibration curve to values from all sample solutions, we subtracted the value of the lubricant from the values of the wear samples to determine the total amount of wear loss.

2.3.3. Particle size distribution

To determine the size distribution of PVA hydrogel wear particles generated from the wear test, we measured the wear particle solution with a particle size measurement system (ELSZ-0S; Otsuka Electronics) that utilizes dynamic light scattering. Most wear particles indicate not so much a spherical morphology as a three-dimensional elongated shape. A detected wear particle, therefore, shows up as an equivalent sphere because this method hypothesizes to measure a fluctuation scale length of a spherical Brownian particle. We diluted the washed wear particle solution to 3 mL and determined the distribution of PVA wear particles between 0.6 nm and 6 μm in size.

2.3.4. Particle shape observation

To observe the shape of PVA hydrogel wear particle, we used a high-resolution field emission gun scanning electron microscope (SEM; SU8000; Hitachi High-Technologies). The washed wear particle solution was filtered and trapped using Whatman Nuclepore track-etched membranes (0.05 μm-pore size; 111103; GE healthcare). We dried the filter at 30% relative humidity in a desiccator for at least 3 days and then coated it with platinum in an ion-coater (JFC-1600; JEOL).

2.4. Biological tests

2.4.1. Endotoxin assay

We measured the endotoxin concentration using colorimetry to confirm low content of the endotoxin. This is because presence of high endotoxin content upregulates the activation of immune response system and induces cytokine synthesis. First, we mixed the washed wear particle solution into basal medium (RMPI-1640 containing 10% v/v fetal bovine serum, 100 times dilution of antibiotic antimycotic solution). We then examined the mixture using the limulus amebocyte lysate (LAL) method with a Pierce LAL chromogenic endotoxin quantitation kit (88282; Thermo Scientific) according to the manufacturer's protocol. If the concentration was lower than 14 endotoxin unit (EU, 1 EU = 0.1 ng) per volume, we judged that the medium could be used without endotoxin-induced problems. EU level was decided by reference to the bacterial endotoxins test in the 2011 United States pharmacopeial convention and the volume was the total volume of culture medium with/without PVA wear particles.

2.4.2. Cell culture

To estimate the macrophage immune response to wear particles, we used the PMA-stimulated THP- 1 cell line as the macrophage [23]. We purchased the THP-1 cell line of human acute monocytic leukemia from the Japanese collection of research bioresources cell bank (JCRB0112; 07122011). We seeded THP-1 cells in PMA-containing medium (1 μg/mL of PMA in the basal medium) into a prescribed culture dish and left for 3 days. After PMA stimulation, we exchanged the medium with the new basal medium, left the culture for an additional 3

days and then identified a CD14-positive macrophage, in which CD14 was a kind of marker of macrophage (data not shown). We prepared two control media; the basal medium was the negative control (NegC) and the culture medium with 1 μg/mL of LPS was the positive control (PosC). LPS was an endotoxin for inducing a powerful immune response.

2.4.3. Cytotoxicity assay

We measured the number of macrophages using a cell counting kit-8 (CK04; Dojindo Laboratories) by monitoring mitochondria activity. We seeded THP-1 cells at 2×10^4 cells/well in PMA-containing culture medium into a 96-well polysthylene plate (353072; Corning Life Sciences). After 3 days, we exchanged the basal medium and left the plates for another 3 days. We measured the cell number according to the manufacturer's protocol.

2.4.4. Cytokine assay by ELISA

To evaluate the presence of cytokines in culture medium, we measured IL-1β and TNF-α levels using an enzyme-linked immune sorbent assay (ELISA). We seeded THP-1 at 1×10^6 cells/well in PMA-containing culture medium into a 60-mm-diameter polysthylene dish (3353004; Corning Life Sciences). After 24 h, we collected the culture medium into a low protein binding tube (BM4015; BM Equipment) and stored it at −80 °C. We then used a human IL-1β ELISA Kit (EH2IL1B; Thermo Scientific) and human TNF-α ELISA Kit (EH3TNFA, Thermo Scientific) according to the manufacturer's protocol.

2.4.5. Immunohistochemistry

We stained IL-1β and TNF-α in macrophages to observe the effect with stimulation of PVA wear particles on macrophage immune response. We seeded THP-1 cells at 5×10^5 cells/dish in PMA-containing culture medium into a 35-mm-diameter glass-bottom dish (glass diameter 12 mm; 3911-035; Iwaki). After 24 h, we mixed cells with 4% w/v PFA/PBS(−) for 15 min and washed them three times with PBS(−). The sample was then permeabilized by 0.1% w/v TritonX-100/PBS(−) for 5 min and washed three times with PBS(−). We immersed the sample in 1% BSA/PBS(−) for 1 h, which was used as a blocking agent. After washing three times with PBS(−), we immunostained samples with the primary antibody of either IL-1β or TNF-α for 90 min at room temperature. We then washed the samples with PBS(−) three times and subsequently immunostained them with Alexa Fluor 568 goat anti-mouse IgG antibody and Alexa Fluor 568 goat anti-rabbit IgG antibody, respectively. We subsequently counterstained for F-actin and nuclei in macrophages with phalloidin and DAPI, respectively, and then observed the stained sample using a confocal laser scanning microscope (CLSM; C1; Nikon).

2.5. Statistical analysis

Results of the endotoxin assay, cytotoxic assay, and ELISA were expressed as the mean ± standard deviation (SD). The significance of the difference between each experimental group was evaluated using the two-tailed Welch's *t*-test. The degree of freedom was abbreviated as *df*. If results of pairwise comparisons between two groups were $d > 1$, $P < 0.01$ and $(1-\beta) > 0.8$ simultaneously, we judged the difference as significant in the tangent modulus, where d is Cohen's d, a kind of the effect size [24]; P is the level of significance; and $(1-\beta)$ is the power of the test that had been calculated using the R language.

3. Results

3.1. Tribological assessments

3.1.1. Total amount of wear particles

The total amount of PVA wear particles for each PVA hydrogel was measured using the TOC analyzer. The total amounts of washed wear particles for WP-FT and -CD were 5.9 ± 0.2 mg ($N=4$) and 0.93 ± 0.07 mg ($N=4$), respectively. The total amount of wear in WP-CD group was significantly suppressed compared with that of WP-FT (WP-FT vs WP-CD: $d=52.6$; df=4.65; $P=1.25 \times 10^{-8}$; $(1-\beta)=0.999$). This result indicated a similar tendency to another report in which Suzuki et al. had demonstrated wear property using a ball-on-plate wear test with an alumina ball on PVA-FT or -CD hydrogel [22].

3.1.2. Size distribution of PVA wear particles

We demonstrated particle number distributions of wear particles of WP-FT and WP-CD by the dynamic light scattering method, as shown in Fig. 2. Wear particles 10–50 nm in diameter were dominantly dispersed in both wear test groups. In the particle volume distribution which was calculated by the volume fraction of the particles based on results from the particle number distribution in Fig. 2(a) and (c), each wear particle distribution of range from 5 to 10 μm was similar to that of range of 100–600 nm as shown in Fig. 2(b) and (d), respectively. There were few particles approximately 1 μm in size, which are known to induce the macrophage immune response [25]. There were negligible differences in the distributions of the WP-FT and -CD groups.

3.1.3. Shape observation of PVA wear particles

We examined SEM images of dried PVA wear particles as shown in Fig. 3. Although the range of particles between 0.5 and 5 μm was chiefly observed (Fig. 3(a, b) and (e, f)), a few large-sized particles such as greater than 5 μm were found. We found a number of submicron-sized wear particles approximately 100 nm in each wear particle group (Fig. 3(c) and (g)). Comparing WP-FT with WP-CD, a majority of micron- and submicron-sized particles were similarly shaped. Most micron-sized particles were spherical and elongated, and appeared to be aggregations of nano-sized PVA particles approximately 20 nm in diameter. These nanoparticles in both WP-FT and -CD groups were attached on the flat surface around the pore of the 0.05 μm pore-sized filter as shown in Fig. 3(d) and (h).

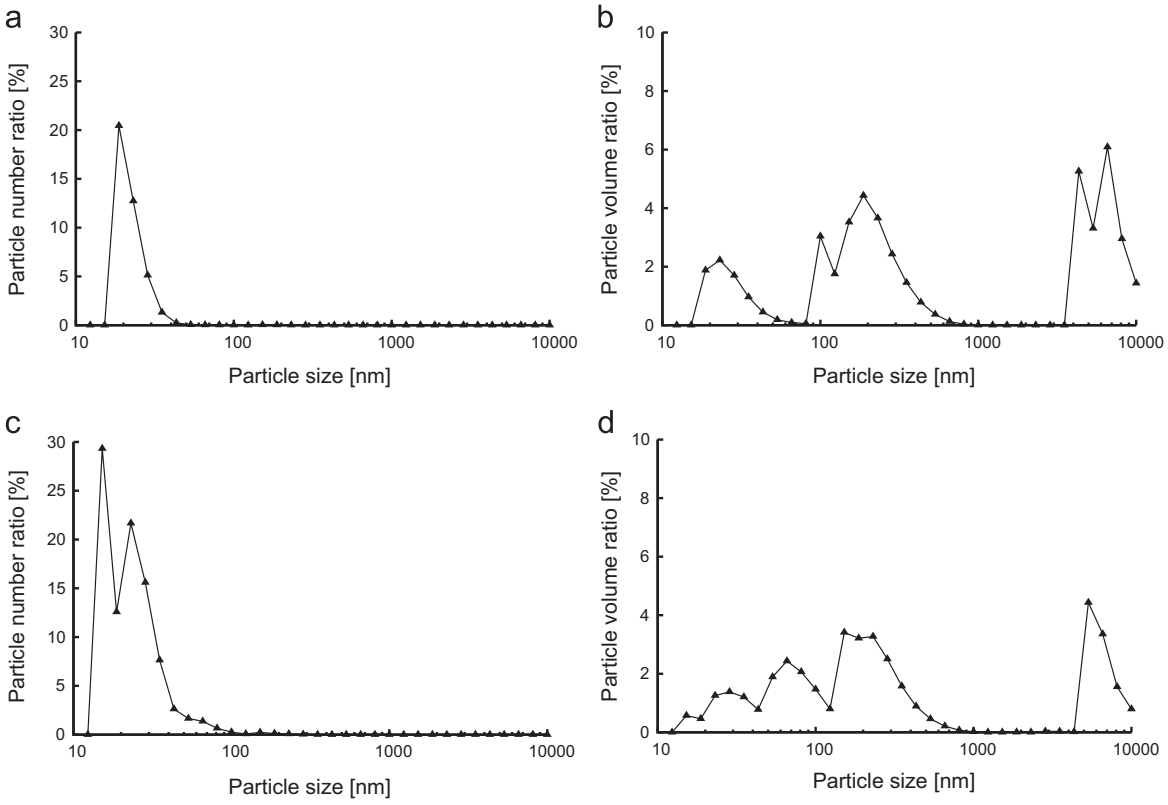

Fig. 2. Particle size distribution of PVA wear particles of PVA-FT (a, b) and PVA-CD (c, d) between 10 nm and 10 μm in diameter; (a, c) particle number distribution; (b, d) particle volume distribution.

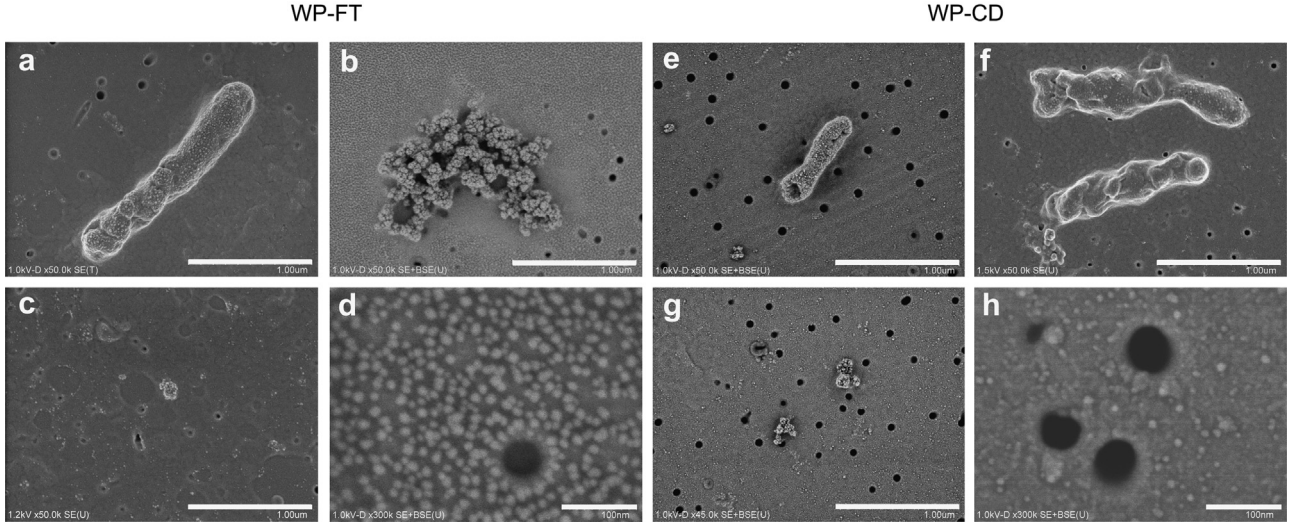

Fig. 3. SEM images of large PVA wear particles of PVA-FT (WP-FT: a–d) and PVA-CD (WP-CD: e–h). Scale bars: a–c, e–g: 1 μm; d, h: 100 nm.

3.2. Biochemical evaluations

3.2.1. Endotoxin assay

We confirmed the endotoxin concentration in each culture medium. Basal medium, as the negative control (NegC), had 0.87 ± 0.39 EU/V ($N=6$) in a total volume of 15 mL. In experimental groups, the WP-FT group had 11.0 ± 2.1 EU/V ($N=9$), and the WP-CD group had 2.3 ± 0.45 EU/V ($N=8$). All media had concentrations below 14 EU/V. We judged, therefore, that all culture media without PosC were appropriate for immunological study of the effect of PVA hydrogel wear particles on macrophages.

3.2.2. Cytotoxicity assay

Fig. 4 shows the results of the macrophage cytotoxicity assay by adding PVA wear particles to the culture medium. The NegC group had $5.76 \pm 0.62 \times 10^4$ cells/cm^2 ($N=9$) and the PosC group had $8.59 \pm 0.54 \times 10^4$ cells/cm^2 ($N=9$). In the experimental groups, WP-FT and WP-CD groups had $6.94 \pm 0.59 \times 10^4$ ($N=7$) and $6.5 \pm 1.1 \times 10^4$ cells/cm^2 ($N=6$),

respectively. Thus, the number of cells per unit area in the PosC group was significantly increased compared to the other groups, as shown in Table 1.

3.2.3. Cytokine synthesis

To estimate the effect of PVA wear particles of PVA-FT and -CD gels on the macrophage immune response, we not only measured cytokines released into the culture medium using ELISA, but also fluorescently immunostained cytokines remaining in their cells and observed the distribution using CLSM. We focused on IL-1β and TNF-α as the most important marker cytokines of the immune response. First, we investigated cytokines released into the medium, as shown in Fig. 5, and summarized results of statistical analysis in Table 2. For IL-1β, the NegC group had 70 ± 66 pg ($N=8$), the PosC group had 410 ± 310 pg ($N=8$), and the WP-FT and WP-CD groups had 83 ± 78 pg ($N=9$) and 54 ± 35 pg ($N=8$), respectively. For TNF-α, the NegC group had 180 ± 210 pg ($N=8$), the PosC group had 4700 ± 3500 pg ($N=8$), and the WP-FT and WP-CD groups had 280 ± 330 pg ($N=9$) and 110 ± 52 pg ($N=8$), respectively. As indicated by

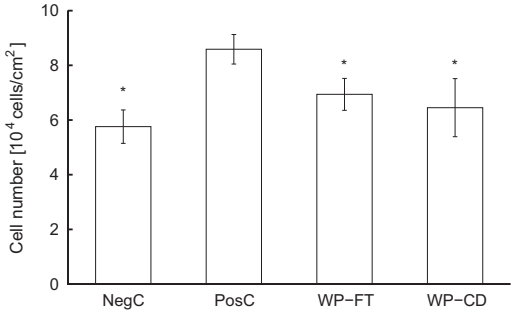

Fig. 4. Cytotoxicity assay of macrophage in culture medium containing each PVA wear particle of PVA-FT (WP-FT) and PVA-CD (WP-CD) for a 24 h culture period. NegC and PosC indicate negative and positive control groups, respectively. Error bar means SD. Asterisks (*)indicate statistically significant respective culture groups compared to PosC, *: $d > 1$, $P < 0.01$, $(1-\beta) > 0.8$.

Table 1
Statistical analysis of cytotoxicity assay between each group.

	d	df	P	$(1-\beta)$
PosC vs NegC	4.91	15.8	9.05×10^{-9}	0.999
PosC vs WP-FT	2.94	15.9	6.23×10^{-6}	0.998
PosC vs WP-CD	2.59	14.4	4.90×10^{-5}	0.983

Table 2
Statistical analysis of cytokine synthesis between each group.

	d	df	P	$(1-\beta)$
IL-1β				
NegC vs PosC	1.57	7.32	0.00763	0.579
NegC vs WP-FT	0.203	9.58	0.343	0.0154
NegC vs WP-CD	0.450	12.9	0.216	0.0323
PosC vs WP-FT	1.53	7.80	0.00707	0.588
PosC vs WP-CD	1.68	7.18	0.00619	0.635
WP-FT vs WP-CD	0.482	11.4	0.171	0.0460
TNF-α				
NegC vs PosC	1.87	7.05	0.00358	0.763
NegC vs WP-FT	0.346	9.92	0.247	0.0269
NegC vs WP-CD	0.512	7.92	0.168	0.0480
PosC vs WP-FT	1.89	7.11	0.00290	0.809
PosC vs WP-CD	1.91	7.00	0.00331	0.781
WP-FT vs WP-CD	0.711	8.46	0.0897	0.102

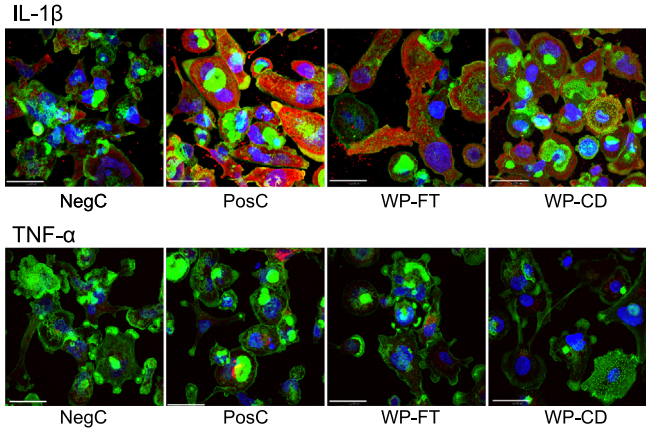

Fig. 6. Immunohistochemical observations of the distribution of two types of cytokines, I and TNF-α, in macrophages by applying PVA wear particles of PVA-FT (WP-FT) and PVA-CD (WP-CD) to PMA-THP1 cells for a 24 h culture period. NegC and PosC indicate negative and positive control groups, respectively. Red: IL-1β or TNF-α; green: F-actin; blue: nucleus. Scale bars: 50 μm.

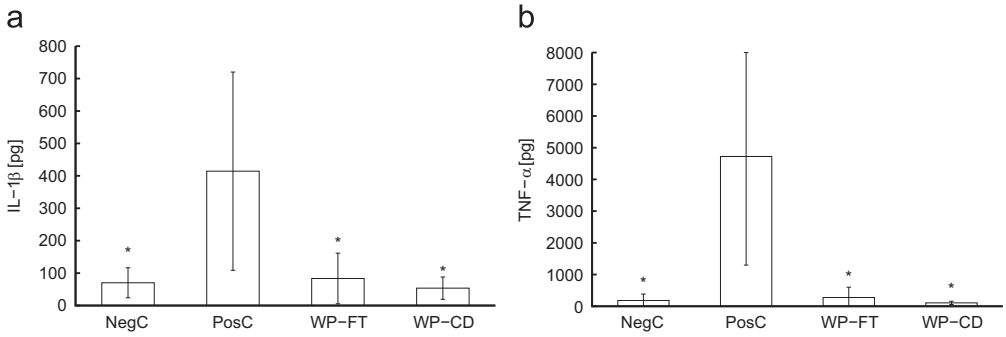

Fig. 5. Cytokine syntheses of IL-1β (a) and TNF-α (b) by applying each PVA wear particle of PVA-FT (WP-FT) and PVA-CD (WP-CD) to macrophages for a 24 h culture period. NegC and PosC indicate negative and positive control groups, respectively. Error bar means SD. Asterisks (*) indicate statistically significant respective culture groups compared to PosC, *: $d > 1$, $P < 0.01$, $(1-\beta) > 0.8$.

the *d* values, exposing macrophages to wear particles in both particle-stimulated groups, compared to negative control, did not change cytokine syntheses. Then, positive control group was found to significantly increase biosynthesis of both cytokines as shown in Table 2 because any relevant Cohen's *d* were enough high. Cytokine synthesis of the WP-CD group was lower than that of the WP-FT group.

Second, we observed the cytokine distribution in macrophages as shown in Fig. 6. In PosC, both IL-1β and TNF-α were synthesized, compared to NegC. In contrast, groups exposed to PVA wear particles exhibited low synthesis of each cytokine compared to the positive control.

4. Discussion

To develop a novel artificial cartilage with superior tribological function, we surveyed whether PVA hydrogel wear particles were nontoxic and biocompatible. We conducted a ball-on-plate tribological test with a Co–Cr–Mo ball specimen and two types of PVA hydrogels, PVA-FT and PVA-CD, as a plate sample in a water lubricant that was saturated by the eluted PVA molecules from each PVA hydrogel. To evaluate PVA wear particles, we measured the wear amount and particle size distributions in water, and observed the morphology of the dried wear particles using SEM. The total wear amount of WP-CD was significantly lower than that of WP-FT. Our results indicated a similar tendency to another report by Suzuki et al., which used a ball-on-plate wear test with an alumina ball on PVA-FT or -CD hydrogel [22]. Moreover, in a series of tribological studies of PVA hydrogels, Murakami et al. [26] examined the tribological behavior of two hydrogels in reciprocating friction tests and numerical simulations using a biphasic finite-element method. Although both Young's moduli were similar between PVA-FT and PVA-CD, both the coefficient of friction and water permeability of PVA-CD gel were clearly lower than those of PVA-FT. Since PVA-FT showed comparatively high permeability, the surface of the PVA network in the solid phase of the FT gel sustained the normal force at a higher rate than the fluid phase. Consequently, the FT gel is subject to higher shear strain than the CD gel after a prescribed period of wear. By contrast, the CD gel sustained the normal force for a long time by hydrostatic pressure in the fluid phase. High load support from the fluid phase minimized contact between the solid phase of the PVA gel and the ball, which slowed the prevention of wear. These differences may explain the greater amount of wear of the FT gel compared to the CD gel in the present study. The friction coefficient of PVA-CD being lower than that of PVA-FT supports this speculation [26].

The particle size distributions of WP-FT and -CD exhibited the same tendency, as shown in Fig. 2. Wear particles were predominately less than 500 nm in diameter, with a majority being approximately 20–50 nm in diameter. There were considerable wear particles from about 100 to 500 nm and over 5 μm and in the particle volume distribution as shown in Fig. 2(b) and (d). Interestingly, there were very few number of wear particles around 1 μm in diameter. Since these micron-sized particles were mostly phagocytosed by macrophages, which induces cytokine synthesis and macrophage immune response [25,27], it is speculated that applying the PVA wear particles did not induce a macrophage immune response.

SEM observations demonstrated that dried micron-sized PVA wear particles formed larger-sized particles by close packing of 20- to 50-nm-sized particles. Many of these nanoparticles homogeneously adhered to the particle-capture filter. Although shape study may be recommended only for size ranges at majority (in this case 20–50 nm) in evaluation of wear particle, at the present stage, it should be conducted not only for 20–50 nm but also for a wide range to several micrometers, since there are few reports for hydrogels on the influence of wear particle shape on immune response. We could observe different shapes for larger particle but globular shapes for 20–50 nm particles in SEM images (Fig. 3(d) and (h)). Dynamic light scattering measurements confirmed that the wear particles were mostly nano-sized. Moreover, we were not able to observe any scattering spectrum of the water lubricant in which the PVA hydrogel was soaked until it was saturated with eluted PVA molecules. We believe that the nanoparticles occurred in association with the wear test because microcrystals, which were crosslinked due to hydrogen bonds in the PVA hydrogel, were gradually scraped off by frictional shear deformation. Although the wear particles are small during the early period, large-sized particles also were formed when the sliding distance is lengthened [16]. Thus, many of the micron-sized wear particles were likely generated during the wear test in this study. Notably, a number of the micron-sized particles exhibited a twisted and elongated morphology. This phenomenon occurred due to the severe shear force applied between the sliding surfaces until ejection of the wear particle.

To assess macrophage immune response to PVA wear particles, we used the THP-1 cell line of human acute monocytic leukemia as macrophages. No difference in basic activation of mitochondria in cells was observed after exposing PMA-stimulated THP-1 cells to wear particles of PVA-FT and -CD. This means that the wear particles did not affect changes in cell number. Therefore, PVA wear particles are nontoxic to macrophages.

Since proinflammatory cytokines, which are synthesized by phagocytizing the wear particles, are generally cited as a major factor of osteolysis and aseptic loosening, we investigated the effect of the PVA hydrogel wear particles on macrophage immune response by assessing two types of cytokines: IL-1β and TNF-α. At the results, few particle-stimulated macrophages were positive stained for cytokines compared to the negative control, and there were remarkably fewer positively stained macrophages than the LPS-stimulated positive control, as shown in the results of immunofluorescent staining in Fig. 5. ELISA did not show any significant increase in synthesis of both IL-1β and TNF-α in culture medium, compared to negative control. We confirmed that PVA wear particles did not induce macrophage immune response. Thus, PVA hydrogels not only as a bulk material but also its wear particles, have remarkable biocompatibility. The WP-CD group showed slightly decreased cytokine synthesis compared

to the WP-FT group. This is because PVA-CD has a lower total amount of wear than PVA-FT. Consequently, the immune response in WP-CD was subsequently decreased because of less phagocytosis. Therefore, it is predicated that suppressing wear inhibits macrophage immune response and osteolysis.

Our results indicated that PVA hydrogels have high biocompatibility in vitro. Here the PVA gels were physically crosslinked by numerous hydrogen bonds between PVA molecules [19]. These would indicate that these primitive physically bonded crosslinked PVA hydrogels are safe in mammalian joints because the side chain of the PVA molecule is only a hydroxyl group. Moreover, there are several reports on the biocompatibility of PVA hydrogels [12 and reference therein]. However, these PVA hydrogels do not have sufficient mechanical properties for severe rubbing at high loading, and it may be difficult to sustain a tribological environment with over several MPa of surface pressure over a long period. Recently, several chemically bonded crosslinked PVA hydrogels have been developed. These use various crosslinkers, such as glutaraldehyde [28] and tetraethylene glycol ditosylatecite [29], which are highly toxic. Therefore, it is necessary to wash and detoxify the crosslinkers. It is infrequently focused on genipin which is a biocompatible and nontoxic crosslinker [12,30]. As discussed above, because sufficient tribological function of the biocompatible PVA hydrogels has yet to be attained at severe conditions, the mechanical properties and tribological functionalities of PVA hydrogel should be enhanced in future studies.

5. Summary

We surveyed the effect of PVA hydrogel wear particles on the immune response of THP-1 macrophages using two types of PVA hydrogels, PVA-FT and PVA-CD gels, to investigate the possibility that these particles lead to aseptic loosing of the artificial cartilage implant system. We collected wear particles of PVA-FT and -CD gels using a ball-on-plate tribological test in water lubricant with a Co–Cr–Mo ball and PVA gel plates. Results of the wear test showed that the total amount of WP-CD was significantly lower than that of WP-FT. This is likely because the PVA-CD gel has a lower coefficient of friction and lower water permeability than the PVA-FT gel. The size distributions of PVA-FT and -CD wear particles were similar. Most of the particles were nanoparticles up to 50 nm in diameter. Considering the particle volume distribution, there were considerable micron-sized particles over 5 μm in diameter. There were very few micron- and submicron-sized wear particles around 1 μm in diameter. In SEM observations of dried PVA wear particles, both distributions of WP-FT and -CD were similar. Micron-sized wear particles were chiefly formed by close packing of 20- to 50-mm-sized particles. Biochemical and immunological evaluations revealed no cytotoxic effects of wear particles on macrophages. Cytokine synthesis of both wear particle-stimulated groups was significantly lower than that of the LPS-stimulated positive control. Thus, PVA wear particles do not affect the macrophage immune response. If the lubricity of the PVA hydrogel can be further improved, the wear will be decreased, and the risk of osteolysis will be reduced.

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