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Effect of particle size on biological response by human monocyte-derived macrophages

H. Chikaura[a], Y. Nakashima[a], Y. Fujiwara[b], Y. Komohara[b], M. Takeya[b], Y. Nakanishi[a,*]

[a]*Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan*
[b]*Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8555, Japan*

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Abstract

Ultra-high molecular weight polyethylene (UHMWPE) wear particles from artificial joints induce osteolysis and the subsequent loosening of implants. Studies have reported that particles in the size range of 0.1–10 μm are the most biologically active in macrophage immune response. To develop prosthetic joints with greater longevity and durability, it is crucial to understand the deleterious effects of wear particles. In this study, to evaluate the effects of particle size on the activities of human monocyte-derived macrophages (HMDMs), seven differently sized particles of polymethylmethacrylate (PMMA), in the range of 0.1–20 μm, were prepared. Viability and the secretion of cytokines were evaluated after phagocytosis of each size particles by HMDMs. Differences in the viability of HMDMs after phagocytosis of particles sized 0.16–9.6 μm were statistically significant. Proinflammatory cytokine production of both tumor necrosis factor-α and interleukin-6 by HMDMs was strongly induced by 0.8 μm PMMA particles. Consistent with the fact that macrophages are known to respond to pathogens measuring approximately 1.0 μm in size, in this study, PMMA particles measuring 0.8 μm in size induced an immune response. This work provides fundamental data for the designing of surface profiles of prosthetic joints, Which may expect the lower incidence of immune response.

Keywords: Particle size; Biological response; Artificial joint; Macrophage; Cytokines

1. Introduction

Several studies have reported the phagocytosis of ultra-high molecular weight polyethylene (UHMWPE) wear particles from artificial joints by macrophages [1–5]. Macrophages release cytokines that stimulate osteoclasts, thereby resulting in bone resorption [2,6,7]. UHMWPE wear particles induce osteolysis and the subsequent loosening of implants, which are the main factors of aseptic revision. Phagocytosis of particles by macrophages represents an important component of the cellular response to implants. UHMWPE particles within a size range of 0.1–10 μm are known to be the most biologically active [8–10]. In particular, particles measuring less than 1.0 μm in size are thought to induce biological responses and stimulate macrophages to produce inflammatory mediators such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [2,4,11,12]; these study findings indicate that the size of the wear particles is an important parameter. In this regard, previous studies have proposed that the nano-level surface texture of the Co–Cr–Mo alloy would ensure both, a larger size of UHMWPE wear particles and minimization of the wear of UHMWPE, which is expected to inhibit the biological activity of macrophages [13]. However, it is not clear that particle size affects on biological response. With an aim to design a surface profile to ensure lower incidence of osteolysis and aseptic loosening of implants, this study investigated the effect of the particle morphology, especially the particle size, on immune responses of human monocyte-derived macrophages. Furthermore, this study attempted to determine the particle size

*Correspondence to: Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Cyuo-ku, Kumamoto 860-8555, Japan.
E-mail address: y-naka@mech.kumamoto-u.ac.jp (Y. Nakanishi).
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that induces the most hazardous biological response by analyzing viability and the secretion of proinflammatory cytokines in macrophages after they phagocytosed particles of each size.

2. Materials and Methods

2.1. Preparation of narrow-dispersion polymethylmethacrylate (PMMA) particles

To unveil the relationship between particle size and biological response, narrow-dispersion (PMMA) particles (Soken Chemical & Engineering Co., Ltd., Japan) were used instead of UHMWPE wear particles. Spherical particles with a mean size of 0.16, 0.43, 0.8, 1.6, 5.6, 9.6 and 19.3 μm were prepared. The 0.16 and 0.43 μm particles were composed of cross-linked PMMA and the others were composed of non-cross-linked PMMA. Fig. 1 showed the scanning electron micro scope (SEM; JSM-6390LV, JEOL Ltd., Japan) images of PMMA particles. Particles of each size were washed with 70% ethanol. The particles were suspended in phosphate-buffered saline (PBS (-)), which made by dissolving sodium dihydrogenphosphate dihydrate (199-02825, Wako Pure Chemical Industries, Ltd., Japan), disodium hydrogenphosphate 12-Water (196-02835, Wako Pure Chemical Industries, Ltd., Japan) and sodium chloride (192-13925, Wako Pure Chemical Industries, Ltd., Japan) in distilled water, in order to obtain an initial concentration of 50 μg/μL. To disperse the aggregated fine particles, the suspensions were treated with ultrasonic waves using Bioruptor (UCD-250, Cosmo Bio Co., Ltd., Japan).

2.2. Preparation of macrophages

Human monocyte-derived macrophages (HMDMs) obtained from healthy volunteer donors were used in the present study. Informed written consent was obtained from all donors. The preparation procedure for the cells is shown in Fig. 2. Peripheral blood (35 mL) was collected from healthy donors. Fifteen milliliter of PBS (−) with 1 mM ethylenediamine-N, N,N′,N′-tetraacetic acid, disodium salt, dihydrate (EDTA 2NA; 34501861, Dojindo Molecular Technologies, Inc., Japan) was

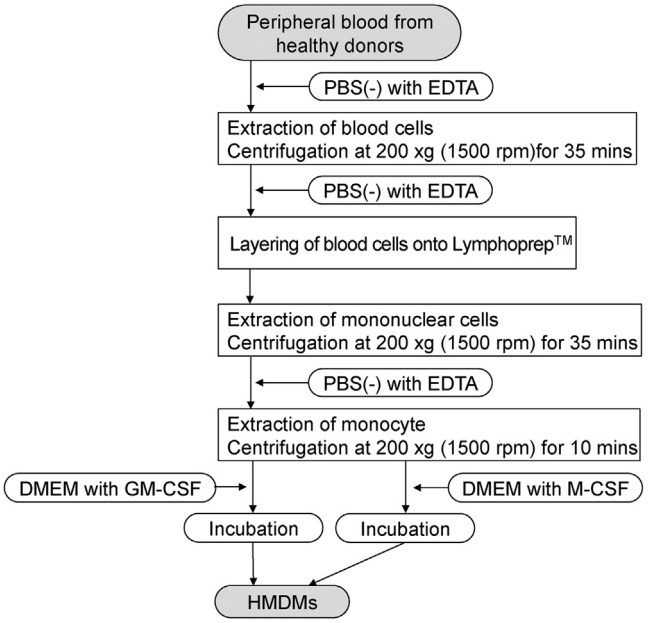

Fig. 2. Procedure for the preparation of HMDMs.

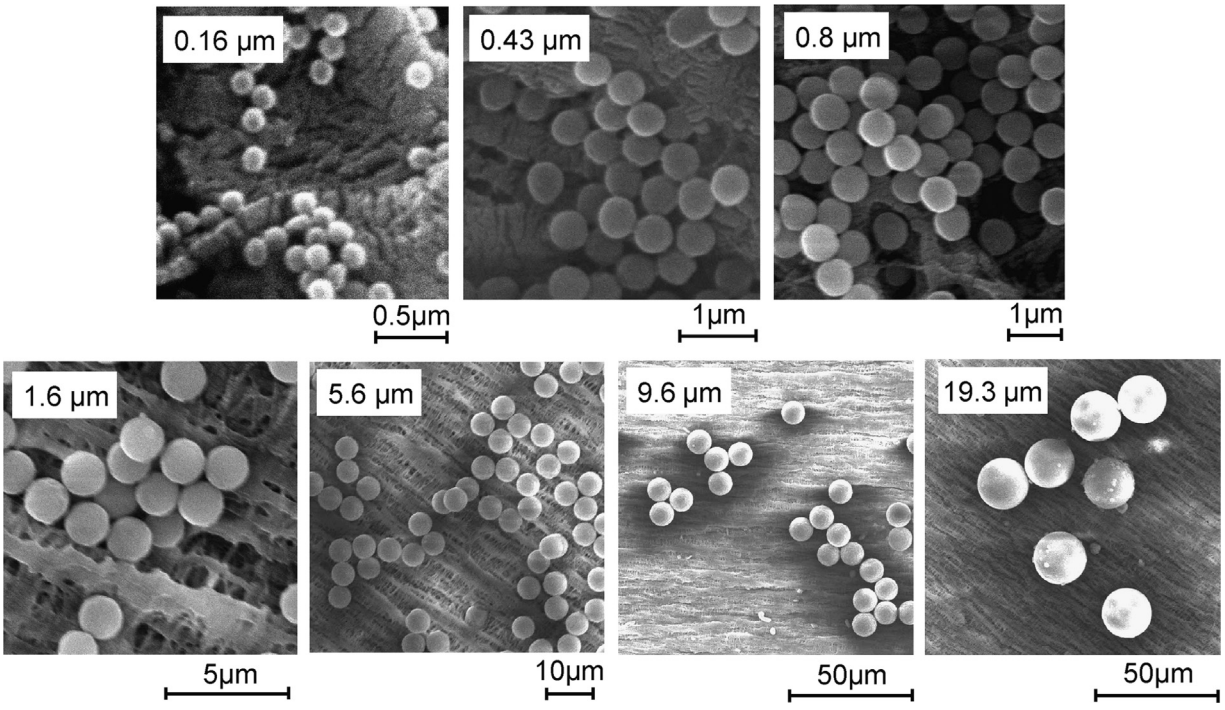

Fig. 1. SEM image of PMMA particles. Particle sizes were 0.16–19.3 μm.

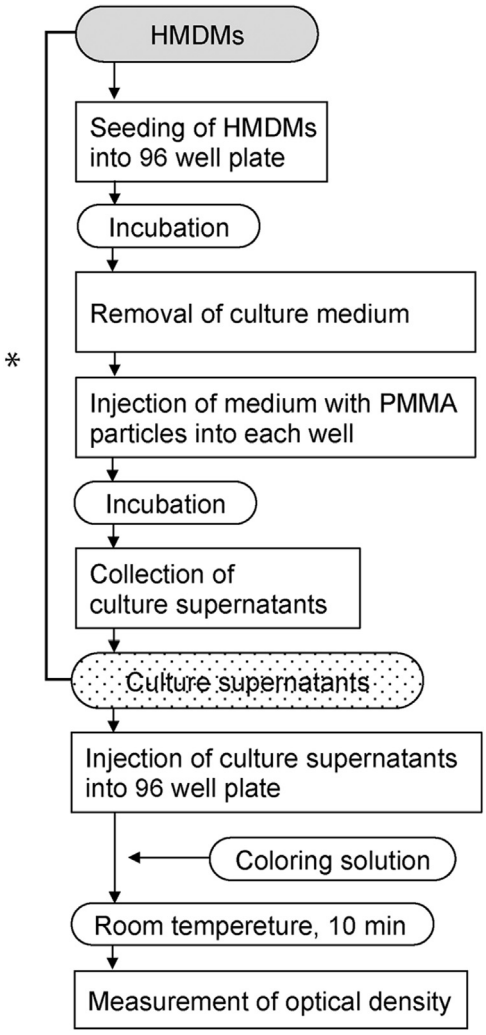

Fig. 3. Procedure detailing the evaluation of HMDMs activation. Asterisk (*) shows the method for collecting culture supernatants.

added to the blood. The blood cells were extracted by centrifugation at 200*g* (1500 rpm) for 35 min using the general purpose refrigerated centrifuge (5920, KUBOTA Co., Japan). Thirty five milliliter of blood cells diluted by PBS (-) with 1 mM EDTA blood cells were layered on top of 10 mL of Lymphoprep™ (1114547, Axis-Shield Diagnostics Ltd., UK). The layered blood cells were centrifuged at 200*g* (1500 rpm) for 35 min, following which three separate components were obtained: blood platelets, mononuclear cells, and red blood cells. Of these components, the mononuclear cells were extracted. The mononuclear cells (50 mL) was diluted with PBS (−) with 1 mM EDTA and then centrifuged at 200*g* (1500 rpm) for 10 min, following which two separate layers were obtained: the lymphocyte layer and the monocyte layer. CD14+ monocytes were purified by positive selection via magnetic-activated cell sorting technology (130-150-201, Miltenyi Biotec, Germany) as described previously [14]. The monocytes were added to 20 mL of Dulbecco's modified Eagle's medium (DMEM; 041-29775, Wako Pure Chemical Industries, Ltd., Japan) supplemented with 2% fetal bovine serum (FBS; 172012-500ML, 12E183-A, Sigma), 100 U/mL

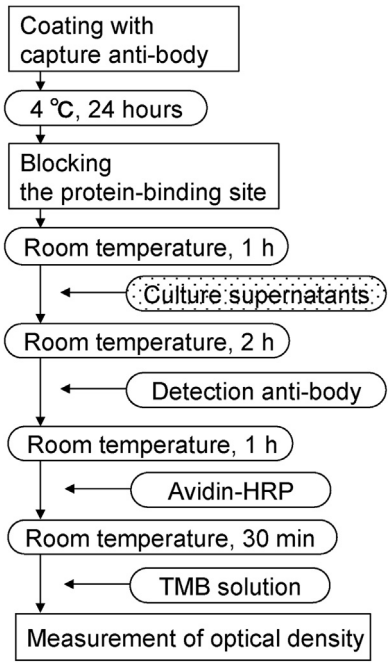

Fig. 4. The procedure of ELISA for the evaluation of proinflammatory cytokine secretion.

penicillin and 100 μg/mL streptomycin (168-23191, Wako Pure Chemical Industries, Ltd., Japan). The culture medium was divided into two 100 mm PRIMARIA™ tissue culture dishes (353803, BD Falcon, USA). Ten nanogram/milliliter granulocyte-macrophage colony-stimulating factor (GM-CSF; 075-04114, Wako Pure Chemical Industries, Japan) or 50 ng/mL macrophage colony stimulating factor (M-CSF; 139-13613, Wako Pure Chemical Industries, Japan) was applied to each dish and incubated for 7 days to differentiate the monocytes into macrophages. Macrophages that were mixed each chemical induced macrophage were used as HMDMs in this study.

2.3. Phagocytosis of PMMA particles by HMDMs

Fifty microgram/microliter PBS (−) containing PMMA particles was diluted to 1.0 μg/μL with culture medium: DMEMs supplemented with 2% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. HMDMs were seeded on an 8-well chamber slide (354118, Corning Inc., USA) at a density of 1×10^5 cells/well. One hundred microliter of culture medium containing PMMA particles (100 μg of PMMA particles of each size) was injected into each well. After exploring the particles for 5 h, phagocytosis activity of HMDMs stained with hematoxylin (1.04302.0025, Merck Millipore, Germany) and eosin (1.15935.0025, Merck Millipore, Germany) was observed with a light microscope (BX51, Olympus Co., Japan).

2.4. Effect of the amount of PMMA particles and the size of the particles on cell viability

To investigate the cytotoxicity of PMMA particles on HMDMs, the lactate dehydrogenase (LDH) cytotoxicity assay

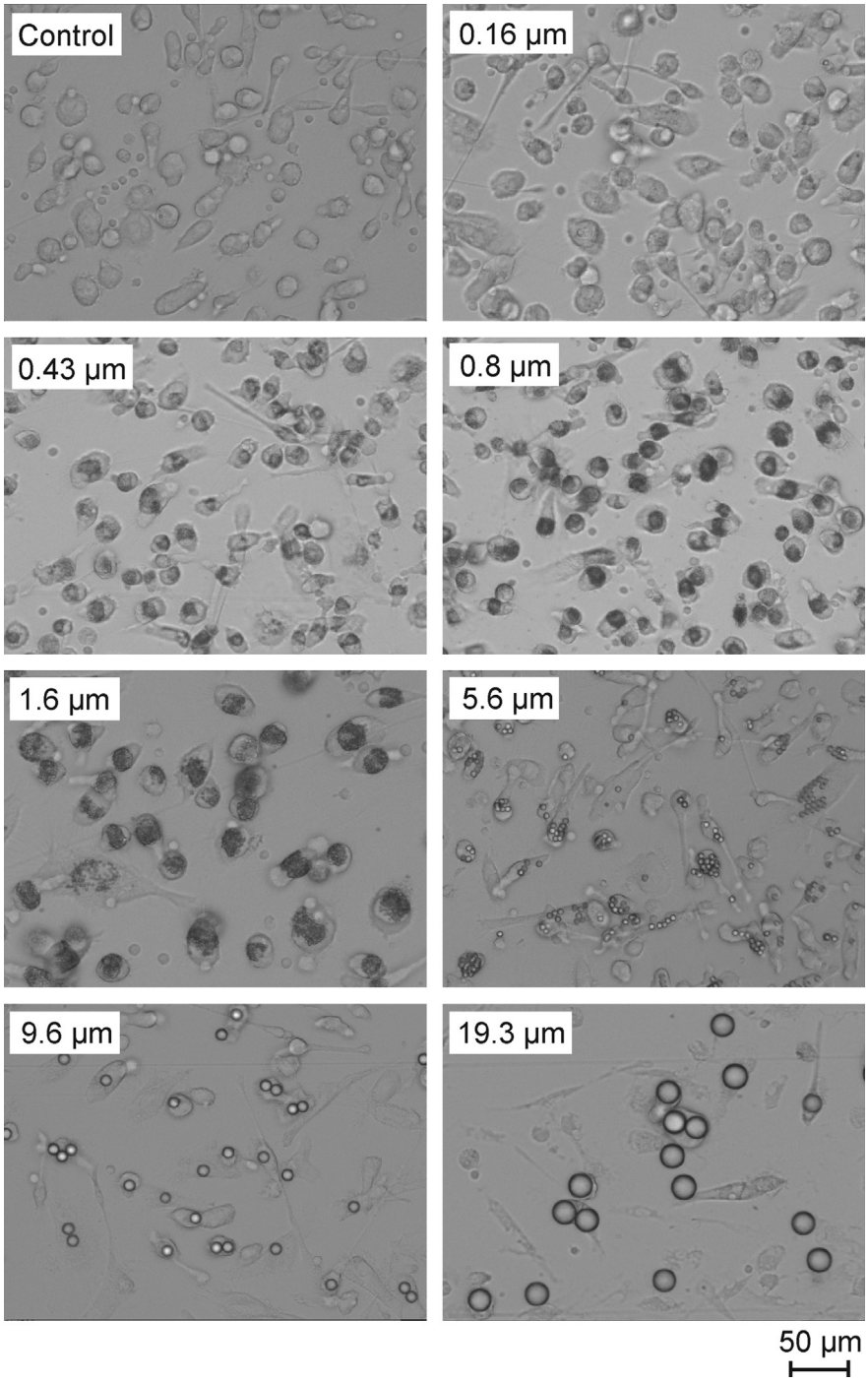

Fig. 5. Optical microscopic images of HMDMs cultured with PMMA particles of each size for 5 h using an inverted phase-contrast microscope. The particle sizes were 0.16, 0.43, 0.8, 1.6, 5.6, 9.6 and 19.3 μm, and a control image means applying no particles as control.

was carried out. This test procedure is shown in Fig. 3. HMDMs were seeded on a PRIMARIA™ 96-well plate (353873, Corning Inc., USA) at a density of 1×10^4 cells/well and incubated for 2 or 3 days to ensure sufficient cell adhesion. An aliquot of 50 μg/μL of PMMA particle in PBS (−) was diluted to 1.0, 3.0 and 5.0 μg/μL with culture medium, respectively. After the culture medium was removed, 100 μL of culture medium with each concentration of PMMA particles was injected into each well. Therefore, we applied 100, 300 and 500 μg/well of PMMA particles to HMDMs, and the cells were incubated for 24, 48, and 72 h. After incubation, culture supernatants in each well were collected and cell viability was analyzed by the LDH cytotoxicity Test kit (299-50601, Wako Pure Chemical Industries, Ltd., Japan). In this test, the supernatant of cells that were incubated without PMMA particles was used as the negative control. For the positive control, culture supernatant of cells incubated with culture medium containing 1% polyoxyethylene sorbitan monolaurate (28353-85, Nacalai Tesque, Inc., Japan) for 1 h after incubation with culture medium alone for 23 h was used.

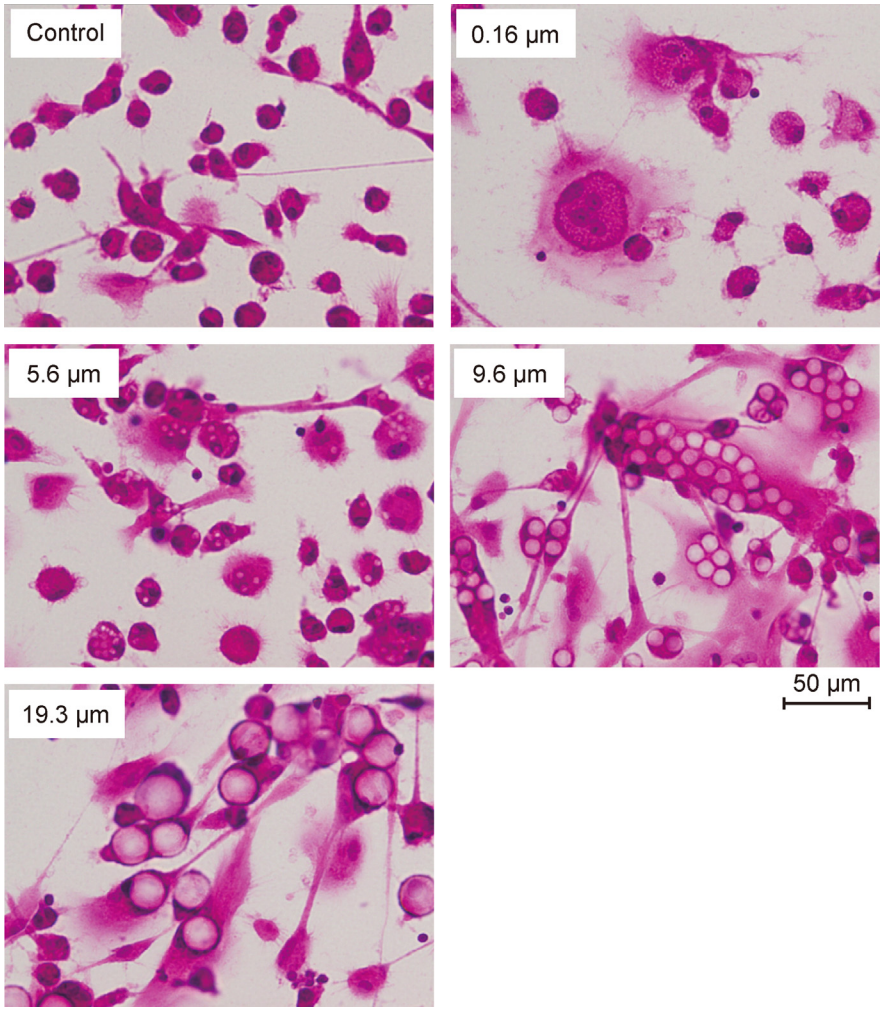

Fig. 6. Optical microscopic images of HMDMs cultured with PMMA particles of each size for 5 h using an inverted phase-contrast microscope. HMDMs were stained with hematoxylin and eosin. The particle sizes were 0.16, 5.6, 9.6 and 19.3 μm, and a control image means applying no particles as control.

Each culture supernatant was then added to a 96-well plate. Subsequently, the coloring solution was applied to each well and it was allowed to react for 10 min at room temperature, following which the optical density of each well was measured. The negative control indicated 100% cell viability and the positive control indicated 0% cell viability.

2.5. Effect of particle size on proinflammatory cytokine production

A flow chart of the method for collecting culture supernatants was the same as that asterisk (*) shown in Fig. 3. For evaluating the effects of each particle size on biological responses, the proinflammatory cytokines, which were IL-1β, IL-6 and TNF-α, in the supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) kits: human IL-1β ELISA kit (88-7010-22, eBioscience, USA) , human IL-6 ELISA kit (88-7066-22, eBioscience, USA) and human TNF-α ELISA kit (88-7346-88, eBioscience, USA). HMDMs were seeded on a PRIMARIA™ 96-well plate at a density of 1×10^4 cells/well. Two hundred microliter of culture medium containing PMMA particles at a concentration of 1.5 μg/μL (approximately 300 μg/well of PMMA) was added to each well in incubated for 24, 48, 72 h. After incubation, culture supernatants in each well were collected. The culture supernatant of cells incubated without particles was used as the negative control, while the culture supernatant of cells incubated with culture medium containing 0.1 μg/mL lipopolysaccharide (LPS) solution was used as the positive control.

The procedure of ELISA for evaluating the production of IL-1β, IL-6, and TNF-α was shown in Fig. 4. Each well of a Maxisorp nunc-immuno 96-well plate (442404, Thermo Fisher Scientific Inc., USA) was coated with a capture anti-body and incubated for 24 h at 4 °C. The remaining protein-binding sites in the coated wells were blocked by adding blocking buffer, followed by incubation for 1 h at room temperature. The collected culture supernatant was added to each well and incubated for 2 h at room temperature. A detection antibody was added to each well and incubated for 1 h at room temperature. Avidin-HRP was added to each well and incubated for 30 min at room temperature, following which TMB solution was added. Finally, the optical density was measured at 450 nm.

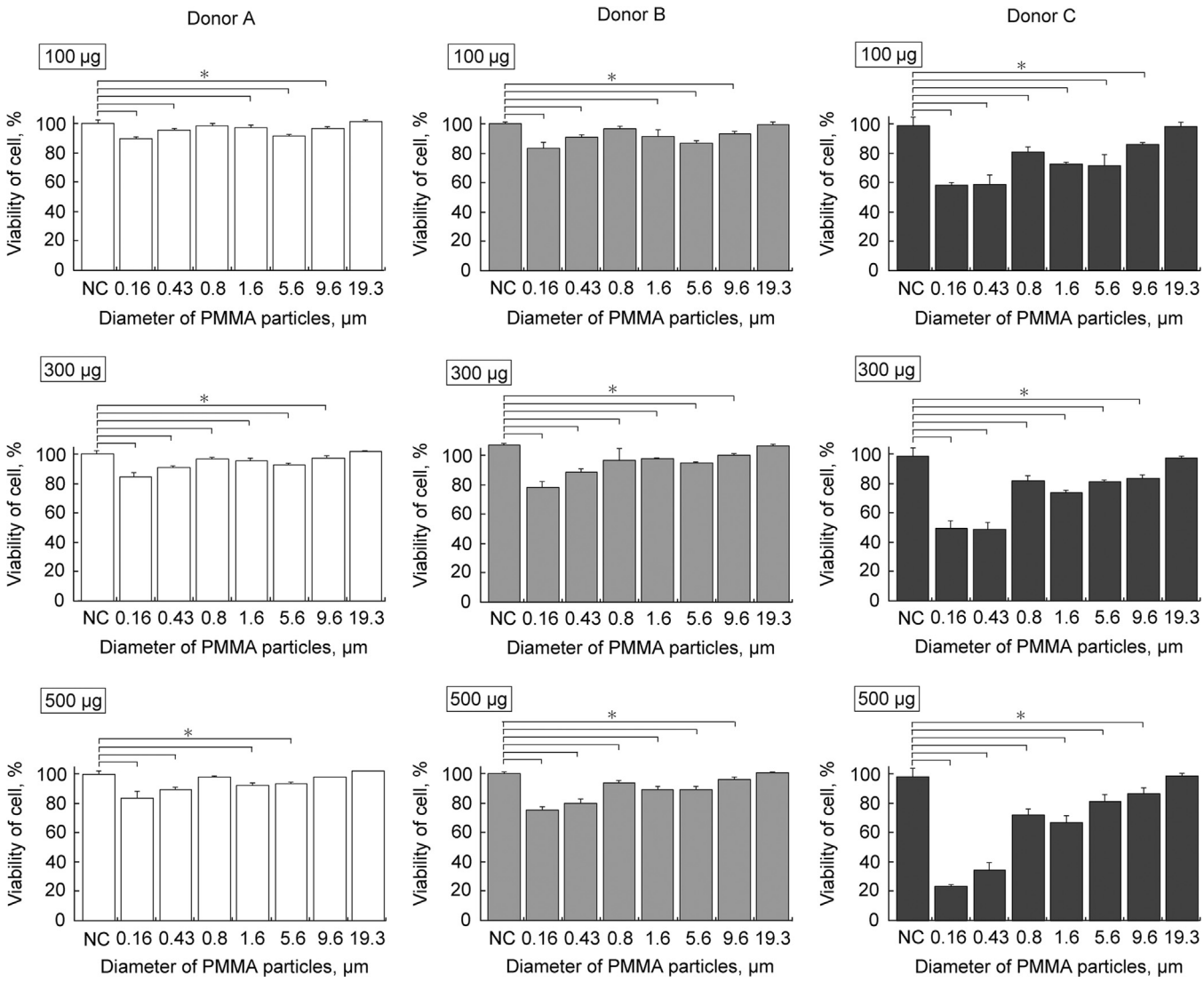

Fig. 7. Viability of HMDMs obtained from three donors after 24 h of incubation with PMMA particles of different sizes. The particles sizes were 0.16, 0.43, 0.8, 1.6, 5.6, 9.6 and 19.3 μm, and the amounts of the injection were 100, 300, and 500 μg, respectively. NC denotes negative control. The data are presented as mean + SD. Statistical significance was evaluated by non-repeated measures one-way analysis of variance (ANOVA); asterisk (*) means $p < 0.05$.

3. Results

Phagocytosis of PMMA particles by HMDMs is shown in Fig. 5. The images of HMDMs after applying each size group of the PMMA particles to the induced HMDMs for 5 h were taken using an inverted phase-contrast microscope. Fig. 6 shows the images of hematoxylin and eosin-stained HMDMs. It was observed that the phagocytosis of PMMA particles by HMDMs was not dependent on the size of the PMMA particles. It was also observed a higher number phagocytosis of the relatively smaller-sized PMMA particles by HMDMs.

Fig. 7 shows the effect of various particle sizes on the viability of HMDMs. The differences in the viability of cells that exposed PMMA particles measuring 0.16–9.6 μm in size were statistically significant among all donors.

Secretion of the proinflammatory factors, IL-1β, IL-6 and TNF-α, by HMDMs stimulated by PMMA particles of different sizes is shown in Fig. 8. In case of the phagocytosis of 0.8 μm PMMA particles, IL-6 and TNF-α were produced at significantly higher levels. Furthermore, secretion of IL-1β was activated by the phagocytosis of 0.8 μm particles, depending on the donor. In contrast, in the case of particles of other sizes, secretion of cytokines was not affected.

Fig. 9 shows the relationship between the secretion of proinflammatory cytokines and the incubation time. The particles did not show any statistically significant differences with respect to proinflammatory cytokine secretion at each incubation time. This indicates that HMDMs were stimulated with the particles within 24 h of incubation. Only particles measuring 0.8 μm in size showed significantly enhanced cytokine secretion.

4. Discussion

This study investigated the particle morphology, especially the particle size, which induces the most hazardous biological response by analyzing cell viability and the secretion of

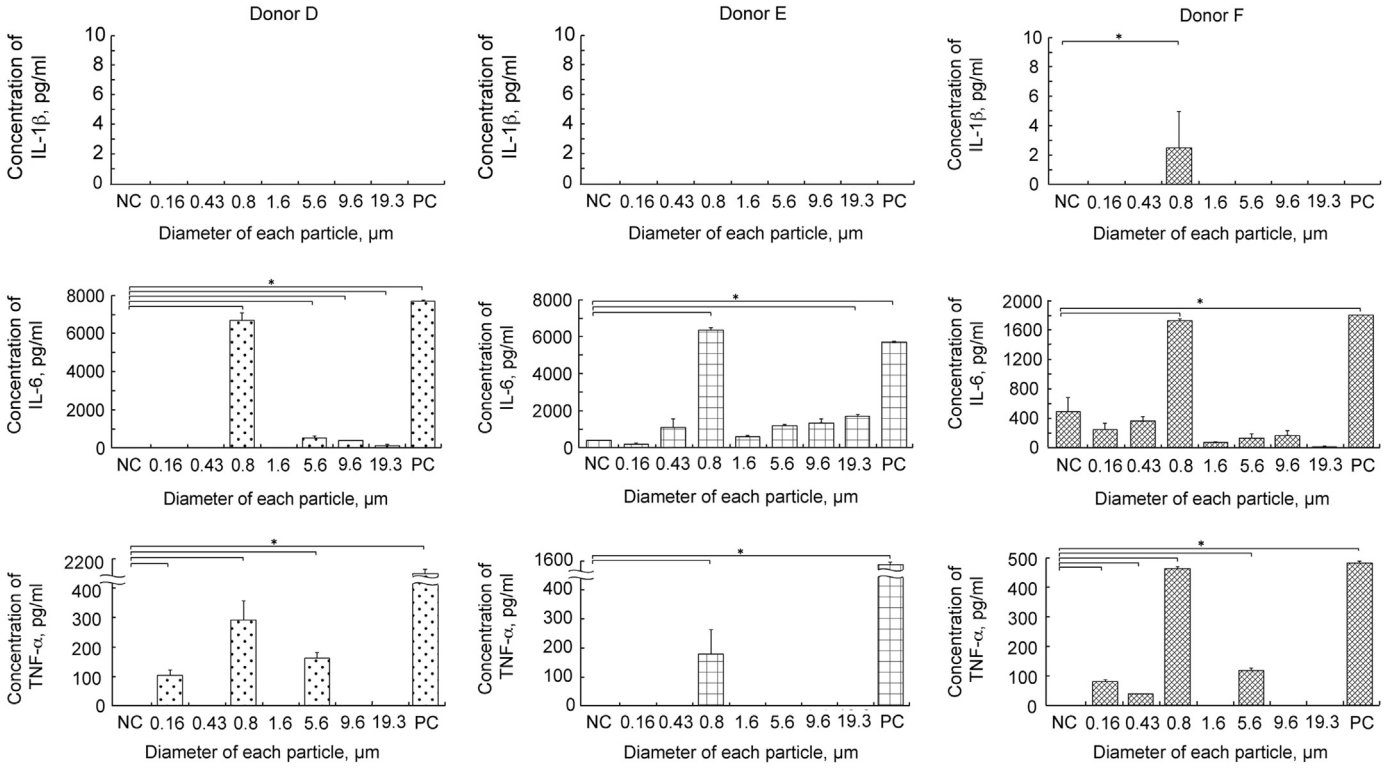

Fig. 8. Effect of the sizes of the PMMA particles on secretions of IL-1β, IL-6 and TNF-α from the HMDMs obtained from three donors after 24 h of incubation. NC and PC denote negative and positive control, respectively. The data are presented as mean + SD. Statistical significance was evaluated by non-repeated measures one-way analysis of variance (ANOVA); asterisk (*) means $p < 0.05$.

proinflammatory cytokines in HMDMs. PMMA is easy to obtain the range of submicron to micron size of narrow distribution particles. The sizes from 0.1 to 20 μm of spherical PMMA particles, which collected from artificial joint [1,15], were used instead of the UHMWPE wear particle in this study. The surface properties such as zeta potential, hydrophobic surface or hydrophilic surface, morphology and surface area affect amount of protein adhesion and subsequently the phagocytosis capacity [16–19]. On the other hand, particle morphology also affects secrete the proinflammatory regardless protein adhesion [20,21].

In this study, the amounts of PMMA particles applied to HMDMs were 100, 300, and 500 μg/well. These amounts of PMMA particles were estimated to be the same as those of UHMWPE wear particles released from the Charnley hip prosthesis in a period of 1 month [22]. Figs. 7–9 indicated that the immune responses were dependent on the size of the PMMA particles. In this study, proinflammatory cytokine production by HMDMs was strongly induced by 0.8 μm PMMA particles. Macrophages are known to respond to pathogens measuring 0.8 μm in size. In this study, PMMA particles measuring approximately 1.0 μm in size also induced an immune response. Therefore, macrophages are considered to have the capacity to recognize a foreign body of an appropriate size and stimulate the relevant biological responses.

Researchers have suggested that the cellular responses to particles may vary with the number and surface area of the particles [4,7,23]. Although a fixed amount of particles was adopted in this study, the total number of particles and the surface area of the particles varied depending on the particle size. For example, for the PMMA particle amount of 300 μg/well, the surface area was 9.5×10^9 μm^2 and the number of particles was 1.2×10^{11} for 0.16 μm particles; however, in the case of 19.3 μm particles, the surface area was 7.8×10^7 μm^2 and the number of particles was 6.7×10^4. It is also necessary to investigate that the effect of these parameters on biological response.

The PMMA material used in this study was different from implant materials such as UHMWPE, however, these particles were recognized as similar size foreign body and subsequently phagocytosis by HMDMs, which in turn stimulate different biological responses. It is, however, unclear whether macrophages have the capacity to recognize particle size. Based on our results, it appears that the magnitude of the biological responses, which are cell cytotoxicity and cytokine secretion, may vary depending on the particle size. Particles larger than 1 μm in size regulated the production of proinflammatory cytokines, which indicated a reduction in the inhibition of biological activation; this, in turn, resulted in a reduction in bone resorption.

5. Conclusions

Macrophages phagocytosed PMMA particles of various sizes (i.e., 0.16, 0.43, 0.8, 1.6, 5.6, 9.6, and 19.3 μm); however, their effects on macrophages were different. PMMA particles measuring 0.16–9.6 μm in size affected the viability of HMDMs. Only 0.8 μm PMMA particles stimulated the HMDMs to enhance the

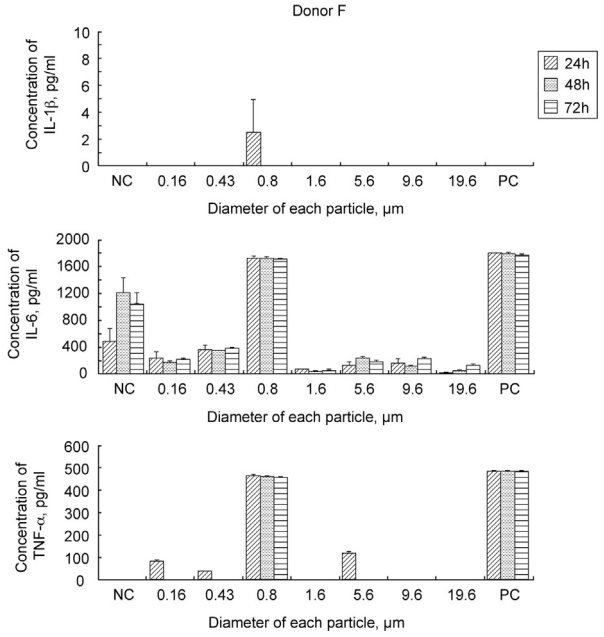

Fig. 9. Effect of the sizes of the PMMA particles on secretions of IL-1β, IL-6, and TNF-α from the HMDMs after 24, 48 and 72 h of incubation. NC and PC denote negative and positive control, respectively.

secretion of IL-6 and TNF-α. PMMA particles larger than 1.0 μm may inhibit immune responses. The data obtained in this study may be crucial in the development of artificial joints that inhibit these biological responses.

Acknowledgments

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