Gene Expression Profile of Macrophage-Like U937 Cells in Response to Polyethylene Particles

A Novel Cell-Particle Culture System

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Abstract: We investigated the gene expression profiles of U937 cells after contact with polyethylene particles. U937 cells were differentiated with phorbol 12-myristate 13-acetate, and cocultured with either retrieved polyethylene particles or commercially produced polyethylene particles (Ceridust 3615, Clariant Japan, Tokyo, Japan). To achieve consistent contact with the polyethylene particles, we used a rotating device. Phagocytosis of the polyethylene particles or retrieved polyethylene particles by differentiated U937 cells stimulated the release of cytokines including interleukin 1β, interleukin 6, interleukin 8, and vascular endothelial growth factor. Microarray analysis revealed that the expression of *IL8*, *CCL4*, *CXCR4*, and some other genes was up-regulated after contact with retrieved polyethylene particles. This study first reports the gene expression profiles of U937 cells after contact with polyethylene particles. We believe that this experimental model is applicable to all other particulate materials. **Key words:** wear debris, polyethylene, macrophage, cytokine, cDNA microarray.

Periprosthetic osteolysis is one of the major problems of joint arthroplasty. Wear particles from articular surfaces are phagocytosed by macrophages, leading to inflammatory cytokine release, which accelerates osteoclast formation and bone resorption. Interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor–α (TNF-α) are well-known "osteolytic cytokines" [1]. It has also recently been reported that interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF) participate in periprosthetic osteolysis. Interleukin 8 stimulates osteoclastogenesis via receptor activator of NF kappa B ligand–dependent and RANKL-independent mechanisms [2,3]. Clinically, IL-8 concentrations are high in tissues around loosened hip prostheses [4] and in the serum and synovial fluid of patients with aseptic loosening [5]. Miyanishi et al [6] reported that VEGF mRNA expression by macrophages increased after contact with titanium particles and that VEGF stimulated the chemotactic activity of macrophages. Polyethylene (PE) is widely used for artificial joint surfaces, and submicronic PE particles are the main component of wear particles [7,8]. There are several problems in the in vitro study of cell-particle interactions. One is the choice

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Submitted January 30, 2007; accepted March 4, 2007.
Benefits or funds were received in partial or total support of the research material described in this article from the New Energy and Industrial Technology Development Organization (Kanagawa, Japan).
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0883-5403/07/1906-0004$32.00/0
doi:10.1016/j.arth.2007.03.013

of cells. Monocyte-derived macrophages (MDMs) have been mainly used [9-11], but the isolation of human MDMs requires donors, and there can be biologic variation among individuals [11]. A cell line has the advantages of availability and homogeneity. U937 is a cell line derived from a human histiocytic lymphoma [12], and its differentiation can be induced by phorbol 12-myristate 13-acetate [13]. Matheson et al [14] reported that differentiated U937 cells show similar biologic activity to that of MDMs in the degradation of polycarbonate-based polyurethanes. Another problem is the low relative density and hydrophobic nature of PE. To achieve satisfactory cell-particle contact, several methods have been reported, including (1) attaching the PE particles to culture dishes or coverslips [15-17]; (2) inverted cell-culture systems [11,18]; (3) rotating the cell-particle mixture [19]; and (4) the use of detergent [20]. A shaking or rotating culture system is suitable for large-scale culture and is applicable not only to PE particles but also to all other particulate materials, such as metals, ceramics, and cements. The purpose of this study was to investigate the biologic activity of differentiated U937 cells when in contact with PE particles and to profile their gene expression using cDNA microarray analysis.

Materials and Methods

Polyethylene Particles

We used 2 types of PE particles. One was a commercially produced PE particle of mean size 6.5 to 8.5 μm (Ceridust 3615). Ceridust 3615 was a generous gift of Clariant Japan (Tokyo, Japan). The other type was "retrieved PE particles" isolated from periprosthetic tissues obtained at revision surgery. Periprosthetic tissues were minced and immersed in a chloroform-methanol (2:1) solution overnight, and then immersed in xylene for 5 hours, according to the method described by Minovic et al [21]. After the tissue had been rinsed with distilled water, about 5 g was placed into a polypropylene tube to which 12 mL of 5 mol/L sodium hydroxide was added, and the tube was incubated at 65°C for 3 hours. This solution (7 mL) was placed into a 15-mL polypropylene conical tube, and 5 mL of 50 wt% sucrose was added, after which the tube was centrifuged at 3000 rpm for 1 hour. The PE particles rose to the top of the tube and were carefully pipetted into a clean tube. These particles were suspended in distilled water, sonicated for 5 minutes, and heated for 30 minutes at 80°C. This solution (7 mL) was placed into a fresh tube, and 4 mL of isopropanol (0.96 g/cm^3) and 2 mL of lower-density isopropanol (0.90 g/cm^3) were added before the tube was centrifuged at 3000 rpm for 2 hours. The white band that formed between the different isopropanol densities was carefully pipetted into a clean vial. The particles obtained were observed using a scanning electron microscope (S-4700, Hitachi, Tokyo, Japan). The morphology of the PE particles was either elongated or shred-like (not shown). The mean size of 100 randomly chosen PE particles was 1.71 μm (range, 0.20-3.66 μm) in length and 2.58 μm (range, 0.24-8.33 μm) in width.

Maintenance and Differentiation of U937 Cells

U937 cells were purchased from the American Type Culture Collection (Manassas, Va). These cells were nonadherent and were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and antibiotics (proliferation medium). The cells were cultured in humid air containing 5% carbon dioxide at 37°C. The differentiation medium was proliferation medium with the addition of 100 nmol/L phorbol 12-myristate 13-acetate. U937 cells were cultured in differentiation medium for 72 hours, followed by 24 hours cultivation in proliferation medium. After this process, most cells became adherent and formed many cell aggregates. The remaining nonadherent cells were discarded by washing, and the adherent cells were trypsinized and collected by centrifugation.

Coculture with PE Particles

Polyethylene particles were added to 3×10^6 cells (10 mL of proliferation medium for each sample). The concentrations of the PE particles ranged from 0.1 to 0.5 mg/mL. Particle-free medium was used as the control. To achieve consistent contact between the cells and floating PE particles, we modified the rotation method described by Rader et al [19]. The cell-particle mixture (10 mL) was placed into a 50 mL polypropylene conical tube with a ventilation aperture and rotated for 6 hours (10 rotations per minute).

Transmission Electron Microscopy

After contact with 0.5 mg/mL PE (Ceridust 3615), the cells were collected by centrifugation. The cell pellets were fixed with glutaraldehyde and osmic acid and then embedded in epoxy resin and sectioned by ultramicrotomy (thickness, 80 nm). The specimens were observed with a transmission electron microscope (H-7000, Hitachi, Tokyo, Japan).

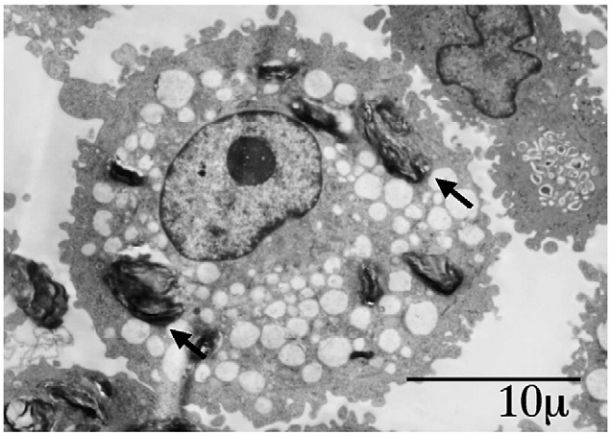

Fig. 1. Polyethylene particles (Ceridust 3615) phagocytosed by U937 cells. Arrow indicates PE particle.

Quantitation of Secreted Cytokines

After incubation with PE, the cell-particle mixture was transferred to a polystyrene culture dish and left for 2 hours to allow cell adhesion. Then the supernatant, containing the particles and floating cells, was discarded, and 10 mL of fresh proliferation medium was added. The supernatant was collected at the indicated times when the medium was changed. Cytokines secreted into the supernatant were quantified using an enzyme-linked immunosorbent assay method. Enzyme-linked immunosorbent assay kits for these cytokines were purchased from R&D Systems (Minneapolis, Minn). Interleukin 1β, IL-6, and TNF-α were first assayed after contact with Ceridust 3615. Then IL-1β, IL-6, IL-8, and VEGF were assayed after contact with either Ceridust 3615 or retrieved PE particles.

cDNA Microarray Analysis

After 6 hours' rotation and 2 hours' cell adhesion, total RNA was extracted from the adherent cells using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA chips, labeling reagents, and the array scanner were purchased from TaKaRa Bio (Shiga, Japan). Microarray analysis was performed according to the instruction manuals provided by TaKaRa Bio. In brief, 20 μg of total RNA from the PE-free or PE-treated samples was reverse transcribed and labeled with Cy3 or Cy5 fluorescent dye (Amersham Biosciences, Buckinghamshire, UK). Equal amounts of Cy3- and Cy5-labeled cDNAs were mixed in the hybridization buffer and were hybridized to a TaKaRa IntelliGene Human Cytokine CHIP, which contains approximately 550 human cytokine-related genes (gene list available from www.takara-bio.co.jp). After hybridization, the chip was washed and laser scanned with the Affymetrix 418 Array Scanner (TaKaRa Bio, Shiga, Japan). The scanned images were processed using the microarray image analyzing software ImaGene

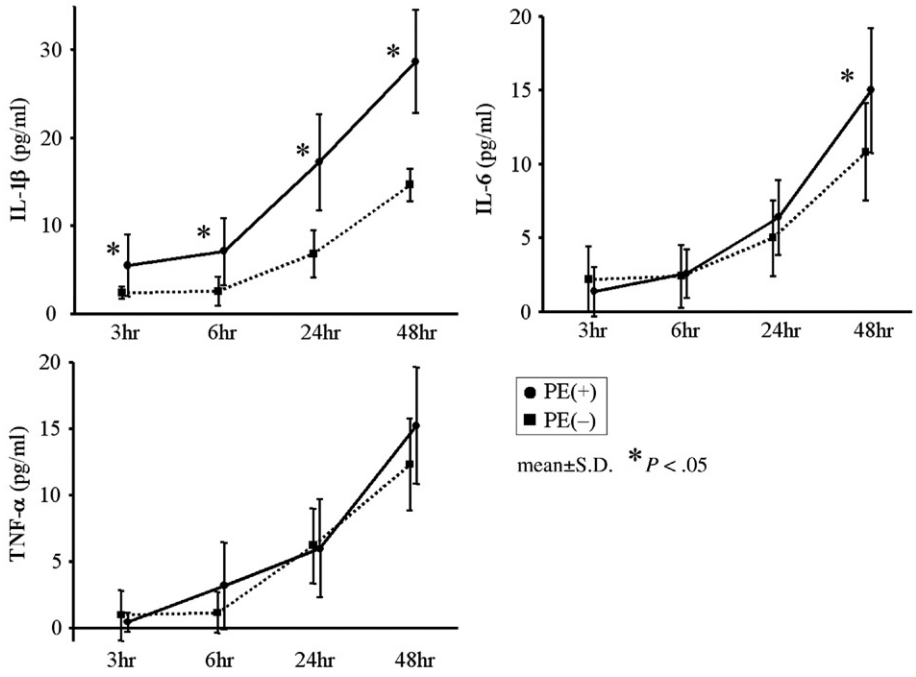

Fig. 2. Cytokine release after contact with 0.5 mg/mL Ceridust 3615. Data are mean ± SD (n = 8). *Significantly higher than PE-free (PE [–]) control cells ($P < .05$).

Table 1. Gene Expression After Contact with Ceridust 3615 or Retrieved PE Particles

Gene Name	Expression Ratio	
	Ceridust 3615	Retrieved
Interleukin 8	1.03	2.33
Chemokine (C-C motif) ligand 4 (*MIP1B*)	0.80	2.55
Inhibin, βA	1.17	2.60
Chemokine (C-X-C motif) ligand 2 (*GRO2*)	1.07	2.62
Similar to regulator of G-protein signaling 2	1.03	2.91
V-fos FBJ murine osteosarcoma viral oncogene homolog	1.02	3.32
Chemokine (C-X-C motif) receptor 4	1.37	3.51
FBJ murine osteosarcoma viral oncogene homolog B	1.05	6.58

Gene expression ratio of each gene after contact with 0.5 mg/mL PE particles to PE-free controls. Genes up-regulated more than twofold are listed.

(BioDiscovery, El Segundo, Calif). The analysis was repeated, reversing the fluorochrome labeling.

Results

On transmission electron microscopy analysis, many particles were seen in the differentiated U937 cytoplasm (Fig. 1). These amorphous particles were believed to be phagocytosed PE particles (Ceridust 3615). At least half of the cells had phagocytosed PE particles. We also identified PE particles phagocytosed into cells, using polarized-light microscopy (not shown).

Cytokine release was assayed at 3, 6, 24, and 48 hours after contact with 0.5 mg/mL Ceridust 3615 (Fig. 2). The concentration of IL-1β was significantly elevated, ranging from twofold to 2.8-fold higher than that of the control cells at all time points. The concentration of IL-6 was significantly higher at 48 hours but not significantly different at the other time points (3-24 hours). Tumor necrosis factor–α showed no significant change at any time point.

cDNA microarray analysis showed that 8 genes, including IL-8 were up-regulated after contact with retrieved PE particles and that their expression was more than twofold higher than that after PE-free incubation. This, however, was not the case after contact with Ceridust 3615 (Table 1). The expression of no genes was down-regulated to less than half of their expression in particle-free culture. Interleukin 1β and VEGF spots were detected on DNA chips but showed no significant difference with/without either type of PE particle. We detected no IL-6 or TNF-α spots because the fluorescent signals for these transcripts were equal to or below the background fluorescence.

Interleukin 1β, IL-6, IL-8, and VEGF concentrations were quantified at 6 and 24 hours after contact

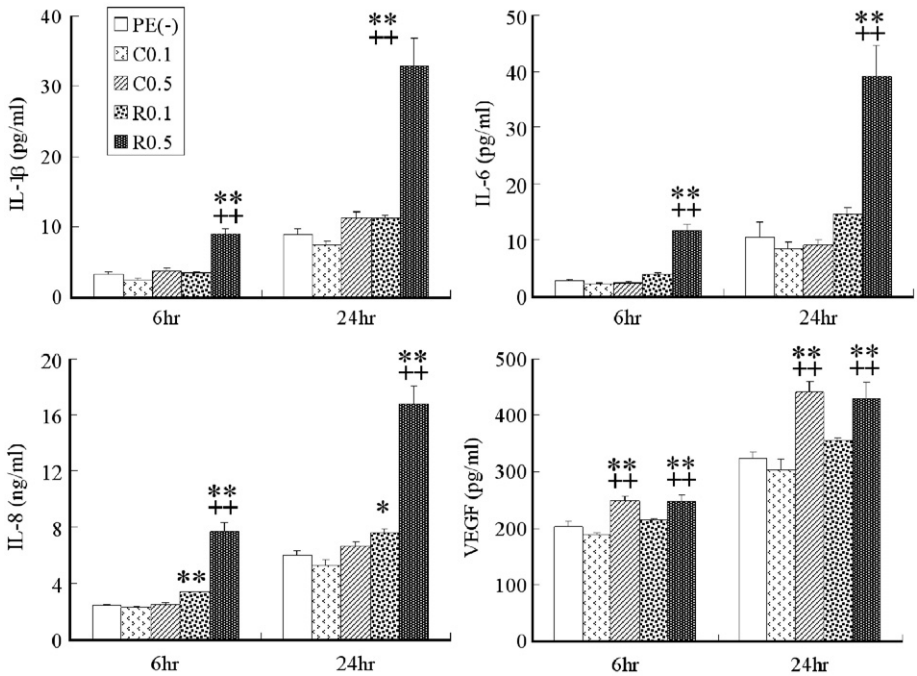

Fig. 3. Cytokine release after contact with Ceridust 3615 (C) or retrieved PE particles (R). Concentrations of particles were 0.1 or 0.5 mg/mL. Data are means ± SD (n = 4). The symbols *, **, ++ indicate significantly higher than PE (–) (*$P < .05$, **$P < .01$) or 0.1 mg/mL (++$P < .01$).

with Ceridust 3615 or retrieved PE particles (Fig. 3). When the cells were in contact with 0.5 mg/mL retrieved PE particles, the concentrations of all the cytokines assayed were significantly higher than the corresponding values in the particle-free culture. At 0.1 mg/mL retrieved PE particles, only IL-8 showed a significant change in secretion. In contrast, at 0.1 mg/mL Ceridust 3615, no cytokine assayed showed a significant difference in secretion relative to that in culture without particles. However, at 0.5 mg/mL Ceridust 3615, the concentration of VEGF was significantly higher than that in the control. The concentrations of these cytokines increased from 6 to 24 hours, and they showed similar patterns at 6 and 24 hours.

Discussion

To compare cell reactions when they contact "different" particles is a challenging process. Most of the components of artificial joints, such as metals, ceramics, and polymethylmethacrylate cements, have a relative density of more than 1 (water). However, PE differs from other components in that it floats on water and is strongly hydrophobic unless coated with protein or other hydrophilic substances. Therefore, to achieve stable contact between cells and PE, we needed to design a suitable culture system. In this study, we aimed (1) to compare all particulate materials using 1 culture system; (2) to incubate relatively large numbers of cells to obtain micrograms of RNA; and (3) to compare the gene expression profiles of these cells. To achieve the first aim, we used a rotation device to gently mix the cells and "floating" PE. To achieve the second aim, we expanded this system to a larger scale (original, 400 μL per sample; this study, 10 mL per sample) [19]. We used the U937 cell line because it shows rapid proliferation and it is easy to obtain the millions of cells we needed.

Under transmission electron microscopy and polarized-light microscopy, the differentiated U937 cells showed vigorous phagocytosis of the PE particles. Although it has been reported that smaller (submicronic to 2 μm in diameter) particles more effectively stimulate cytokine production [17,20], the differentiated U937 phagocytosed relatively large Ceridust 3615 particles (6.5-8.5 μm in diameter) and secreted more IL-1β and IL-6 than did the PE-free control cells.

We performed gene expression profiling using a cDNA microarray and quantified the expression of cytokines associated with osteolysis to compare the proinflammatory effect of 2 types of PE particles. Microarray analysis revealed that the expression of 8 cytokine-related genes, including those of chemokines, chemokine receptor, and signal transduction factors, was up-regulated by retrieved PE particles. Interleukin 8 was up-regulated at the levels of both mRNA expression and cytokine secretion. Conversely, the secretion of cytokines IL-1β and VEGF was stimulated, whereas their mRNA expression was not. The expression of IL-6 and TNF-α was not detected with the DNA chips. We consider that cDNA microarray analysis has some technical limitations. Firstly, the secretion of some cytokines did not correlate with their microarray results (mRNA expression). In this study, genes up-regulated more than twofold were assumed to be "significantly up-regulated genes." This may have caused us to miss subtle changes in expression. Secondly, the signals of genes with low-level expression were undetectable because the DNA chip itself has some fluorescence. Despite these difficulties, some new candidate genes associated with osteolysis were identified.

Chemokine (C-C motif) ligand 4 (CCL4) is also known as macrophage inflammatory protein 1β. Hashimoto et al [22] reported that multiple myeloma cells secrete CCL4 and that there is a correlation between CCL4 secretion and the extent of multiple myeloma bone lesions. Stromal cell–derived factor 1 (SDF-1) is a specific ligand for chemokine (C-X-C motif) receptor 4 (CXCR4). Yu et al [23] reported that CXCR4 is strongly expressed in osteoclast precursors and mediates their SDF-1–induced chemoattraction and matrix metalloproteinase 9 expression. It is unclear whether both the chemokine and the chemokine receptor are directly involved in periprosthetic osteolysis, and further investigations are required.

The secretion of IL-8 and VEGF after contact with Ceridust 3615 or retrieved PE particles showed different patterns. Only IL-8 showed a significant difference in cells mixed with 0.1 mg/mL retrieved PE particles relative to that in the PE-free control cells. This result means that of these cytokines, IL-8 secretion is more sensitive to retrieved PE particles. Conversely, the concentration of VEGF in cells exposed to 0.5 mg/mL Ceridust 3615 was equal to that in cells exposed to 0.5 mg/mL retrieved PE particles.

In conclusion, this study is the first to report the gene expression profiles of differentiated U937 cells after contact with PE particles. Retrieved PE particles stimulated greater cytokine mRNA expression and cytokine release by differentiated U937 cells than did Ceridust 3615. Further study is required to investigate the dose/material effects on these gene expression

profiles (especially by non-PE materials such as metals). We believe that this experimental model is convenient and will facilitate the clarification of the mechanisms of particle-induced osteolysis.

Acknowledgment

This study was financially supported by the New Energy and Industrial Technology Development Organization (Kanagawa, Japan). We thank Makio Fujoika for technical help with electron microscopy.

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