The effect of polyethylene particle phagocytosis on the viability of mature human macrophages

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Abstract: Macrophages are the major cell type observed in the inflammatory membrane retrieved at implant revision surgery. In this study, mature human monocyte-derived macrophages (MDM) were adapted to a previously established in vitro model to examine the influence of high-density polyethylene (HDPE) particulate (4–10 μm) on MDM viability. HDPE particles were suspended in soluble type I collagen, which subsequently was solidified on glass coverslips. Mature human macrophages, derived from differentiating peripheral blood monocytes on polystyrene for 10 days, were incubated in culture media on collagen controls and collagen–particle substrata for 31 days. Histologic analysis demonstrated that MDMs were in contact with the particles at 2 h. The majority of the particles were associated with the cells within 24 h. Based on electron microscopy, those cells associated with the particles appeared to be morphologically activated rather than necrotic or apoptotic. Assessment of cell viability revealed no differences among the groups at 24 h, but at 31 days significantly more viable cells and higher DNA values were found associated with the particle groups versus the collagen controls. The histologic results validate human mature MDMs as a clinically relevant cell type for study of the role of polyethylene particulate in aseptic loosening. The cell viability results indicate that phagocytosis of HDPE is not toxic to MDMs but in fact prolongs MDM survival. The long-lived MDMs may play a role in perpetuating chronic inflammation surrounding implants. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 61: 619–627, 2002

Key words: monocyte-derived macrophage; polyethylene; phagocytosis; histology; cell viability

INTRODUCTION

Aseptic loosening is the major complication of long-term orthopedic implants, often leading to technically challenging and costly revision surgery. Analysis of revision membranes has shown the presence of numerous PE particles and macrophages associated with the particles. Current theory suggests that the engulfing of PE particles by macrophages results in cell activation and secretion of pro-inflammatory mediators. These bioactive molecules perpetuate chronic inflammation, resulting in bone loss and implant failure. It has been proposed that macrophages are capable of resorbing bone directly and that they activate osteoclastic bone resorption and/or differentiate into bone resorbing osteoclasts under the influence of wear debris. However, the precise mechanism of bone loss still remains to be elucidated.

In an earlier study, a model system using a mouse macrophage cell line (IC-21) was developed. Particles of HDPE were embedded in a collagen matrix to facilitate macrophage phagocytosis. The model system later evolved to use differentiating human monocytes in which human monocytes isolated from whole blood were exposed to collagen ± HDPE immediately after isolation. Initially, the human monocytes interacted with the collagen ± HDPE, and then they differentiated in culture. This culture system increased the relevance of the study but was a difficult system to characterize because cells were present in different states of differentiation at early time points. Hence the system simultaneously was looking at the effect of particles on the differentiating process and at the phagocytic mechanism.

Studies have shown that different macrophage populations (i.e., mouse P388D1, IC-21, and peritoneal...
macrophages) release PGE2 and IL-1 and resorb bone to a different degree when exposed to wear particles. The state of differentiation of the monocyte–macrophage also has been shown to play an important role in cell behavior; for example, Wewers et al. found that human alveolar macrophages released less IL-1β than human monocytes (3.5 ± 0.8 ng/10⁶ macrophages versus 13.3 ± 0.8 ng/10⁶ monocytes) when stimulated by lipopolysaccharide. Labow et al. also have shown that mature human macrophages have much higher protein levels and esterase activity compared with differentiating human monocytes. Aseptic loosening is a chronic process, with many mature macrophages interacting with particulates. Mature MDMs therefore would be favored as a clinically relevant cell type in an analysis of cellular responses following a PE particle challenge.

The objective of this study was to focus on implementing an in vitro cell system of mature human MDM, rather than differentiating human monocytes, to study the phagocytosis of PE particulates. In such a model, human monocytes isolated from whole blood first were matured to fully differentiated macrophages by culturing them on tissue culture polystyrene plates for 14 days. The mature MDMs then were trypsinized and replaced with collagen ± HDPE. The mature MDM–PE interactions were analyzed histologically by light and electron microscopy, and cell viability following long-term exposure to PE particulates was investigated.

MATERIALS AND METHODS

Particle preparation and characterization

Particles of high-density polyethylene (HDPE, size: 4–10 μm) were obtained from Shamrock Technologies, Newark, NJ. Chemical characterization and endotoxin tests of the particles were previously performed by Voronov et al. The particles had chemical characteristics similar to ultra-high molecular weight polyethylene (UHMWPE) and were free of endotoxin. The particles chosen for this study were sterilized with 2.5 Mrad of γ-irradiation in air. The particles were suspended in 0.01% collagen type I solution at a concentration of 10 × 10⁶ particles/mL. The particle concentration was counted using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL).

Coverslip preparation

Microscope coverslips were sterilized by dry heat at 200°C for 2 h. Each square coverslip (22 × 22 mm², Fisher Scientific, Whitby, ON.) was coated with 10 μL of particle suspension (∼100,000 particles). Coverslips coated with collagen alone were used as negative controls.

Cell cultures

Human blood was collected from healthy volunteers (University of Toronto, ethical protocol #2015). Human monocytes were isolated using Ficoll-Paque (Pharmacia, Biotech) density gradient centrifugation, as previously described except for the following modification. Approximately 2 million monocytes/well were seeded onto 12-well plates. After a 2-h incubation, the well contents were removed, and 2 mL of fresh media were added. The cells then were cultured for 10 days with a complete medium replacement every other day. At 10 days, MDMs were harvested with 0.25% trypsin (Gibco BRL, Burlington, ON) supplemented with 16.7 mM of EDTA, as described by Labow et al. Approximately 5.0 × 10⁶ MDMs were seeded on each of the particle/collagen coverslips. Two hours after seeding, the well contents were replaced with fresh media. This time point was defined as time zero, and the cells were maintained for up to 31 days.

Histology

The cultures were terminated at 2 and 24 h and at 31 days, washed with PBS buffer, fixed in a mixture consisting of 100% methanol and 10% formaldehyde (2:1 in volume) for 2 h, and stained with hematoxylin and eosin (H & E). The coverslips then were mounted on microscope-grade glass slides and photographed under both regular and polarized light.

The cells, incubated for 2 and 24 h, were fixed with 2.5% glutaraldehyde in 0.1M of sodium cacodylate for 4 h at 4°C, dehydrated in graded alcohols (from 30 to 90%), critical-point dried from CO₂, sputter-coated with platinum, and examined in a Hitachi (model 2500, Mito, Japan) scanning electron microscope (SEM) at the Faculty of Dentistry, University of Toronto.

The cells, incubated for 24 h, were fixed with 2.0% glutaraldehyde in 0.1M of sodium cacodylate buffer, postfixed with 1% osmium tetroxide in the 0.1M of sodium cacodylate buffer, dehydrated in graded acetone (from 50 to 100%), embedded in Epon, sectioned, and examined using a Phillips/201 (N.V. Phillips, Gloeilampenfabrieken, Eindhoven, Netherlands) transmission electron microscope (TEM) in the Pathology Department of the Hospital for Sick Children.

Cell viability

A live/dead staining kit (Molecular Probes, Oregon) provided two probes, calcein and ethidium, for detecting two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity, respectively. Following trypsinization and prior to seeding on the coverslips, approximately 1 × 10⁶ MDMs were washed twice
with PBS, stained in 200 μL of ethidium solution (8 mM of EthD-1) for 10 min, and fixed in 200 μL of 1% paraformaldehyde for 30 min. The samples were examined immediately by FACS analysis at the Medical Science Building, University of Toronto.

Following 24 h of incubation, adherent cells on the coverslips were rinsed with PBS, double stained with ethidium and calcine (4 mM of EthD-1 and 2 mM of calcine) for 45 min, and mounted on microscope glass slides. Under fluorescent examination, viable cells appeared green while necrotic cells appeared red. Five microscopic fields (magnification ×200) were chosen to determine the percentages of live/dead cells in experimental and control groups. Fluorescent images were correlated with polarized images to visualize PE particles using the Northern Eclipse computer software (EMPIX Image Inc., Mississauga, ON) to determine the percentages of live/dead cells associated with particles.

DNA analysis

At time zero, day 7, and day 31, adherent cells were rinsed with PBS and combined with lysis buffer (0.05% TritonX-100/10 mM of EDTA/PBS). The DNA contents were then determined using a fluorometric assay with Hoechst dye (Fisher H33258), as described by Labow et al. Each DNA sample was assayed in duplicate.

Statistical analysis

A Student’s t test built in Microsoft Excel software was applied to analyze the difference of 24-h cell viability between HDPE and controls. Due to variability in DNA values from one experiment to another (associated with blood-donor variability), a nested model was applied to determine differences in DNA content between time zero and 31-day measurements, and Scheffe’s test was used to determine the differences between conditions at each time point. The analyses were carried out using Statistical Analysis System (SAS) software (SAS Institute Inc., NC). Statistical significance for DNA was determined at the 0.02 level, with the 0.03 level being assigned as marginal significance because of the small sample size. Three separate cell isolations from three different donors were carried out, with each experimental parameter (collagen or HDPE) assessed in triplicate.

RESULTS

Monocyte differentiation

Human peripheral blood monocytes, cultured on polystyrene, were observed to flatten and gradually spread within the first few h. This was followed by an increase in cell size and granularity. By 10 days, the cells were about twice as large as the original monocytes, coupled with membrane extension and ruffling, thus assuming a typical macrophage appearance. After trypsinization, approximately 1 × 10^6 MDMs were stained with ethidium and analyzed by flow cytometry. The forward scatter mode showed that approximately 90% of cells were large and granular [area D in Fig. 1(a)], corresponding to the microscopic examination at 10 days. Ethidium staining revealed that less than 10% of MDMs [area K in Fig. 1(c)] were ethidium positive while more than 90% of MDMs [Fig. 1(b,c)] excluded ethidium (SD = 0.02, n = 2). As ethidium exclusion was an indication of viable cells, these ex-

Figure 1. FACS analysis of MDMs after trypsinization: (a) forward scatter [D: large and granular cells]; (b) ethidium staining [C: ethidium-positive cells (dead cells)]; (c) ethidium staining [K: ethidium positive cells (dead cells)]. Note that the majority of MDMs are large and granular and that there is only a small proportion of dead cells after trypsinization.
Experiments demonstrated that more than 90% of MDMs were viable after trypsinization.

### Histology

At 24 h of incubation, H & E staining showed good cell density with 500,000 cells per square coverslip. There was variability in cell size, with some cells well spread out and others rounded up. The cells appeared healthy [Fig. 2(a)]. The same microscopic view examined under polarized light demonstrated that the majority of the particles was seen in contact with cells [Fig. 2(b)].

Upon SEM analysis at 2 h after incubation with HDPE particles, MDMs were actively contacting particles. In Figure 3(a,b), two cells were seen in contact with several particles. Both cells appeared morphologically healthy. At 24 h, the cells still appeared to be very motile and able to move toward more particles [Fig. 3(c)].

TEM analysis was performed at 24 h of incubation. Four particles located within a cross-section of a single cell were observed in Figure 4(a). TEM provided evidence suggesting that internalization of the PE particles had occurred. Close examination of Figure 4(a,b) revealed many vacuoles, endoplasmic reticulum, and lysosomes distributed around the particles. No chromatin condensation or mitochondrial dilation, which are morphologic features of apoptosis, was observed in either particle or collagen groups at 24 h.

### Cell viability

At 24 h, adherent MDMs on the coverslips were double stained with calcein and ethidium. Fluorescent examination showed that cell viability was similar in both the HDPE groups and the collagen controls (94% versus 92%, n = 5, p = 0.4). The fluorescent images were correlated with the polarized images and revealed that within cultures containing HDPE particles, the cells that had engulfed particles were as viable as the cells without particles (96% versus 94%, n = 5, p = 0.4).

It was noted in cultures that after 18 days cells decreased more rapidly in collagen versus HDPE. On day 31, there were 2.5 times more cells in the particle-challenged group versus the collagen controls, based on H & E staining and cell counting (n = 3, Fig. 5). Multinucleated giant cells associated with several HDPE particles were found in the HDPE cultures (Fig. 6) and appeared more frequently in HDPE cultures than in collagen controls.

### DNA analysis

DNA content was measured in order to estimate cell number since previous studies have shown a direct correlation between these two parameters. Statistical analysis of pooled DNA data from three different donors showed that DNA content decreased with incubation time in both particle and control groups, with a 40 to 50% reduction at day 7 and an 85 to 95% reduction by day 31 (p = 0.0001, Table I). At time zero, DNA content was significantly greater in controls versus particle groups (p = 0.0001, Table I). No difference was seen between the groups at 7 days. Interestingly, by 31 days, more DNA was noted in HDPE versus controls (p = 0.03, Table I).

### DISCUSSION

An in vitro cell system used previously with a mouse cell line and differentiating human mono-
cytes was adapted for use with mature human MDMs. Both the current study (Fig. 1) and the literature provide evidence that human monocytes are able to differentiate into mature MDMs after being cultured on polystyrene for 10 days. Mature MDMs were harvested by trypsin, as described in previous work by Labow et al. Trypsinized MDMs were established on glass coverslips coated with the HDPE particle–collagen solution. This allowed for conventional H & E staining, SEM, and TEM analyses of the

Figure 3. SEM analysis of MDMs exposed to HDPE particles (open arrows): (a) and (b) 2-h incubation, cells in contact with HDPE particles; (c) 24-h incubation, a well-spread MDM, possibly containing particles (arrows). Note that all cells associated with HDPE particles appear morphologically healthy.
macrophages exposed to the polyethylene particles. MDMs were seen in contact with the particles as early as 2 h after the initial incubation [Fig. 3(a,b)].

Contact with the particles did not appear to cause a toxic effect since the cells were normal in appearance (i.e., integrity of cell membrane and nucleus). At 24 h, H & E staining in combination with normal and polarized light microscopy demonstrated that the majority of the particles was associated with MDMs [Fig. 2(a,b)], suggesting that a good particle–cell interaction had been achieved by 24 h. Hence, this time point was chosen for TEM analysis. TEM showed that particles were located within a section of the MDMs [Fig. 4(a,b)], indicating that phagocytosis of HDPE had occurred. The MDMs with four engulfed particles appeared to contain numerous vacuoles, endoplasmic reticulum, and lysosomes. This suggests that mature MDMs have greater phagocytic and degradative capacity compared with differentiating human monocytes because the latter exhibited a smooth surface topography (SEM), encroached nuclei (TEM), and fewer lysosomes (TEM) when challenged with the same dose of HDPE particles. Many lysosomes around the internalized particles [Fig. 4(b)] may imply that the particles actually stimulated the formation of lysosomes, which is a feature of macrophage activation. Lack of chromatin condensation or mitochondrial dilation in both the particle and the collagen groups suggests the absence of apoptotic cells in the cultures at 24 h.

The HDPE particles activated the cells without creating significant cytotoxicity, evidenced by the fact that no difference in cell viability was observed be-

Figure 4. TEM analysis of MDMs exposed to HDPE particles for 24 h. PE particles (*), nucleus (N), Golgi apparatus (G), mitochondria (m), lysosomes (L), Microvilli (arrowheads at the perimeter of the cell). Original magnification ×11,920. Note that both cells appear activated with increased lysosomes while there is little evidence of cytotoxicity.

Figure 5. H & E staining of 31-day MDMs. (a) collagen controls; (b) virgin HDPE. Original magnification ×200. Note the greater cell density and cell spreading in virgin HDPE versus controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
tween HDPE and collagen controls following 24 h of exposure to HDPE particles. Also, at 24 h, within the HDPE culture itself, cells that had engulfed particles were as viable as cells without particles, with approximately 95% of MDMs viable following exposure to HDPE. The viability of MDMs reported in this study (95%) was much greater than the 50% cell viability with elevated particle volumes reported in an earlier work. It is certainly possible that particle concentration is important in determining MDM viability because increased cell death has been observed with increasing particle concentrations of Al$_2$O$_3$ and ZrO$_2$. Although the *in vivo* particle–cell ratio still is unknown, histologic analyses of the inflammatory membrane show that the majority of PE particles are associated with cells, and the cells with engulfed PE particles appear to be activated rather than necrotic.

Figure 6. H & E staining of MDMs exposed to HDPE for 31 days. (a) Polarized light: HDPE particles associated with a giant cell (open arrow). All HDPE particles are associated with MDMs or giant cells at 31 days. (b) Normal light: many nuclei in a giant cell (closed arrow). The presence of multiple nuclei clearly supports the existence of foreign-body giant cells or osteoclast-like cells in the culture. Original magnification ×400.

TABLE I

<table>
<thead>
<tr>
<th>Blood Donor</th>
<th>DNA Values (ng, mean ± SD)</th>
<th>Time Zero</th>
<th>7 Days</th>
<th>31 Days</th>
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<td></td>
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<td>HDPE</td>
<td>Control</td>
<td>HDPE</td>
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<td>3904 ± 147</td>
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<td>n/a</td>
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<tr>
<td>Donor 2</td>
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<td>2496 ± 110</td>
<td>1576 ± 182</td>
<td>1472 ± 77</td>
</tr>
<tr>
<td>Donor 3</td>
<td>2608 ± 257</td>
<td>2136 ± 105</td>
<td>1104 ± 24</td>
<td>1152 ± 24</td>
</tr>
</tbody>
</table>

DNA Decreases with Time in MDM Cultures. Statistical Analysis of Pooled DNA Data Showed More DNA in Controls Versus HDPE (*p* = 0.0001) at Time Zero but More DNA in HDPE Versus Controls at Day 31 (*p* = 0.03). Controls had collagen coating only; HDPE had collagen plus HDPE particles; each donor group had an *n* = 3; n/a: sample groups were not analyzed.
viability in the presence of HDPE particulate in the long-term culture. There were significantly more cells in the HDPE cultures versus the collagen controls at 31 days. This cannot be explained by differences in MDM proliferation. Although previous studies showed the proliferation of mouse macrophages (IC-21) in collagen ± PE cultures, mature human MDMs have little or no proliferative capacity as they are fully differentiated. The latter situation also is confirmed in this study by decreased DNA/cell number over time, with less than 10% of initial DNA/cell at 31 days in both particle and control groups (Table I). Prolonged MDM survival is a more likely explanation for the greater cell number in HDPE cultures than in collagen controls at 31 days.

Prolonged MDM survival may be due to MDM activation after the phagocytosis of collagen and/or PE. Others have shown that any factor (i.e., lipopolysaccharide, colony-stimulating factor, or interleukin-1β) capable of activating monocytes/macrophages prolongs their survival and prevents apoptosis. In this study, activation of MDMs by initial phagocytosis of collagen may have rescued MDMs from apoptosis at early time points because TEM showed no cells with morphologic features of apoptosis in either collagen controls or collagen–HDPE at 24 h. This also was supported by previous studies with annexin V binding and propidium iodide staining, which showed very few apoptotic monocytes (less than 1%) in both collagen controls and collagen–HDPE at 24 and 48 h [data not shown]. In contrast, other work showed 23% apoptotic monocytes in simple monocyte cultures without collagen at 48 h. In the long-term cultures, after the degradation of collagen, the HDPE in the collagen–HDPE cultures may continue to stimulate MDM survival and prevent apoptosis.

MDMs challenged with PE particulate could live longer, and this may have an important implication in terms of bone resorption around implant sites because macrophages are believed to be capable of resorbing bone directly, activating osteoclastic bone resorption, and/or differentiating into bone-resorbing osteoclasts under the influence of wear debris. Given MDM’s bone-resorbing capability, prolonged MDM survival due to PE phagocytosis at implant sites may provide a prolonged state of bone-resorbing activity, particularly over the long periods of time observed with joint replacement failure. The increased periprosthetic MDM life span potentially may lead to more bone loss. This hypothesis provides another novel explanation for periprosthetic osteolysis.

CONCLUSIONS

Mature human MDMs have successfully been adapted into the previously established model to investigate the long-term effect of PE phagocytosis on cell viability. The important and novel finding of this study was the prolonged human MDM survival after long-term PE stimulation in vitro. Previous work has shown that the surface chemistry of PE influences monocyte cytokine secretion. The use of this cell system, a model of mature human MDMs, will assist in the controlled analysis of the effect of PE surface chemistry as well as that of particle type, size, and dose on MDM activation. By understanding MDM activation, it may be possible to link this PE-generated activation to periprosthetic bone resorption, which ultimately will be beneficial to the clinical treatment of aseptic loosening and the extension of the clinical application of joint implants.

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