Gene expression analysis of osteoblastic cells contacted by orthopedic implant particles

Dominique P. Pioletti,[1,2,3]* Lorenzo Leoni,[4]* Davide Genini,[4] Hiroshi Takei,[5] Pinyi Du,[6] Jacques Corbeil[4,6,7]
[1]*Department of Bioengineering, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093*
[2]*Bone Biophysics Group, Hôpital Orthopédique de la Suisse Romande, Av. Pierre Decker 4, 1005 Lausanne, Switzerland*
[3]*Institute for Biomedical Engineering, Swiss Federal Institute of Technology, SGAAB, 1015 Lausanne, Switzerland*
[4]*Department of Medicine and the Stein Institute for Research on Aging, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093*
[5]*Department of Orthopedics, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093*
[6]*Center for AIDS Research, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093*
[7]*Veterans Medical Research Foundation, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093*

Received 27 July 2001; revised 10 December 2001; accepted 10 January 2002

Abstract: Particles generated from orthopedic implants through years of wear play an essential role in the aseptic loosening of a prosthesis. We have investigated the biocompatibility of these orthopedic particles on different osteoblast-like cells representative of different stages of osteoblast maturation. We found the particles induced a caspase-dependent apoptosis of osteoblasts, with less mature osteoblasts being the most susceptible. An analysis of gene expression was performed on the less mature osteoblasts, which were in contact with the particles. We found that the particles had a profound impact on genes that code for inflammatory cytokines and genes involved in controlling the nuclear architecture. Results from this study suggest that the peri-implant osteolysis after a total joint replacement can be due in part to a decrease of bone formation and not solely to an overstimulation of bone resorption as is generally proposed. Development of new drugs that promote normal bone formation and osteoblast survival would possibly control peri-implant osteolysis, resulting in a better prognosis for patients with orthopedic implants. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 61: 408–420, 2002

Key words: genomic study; osteoblast; particle; apoptosis; cytokine

INTRODUCTION

The peri-implant osteolysis after a total joint arthroplasty (TJA) is responsible for the majority of orthopedic implant loosening.[1] Failure rates of hip arthroplasty can exceed 30% after 15 years for patients <50 years old,[2] thus, requiring a revision surgery where the old implant is replaced by a new one. Joint disorders treated with TJA can reasonably be expected to give satisfactory results for 20 to 30 years with an initial and revision surgery, but a subsequent procedure cannot be performed in a routine manner. The current trend is to propose TJA to younger patients with the caveat that a second surgery is likely to be required.

The response of cells to particles has been identified as a major cause of implant loosening.[3] These particles are generated through years of wear. Any of the materials (metallic, polyethylene, methacrylate, ceramic) used for the implants or even the calcium phosphate cement[4] can be involved. In cases of loose Ti implants, the mean concentration of Ti particles retrieved from tissue surrounding the implants can reach 0.1% of the dry tissue weight.[5]

Normal bone function is assured when there is an equilibrium between bone formation and bone resorption. Bone resorption has been mostly studied in rela-

*These authors have contributed equally.

Correspondence to: D.P. Pioletti; e-mail: dominique.pioletti@epfl.ch

Contract grant sponsor: Swiss Biology and Medicine Foundation
Contract grant sponsor: Leenaards Foundation; contract grant number: 309
Contract grant sponsor: National Institutes of Health; contract grant number: GM23200
Contract grant sponsor: Veterans Medical Research Foundation
Contract grant sponsor: UCSD Center for AIDS Research Genomic Core Laboratory; contract grant number:AI36214

tion with wear particles. Macrophages,[6] monocytes,[7] or giant cells[8] in contact with particles can produce potent osteolytic factors. Effects of implant particles on bone formation have been neglected and need to be explored with a particular emphasis on biocompatibility testing.

Particles from orthopedic implants have been shown to influence the expression of some extracellular matrix proteins in osteoblasts.[9] It has been proposed that biocompatibility testing should include apoptosis studies.[10] Apoptosis, or programmed cell death, is characterized by the activation of cysteine proteases called caspases, which cleave proteins essential for the survival of the cell. A member of this family, caspase-3, has been identified as a key mediator of apoptosis of mammalian cells.[11] Furthermore, these caspases activate the endonuclease responsible for the internucleosomal cleavage of genomic DNA.[12] The TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) can detect the amount of internucleosomal DNA fragments and allow for apoptosis quantification. Apoptosis quantification is important as it is the normal fate for the majority of osteoblasts.[13] Moreover, an *in vitro* study showed that Ti particles can induce apoptosis in osteoblasts.[14] The analysis of this apoptotic pathway and the stimuli that can trigger it warrants investigation.

The recent advances in molecular biology techniques allow for a more comprehensive study of the biological alterations induced by wear particles in physiologically relevant cells such as osteoblasts.[15] In this study, we quantified the apoptotic response to titanium (Ti) and polymethylmethacrylate (PMMA) particles in three osteoblast-like cells (human MG-63, rat osteoblast, and human SaOS-2). These are representative of the different maturation stages of the osteoblasts. The choice of the particles type was based on the fact that Ti is a widely used metal for orthopedic implants and that it generates a large amount of particles[5] that directly interact with osteoblasts during different stages of their differentiation. PMMA, the bone cement used to seal orthopedic implants, also generates particles.[16] A gene expression analysis was performed for the MG-63 osteoblast-like cells while in contact with these particles.

Our results demonstrate that implant particles induced and promoted apoptosis, especially in less mature osteoblasts, and that caspase-3 was involved in this process. The particles had a strong gene expression effect in osteoblasts with an overall trend favoring bone resorption. These results clearly suggest that wear particles from orthopedic implants are involved in the osteolysis not only by favoring bone resorption, as it is generally accepted, but also by inhibiting bone formation and interfering with osteoblast proliferation. Future drugs development aimed to control the peri-implant osteolysis should enhance the functioning and survival of osteoblasts when particles are present.

MATERIALS AND METHODS

Particles

Commercially pure titanium particles of –325-mesh nominal diameter were purchased (Aldrich, Milwaukee, WI). The PMMA particles were a gift from Zimmer (Worthington, OH). Particle size distributions were performed by laser diffraction using a Malvern MasterSizer equipment. The values obtained for the different powders are given in Figure 1. The particles, sterilized by overnight UV irradiation, were mixed with the culture medium under sterile conditions at a concentration of 0.1% (w/w). This concentration can be considered as representative of the particle concentration found in the surrounding tissue of loose implant in biopsy study.[5] The particle solutions were sonicated for 20 min in a sealed sterile container to minimize their agglomeration before being added to the cell culture. Endotoxin contamination of particles was negligible as tested by limulus assay (QCL-

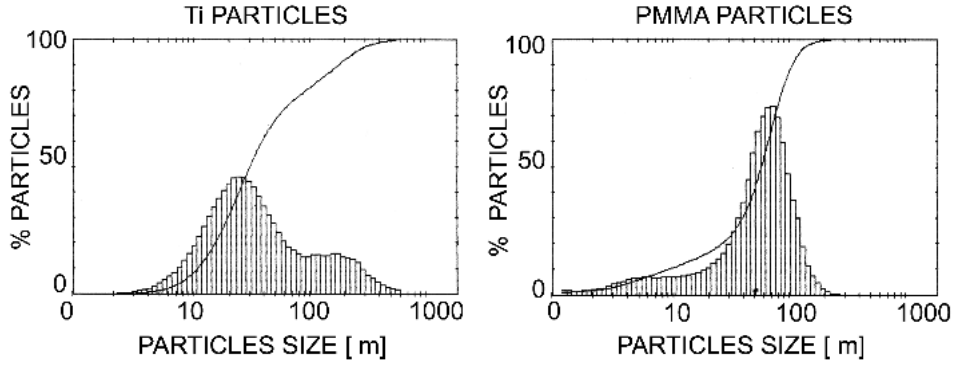


Figure 1. Frequency plot showing the size distribution of the Ti and PMMA particles used. The plain line represents the cumulative particle percentage. The size distribution was measured by laser diffraction (Malvern). For the Ti particles, 10% of the particles had a diameter smaller than 10.7 μm, 50% smaller than 30.2 μm, and 90% smaller than 176.3 μm. For the PMMA particles, 10% of the particles had a diameter smaller than 8.9 μm, 50% smaller than 56.9 μm, and 90% smaller than 105.6 μm.

1000 Chromogenic LAL; BioWhittaker, Emerainville, France).

Cell cultures

The gene expression of the osteoblast has been shown to depend on its maturation stage.[17] The osteoblast maturation can be evaluated through the production of alkaline phosphatase.[18] Three types of osteoblast-like cell lines were used spanning three different stages of osteoblast maturation. The human MG-63 osteoblast-like cell (American Type Culture Collection, Rockville, MD) can be considered as the least mature osteoblast used in this study (alkaline phosphatase activity: 2.7 μU/μg protein).[19] The rat osteoblast, isolated in our laboratory from neonatal Sprague-Dawley calvaria rat following the procedure of Puelo,[18] is an intermediate mature osteoblast (alkaline phosphatase activity: 60 μU/μg protein; measurement performed in our laboratory using the Diagnostic kit 245 of Sigma, St. Louis, MO). Finally, the human SaOS-2 osteoblast-like cell (American Type Culture Collection) is the most mature osteoblast (alkaline phosphatase activity: 5264.4 μU/μg protein).[19] The osteoblasts were seeded at a concentration of 50,000 cells/cm^2 in 50-mm petri dishes and incubated for 4 h to allow cell adhesion. The supernatant was removed, and 5 mL of particles solution and normal medium was added, defining the time 0. Cell samples were collected at 4 and 24 h for the gene expression analysis, fibronetin (protein level), osteocalcin (protein level) as well as alkaline phosphatase activity and at 24, 48, and 72 h for the apoptosis, Bcl2/Bax ratio, and caspase activity quantification.

Confocal imaging (MG-63 osteoblast-like cells)

Cells were cultured 24 h with 0.1% Ti particles on coverslips. The cells were fixed 10 min in 4% paraformaldehyde and washed in PBS. To visualize actin filaments, cells were incubated for 1 h at 37°C with Alexa-488-conjugated phalloidin (Molecular Probes, Eugene, OR); tubulin was visualized using anti-B-tubulin monoclonal antibody, followed by incubation with an Alexa anti-mouse secondary antibody (Molecular Probes) as previously described.[20] Coverslips were washed successively in PBS and deionized H_2O for 5 min and mounted in Flouromount (Fisher, Santa Clara, CA). Images were acquired using a confocal laser scanning microscope LSM 510 equipped with an argon laser module (Carl Zeiss Inc., Thornwood, NY) using a 40× 1.3-na oil objective.

Measurement of DNA fragmentation as apoptosis quantification (MG-63 osteoblast-like cells, rat osteoblast, SaOS-2 osteoblast-like cells)

The internucleosomal cleavage of genomic DNA has been demonstrated to be a hallmark of apoptosis.[21] This was used as quantification of osteoblast apoptosis. Before the analysis, cells were fixed in ice-cold 30% ethanol and incubated with 100 μg/mL of RNAse A and 50 μg/mL propidium iodide for 1 h at 37°C. FSC/SSC gateway was used to discriminate between particles and cells. Hypodiploid cells were visualized using a Becton-Dickinson FACScalibur and the program ModFit LT 2.0 (Verity Software House, Topsham). DNA fragmentation was assessed by flow cytometry.

Cellular assay for caspase activity (MG-63 osteoblast-like cells, rat osteoblast, SaOS-2 osteoblast-like cells)

At the indicated time points, cells were washed twice with PBS, and the pellet was resuspended in caspase buffer (50 m*M* Hepes, pH 7.4, 100 m*M* NaCl, 1 m*M* EDTA, 0.1% Chaps, and 5 m*M* dithiothreitol) for 30 min at 4°C. Lysates were then stored at −80°C. The caspases enzymatic assays were performed in 96-well plates. Lysates (10–20 μg of total protein) were mixed with 50 μL of caspase buffer, and reactions were initiated by the addition of 100 μ*M* of the specific substrate. After a 1-h incubation at 37°C, caspase-3-like protease activity was measured with the substrate Ac-DEVD-AFC. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) monitoring fluorescence at excitation and emission wavelengths of 400 and 505 nm, respectively.

Bcl2/Bax ratio (MG-63 osteoblast-like cells)

At the indicated time points, cells were washed twice with PBS and lysed. Proteins were resolved by SDS-PAGE on a 4–20% Tris-Gly gel and transferred to a PVDF membrane. The membrane was probed with specific antibodies against Bcl-XL (mouse monoclonal clone 4; Transduction Lab), Bcl-2 (mouse monoclonal clone 7, Transduction Lab), Bax (rabbit polyclonal; gift from J. Reed of The Burnham Research Institute, San Diego, CA), and actin (mouse monoclonal, Sigma). The bands were resolved with specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL; Amersham). The intensity of each band was quantified by densitometry and compared with the actin band. The intensity of each Bcl-2 family member was then normalized to the control treated group (indicated as 1) at each time point.

Analysis of gene expression (MG-63 osteoblast-like cells)

We used Genefilters (GF211; Research Genetics, Huntsville, AL) and monitored the expression of approximately 4000 genes. The samples (5 μg of total RNA per condition) were processed according to the manufacturer's recommendations. A filter was used twice with the same sample in order to verify the consistency of the results. There was good

concordance between the two measurements. The data were analyzed using the pathway software developed by Research Genetics. Eighty genes were selected by fold modulation over the control and were classified into eight groups (extracellular matrix, cytokines, receptors, enzymes, nuclear architecture regulation, cell adhesion, apoptosis, and others).[17,22,23] Three analyses were performed at 4 and 24 h: control versus Ti, control versus PMMA, and Ti versus PMMA. The difference in gene expression was based on our experimental finding that genes had to be modulated at least 1.5- and 2.5-fold for the 4- and 24-h experiments, respectively, to be significant in the assay used.

We tested the reliability of the Genefilters' results by selecting genes identified as being modulated significantly for analysis and validation by quantitative real time RT-PCR (TaqMan ABI Prism 7700; Applied Biosystem, Foster City, CA). The GAPDH housekeeping gene was used to control for input, thereby, allowing a comparison between samples.

Protein levels of fibronectin and osteocalcin and alkaline phosphatase (ALP) activity (MG-63 osteoblast-like cells)

After a 4-h incubation, the supernatant was removed, and the cells were washed three times with PBS. One milliliter of particles solution (serum free) and medium (serum free) was added defining the time 0. Supernatant and cell lysate (obtained with 1% Triton-X) were collected at 4 and 24 h. The lysate was sonicated for 30 s on ice. Before measuring, supernatant and lysate were centrifuged at 800*g* for 2 min.

A volume of 100 μL (respectively, 25 μL) of supernatant of the different samples was used to determine the level of fibronectin (respectively, osteocalcin) with a commercial enzyme-linked immunoassay (Biomedical Technologies Inc., Stoughton, MA). In parallel, a volume of 2 μL of lysate sample was added to *p*-nitrophenyl phosphate solution (Sigma) within a 96-well plate at 37°C for 3 min. *p*-Nitrophenol is produced in the presence of ALP, and the absorbance can be measured with a plate reader at 405 nm. The change in rate of absorbance is directly proportional to the activity of ALP. Data for fibronectin and osteonectin and ALP activity were normalized by the total cell protein (DC protein Assay Kit; Bio-Rad, Hercules, CA). Experiments were performed in triplicate, and measurements were made in duplicate.

RESULTS

Confocal imaging (MG-63 osteoblast-like cells)

The views of the MG-63 osteoblasts at different z-levels clearly showed that the cytoplasm contained the Ti particles, representing phagocytosis. A large number of particles were also found adjacent to the nucleus (Fig. 2). Rhodamin-phalloidin staining showed that the organization of the actin filaments was profoundly altered when particles were present (data not shown).

Apoptosis and caspase activity (MG-63 osteoblast-like cells, rat osteoblast, SaOS-2 osteoblast-like cells)

The particles induced apoptosis in osteoblast cells as demonstrated by the appearance of hypodiploid DNA-containing cells, which were measured by flow cytometry in permeabilized cells stained with propidium iodide (Fig. 3). The fluorometric measurement of caspase-3-like activity using the synthetic substrate Ac-DEVD-AFC confirmed the DNA content results (Fig. 4). The Ti particles had the greatest effect upon less mature MG-63 and mature SaOS-2 osteoblast-like cells, whereas the rat osteoblasts appeared more resistant. The PMMA particles were less potent to induce apoptosis than the Ti particles. Control latex particles did not induce apoptosis or caspase activity despite being phagocytosed (results not shown).

Bcl2/Bax ratio (MG-63 osteoblast-like cells)

Different biological responses were observed between materials when expression of Bcl-2 family members was monitored in particle-treated MG-63 cells (Fig. 5). Ti particles induced a slight downregulation of the Bcl-XL levels after a 24-h incubation. Conversely, PMMA induced a strong induction of the Bcl-XL levels that was prolonged up to 48 h postincubation, thus favoring survival. The Bcl-2 level also changed when cells were incubated with Ti and PMMA particles but its relative level of expression, when compared with Bcl-XL, was much lower. Finally, Bax levels, although somewhat increased in PMMA-treated cells at 24 h concomitantly to the Bcl-XL and Bcl-2 increase, did not change dramatically during the incubation period. It is conceivable that the upregulation of the two anti-apoptotic proteins Bcl-XL and Bcl-2 observed at 24 and 48 h in PMMA-treated cells may be related to the delay in activation of caspases.

Gene expression (MG-63 osteoblast-like cells)

Modulation of gene expression after a 4-h co-incubation with Ti particles

After a 4-h co-incubation of Ti particles with MG-63 osteoblast-like cells, 853 genes of the 3964 queried had

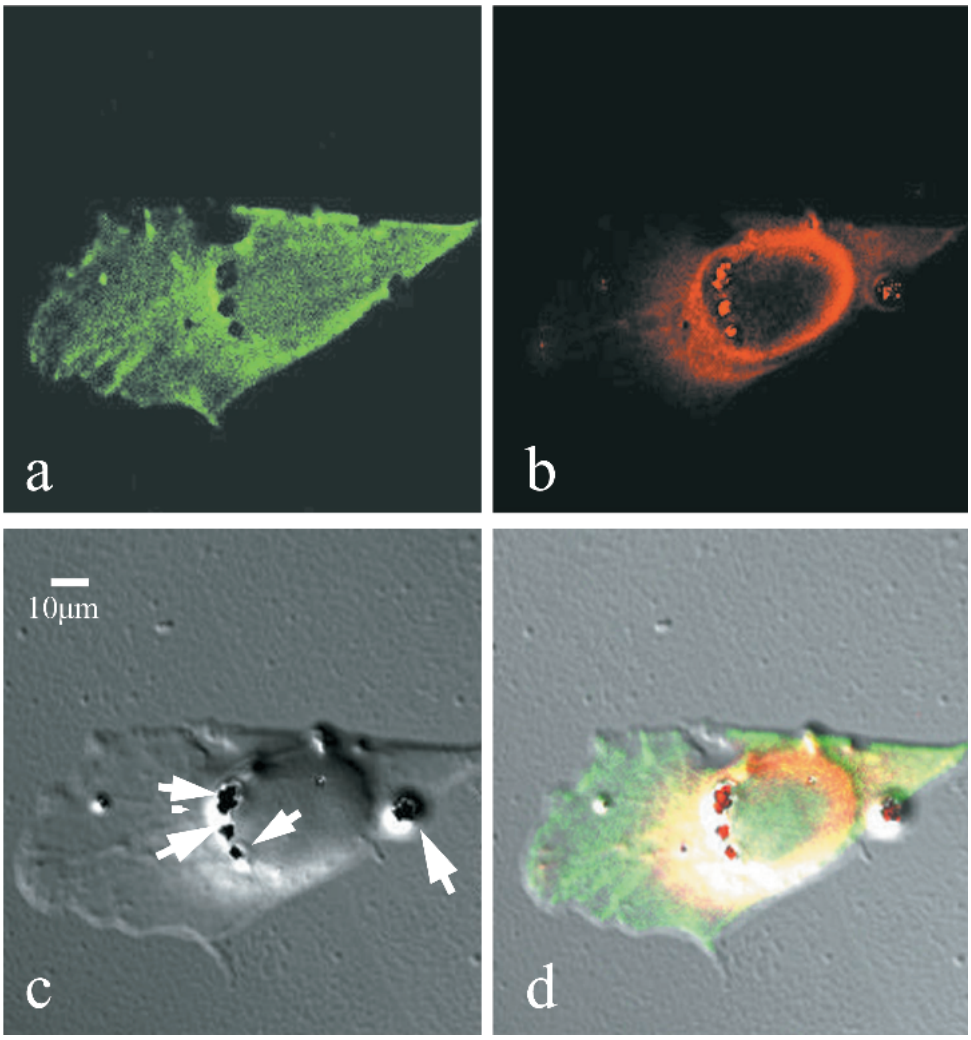
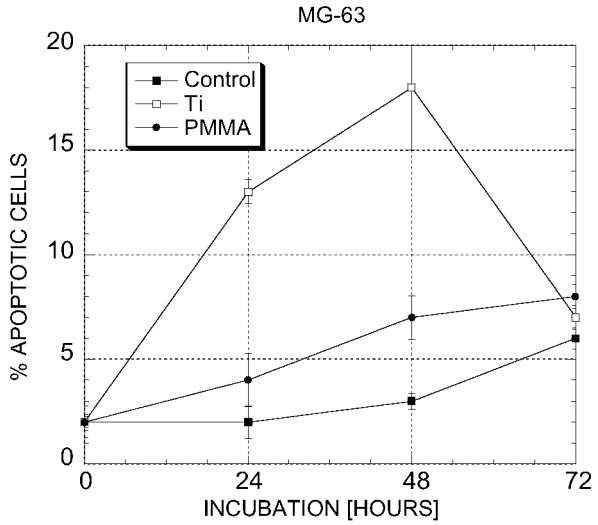
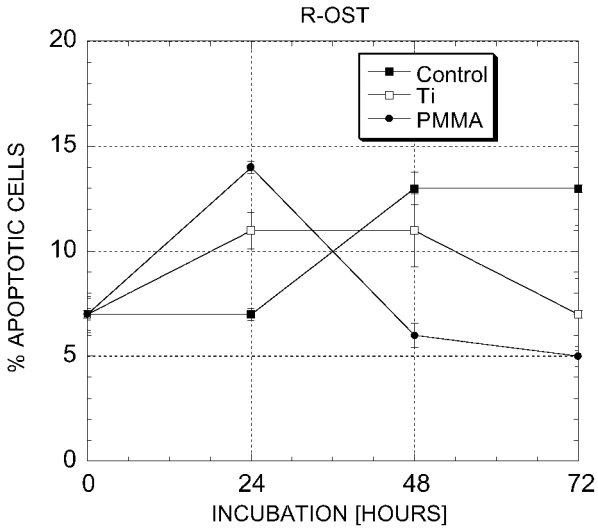
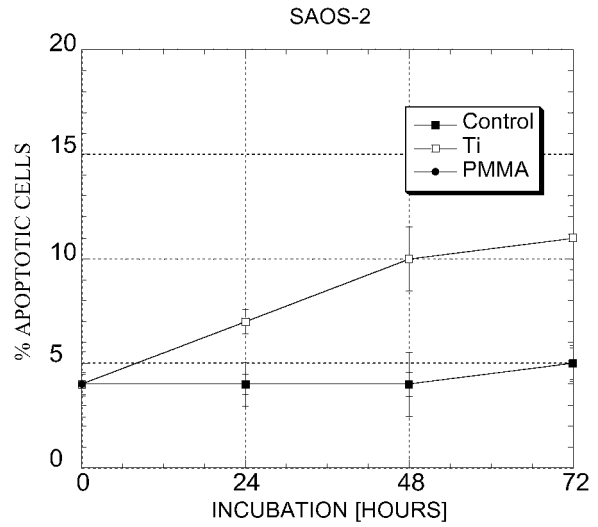


Figure 2. MG-63 osteoblast-like cells were cultured 24 h with 0.1% Ti particles, cells were then fixed, and the cytoskeletal alterations were visualized by staining with an antitubulin antibody (red, microtubules) and FITC-labeled phalloidin (green, actin microfilaments). (a) Confocal fluorescence image showed the distribution of the actin microfilaments of a MG-63 cell. Three dark spots at the center of the cell clearly show the lack of actin filaments in correspondence to the "ingested" Ti particles. (b) Confocal fluorescence image shows the distribution of the microtubules of an MG-63 cell. The three bright red spots at the center of the cells represent the Ti particles. (c) Nomarsky phase contrast image of the same cell demonstrates the presence of the Ti particles ingested by the cell, indicated by the white arrows. (d) A combined image of the panels a, b, and c illustrates the colocalization of the beads with the cytoskeletal alterations.

their expression modulated significantly. Of these, 32 were of the 80 initially selected genes. Twenty-one genes were upregulated, and 11 were downregulated in the Ti-treated group compared with the control group. Little effect on the gene expression of extracellular matrix proteins (type I collagen, osteonectin, osteopontin, and alkaline phosphatase) was observed, except for an upregulation of the fibronectin and osteocalcin genes (Table I). The Ti particles had a strong impact on cytokine gene expression with an upregulation of the bone resorption inducers, interleukin 1α and 1β,[23] and TGF-β (stimulates osteoblasts growth and differentiation[24]), and a downregulation of fibroblast growth factor (promoter of osteoblast survival[25]) gene (Table II). Gene expression for receptors of TNF (bone resorption inducer[23] and proapoptotic agent[25]), interleukin 1 antagonist, insulin-like growth factor (stimulates osteoblast proliferation and differentiation[26]) and tyrosine-protein kinase (stimulate cellular proliferation[27]) were downregulated, while TNF and laminin receptor genes expression were upregulated (Table III). A downregulation of the tissue inhibitor of metalloproteinase (TIMP-1) gene expression was detected (Table IV). This enzyme neutralizes the effect of MMP-1, which can degrade type I collagen.[28] In conglomerate, the profile of genes modulated in MG-63 osteoblasts in contact with Ti particles appears to favor bone resorption. The Ti particles also had a profound impact on the gene expression of proteins that regulate the nuclear architecture (Table V).





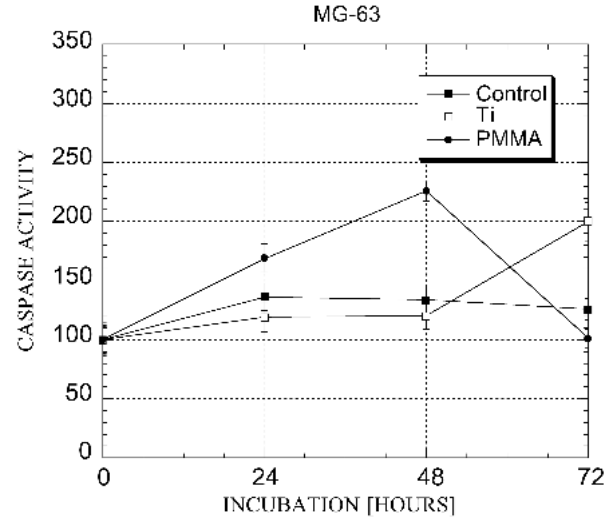


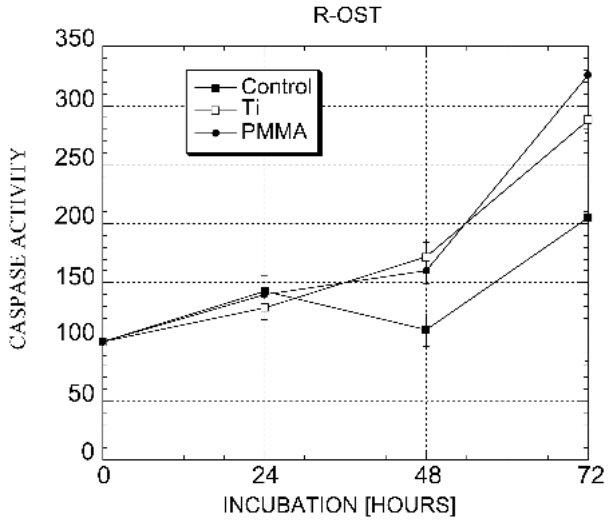
An upregulation of the Pou and NuMA genes and a downregulation of the HMG and SATB1 genes were observed. These genes belong to the nuclear matrix gene family.[22] A downregulation was induced in the actin-depolymerizing gene expression as well. The Ti particles also modulated the expression of genes that code for cell adhesion proteins with a downregulation of the integrin β_1 ($\alpha_2\beta_1$ is the major receptor for type I collagen[29]) and an upregulation of laminin γ_1 (Table VI). The apoptosis-related genes p53-binding protein gene was upregulated, the anti- apoptotic Bcl-2 was downregulated, possibly rendering the cells more prone to undergo apoptotic cell death (Table VII). The Ti particles had no effect on proliferation genes (c-fos, c-myc, c-jun) (Table VIII).

Modulation of gene expression after 24 h co-incubation with Ti particles

After a 24-h incubation of MG-63 osteoblast-like cells with Ti particles, a less pronounced effect was observed on gene modulation than at the 4-h time point. Twenty-six genes of the 80 initially selected genes had their expression modulated. Three genes were upregulated, and 23 were downregulated in the Ti group compared with the control group. For the extracellular matrix protein group, no significant effect was observed except a downregulation of type XI collagen (Table I). The largest difference in gene expression, as compared with the 4-h results, were found in the cytokines group with a downregulation of the macrophage-stimulating 1 and PDGF genes. A strong downregulation of TGF-β inducible early protein, and PDGF were also measured (Table II). Some changes between the 4- and 24-h incubation were found in the receptor group with a downregulation in the gene expression of the receptors for interleukin 10, colony-stimulating factor 3, TGF-β, BMP type II, steroid hormone, epidermal growth factor, and tyrosine-protein kinase (Table III). MMP-7 gene expression was also downregulated (Table IV) as well as the Pou and

Figure 3. Percentage of apoptotic cells in the three osteoblast-like cells used and with the two particle types tested. Results are expressed as the means ± standard deviation (error bars) and are representative of four independent experiments. The Ti particles strongly induced apoptosis in the MG-63 osteoblast-like cells, whereas the PMMA particles had a less remarkable effect when compared with control. This trend increased until 48 h of incubation, and then no difference was found between the two types of particles and the control. The induction of apoptosis by the particles was less for the rat osteoblast than for the other cell types. Control cells presented a higher level of apoptotic cells than both particle groups after 48 h of incubation. The Ti particle was the only one to induce apoptosis in the SaOS-2 osteoblast-like cells. Apoptosis increased with incubation time.





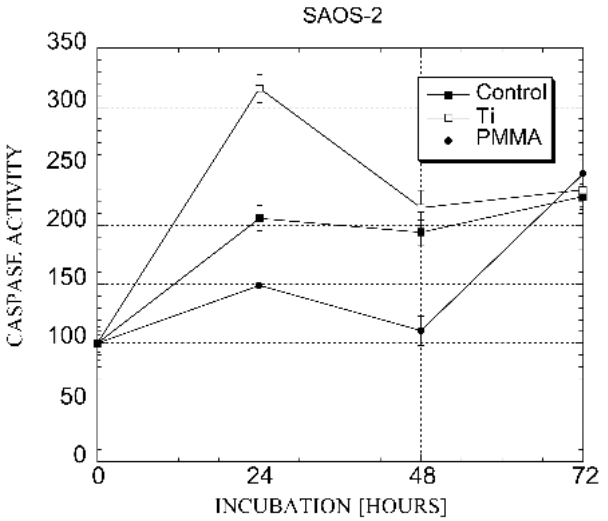


Figure 4. Caspase-3-like activity for the three osteoblast cell lines used and with the two particle types tested. Results are expressed as the means ± standard deviation (error bars) and are representative of four experiments. The caspase activity of the MG-63 osteoblast-like cells in contact with PMMA particles increased over time reaching a peak at 48 h and then returned to its initial level. Unlike the PMMA particles, the Ti particles increased the caspase activity of osteoblasts up to the last timepoint of 72 h. The caspase activity of the control group remained stable for the duration of the experiment. The rat osteoblasts had a similar caspase activity for the two particles types with a constant increase over time. The control group followed the same pattern with a lower value of caspase activity. The caspase activity of SaOS-2 cells peaked after 24 h and then decreased and remained stable until 72 h. PMMA particles decreased the caspase activity of SaOS-2 cells when compared with control. However, after 72 h of incubation, no difference were found between the caspase activity of the SaOS-2 in contact with the two particle types and the control group.

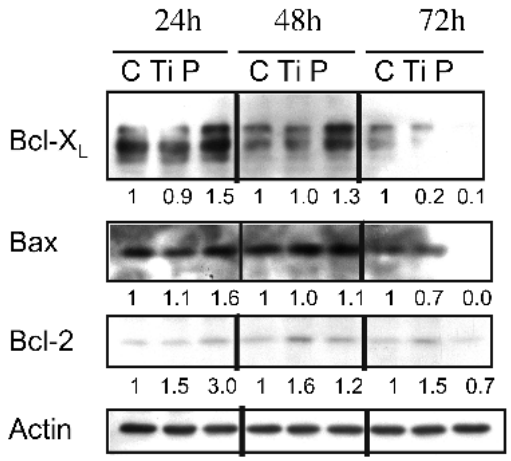


Figure 5. Particle-induced modulation of Bcl-2 family members expression for the MG-63 osteoblast-like cells.

YY1 genes (Table V). No differences with control were observed for the expression of genes in the adhesion group except for a downregulation of integrin α_M (Table VI). For the apoptotic genes at 24 h, only BCL-2 was downregulated (Table VII). The proliferation gene c-myc was downregulated as well as two heat shock proteins (1 and 2), fast kinase, and serine/threonine protein kinase SAK genes (Table VIII).

Comparison of gene modulation induced by Ti and PPMA particles at both 4 and 24 h

After a 4-h incubation of MG-63 osteoblast-like cells with PMMA and Ti particles, no modulation difference was observed except for the extracellular matrix group. A downregulation was observed for TGF-β, TGF-β receptor, steroid hormone receptor, tyrosine-protein kinase receptor, YY1, BCL2, heat shock protein

TABLE I

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Collagen, type I, alpha-2	+1.14	+1.10	−1.03	−1.91	−2.35	−1.25
Human pro-a2 chain of collagen type XI (COL11A2) gene, complete cds	−1.21	−1.37	−1.11	**+3.06**	**+3.30**	+1.07
Fibronectin 1	**−1.58**	−1.45	+1.04	+1.04	−1.09	−1.13
SPARC/osteonectin	+1.29	−1.03	−1.32	−2.27	−2.47	−1.08
Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I)	−1.35	**−1.52**	−1.12	−1.51	−1.38	+1.09
Biliary glycoprotein (BGP) (ostoecalcin)	**−1.52**	−1.16	+1.3	+1.32	+2.39	+1.81
Alkaline phosphatase, liver/bond/kidney	−1.19	+1.15	+1.37	+1.06	+1.17	+1.09

TABLE II

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Homo sapiens TNF-related ligand TRANCE mRNA, partial cds	−1.17	−1.2	−1.03	+1.61	**+2.66**	+1.65
Bone morphogenetic protein 2 (BMP2)	+1.31 (+1.61)	**+1.82** (+1.40)	+1.39	+1.60	+2.09	+1.30
Transforming growth factor beta (BMP6)	−1.30	−1.32	−1.01	−1.70	−2.02	−1.10
Transforming growth factor, beta 3	**−1.97**	−1.42	+1.38	+1.40	+1.70	+1.21
Human TGF-beta inducible early protein (TIEG) mRNA, complete cds	**−1.86** (+1.11)	−1.43 (−1.21)	+1.30	**+4.90** (+1.04)	**+5.28** (−2.95)	+1.07
Macrophage stimulating 1 (hepatocyte growth factor-like)	**−1.57**	**−1.91**	**−2.13**	**+3.0**	**+4.98**	−1.22
Human PDGF associated protein mRNA, complete cds	−1.29	+1.04	+1.36	**+4.02**	**+2.76**	−1.45
Platelet-derived growth factor PDGF-A	**−1.75**	−1.43	+1.22	+1.74	+1.44	−1.20
Interleukin 1 α	**−1.59**	−1.47	+1.08	−1.98	−1.45	+1.37
Interleukin 1 β	**−1.56**	−1.46	+1.07	−1.69	−1.32	+1.27
Interleukin-1 β convertase precursor	−1.30	−1.28	+1.01	+1.29	+1.34	+1.04
Interleukin 6 (B cell stimulatory factor 2)	−1.12	−1.04	+1.11	−1.24	−1.37	−1.10
Fibroblast growth factor 7 (keratinocyte growth factor)	**+1.51**	+1.30	−1.13	−1.96	−1.64	+1.15
Granulocyte colony-stimulating factor induced gene	−1.0	**−1.82**	**−1.80**	−1.09	−1.66	−1.52

TABLE III

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Interleukin 1 receptor antagonist	**−1.80**	**−2.10**	−1.17	−1.90	−1.72	+1.10
Interleukin 4 receptor	+1.10	−1.15	−1.28	−1.24	−1.33	−1.07
Interleukin 10 receptor	+1.21	**+1.50**	+1.25	**+3.46**	**+3.37**	−1.02
Colony stimulating factor 3 receptor (granulocyte)	+1.01	+1.32	+1.33	**+4.05**	**+4.87**	+1.2
Homo sapiens TNF receptor-1 associated protein (TRADD) mRNA, 3′ end of cds	**+1.5**	−1.45	**−2.16**	−1.56	**−2.74**	−1.76
Human TNF receptor associated factor 6 (TRAF6) mRNA, complete cds	−1.35	**−1.5**	−1.09	−1.98	−2.08	−1.05
Prostaglandin E receptor 2 (subtype EP2), 53kD	−1.40	−1.33	+1.06	+1.31	+1.08	−1.20
Transforming growth factor, beta receptor II (70–80 kD)	**−1.51**	−1.01	**+1.5**	**+4.06**	**+3.52**	−1.15
Insulin-like growth factor 2 receptor	**−1.67**	**−2.15**	−1.28	−1.4	−1.55	−1.10
Bone morphogenetic protein receptor, type II (serine/threonine kinase)	+1.42	**+1.69**	+1.19	**+3.63**	**+3.16**	−1.15
Steroid hormone receptor err1	−1.20	**+1.58**	**+1.91**	**+3.71**	**+3.53**	−1.05
1.05						
Epidermal growth factor receptor	−1.12	+1.26	+1.42	**+3.18**	**+3.74**	+1.17
Tyrosine-protein kinase receptor eph precursor	**−2.1**	−1.40	**+1.50**	**+4.87**	**+3.93**	−1.24
Laminin receptor (2H5 epitope)	**+1.71**	+1.02	**−1.67**	−1.65	−1.40	+1.18
Human clone pSK1 interferon gamma receptor accessory factor-1 (AF-1)	+1.32	+1.08	−1.22	−1.13	−1.9	−1.68

TABLE IV

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Matrix metalloproteinase 2 (gelatinase A; collagenase type IV) (MMP2)	−1.32	−1.25	−1.05	−1.56	**+2.69**	+1.72
Matrix metalloproteinase 7 (matrilysin, uterine) (MMP7)	−1.26	−1.03	+1.21	**+3.10**	**+5.00**	+1.61
Matrix metalloproteinase 10 (stromelysin 2) (MMP10)	−1.27	−1.3	−1.01	−1.82	−1.04	+1.73
Matrix metalloproteinase 13 (collagenase 3) (MMP13)	−1.17	−1.45	−1.23	−1.76	−1.71	+1.03
TIMP1	**+1.54**	+1.20	−1.28	+1.06	−1.68	−1.78
tissue inhibitor of metalloproteinase 2 (TIMP2)	+1.10	−1.18	−1.30	−1.32	−1.63	−1.23

TABLE V

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Homo sapiens HMG box containing protein 1 mRNA, complete cds	**+1.58**	+1.31	−1.20	−1.24	−2.13	−1.70
SATB1	**+1.51**	−1.06	−1.42	−1.60	−1.75	−1.09
POU homeobox protein	**−1.64**	−1.11	+1.46	**+4.03**	**+6.28**	+1.55
Human YY1-associated factor 2 (YAF2) mRNA, complete cds	−1.36	+1.18	**+1.62**	**+2.60**	**+3.05**	+1.17
Clone PEBP2aA1) core-binding factor, runt domain, alpha subunit 1 (CBFA1)	−1.15	**−1.63**	−1.39	−2.40	−1.83	+1.31
H. sapiens NuMA gene (Clone T33)	**−1.52**	+1.07	−1.41	−1.24	−1.36	+1.02
Lamin B receptor	**−1.79**	**−1.53**	+1.16	−1.60	+1.00	+1.58

TABLE VI

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	+1.19	−1.24	−1.49	−1.36	−1.70	−1.24
Integrin, beta 1	**+1.56** (+1.33)	−1.3 (−1.28)	**−2.04**	−1.19 (−2.12)	−1.82 (+1.12)	−1.53
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	+1.45	+1.06	−1.37	−1.17	**+3.91**	−2.01
Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	−1.48	−1.30	+1.13	+1.22	+1.28	+1.04
Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	+1.04	−1.25	−1.3	+1.79	+1.26	−1.41
Integrin, alpha M (complement component rece. 3, alpha; CD11b (p170))	+1.26	+1.31	+1.04	**+3.08**	+2.20	−1.39
Integrin, alpha 7B	−1.07	−1.28	−1.18	−1.07	−1.45	−1.36
Integrin beta-5 subunit	+1.01	−1.1	−1.11	−1.71	−1.51	+1.12
Homo sapiens integrin cytoplasmic domain associated protein (Icap-1a)	−1.02	+1.1	+1.12	−1.50	−1.31	+1.14
Human metalloprotease/ disintegrin/cysteine-rich protein precursor (MDC9)	+1.27	+1.20	−1.05	−1.87	−1.87	−1.00
Integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1)	−1.13	−1.05	+1.06	−2.12	−1.41	+1.50
Laminin, gamma 1 (formerly LAMB2)	**−1.57**	**−1.51**	+1.04	+1.25	+1.16	−1.07
Laminin, beta 2 (laminin S)	−1.33	**−1.74**	−1.31	−1.67	−1.95	+1.04

TABLE VII

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Human Bcl-2 related (Bfl-1) mRNA, complete cds	**+1.77**	**+3.02**	**+1.76**	**+3.21**	+2.01	−1.59
Nucl. fact of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NF-κβ)	−1.43 (−1.88)	−1.02 (−1.20)	+1.40	−1.40 (−1.01)	+1.00 (−1.12)	+1.39
Mouse double minute 2, human homolog of; p53-binding protein	**−1.67**	−1.40	+1.19	−1.15	−1.02	+1.12
Hum cysteine protease CPP32 isoform alpha mRNA, complete cds (caspase-3)	−1.03	−1.15	−1.11	−1.00	−1.17	−1.18

1, and serine/threonine protein kinase SAK gene expression. An upregulation of macrophage stimulating 1, granulocyte colony-stimulating factor, TNF receptor 1, lamin receptor, integrin β_1, and c-fos genes expression was induced by the PMMA particles compared with Ti. After 24 h of coincubation, there was no noticeable difference in expression of the 80 selected genes by the two types of particles.

Protein levels of fibronectin and osteocalcin and alkaline phosphatase activity (MG-63 osteoblast-like cells)

A slight increase of fibronectin and osteocalcin production by the Ti group at 4 h in comparison to control at 4 h was observed corresponding to the Genefilters results (Fig. 6). However, these differences were not

TABLE VIII

Gene Name	C-Ti 4 h	C-PMMA 4 h	Ti-PMMA 4 h	C-Ti 24 h	C-PMMA 24 h	Ti-PMMA 24 h
Heat shock factor protein 2	**−1.56**	**−1.52**	+1.02	**+4.63**	**+6.18**	+1.33
Heat shock 760 kd protein 1	−1.41	+1.12	**+1.59**	**+4.21**	**+4.21**	−1.00
Transcription factor p65	**−1.58** (−1.68)	−1.48 (−1.47)	+1.06	**+3.18** (+1.03)	**+3.51** (−1.13)	+1.10
H. sapiens mRNA for FAST kinase	+1.22	+1.41	+1.15	**+3.91**	**+3.51**	−1.27
H. s. intermediate conductance calcium-activated potassium channel (hKCa4)	−1.36	+1.06	+1.44	**+4.06**	**+5.26**	+1.29
Homo sapiens mRNA for serine/threonine protein kinase SAK	**−1.58**	−1.03	**+1.53**	**+4.08**	**+6.21**	+1.52
Human protein kinase (MLK-3) mRNA, complete cds	**+1.82**	**+2.09**	+1.15	**+4.36**	**+3.31**	−1.31
40S ribosomal protein S15A	**+1.51**	**+1.50**	−1.00	**−2.65**	−1.52	+1.71
Human NADH:ubiquinone oxidoreductase MLRQ subunit mRNA, complete cds	+1.25	+1.45	+1.14	**−2.84**	−1.77	+1.60
H. sapiens RFXAP mRNA	+1.12	−1.42	**−1.60**	**−2.50**	−1.75	+1.42
Human mRNA for c-myc binding protein, complete cds	−1.10	−1.32	−1.19	**+2.64**	**+3.54**	+1.34
P55-c-fos proton-oncogene protein	−1.04	**−1.59**	**−1.53**	−1.50	−1.88	−1.25
Human c-jun proto oncogene (JUN), complete cds, clone hCJ-1	+1.05	−1.21	−1.28	+1.39	+1.45	+1.04
Capping protein (actin filament), gelsolin-like	+1.42	+1.02	−1.39	−1.58	−1.57	+1.00
Actin depolymerizing factor [human, fetal brain, mRNA, 1452 nt]	**+1.57**	**+1.73**	+1.10	−1.62	−1.37	+1.18

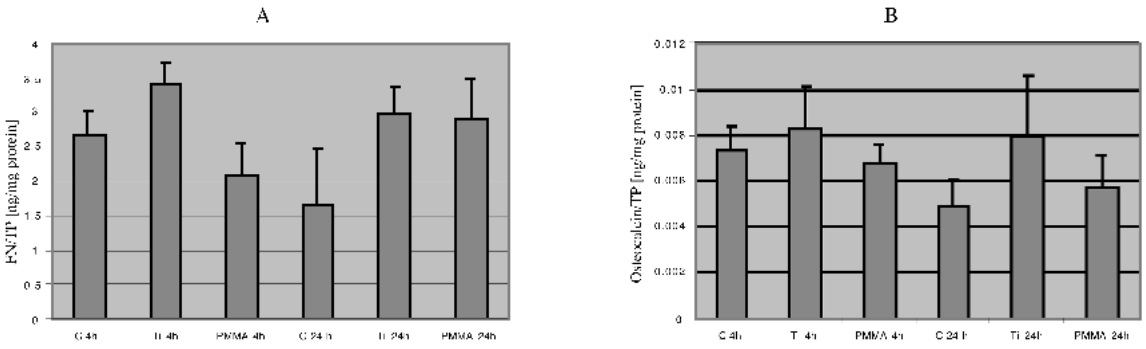


Figure 6. Mean production ± SD (n = 3) of fibronectin (A) and osteocalcin (B) normalized by the total protein (TP) for MG-63 osteoblast-like cells. The differences were not statistically significant.

statistically significant. Indeed, no statistical difference was observed between the groups for fibronectin and osteocalcin production. Also, no statistical difference was observed between all the samples for the ALP activity, which followed the trend given by the Genefilters results (Fig. 7).

DISCUSSION

The cells reaction to orthpedic implant particles has been shown to be involved in implant loosening. Most of the previous works studied the process of bone resorption related to the presence of particles. Effects of particles on bone formation have been negleted. In the present study, we investigate the osteoblasts reaction to Ti and PMMA particles.

Orthopedic implant particles induced apoptosis in osteoblasts in our *in vitro* model system, and caspase-3 was involved in this process. This finding extends the results obtained from previous studies with rat osteoblasts using the TUNEL assay.[14] A higher percentage of apoptosis induced by the particles was observed in the MG-63 osteoblast-like cells. The increased susceptibility to apoptosis of the less mature osteoblast could have important consequences for bone remodeling. Indeed, if less mature osteoblasts undergo apoptosis *in vivo* as our results may indicate, the number of mature osteoblasts that have to synthesize new bone will obviously thereby decrease, favoring the resorption process. Particle-induced apoptosis in osteoblast may thus be partly responsible for the osteolysis surrounding orthopedic implants.

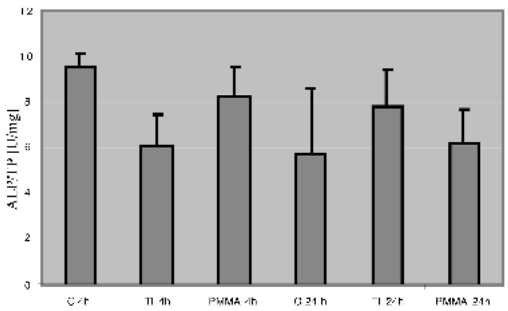


Figure 7. Mean ALP activity ± SD (n = 3) normalized by the total protein (TP) for MG-63 osteoblast-like cells. The differences were not statistically significant.

The apparent discrepancy between the levels of caspase activity (Fig. 4) and the percentage of apoptotic cells (Fig. 3) can be explained by the nature of the assays. The percentage of apoptotic cells is a cumulative measurement of cells currently undergoing apoptosis as well as cells that are dead but not completely degraded. On the other hand, the measurement of caspase activity is a snapshot of current enzymatic activity, but the activated caspases will initiate a proteolytic cascade that will eventually lead to their complete cleavage and inactivation. At the protein level using Western blot analysis, different biological responses were observed between materials when expression of Bcl-2 family members was monitored in particle-treated MG-63 cells. The particles did alter the relative levels of the Bcl-2 family members in the treated cells, but the intensity of the changes do not seem to be sufficient to explain the induction of an apoptotic program.

Twelve of the 18 genes tested by real time RT-PCR confirmed the results obtained in the cDNA Genefilters. Differences may occur because cDNA microarray does not discriminate between highly related genes (>80% homology). In fact the signal of related genes contribute in an additive manner to the measurements. This is a limitation of the technology and why validation by quantitative RT-PCR was performed. Protein production of fibronectin by ELISA kit, osteocalcin by EIA kit, and alkaline phosphatase activity by *p*-nitrophenyl phosphate solution gave trends similar to the one observed with Genefilters.

Ti particles had a stronger effect on transcriptional induction of genes than PMMA particles. This effect could be partially explained by the slightly smaller size distribution of the Ti particles compared with the PMMA ones. It should be noted that the mean size of

the particles used in this study was larger than particles retrieved from peri-implant tissues obtained at revision of failed prosthesis.[30,31] Ninety percent of the used Ti and PMMA particles had a diameter >10 μm, rendering phagocytosis by the cells difficult. Therefore, it is possible that most of the effects quantified in this study could be due more to cell-particle contact than to phagocytosis. However, because of the large distribution of particles size, it is difficult to draw a definitive conclusion on the size effect in this study. A minority of particles (smallest) could induce the most stricking effect.

A profound effect of particles on genes that regulate the nuclear and cell architecture was noted. This observation was confirmed by confocal imaging, which demonstrated a reorganization of actin filaments when particles were present. This effect on architecture could be explained by particles being phagocytosed by osteoblasts imposed deformations on the cytoskeleton. These deformations can modulate the expression of genes involved in controlling the architecture of the cell as described previously.[32]

The most striking effect of the particles was on the modulation of cytokine genes, which was noticeable after only 4 h of exposure of the cells to the particles. It is difficult to interpret this effect on bone remodeling. However, there was a trend toward downregulation of cytokines or cytokine receptors involved in bone formation (FGF, TIMP-1, ILGF) and an upregulation of cytokines involved in bone resorption (IL-1α, IL-β, TNF-α). Thus, it is likely that the contact of particles with osteoblasts would favor the bone resorption *in vivo.*

Interestingly, this study showed that the expression of the integrin β_1, a major integrin responsible for osteoblast adhesion, was downregulated. Because osteoblasts are anchorage dependent cells, downregulation of adhesion proteins can be responsible for cell dysfunction. Especially for less mature osteoblasts, it has been shown that during early maturation the osteoblast adhesion proteins are usually upregulated.[17] The downregulation of integrin could explain our recent finding that the adhesion strength of osteoblasts in contact with particles was decreased.[33] The succession of events when osteoblasts are in contact with particles can therefore be proposed: osteoblast-particle contact, phagocytosis of particles, decrease of cell adhesion, and subsequent induction of apoptosis. However, this hypothetical schema needs further validation.

Our study demonstrates the undeniable impact of orthopedic implant particles on osteoblasts. The peri-implant osteolysis after a total joint replacement could in part be attributed to a decrease of bone formation and not only to an overstimulation of bone resorption as it was generally believed. Consequently, development of new drugs designed to control peri-implant osteolysis should target the osteoblasts by promoting their survival, increasing their adherence, favoring the production of extracellular bone matrix, and decreasing the synthesis of osteolytic cytokines.

The authors thank Davey Smith for critical review of this manuscript.

References

1. Clarke IC, Campbell P, Kossovsky N. Debris-mediated osteolysis—A cascade phenomenon involving motion, wear, particulates, macrophage induction, and bone lysis. In: St. John KR, editor. Particulate debris from medical implants: mechanisms of formation and biological consequences, ASTM STP 1144. Philadelphia: American Society for Testing and Materials; 1992. p 7–26.
2. Amstutz H, Dorey FJ, Finerman GAM. The cemented T-28/TR-28 prosthesis. In: Finerman GAM, Dorey FJ, Grigoris P, McKellop HA, editors. Total hip arthroplasty outcomes. New York: Churchill Livingston; 1998. p 55–63.
3. Friedman RJ, Black J, Galante JO, Jacobs JJ, Skinner HB. Current concepts in orthopaedics biomaterials and implant fixation. J Bone Joint Surg 1993;75-A:1086–1109.
4. Pioletti DP, Takei H, Lin T, Van Landuyt P, Ma QJ, Kwon SY, Sung KL The effects of calcium phosphate cement particles on osteoblast functions. Biomaterials 2000;21:1103–1114.
5. Salvati EA, Foster B, Doty SB. Particulate metallic debris in cemented total hip arthroplasty. Clin Orthop 1993;293:160–173.
6. Glant TT, Jacobs JJ. Response of three murine macrophage populations to particulate debris: bone resorption in organ cultures. J Orthop Res 1994;12:720–731.
7. Lee SH, Brennan FR, Jacobs JJ, Urban RM, Ragasa DR, Glant TT. Human monocyte/macrophage response to cobalt-chromium corrosion products and titanium particles in patients with total joint replacements. J Orthop Res 1997;15:40–49.
8. Goodman SB, Fornasier VL. Clinical and experimental studies in the biology of aseptic loosening of joint arthroplasties and the role of polymer particles. In: St. John KR, editor. Particulate debris from medical implants: mechanisms of formation and biological consequences, ASTM STP 1144. Philadelphia: American Society for Testing and Materials; 1992. p 27–37.
9. Yao J, Cs-Szabo G, Jacobs JJ, Kuettner KE, Glant TT. Suppression of osteoblast function by titanium particles. J Bone Joint Surg 1997;79-A:107–112.
10. Eloy R, Weill N. Cytotoxicity part II: apoptosis. In: Braybrook JH, editor. Biocompatibility assessment of medical devices and materials. London: John Wiley & Sons; 1997. p 124–128.
11. Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 1997;278:294–298.
12. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–446.
13. Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. J Bone Miner Res 1998;13:793–802.
14. Pioletti DP, Takei H, Kwon SY, Wood D, Sung K-LP. The cytotoxic effect of titanium particles phagocytosed by osteoblasts. J Biomed Mater Res 1999;46:399–407.
15. Puelo DA, Preston KE, Shaffer JB, Bizios R. Examination of osteoblast-orthopaedic biomaterial interactions using molecular techniques. Biomaterials 1993;14:111–114.
16. Horowitz SM, Gonzales JB. Inflammatory response to implant

particulates in a macrophage/osteoblast coculture model. Calc Tissue Int 1996;59:392–396.
17. Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. Transcriptional control of osteoblast growth and differentiation. Physiol Rev 1996;76:593–629.
18. Puleo DA, Holleran LA, Domerus RH, Bizios R. Osteoblast response to orthopaedic implants in vitro. J Biomed Mater Res 1991;25:711–723.
19. Rifas L, Fausto A, Scott MJ, Avioli LV, Welgus HG. Expression of metalloproteinases and tissue inhibitors of metalloproteinases in human osteoblast-like cells: differentiation is associated with repression of metalloproteinase biosynthesis. Endocrinology 1994;134:213–221.
20. Sanders MC, Wang YLJ. Exogenous nucleation sites fail to induce detectable polymerization of actin in living cells. J Cell Biol 1990;110:359–365.
21. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998;391:43–50.
22. Bidwell JP, Alvarez M, Feister H, Onyia J, Hock J. Nuclear matrix proteins and osteoblast gene expression. J Bone Miner Res 1998;13:155–167.
23. Gowen M. Cytokines and cellular interactions in the control of bone remodelling. In: Heersche JNM, Kanis JA, editors. Bone and mineral research, Vol. 8. New York: Elsevier Science; 1994. p 77–114.
24. Roodman GD. Role of cytokines in the regulation of bone resorption. Calcif Tissue Int 1993;53:S94–S98.
25. Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology 1997;138:3849–3858.
26. Chevalley TH, Rizzoli R, Manen D, Caverzasio J, Bonjour J-P. Arginine increases insulin-like growth factor-1 production and collagen synthesis in osteoblast-like cells. Bone 1998;23:103–109.
27. Siddhanti SR, Quarles LD. Molecular to pharmacologic control of osteoblast proliferation and differentiation. J Cell Biotech 1994;55:310–320.
28. Takagi M, Santavirta S, Ida H, Ishii M, Mandelin J, Konttinen YT. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in loose artificial hip joints. Clin Orthop 1998;352: 35–45.
29. Xia G, Wang D, Bensar MD, Karsenty G. Role of alpha-2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 1988;273:32988–32994.
30. Lee JM, Salvati EA, Betts F, DiCarlo EF, Doty SB, Bullough PG. Size of metallic and polyethylene debris particles in failed cemented total hip replacements. J Bone Joint Surg Br 1992;74: 380–384.
31. Maloney WJ, Smith RL, Schmalzried TP, Chiba J, Huene D, Rubash H. Isolation and characterization of wear particles generated in patients who have had failure of a hip arthroplasty without cement. J Bone Joint Surg Am 1995;77:1301–1310.
32. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science 1997;276:1425–1428.
33. Kwon SY, Takei H, Pioletti DP, Lin T, Ma QJ, Akeson WH, Wood DJ, Sung KLP Titanium particles inhibit osteoblast adhesion to fibronectin-coated substrates. J Orthop Res 2000;18: 203–211.