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[‡]*In memory of Florencia Alvarez (Flor) whose lovely life, plenty of fortitude and spiritual values, we had the honor of sharing.*

TIME LAPSE MICROSCOPIC EVALUATION OF THE INTERACTIONS BETWEEN BIODEGRADABLE Mg PARTICLES AND CELLS[‡]

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ABSTRACT

Mg-based implants have promissory applications as biodegradable materials in medicine for orthopedic, dental and cardiovascular therapies. During wear and degradation micro-debris are released. Time lapse multidimensional microscopy (MM) is proposed here as a suitable tool to follow, in fixed intervals over 24h periods, the interaction between cells and particles. Results of MM show interactions of macrophages (J774) with the magnesium particles (MgPa) that led to modifications of cell size and morphology, decrease in duplication rate and cell damage. Corrosion products were progressively formed on the surface of the particles and turbulence

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was generated due to the hydrogen development. Changes were more significant after treating MgPa with KF. In order to complement MM observations, membrane damage (LDH) and mitochondrial activity (WST-1) assays with macrophages and osteoblasts (MC3T3-E1) were compared. More significant concentration-dependent effect was detected for macrophages exposed to MgPa than for osteoblasts. Accordingly, complementary data showed that viability and cellular cycle seem to be more altered in case of macrophages. Additionally protein profiles and expression of proteins associated to adhesion process changed in the presence of MgPa. These studies revealed that time lapse MM is a helpful tool to monitor the changes at real time and *in situ* of biodegradable materials and the biological surrounding. This microscopic information is useful to complement studies related to biodegradable biomaterials.

Keywords: biodegradation, magnesium, multidimensional microscopy, macrophage, osteoblast

1. INTRODUCTION

Orthopedic implants frequently generate debris that contributes to their aseptic loosening (Goodman, et al., 1996; Jasty, et al., 1986; Maloney & Smith, 1995; Mirra, et al., 1976; Schmalzried, et al., 1993). Periprosthetic granulomatous tissues retrieved from failed implants have been histologically analyzed and the presence of macrophages, lymphocytes, foreign body giant cells and abundant particulate debris were detected (Dumbleton, 1981; Freeman, et al., 1982; Goldring, et al., 1993; Harris, et al., 1976; Horowitz & Purdon, 1995; Johanson, et al., 1987; Kang Jung, et al., 1994; Maloney & Smith, 1995; Santavirta, et al., 1991; Schmalzried, et al., 1993). It is known that macrophages phagocytose orthopedic biomaterial particles and

release the pro-inflammatory mediators in response to particulate debris (Davis, et al., 1993; Gelb, et al., 1994; Glant & Jacobs, 1994; Horowitz, et al., 1988; Horowitz & Gonzales, 1996; Nakashima, et al., 1999; Trindade, et al., 1999) . Furthermore, macrophages interact with other cells present at the bone-implant interface to increase the release of pro-inflammatory mediators (Lind, et al., 1998) and also may be involved in adhesion-dependent spontaneous apoptosis after the interaction with biomaterials (Zachman, et al., 2013). These effects have been recurrently analyzed in corrosion resistant debris (Caicedo, et al., 2009; Posada, et al., 2015). However, only few reports are related to debris released by degradable metals (Badar, et al., 2013; Bondarenko, et al., 2011; Roth, et al., 2014).

Mg-based materials are considered promising for [orthopedic](#), dental, and cardiovascular applications (Goodman, et al., 1996). The effect of Mg particles on UMR-106 cells was recently reported and [toxic effects were](#) particularly analyzed (Di Virgilio, et al., 2011; Grillo, et al., 2011). Cytotoxicity was found after exposures to magnesium particles (MgPa) at concentrations $\geq 1000 \mu\text{g/ml}$. On the other hand, Roth and coworkers (Roth, et al., 2014) reported that Mg debris are biocompatible and do not seem to restrict the immune function of macrophages at concentrations $\leq 500 \mu\text{g/ml}$. Nevertheless, the analysis is complex because it is difficult to obtain the mass/volume relationships of the particles that are in contact with cells and degrade *in vivo*. Besides, Mg leach liquor seems to be an effective anti-resorptive agent for the treatment of particle-induced osteolysis (Zhai, et al., 2014). Thus, depending on the characteristics and concentration of Mg-containing products benefic or detrimental effects may be found.

Biocompatibility of Mg-containing metallic materials is mostly tested *in vitro* by replacing the supernatant culture medium of the cell culture by extracts. These

extracts are obtained after immersing the metal sample in the culture medium during selected periods. However, the use of magnesium particles (MgPa) is closer to the *in vivo* situation than the use of Mg salts or extracts because there are factors like local generation of bubbles, the space and time dependence of the concentration of corrosion products as well as the direct contact of the cells with the particles and corrosion products that cannot be taken into account when extracts and Mg salts are used.

Considering that macrophages are one of the main biological actors that interact at the metallic debris/biological interface, it is interesting to investigate the effect of the interaction of Mg particles (with and without protective treatments with KF) *in situ* with the macrophages.

There are several reports related to the interaction of cells with corrosion resistant debris (Gu, et al., 2012; Hart, et al., 2010; Ogunbileje, et al., 2014; Zhai, et al., 2014). However, to our best knowledge, the interaction of cells with degradable particles and with the corrosion products that are forming when the particles are in contact *with the biological medium has not been analyzed at real time in situ*.

Considering the biodegradable nature of Mg and its alloys that generates particles during their degradation, it can be inferred that the amount of debris will be enhanced in relation to the traditional implants (Co-Cr, Ti and Ti alloys) (Hart, et al., 2010). The direct assays with cells in contact with the particles using time-lapse multidimensional microscopy (MM) allow us to mimic some aspects of the real situation and to obtain qualitative information about the changes at real time and *in situ* as a first step previous to *in vivo* assays.

It has been reported that debris dimension is an important factor when the effect of the particles is assayed. Debris size is frequently in the 5µm - 70µm range (Reddy, et

al., 2014). Mg particles, mesh 325, were used in this work and, accordingly, their diameters are different and shorter than 70 μm . Consequently, due to their dissimilar volumes their interaction with cells is probably different. Additionally, it should be taken into account that, during the corrosion process, the mass of the particles decreases and some of them may be completely dissolved (Di Virgilio, et al., 2011). To follow this dynamic process at real time we used here MM. Thus, the effect of the degradation process that is, the formation of corrosion products, including bubbles development, could be detected and the different stages of these processes and their effect on cell growth were reported.

Several cellular and physicochemical processes involve fast transformations which could be followed by 3D time-lapse microscopy (also called 4D microscopy or multidimensional microscopy (MM)). Faster, more robust and more sensitive equipments and algorithms for image and time-lapse processing were published in recent years (Baradez & Marshall, 2011; Collins, 2007; Held, et al., 2010; Huth, et al., 2011). In time-lapse microscopy images are recorded in fixed intervals over extended periods of time. 4D imaging should collect focal planes in time-lapse mode as rapidly as possible, without perturbing the sample by strong illumination or the use of staining. The original stack of 2D images is reconstructed into a 3-D image that changes with time. This type of microscopy offers great functionality for biological, cytomorphological and physicochemical examinations. Temperature stability, minimum exposure of specimens to light (protection from photobleaching) and highly sensitive recording techniques are frequently required to follow these processes. Thus, a climate chamber, cell chamber, CO₂ controller and incubators must be included in the station when biological samples are analyzed. The resulting images of movies can provide direct insights into the behavior of cells including duplication,

cell death and migration among other processes (Baradez & Marshall, 2011; Huth, et al., 2011).

MM is proposed here as a suitable tool to follow, in fixed intervals over 24h periods, the interactions of particles without or with fluoride coatings (MgPa and MgPa-F, respectively) with macrophages. Turbulence due to hydrogen development, gradual formation of corrosion products and interactions with cells were evaluated *in situ*, at real time, by MM.

These MM observations were complemented by a comparative study of the response of a macrophage cell line (J774) and an osteoblast cell line (MC3T3-E1) in relation to membrane damage (LDH), mitochondrial activity (WST-1) after the exposure to MgPa and MgPa-F. Additional information about the changes of expression of proteins and cellular cycle before and after the exposures were also provided.

2. Materials and Methods

2.1. Cells culture

Mouse osteoblasts (MC3T3-E1 cell line) and mouse macrophages (J774 cell line) were originally obtained from the cell Bank of the Animal Cell Culture Facility of CIB (Centro de Investigaciones Biológicas, CIB, Madrid, Spain). Cells were grown in T-25 flasks with D-MEM culture medium (GIBCO-BRL, LA, USA) supplemented with 10% inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate (complete culture medium, (CCM)) at 37 °C in a 5% CO₂ humid atmosphere. Cells were counted in an improved Neubauer.

2.2. Mg particles and fluoride treatment

MgPa (99.8%, 325 mesh, $58.9 \mu\text{m} \pm 20.7 \mu\text{m}$) were supplied by Alfa Aesar (Ward Hill, MA, USA) and were used to simulate Mg debris. Their chemical composition is reported in [Table 1](#).

Some MgPa were treated with KF to test the effect of this corrosion protection treatment. Fluoride layers were generated on MgPa (MgPa-F) by immersion in 0.2 M KF solutions for 1 h at room temperature. The KF dose employed was selected taking into account the dose used for previous electrochemical studies that showed an inhibitory effect for KF-treatment (Pereda, et al., 2011). After the immersion period, MgPa-F were centrifuged and washed twice with sterile water, then suspended in CCM and subsequently added to the cell cultures. MgPa were used in the 250-2000 $\mu\text{g/ml}$ concentrations range. In experiments at real time, *in situ*, the concentrations used were 1000, 1500 and 2000 $\mu\text{g/ml}$.

Incorporation of F after KF treatment was detected by mass spectroscopy (Maldi-TOF) analysis (data not shown) and it was the evidence of the presence of fluoride layer on the MgPa-F.

All the chemicals used in the experiments were of analytical grade. Milli Pore-MilliQ water was used to prepare the solutions.

2.3 Multidimensional microscopy *in vivo*

Time-lapse microphotographs are very useful to follow the formation of corrosion products as well as cells/MgPa interactions [at real time](#). MM analysis was used to observe the interaction of J774 macrophages with Mg micro-particles at 1000, 1500 and 2000 $\mu\text{g/ml}$, [and their corrosion products by the time-lapse](#) sequence during 24 h. Cells cultured into 8 multi-well plate with controlled parameters ([37°C temperature, 5% CO₂ and humid atmosphere](#)) were observed by Multidimensional microscope

(Leica DMI6000B /AF6000 LX) connected to a CCD Hamamatsu C9100-02 high resolution monochrome camera with a high speed acquisition shot (30 images/s) at real time.

2.4. LDH leakage assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells that is involved in the conversion of lactate to pyruvate with parallel reduction of Nicotinamide adenine dinucleotide (NAD). LDH is rapidly released into the cell-culture supernatant when the plasma membrane is damaged. To evaluate and quantify cell death and cell lysis, LDH activity was measured in the supernatants of cell cultures by an enzymatic assay using the Cytotoxicity Detection Kit (Roche Diagnostics, Barcelona, Spain) as it is described in (Lozano, et al., 2013). In this reaction the tetrazolium salt is reduced to a colored compound, formazan. The increase in supernatant LDH activity directly correlates to the amount of formazan formed over time.

For this analysis 2.5×10^3 cells/well were cultured in 24 multi-well plate and grown at 37 °C in 5% CO₂ humid atmosphere, in CCM, for 24 h. Then, CCM was replaced by other with different concentrations of MgPa or MgPa-F treatment. After 24 h exposition the enzymatic assays were performed on the supernatants according to the LDH kit (Roche Diagnosis) protocol. CCM was used as background control. LDH activity was measured based on differential absorbance at 490-655 nm using a Bio-Rad iMark microplate reader. Cell cultures controls (untreated cells, without particles) were also performed and a background control with CCM was used as absorbance blank. A relative cytotoxicity percentage was calculated as $[(A-B)/A] \times 100$, where A and B correspond to the absorbance of control and treated cells, respectively. Each

experiment was independently repeated three times. Data were analyzed using one-way ANOVA test and multiple comparisons were made using p values corrected by the Bonferroni method.

2.5. WST-1 assay

This assay measures the cleavage of tetrazolium salt (WST-1) (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Roche Diagnostics, GmbH, Mannheim, Germany) to formazan by dehydrogenase enzymes of mitochondria. The amount of formazan dye formed is directly correlated to the number of metabolically active cells in the culture.

For this analysis 2.5×10^3 cells/well were cultured in 24 multi-well plate and grown at 37 °C in 5% CO₂ humid atmosphere, in CCM, for 24 h. Then, CCM was replaced by other with different concentrations of particles with (MgPa) and without (MgPa-F) KF treatment. After 24 h exposure, CCM was removed, cells were washed with phosphate buffered solution (PBS) and fresh medium containing WST-1 reagent (Cell Proliferation Reagent WST-1, Roche Diagnostics, Barcelona, Spain) was added and incubated for 3 h. Then the plate was shaken for 1 min and the absorbance of the samples was measured at 415-655-nm using a Bio-Rad iMark microplate reader. A background control with CCM was used as absorbance blank. Negative controls (untreated cells) were run simultaneously in cultures without particles. A relative cytotoxicity percentage was calculated as $[(A-B)/A] \times 100$, where A and B are the absorbance of control and treated cells, respectively. Each experiment was independently repeated three times. Data were analyzed using one-way ANOVA test and multiple comparisons were made using p values corrected by the Bonferroni method.

2.6 Flow cytometry

A comparative study of the effect of MgPa and MgPa-F and their degradation products on cell cycle and cellular viability of J774 and MC3T3-E1 cell lines analyzed by flow cytometry (FC) analysis (XL Flow Cytometer, Beckman Coulter Corp) were included to complement MM information with illustrative purpose. Propidium iodide method which is a DNA intercalating fluorescent agent for DNA staining was used. Briefly, cells (5×10^5 cells/well) were cultured in 12 multi-well plate at 37 °C in 5% CO₂ humid atmosphere, in CCM, for 24 h. Then, CCM was replaced by other with 1000 µg/ml of MgPa or MgPa-F and cultured for 24h. After the exposure time, cell cultures were processed for flow cytometry analysis as described in [Lozano, 2013 #1087; Zachman, 2013 #1148]. For comparative purposes we included the percentage of each cell in each cycle phase (subG₁, G₁, S, G₂/M) that was calculated using CXP software (Beckman Coulter Corp).

2.7 Confocal immunofluorescence microscopy

Data obtained by confocal immunofluorescence microscopy (CIM) were included to show the changes of vimentin and ICAM-1 after exposures to MgPa complementing the MM information with descriptive objective. With this purpose MC3T3-E1 osteoblast were seeded on sterile glass cover slips with 1 ml of CCM within a 12-well culture plates at a cell density of 2.5×10^3 cell/well and grown at 37°C in 5% CO₂ humid atmosphere for 24 h. Then, CCM was replaced by other with 1000 µg/ml MgPa and cells were maintained in culture for 24 h. For immunodetection assays, cells were fixed with 4% paraformaldehyde and with 2% of milk powder to block nonspecific binding of the primary antibody. The antibody anti-vimentin (Mouse

Monoclonal anti-mouse) (Sigma-Aldrich Cat.No. V5255) and anti ICAM-1 (rabbit polyclonal anti-mouse) (Santa Cruz, BioTechnology, INC. USA Cat.No. sc-1511) were used as primary antibodies in a 1:200 and 1:1000 dilutions in 2% powder milk in PBS, respectively. Subsequently, incubations for 1 h at room temperature with each antibody were made. Alexa 488 anti-rabbit (Molecular Probes Cat.No. A11008) 1:400 dilution in 2% powder milk in PBS, was used as secondary antibody for ICAM-1 detection (green fluorescence) and Alexa 568 anti-mouse (Molecular Probes Cat.No. A11004) 1:400 dilution was used as secondary antibody in a solution of 2% powder milk in PBS for vimentin detection (red fluorescence). Both were visualized by confocal microscopy (LEICA SP2 Confocal Scanning Microscopy). Cell nuclei were also stained with Hoechst 33258 (Sigma-Aldrich) reagent by incubation for 10 min at room temperature with 1 ml of 2 µg/ml Hoechst 33258 (Doyle, et al., 1995) in PBS and cell fluorescence was visualized by confocal microscopy.

2.8 Proteomic *profile* analysis

A comparative assay of the proteomic profiles of osteoblasts (MC3T3-E1) and macrophages (J774) before and after exposition of cells to 1000 µg/ml of MgPa and MgPa-F was made.

Briefly 5×10^5 cells/well were cultured in 12 multi-well plate and grown at 37 °C in 5% CO₂ humid atmosphere, in CCM, for 24 h. Then, CCM was replaced by other with 1000 µg/ml of particles with (MgPa-F) and without (MgPa) KF treatment and maintained in culture for 24 h.

Osteoblasts and macrophages cell cultures were harvested by scraping to avoid the proteolysis of proteins by tripsin. Cell suspensions were lysed at 4°C by the addition of 1 x RIPA lysis buffer (Santa Cruz Biotechnology). After 1 h incubation at 4°C in the

darkness, the samples were centrifuged at 5000g for 20 min at 4°C and the supernatants were recovered and stored at -20°C until use. The protein concentrations of the samples were determined using the DC protein assay kit from Bio-Rad. 50 µg of total proteins were analyzed for each sample in duplicate by two dimensional (2D) electrophoresis. The samples were processed and proteins were identified as described by Lozano et al. (Lozano, et al., 2013).

3. RESULTS

3. 1 *Turbulence by H₂ bubbles and corrosion products formation on the MgPa surface*

MgPa are corroded in the presence of chloride-containing biological medium. The reported corrosion reaction steps include the increase of pH and the formation of hydrogen bubbles. (Mueller, et al., 2009) The corrosion process was followed at real time by MM. The generation and detachment of bubbles (Fig. 1, circles) that induced displacements of the surrounding particles (arrows) could be followed by MM. Consequently, there was turbulence in the vicinity that probably affects the surrounding cells.

In the sequence of Fig. 1S it can be noticed that a grey layer (probably Mg(OH)₂ (Mueller, et al., 2009)) progressively covered MgPa.

3. 2 *Interaction of macrophages with MgPa during the corrosion layer formation*

In the absence of MgPa particles, macrophages grow normally. It could be detected an increase in cell number in the 24 h assay, as can be observed in Figure 2S.

A different cellular behavior was noticed when MgPa were added to the cell culture (sequence of Fig. 2, supplementary information in Fig. 3S). Some macrophages closely interacted with the corrosion products, moved the position of the particles (cells contacted the particles and particles were slowly moved) and displayed cellular morphological changes. The initial condition is shown in Fig. 2a. Bright cells and particles without corrosion products can be seen. In Fig. 2.b one of the cells on the right died and the division process can be observed in the other cell. In addition, bright corrosion products can be detected on the borders of some of the MgPa. In the same Figure two cells can be clearly distinguished on the right and four in the centre due to the division process. These cells interact with the MgPa and with sub-micron particles of the surrounding area. In Fig. 2c (left side) morphological changes and cell damage can be noticed after cell/MgPa contact. Subsequently, all the cells on the right died (Fig. 2d). When Figs. 2a and 2d are compared it can be noticed that the position of some of MgPa changed after the interaction with cells (no bubble formation associated to the movement of the MgPa was detected in this frame). Figure 2d shows cells that seem to be alive but are smaller and shadowy than those of the initial $t=0$ time (Fig. 2a). Some cells that were not very close to the particles also died a few minutes after their duplication.

3. 3. *Interaction of macrophages with submicron particles*

The interaction of macrophages with MgPa and MgPa-F was also observed in the assays. Successive stretching and retracting processes of pseudopodia to engulf sub-micron particles could be followed by the time-lapse sequence in both cases, MgPa and MgPa-F (Fig. 3).

3.4 Alterations of cell cycles and viability of J774 and MC3T3-E1

To complement microscopic observations cell cycles corresponding to J774 and MC3T3-E1 cell lines were studied by flow cytometry and a qualitative comparison was reported here (Table 2). We wanted to know if different cell lines were similarly affected by the presence of the MgPa and MgPa-F. Our results showed considerable differences for both cell lines cycles when results with and without particles were compared. However, they were more meaningful in the case of macrophages. Thus, when MgPa were present, the SubG₁ phase increase (with respect to the control) was higher for J774 than for MC3T3-E1. Conversely, when MgPa were present, S phase markedly decreased in case of J774 while for MC3T3-E1 remains close to the control value. Moreover, relative viability values after 24 h exposure to MgPa revealed reductions for both cell lines.

Notable differences in cell cycle phases after exposure to MgPa-F were also observed. Comparing results with those obtained for MgPa and the respective control, (Table 2) in the case of macrophages the exposition to the MgPa-F particles produce an important increase in the percentage of cells in SubG₁ phase and a substantial decrease in S phase. No meaningful changes were observed in the case of osteoblasts.

Relative viability of both cell lines decreased in presence of particles being macrophages more sensitive than osteoblasts, showing the lowest viability value in the presence of MgPa-F. Consequently, the response of these two cell lines was different when they were exposed to the particles.

In agreement, the analysis of the microphotographs obtained by MM at real time revealed a high decrease in the number of cells during exposure to MgPa or MgPa-F (Table 2, Fig 2 and 4). Interestingly, it can be noticed that those cells that were close

to corroding particles (squares) were seriously damaged while those that were far from the corroding area were not significantly altered (circles in Fig. 4a and b). The slowly movement of particles by the cells could also be observed (Fig.4, rectangles recorded at the initial and final positions after several minutes of movement).

3.5 Alterations of MC3T3-E1 adhesion process by MgPa. Confocal immunofluorescence microscopy

Adhesion process is frequently analyzed when the interaction of cells with biomaterials is studied (Zachman, et al., 2013). During the harvesting step it was noticed that removal of MC3T3-E1 cells using trypsin to obtain cell suspensions was more difficult in case of cells growing with MgPa. Under this condition these cells showed stronger resistance to be removed than those without particles that could be detached easily. Consequently these changes may suggest alterations in the adhesion process in the presence of MgPa.

CIM was used to make a qualitative investigation about the influence of the presence of MgPa on cell adhesion to the substrate. For this assay, osteoblasts (MC3T3-E1) were firstly incubated with the primary antibodies (anti-ICAM-1 and anti-vimentin) and subsequently with the fluorescent secondary antibodies.

Micrographs obtained in the absence (left) and in the presence (right) of 1000 µg/ml MgPa are shown in Fig. 5. The cells were incubated with the primary antibodies to the adhesion protein ICAM-1 and the intermediate filament protein vimentin and the specific immune interaction was detected by the respective secondary antibodies, (green for ICAM-1 and red for Vimentin), as described above. The results obtained for the dual detection of anti ICAM-1 and anti vimentin are shown in Fig. 5 (see Fig. 4S for individual detection of ICAM-1 and Vimentin). It can be noticed that those cells

exposed to MgPa showed differences in the expression of the ICAM-1 and vimentin proteins involved in the mechanism of cell adhesion. Particularly, the expression of ICAM-1 (green) when cells were in contact with MgPa was particularly increased with respect to the control without particles.

3.6 Proteomic analysis *profile* before and after the exposure to MgPa

Proteomic *profile* analysis of cells under study was used to investigate the effect of MgPa on protein expression (Alcaide, et al., 2012; Vandekerckhove & Weber, 1978). The analysis of J774 macrophage lysates in the absence and presence of MgPa (1000µg / ml) showed several differences between identified proteins from their peptide fingerprinting. After the exposure to MgPa, results showed differences in the expression of proteins involved in cytoskeleton such as tubulin, gamma actin and elongation factor G3PDH (Table 1S). The exposure MgPa produced a decreased in Tubulin expression and in heat shock proteins, HSP90 and HSP8. On the other hand, gamma actin expression together with Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and actin regulatory protein (isoform 2) increased after exposures to MgPa. Besides, shift in the acidic direction of the isoelectric point of gamma actin was also observed.

Proteomic *profile* analysis of MC3T3-E1 lysates also showed changes in cytoskeletal proteins. In the presence of MgPa the expression of tubulin and peptide elongation factor 1 α 1 decreased, while expression of γ -actin increased and shift in the acidic direction (Table 1S).

3.7 Comparison of LDH and WST-1 assays for J774 and MC3T3-E1 with MgPa and MgPa-F

Fig. 6 shows a marked difference in the levels of LDH for J774 and MC3T3-E1 cell lines in response to MgPa presence (24 h exposure). A concentration dependent impact with high deleterious effect for J774 in contact with MgPa for concentrations ≥ 1000 $\mu\text{g/ml}$ was found while MC3T3-E1 cells were not significantly affected (Fig. 6a).

Alternatively, WST-1 assay showed concentration dependent adverse effects for both J774 and MC3T3-E1 cell lines (Fig. 6b). Significant decrease of mitochondrial activity was found for concentrations $\geq 500 \mu\text{g/ml}$, more detrimental in case of J774 (≤ 25 % for ≥ 1500 $\mu\text{g/ml}$).

Damage of cell membranes was dependent on the concentration of particles when certain threshold values were exceeded, both, in the presence and absence of F-coating on the particles. Thus, in case of J774 after exposures to MgPa-F significant damage was found for concentrations ≥ 1000 $\mu\text{g/ml}$ and it was less deleterious than for exposures to MgPa (Fig. 6a). MC3T3-E1 cells were almost not affected, except for 2000 $\mu\text{g/ml}$ MgPa-F.

WST-1 assay showed concentration dependent adverse effects for both J774 and MC3T3-E1 (Fig. 6b). A more important decrease in mitochondrial activity was found for concentrations ≥ 1000 $\mu\text{g/ml}$ in case of J774 in contact with MgPa-F than after exposures to MgPa.

4. DISCUSSION

Biocompatibility of Mg-based materials is frequently analyzed employing extracts obtained from Mg degradation (Mao, et al., 2013; Scheideler, et al., 2013; Yang, et al., 2013). However, this type of assay does not provide information about the

interaction of cells with particles and corrosion products that are gradually formed *in situ* (Di Virgilio, et al., 2011; Grillo, et al., 2011). Degradation of Mg and interaction with cells have been previously analyzed *ex situ* (Zhang, et al., 2009). Formation of $\text{Mg}(\text{OH})_2$ and hydroxiapatite on Mg and Mg-based alloys was reported. In a previous work we have shown that MgPa degraded and affect the growth of osteoblastic cells in culture. (Di Virgilio, et al., 2011). To achieve a better understanding of the processes occurring at the particle/cell interface *in situ* a real time evaluation was made by MM to assess macrophages response after exposure to particles, allowing the simultaneous evaluation of corrosion processes in particles and the concomitant cellular response. These observations were complemented by other biological assays including CIM to test effects on cells of the Mg particles and the corrosion products derived from them.

The gradual corrosion of MgPa that were progressively covered by a grey layer that may be probably $\text{Mg}(\text{OH})_2$ (Mueller, et al., 2009) could be followed by MM. Additionally, formation of hydrogen bubbles, derived from Mg degradation (Kirkland, 2012) that generated turbulence and moved the position of particles, could also be detected. Marked differences between the J774 control cells and those exposed to MgPa were noticed. A decrease in the cell number and an important number of dead cells were observed when the particles were present. Several macrophages closely interacted with MgPa and MgPa-F displayed changes in size morphology and brightness. Interactions of cells with larger MgPa resulted in cell damage and eventually lead to their death, particularly in areas where MgPa were corroding. Repeated stretching and retracting processes of pseudopodia to engulf sub-micron particles could also be followed by the time-lapse sequence of MM in both cases, MgPa and MgPa-F. Cells do not seem to be affected after these processes.

Meanwhile, cells of the control assay, without particles, divided and remained bright and alive during the whole observation time.

The results of cell cycle and viability during the exposure to the particles were provided to complement MM information and to identify biological alterations. Important changes were observed after exposures to 1000 µg/ml MgPa: macrophage viability decreased greatly, SubG1 increased while S phase decreased. As it was described previously (Luo, et al., 2014) subG1 population is indicative of apoptotic cells. Exposition of macrophages to MgPa during 24h induced an apoptotic response that finally led to cell death, probably due to changes induced by the corrosion process: pH increase, release of corrosion products and bubbles formation with concomitant turbulence.

Proteomic [profile](#) analysis revealed a significant change in the gamma actin expression pattern in presence of MgPa in osteoblasts and macrophages cell lysates. In both cell cultures, [an increase in the expression of](#) a form of this protein with a lower Ip was detected. Gamma actin acidification has been related to alterations in the cell cytoskeleton as well as changes in cell morphology (Lozano, et al., 2013). Actin is a monomeric subunit of cytoskeleton microfilaments, and its polymerization provides structural support and regulates contractility, cytokinesis, phagocytosis, adhesion and cell morphology (Vandekerckhove & Weber, 1978). It was found their polymerization is dependent on the presence of Ca and Mg cations. (Bergeron, et al., 2010; Vandekerckhove & Weber, 1978) . These proteins are also very important in the mobility of migratory cells, which modify the actin filaments forming lamellipodia with free extensions called filopodia. Lamellipodia are frequently developed by osteoblastic cells growing on rough surfaces (Sawase & Watanabe, 2015). Consequently, the alterations in the adhesion process may be related to

change in gamma actin [expression](#) by the presence of MgPa. These results were complemented by those obtained with CIM, whereby [differences](#) were visualized in [the expression of proteins](#) related to adhesion [processes such as](#) ICAM1 and vimentin.

It is known that ICAM-1 is an intercellular adhesion protein, associated with the cytoskeleton, which is involved in intracellular signaling activation. Vimentin is a protein of the intermediate filaments that forms the cell cytoskeleton and contributes to support cell membranes. Osteoblasts adhere to opposing cells through these particular adhesion molecules (vimentin and ICAM1) on their surface and these adhesion molecules not only function as glue with opposing partners but transduce activation signals that facilitate the production of bone-resorbing cytokines (Tanaka, et al., 1995). Thus, alterations in vimentin and ICAM1 expressions by the presence of MgPa detected by CIM are probably related to changes in the adhesion processes. They may be promoted by Mg ions that [possibly](#) mediate the adhesion of cells to the substrate, probably by decreasing the negative charge of the surface, hence reducing the surface repulsive electrostatic forces (Kleine, et al., 2003; Takeichi & Okada, 1972).

To test the influence of other ions on the biological response of cells, experiments with MgPa treated with KF were made. A weak protection of the F-containing layer was confirmed by MM. Corrosion process was noticed in several particles that were progressively covered by corrosion products, similarly to those observed [in case of](#) MgPa. In addition, higher reduction in the viability of cells than that observed in the control cells or in those cells exposed to MgPa was detected during [cells/MgPa-F](#) MM observations. This deleterious effect was followed by [other techniques to complement MM information. Results showed changes](#) in all the phases of cell cycle

as well as in viability in case of macrophages after exposures to 1000 $\mu\text{g/ml}$ MgPa-F. SubG1 phase increased and S phase and viability decreased. Accordingly, it was observed that cells that were close to MgPa-F were strongly affected and alterations frequently led to the reduction of cell number with respect to the control and to cell death. However the effect of MgPa-F on macrophages was more significant than that on MC3T3-E1, since viability of J774 was lower and cell cycle was more affected. Variations were more important than those observed after exposures to MgPa without treatment and, consequently, F-dependent effects may be related with them. In this sense, F⁻ ions do not affect the lysosomal activity when added up $1 \times 10^{-3} \text{M}$ to the cell culture as KF (Grillo, et al., 2011). However, present results seem to indicate that in the case of MgPa-F either F⁻ local concentration (close to the particles) may be high ($\geq 1 \times 10^{-3} \text{M}$) at certain sites or the products of their interaction with Mg affect mitochondrial activity and, ultimately, cell proliferation. It is known that the F⁻ may induce oxidative stress and damage at DNA level (Zhang, et al., 2008). Free radicals can enter the cell nucleus and cause changes in DNA, such as chains breaks. Furthermore, F⁻ can form covalent bonds with mammalian DNA and cause damage at the cellular level (Guo, et al., 2003; Gutowska, et al., 2010). It was reported that incubation of THP-1 macrophages in F⁻-containing solutions caused a decrease in ATP synthesized by the cells and an increase in the formation of reactive oxygen species (ROS). Besides, an increase in cell death after the increase of F⁻ concentration in the medium was noticed. It was suggested that F⁻ could destabilize the normal functioning of the cell respiratory chain, which induce lipid peroxidation and apoptosis of cells (Hirano & Suzuki, 1996; Wang, et al., 2004). In addition, cytotoxic effect of F⁻ in rat alveolar macrophages was also reported while Anuradha *et al* (Anuradha, et al., 2000) argue that F⁻ causes the activation of kinases that

induce apoptosis. Gong et al (Gong, et al., 2014) studied the effect of F^- on the viability of MC3T3-E1 cells and reported that the NaF reduced cell viability in a concentration-dependent manner and promotes apoptosis. They suggest that this is due to the direct action of F^- on the expression of the proteins of the bcl-2 family.

It is known that treatments with KF are weakly protective unlike other treatments with HF (Wolters, et al., 2015). It was reported (Pereda, et al., 2011) that when KF treated MgPa are immersed in chloride-containing media, chloride ions induce the dissolution of F-containing layer, and subsequently the F content of the layer decreases. Thus, the layer becomes more labile to the corrosion attack, corrosion products are formed and fluoride-containing ions are released together with Mg ions. Thus, changes in the biological activity of cells after exposures to MgPa-F in relation to exposures to MgPa without treatment could be related to the release of F-containing degradation products.

CONCLUSIONS

To achieve a better understanding of the processes occurring at the biodegradable metallic debris/cell interface *in vivo*, microscopic evaluation of corrosion processes in Mg-based particles and alterations of the surrounding cells was made by multidimensional microscopy (MM). Development of hydrogen bubbles with concurrent turbulence and formation of Mg-based corrosion products covering the particles were detected. These effects, probably together with pH changes, Mg ions and F^- release (in case of MgPa-F) induced changes in the surrounding cells. MM evaluations made at real time, *in situ* provided important information about the biodegradation of biomaterials that could not be obtained using extracts. This MM analysis was complemented by other biological assays to test cell response.

The behaviors of macrophages and osteoblasts were compared. Complementary measurements showed alterations in cell cycle, and changes in proteomic profile, in proteins implicated in adhesion processes, and in cell number and viability. These changes were sometimes more significant in case of MgPa-F covered by a poorly protective fluoride layer. This more deleterious effect was probably due to the F⁻ containing species released during the corrosion process. Interaction of macrophages with the particles was more deleterious than in case of osteoblasts. These results were in agreement with the MM information, demonstrating that this technique is a helpful tool to monitor the changes of biodegradable materials and the surrounding cells during the degradation process at real time and *in situ* and to achieve a better simulation of the interaction of debris with cells *in vivo*.

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Legends for the figures

Figure 1. Time lapse microphotographs showing the degradation process of MgPa (1000 μ g/ml) with bubble formation (see circles, bubble is absent in Fig. 1a and present in Fig. 1b) and detachment (see rectangles, bubble is present in Fig. 1a and absent in Fig. 1b) as well as displacement of some MgPa after the bubble detachment (arrows).

Figure 2. Time lapse microphotographs of J774 macrophages in contact with 1500 μ g/ml MgPa during a 24h assay: Interaction of macrophages with MgPa, [duplication of cells](#), displacement of particles by the cells, formation of corrosion products on the surface and cell death can be detected within the sequence a-d (see also Fig. 3S).

Figure 3. Time lapse microphotographs showing the interaction of J774 macrophages with sub-micron MgPa during 1500 μ g/ml MgPa-F assay. The stretching and retracting of macrophage pseudopodia to engulf microparticles can be seen, the process is repeated over the exposure period.

Figure 4. Time lapse microphotographs of J774 (a) at the beginning (0h) and (b) 24 h after the exposure to 1000 $\mu\text{g/ml}$ MgPa-F. Cell death can be detected in (b) (ellipses). Magnification (400X). (c-f) time lapse microphotographs showing a detailed sequence for the region within the yellow ellipse. The gradual formation of corrosion products on MgPa-F surface (white arrows in the squares) and the cells progressively damaged in the surroundings (black arrows) can be noticed. Those cells that are far from the corroded particles are unaltered (circles) and interact with the submicron particles of the surrounding area. Macrophages are able to change the position of the particles (rectangles). Magnification (1000X).

Figure 5. MC3T3-E1 immunofluorescence studies. Dual staining and detection by confocal microscopy of ICAM-1 (green) and Vimentin (red) using the specific primary and secondary antibodies described in materials and methods: a) MC3T3-E1 cultures in the absence of MgPa (named control) and b) MC3T3-E1 cultures in the presence of 1000 $\mu\text{g/ml}$ MgPa (see also Fig. 5S).

Figure 6. Effects after 24 h exposure to different concentrations of MgPa (black) and MgPa-F (grey) on plasma membrane integrity evaluated by LDH activity, (panel a), and cell proliferation measured by mitochondrial respiratory activity, evaluated by WST-1, (panel b).

Significant difference at *** $p < 0.001$ without particles vs with MgPa; ___ $p < 0.001$; __ $p < 0.01$; _ $p < 0.05$ MgPa vs MgPa-F.

SUPPLEMENTARY INFORMATION

Figure 1S. Time lapse microphotographs of a MgPa degradation during 24h period. The formation of corrosion products (probably $\text{Mg}(\text{OH})_2$) layer (light grey) on the surface can be seen.

Figure 2S. Control cells growing without MgPa at the beginning of the assay (left) and 24h after (right). Magnification (400X).

Figure 3S. Time lapse microphotographs of J774 macrophages in contact with 1500 $\mu\text{g/ml}$ MgPa during a 24h assay: a-f Interaction of macrophages with MgPa, duplication of cells, displacement of particles by the cells and formation of corrosion products on the surface and dead cells can be observed.

Figure 4S. Confocal microscopy and immunofluorescence studies with primary and secondary antibodies with MC3T3-E1 cells in the absence and in the presence of MgPa: a and b controls without antibodies; c, e, g controls without particles; d, f, h with 1000 $\mu\text{g/ml}$ MgPa. c and d anti-ICAM1 (green); e and f Anti-vimentin (red); g and h anti-ICAM1 and Anti-vimentin.

Figure 1. Time lapse microphotographs showing the degradation process of MgPa (1000 $\mu\text{g/ml}$) with bubble formation (see circles, bubble is absent in a) and present in b) and detachment (see rectangles, bubble is present in a) and absent in b) as well as displacement of some MgPa after the bubble detachment (arrows). Magnification (400X).

Figure 2. Time lapse microphotographs of J774 macrophages in contact with 1500 $\mu\text{g/ml}$ MgPa during a 24h assay: Interaction of macrophages with MgPa, duplication of cells, displacement of particles by the cells, formation of corrosion products on the surface and cell death can be detected within the sequence a-d (supplementary information can be seen in Fig. 3S).

Figure 3. Time lapse microphotographs showing the interaction of J774 macrophages with sub-micron MgPa during 1500 µg/ml MgPa-F assay. The stretching and retracting of macrophage pseudopodia to engulf microparticles can be seen, the process is repeated over the exposure period. Magnification (1000X).

Figure 4. Time lapse microphotographs of J774 (a) at the beginning (0h) and (b) 24 h after the exposure to 1000 $\mu\text{g/ml}$ MgPa-F. Cell death can be detected in (b) (ellipses). Magnification (400X). (c-f) time lapse microphotographs showing a detailed sequence for the region within the yellow ellipse. The gradual formation of corrosion products on MgPa-F surface (white arrows in the squares) and the cells progressively damaged in the surroundings (black arrows) can be noticed. Those cells that are far from the corroded particles are unaltered (circles) and interact with the submicron particles of the surrounding area. Macrophages are able to change the position of the particles (rectangles). Magnification (1000X).

Figure 5. MC3T3-E1 immunofluorescence studies. Dual staining and detection by confocal microscopy of ICAM-1 (green) and Vimentin (red) using the specific primary and secondary antibodies described in materials and methods: a) MC3T3-E1 cultures in the absence of MgPa (named control) and b) MC3T3-E1 cultures in the presence of 1000 µg/ml MgPa (see also Fig. 5S).

Figure 6. Effects after 24 h exposure to different concentrations of MgPa (black) and MgPa-F (grey) on plasma membrane integrity evaluated by LDH activity, (panel a), and cell proliferation measured by mitochondrial respiratory activity, evaluated by WST-1, (panel b).

Significant difference at *** $p < 0.001$ without particles vs with MgPa; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ MgPa vs MgPa-F.

Table 1. Composition of the Mg particles.

Al	Si	Mn	Fe	Ca	Mg
0.030	0.031	0.019	0.015	0.110	Balance

Table 2. Cellular effects of MgPa and MgPa-F (1000 µg/ml) in the different cell cycle phases by flow cytometry.

Cell line	MgPa Surface	SubG ₁ (%)	G ₁ (%)	S (%)	G ₂ /M (%)	Relative Viability (%)
MC3T3-E1	---	3.5	76.6	13.9	6.0	100
MC3T3-E1	MgPa	4.4	72.6	13.2	9.8	78.5
MC3T3-E1	MgPa-F	4.2	75.2	12.2	8.4	65.7
J774	---	3.2	64.6	20.0	12.2	100
J774	MgPa	8.3	84.2	1.7	5.8	65.0
J774	MgPa-F	14.8	71.2	7.6	6.4	35.1

Figure 1S. Time lapse microphotographs of a MgPa degradation during 24h period. The formation of corrosion products (probably $\text{Mg}(\text{OH})_2$) layer (light grey) on the surface can be seen.

Figure 2S. Control cells growing without MgPa at the beginning of the assay (left) and 24h after (right). Magnification (400X)

Figure 3S. Time lapse microphotographs of J774 macrophages in contact with 1500 µg/ml MgPa during a 24h assay: a-f Interaction of macrophages with MgPa, duplication of cells, displacement of particles by the cells and formation of corrosion products on the surface and dead cells can be observed.

Figure 4S. Confocal microscopy and immunofluorescence studies with primary and secondary antibodies with MC3T3-E1 cells in the absence and in the presence of MgPa: a and b controls without antibodies; c, e, g controls without particles; d, f, h with 1000µg/ml MgPa. c and d anti-ICAM1 (green); e and f Anti-vimentin (red); g and h anti-ICAM1 and Anti-vimentin.

Tabla 1S. Differential expression level of proteins identified by 2-DE and MS/MS in J774 and MC3T3-E1 cell cultures in the absence and presence of MgPa

Protein	Expression in presence of MgPa compared to the control without MgPa
J774 cell line	
Heat Shock Protein 90 (HSP 90)	Decreased
Heat Shock Protein 8 (HSP 8)	Decreased
Tubulin $\alpha 1\beta$ chain	Decreased
Peptide elongation factor 1 α 1	Decreased
γ -actin	Increased (IP shift in the acid direction)
protein isoform 2	Increased
<u>Glyceraldehyde 3-phosphate dehydrogenase</u> (G3PDH)	Increased
MC3T3-E1 cell line	
Tubulin β -5	Decreased
Peptide elongation factor 1 α 1	Decreased
γ -actin	Increased (IP shift in the acid direction)
Tubulin β -5	Decreased