UHMWPE oxidation increases matrix metalloproteinase-2 (MMP-2) release in human fibroblast

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MRC-5 human fibroblast were grown polystyrene plates, UHMWPE slices and UHMWPE slices oxidized in air in oven at 120 °C for 80 h. Cell proliferation and cytotoxicity were assessed after 24, 48, and 72 h. Control cells grown on polystyrene doubled their number after 72 h while both cell population grown on UHMWPE and oxidized UHMWPE showed a reduced proliferation rate. Both UHMWPE types seem to induce a cytostatic rather than a cytotoxic effect, in fact, no difference in the LDH release were observed between the three cell populations at 24, 48, and 72 h. Gelatinase activity was assessed in cell medium at every time point using gelatin zymography. Only gelatinase B (MMP-2) activity was present in MRC-5 conditioned medium. Oxidized UHMWPE induced a strong release of MMP-2 after 72 h compared to control and UHMWPE samples. The augmented MMP-2 release in the absence of a cytotoxic phenomenon suggests a possible role for UHMWPE oxidation in the MMPs stimulation observed in the periprosthetic tissues.
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1. Introduction

Wear debris of ultra high molecular weight polyethylene (UHMWPE) prosthetic components is known to induce a host granulomatous reaction which recruits macrophages and multinucleated cells that can start an inflammation process leading to osteolysis and aseptic loosening of the prosthesis. It has been pointed out that the sterilization process using γ-ray in air induces a strong oxidation of UHMWPE, which can enhance the *in vivo* mechano-degradation [1]. Amount and dimension of wear debris seem to result from alterations in the UHMWPE chemical and physical properties induced by the oxidative processes [2].

Nevertheless there are no reports about the direct effects of UHMWPE oxidized surface on the behavior of the cells present in the periprosthetic tissues in the absence of debris. Matrix metalloproteinases (MMPs), a family of proteolytic enzymes capable of remodeling the extracellular matrix (ECM) have been implicated in destructive process observed in loose total hip replacements [3]. In particular, the MMP-2 (gelatinase A) was strongly expressed around joint implants during loosening. The aim of our study was to observe if the interaction with a biocompatible biomaterial such as UHMWPE used in orthopedic implantology was able to induce the production of MMPs in human fibroblast *in vitro* and if oxidative alterations of the biomaterial surface could alter directly the tissutal response to the implant.

2. Materials and methods

A compression molded plate of prosthetic ultra high molecular weight polyethylene (UHMWPE) (Gur 1020, Poly Hi Solidur, Germany) was used. The material, in compliance with ASTM F 648-98 was without additives. The plate was initially sawn to a size suitable for microtomy ($5 \times 3 \times 7\,cm^3$). A PolyCuts Microtome (Reichert-Jung) was used. Microtomy was performed at $20\,mms^{-1}$ in air at room temperature. Slices of about 200 μm were recovered. Some of the slices were oxidized in air in oven at 120 °C for 80 h. The original and oxidized samples were analyzed with infrared spectroscopy in attenuate total reflection (ATR). An FTIR microscope (Perkin Elmer System 2000, Autoimage) equipped with an ATR objective (Germanium, incidence angle of the IR beam 45°, $100 \times 100\,\mu m^2$ nominal surface area) was used. ATR spectra were collected using 64

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scans. The ATR spectra were corrected for wavelength dependence of the beam penetration by a computer program (Atrcorr program, Grams 32, Galactic) assuming 1.5 to be the refractive index of UHMWPE. The absorbance of oxidation products on surface ($1718\,cm^{-1}$), determinate by Microscopy FTIR in attenuated total reflection (ATR) was 0.25.

2.1. Cell culture

Human lung fibroblasts MRC-5 were grown in Dulbecco's Modified EagleMedium (DMEM) supplemented with glutamine, antibiotics and 10% fetal bovine serum (FBS). Cells grown at confluence were trypsinizated and washed with DMEM with 10% FBS. Pelleted cells were resuspended and their viability was assessed using the trypan dye esclusion test. Fibroblasts were seeded on polystyrene plates (control), slices of UHMWPE (PE) and oxidized UHMWPE (PEOX) at a concentration of 5×10^4cells/cm^{-2} and were incubated at 37 °C with CO_2 5% for 24, 48, and 72 h in serum-free DMEM.

2.2. Gelatinase activity

Gelatinase A (MMP-2) and B (MMP-9) activity were determined by gelatin zymography in medium obtained from control cells seeded on polystyrene dishes and from cells seeded on UHMWPE and UHMWPE oxidized after 24, 48, and 72 h. Latent and active gelatinases were detected by zymogram analysis using SDS–polyacrylamide gels copolymerized with 0.2% gelatin [4]. These enzymes become dissociated from tissue inhibitor metalloproteinases (TIMPs) by the presence of SDS during electrophoresis. Removal of SDS following electrophoresis allows the proenzymes to renature in an active or partially active conformation. This permits their detection and the detection of lower molecular weight activated forms. In brief, conditioned medium were mixed with sample buffer and electrophoresed directly without boiling or reduction. Following electrophoresis, SDS was extracted from the polyacrylamide gel with Triton X-100, and the gel was incubated in 0.05 M Tris, pH 7.5, containing 5 mM $CaCl_2$, and 5 mM $ZnCl_2$ at 37 °C overnight. Gels were stained with Coomassie blue and destained. Both proenzyme and active gelatinase were detected as clear bands against the blue background of the stained gelatin. A densitometric analysis of the bands seen on gels was performed using the NIH Image 1.62 program. The statistical analysis of data was performed using the SPSS software for Windows. The Student's *t*-test used was for data coupled with a significance of $p < 0.05$.

2.3. Proliferation test

After 24, 48, and 72 h samples mediums were collected and cells were washed three times with Phosphate Buffered Saline (PBS) and were fixed with a solution of 3.7% formaline and 3% saccarose in PBS. Afterwards, cells were stained for 5 min with acridine orange solution (0.025% w/v) in PBS and washed three times with PBS. Ten fields for each sample were counted under a fluorescence microscope at a magnification of 400 × . Results were expressed as number of cells cm^{-2}.

2.4. Cytotoxicity test

Medium samples were immediately analyzed to check the cell viability analyzed using a commercial kit to evaluate the amount of lactate dehydrogenate with a sensitivity of 3–5 U/L (LDH-optimized lactate dehydrogenase 1.1.1.27 UV Method, Sigma-Aldrich, Milan, Italy). The readings were taken at 340 nm using a spectrophotometer Beckman DU-68.

3. Results

3.1. Surface characterization

The ATR spectrum of PEOx (Fig. 1) showed the presence of OH groups ($3450–3350\,cm^{-1}$) due to alchohol and acid compounds, ester groups ($1740\,cm^{-1}$), ketones ($1718\,cm^{-1}$) and acid ($1710\,cm^{-1}$) [5]. The esters groups ($1740\,cm^{-1}$) were also present on the surface of the PE samples (data not shown). This small level of oxidation depends on the microtoming process [6].

3.2. MMP-2 production and release

Only MMP-2 (gelatinase A) was present in MRC-5 medium in both activate (64 kDa) and inactivate (72 kDa) form. When cells were seeded on PE slices after 24 h it has been observed an statistically significant in MMP-2 release compared to the control samples ($p < 0.05$) (Fig. 2). After 48 and 72 h of incubation basal MMP-2 levels (control samples) were slightly higher than 24 h ones and there was no difference between gelatinase A levels in control and in UHMWPE samples. A similar trend was observed also for PEOX samples with the exception of the 72 h MMP-2 concentration value that was higher than values observed in control and PE samples ($p < 0.001$) (Fig. 2). As shown in Fig. 3, the MMP-2 concentration in PEOX medium after 72 h was almost twice compared to the concentration measured in PE samples at the same time point. Nevertheless the percentage of activation (% active form/active + inactive form) was similar for both samples (about 50% activation).

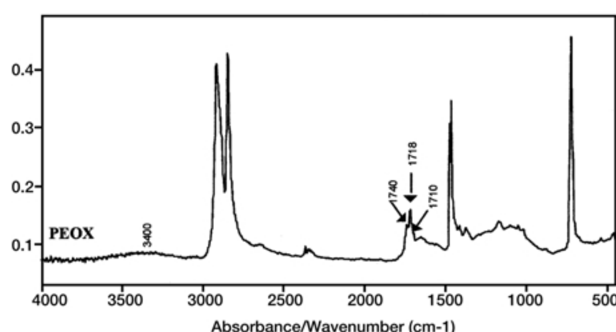

Figure 1 ATR spectrum of UHMWPE thermally oxidized (PEOx).

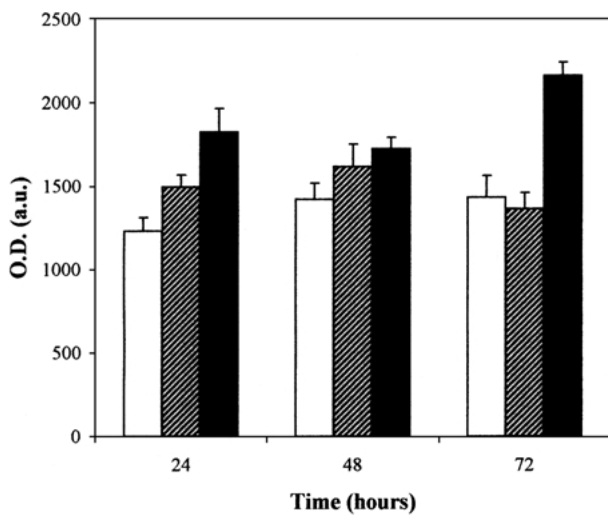

Figure 2 Densitometric analysis of gelatin zymography. MMP-2 expression. Enzyme activity was measured in serum free medium obtained from MRC-5 cells seeded onto polystyrene plates (□), PE (▨) and PEOX (■) slices at 24, 48, and 72 h. The optical density (OD) was expressed as arbitrary units (a.u.) ± S.E: and data were obtained from three separate experiments.

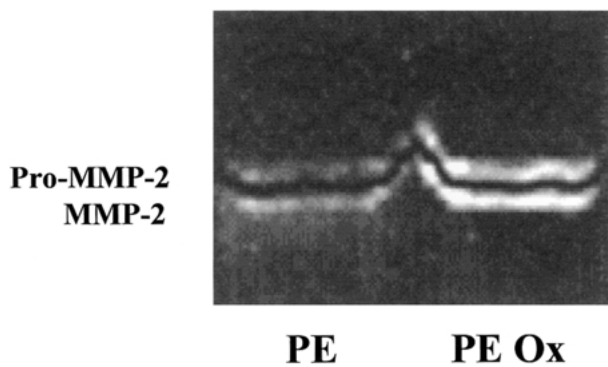

Figure 3 Representative gelatin zymography obtained from 72 h medium conditioned by PE and PEOX treated MRC-5 cells. Both inactive (pro-MMP-2, 72 kDa) and active form (MMP-2, 64 kDa) enzyme form were detected.

3.3. Cells proliferation

The human fibroblasts MRC-5 demonstrated a different proliferation capacity when seeded onto plastic (polystyrene plates) or PE and PEOX. In particular, control samples were able to double their number in 72 h while both cells seeded onto PE and PEOX proliferate slowly and after 72 h did not doubled their number (Fig. 4). No significative differences have been observed between PE and PEOX proliferation rate at any time point.

3.4. LDH release

In order to understand if the cell number reduction observed at 48 and 72 h in PE and PEOX samples was due to a cytotoxic rather than a cytostatic effect LDH release was measured at 24, 48, and 72 h in control, PE and PEOX samples. As shown in Fig. 5, an increase in LDH level released in growth medium occurred at 48 and 72 h compared to 24 h, but no statistically significant differences were observed between the values obtained from Ct, PE and PEOX samples for the same time point.

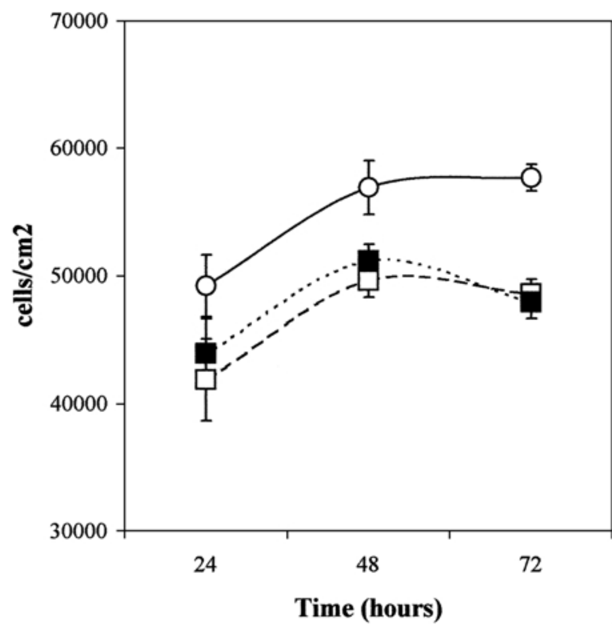

Figure 4 MRC-5 proliferation curve. Data are expressed as number of cells cm^2 ± S.E. ($n = 3$). (○) Control, (□) PE and (■) PEOX samples.

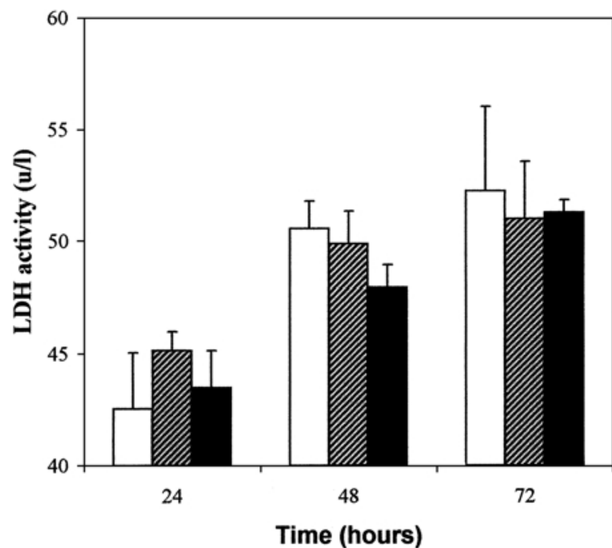

Figure 5 MRC-5 LDH release. LDH activity measured in the cell media was expressed as u/l ± S.E. ($n = 3$), obtained from control (□), PE (▨) and PEOX (■) samples.

4. Discussion

The UHMWPE is a biocompatible polymeric material which has been used for more than 30 years in prosthetic implants especially in the acetabulum and tibial plateau components. In recent years, UHMWPE, despite its chemical inertia and excellent mechanical properties, has posed many clinical problems attributable to a rapid deterioration of the material, seemingly due to its oxidation by processes of sterilization with γ rays in air.

In our experimental model, the surface chemical–physical modifications induced by the oxidation of PE were able to stimulate the human fibroblasts to produce MMP-2. Non-tumor cells such as fibroblasts in normal conditions do not produce MMPs and their interaction with the substrate is mediated principally by a family of transmembrane proteins, the integrins which control the

growth and cellular differentiation [7]. It has been suggested by many studies that MMPs play an important role the variety of tissue destruction, that is metastatic tumor, rheumatoid arthritis, and cartilage breakdown [8]. In addition, it was proved that the macrophages, endothelial cells, and fibroblast usually present in the interface tissue around prosthesis are able to produce MMPs and their tissue inhibitors (TIMPs) [3]. In our experimental model, the oxidation of the PE surface induced MMP-2 release in human fibroblast in the absence of a cytotoxic effect, as suggested by the LDH activity values measured in cell medium. At the same time UHMWPE itself seems to act as a cytostatic agent reducing cell proliferation. This augmented MMP-2 activity is noteworthy because of the ability of this gelatinase, together with MMP-9 and MMP-1 to modulate bone resorption and tissue granulation and formation [9]. Moreover, MMP-1, -2, -9, and -3 mRNA have been detected in interface tissue around implants in loosening total hip arthroplasty [3]. It is possible that the direct interaction between cells and oxidation products on the polymer surface is not the only responsible for MMP-2 augmented production. Even if in our experimental model cells were grown in the absence of serum, a small amount of proteins is produced directly by fibroblast (e.g. fibronectin) and, in our opinion, it is possible that PE oxidized surface binds proteins with a different affinity or a different pool of proteins compared to the not oxidized one. A ''modified'' proteic layer could account for the MMP-2 production modulation [7]. Further analyzes are necessary to understand the biochemical pathway leading to the increased MMP-2 release. This scenario suggests caution in the use of PE oxidized products because of its ability to induce MMP-2 activity in fibroblast that could, alone or together with other proteolytic enzyme, alter the homeostasis of the tissues surrounding the prosthesis and favor the failure of the implant.

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