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The induction of MMP-9 release from granulocytes by Vitamin E in UHMWPE

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Abstract

Ultra-high molecular weight polyethylene (UHMWPE) is a biopolymer widely used in orthopaedic implants and its oxidation is considered as major responsible for inflammation and the prosthesis failure. We have studied the effect on the activation of resting human granulocytes of the addition of Vitamin E (Vit.E, α-tocopherol), a natural biological antioxidant and antinflammatory agent, to UHMWPE. We have measured changes in granulocytes morphology and respiratory burst by flow cytometry using Dihydrorhodamine 123 and matrix metalloproteinase 9 (MMP-9, gelatinase B) release and activity in the growth medium using substrate zymography following contact (60 min at 37°C) with cell grade polystyrene (PS), normal UHMWPE (PE) and Vit.E added UHMWPE (PE Vit.E).

FTIR analyses showed that the surfaces of PE and PE-Vit.E were not significantly different. PS, PE and PE Vit.E did not alter granulocytes morphology and respiratory burst as showed by the mean fluorescence emitted (PS = 12.0 ± 0.1, PE = 13.0 ± 0.4, PE Vit.E = 14.5 ± 0.1). PE Vit.E was able to increase MMP-9 release compared to PS and normal PE (215 ± 16% of the control, $p < 0.001$). The PE Vit.E-induced MMP-9 release was abolished by okadaic acid (0.5 nM), suggesting a direct role of Vit.E in the phenomenon.

Keywords: UHMWPE; Vitamin E; Biocompatibility; Granulocytes activation; MMP-9; dihydrorhodamine 123

1. Introduction

Ultra-high molecular weight polyethylene (UHMWPE) is widely used for manufacturing components of hip, shoulder and knee prostheses as more than half-million of prosthetic operations are carried out in the USA every year and more than one million worldwide [1]. According to ASTM F648-98 standard, any stabilizer (i.e. also antioxidants) must be avoided while manufacturing medical grade UHMWPE [2].[1] Use of UHMWPE for orthopaedic applications shows two major drawbacks: (a) abrasion on the surface of the polymeric components in contact with metal/ceramic components followed by the debris formation which is thought to be at the origin of formation of the loosening membrane and the following substitution of prosthesis; (b) breaking of the hip component and the tibial plateaux, which is possibly related to the sterilization process performed in air with high dose of gamma rays [3].

Sterilization in inert environment has recently come to the solution of the last problem. However, dissolved oxygen is still able to undergo oxidation process and even ethylene-oxide sterilized prosthesis, shelf-ged, or retrieved can be oxidized in some cases. This oxidation is originated during the complex UHMWPE processing to prepare components [4].

It appears clear that in order to extend the useful life of UHMWPE prosthetic component they must be stabilized. This is also the only way to make implant with UMWPE components in young patients possible.

However, changing of the ASTM 648, to make possible the addition of antioxidant requires that FDA

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[1] No stabilisers or processing aids are to be added to the virgin polymer powder during manufacture of a fabricated form.

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or other international organizations approve suitable products. Vitamin E (Vit.E, α-tocopherol) is one of the likely candidates [5] because it is a very efficient stabilizer against oxidation of polyethylene [6], a natural product whose decomposition products have already been tested for biocompatibility with positive results. Thus the FDA classifies Vit.E as generally recognized as safe and it is worldwide approved for use in food contact applications.

One advantage of the use of Vit.E is its low diffusion coefficient, which makes diffusion from the prosthesis to the body extremely long. Moreover, the addiction of Vit.E (0.1 and 0.3%) to UHMWPE grains reduces fatigue cracks [7]. Vit.E is a natural biological antioxidant, which prevents peroxides from accumulating and protects cells from damaging effects of free radicals [8]. Vit.E has also been described as an antinflammatory agent, inhibiting many key events in inflammation such as IL-1β release from activated monocytes [9], monocyte adhesion to endothelial cells [10] and respiratory burst [11]. Moreover, among the non-antioxidant molecular function of Vit.E, its ability has been reported to activate the protein phosphatase 2A (PP2A) [12], an enzyme that modulates protein-kinase C activity.

The Vit.E antioxidative and anti-inflammatory properties have been extensively used to improve the biostability and the biocompatibility of different biomaterials. In fact, Vit.E has been used to produce amphiphilic monomers and polymers [13] and it has been added to poly(etherurethane urea) elastomers [14], acrylic bone cements [15] and cellulose membranes for haemodialysis [16] reducing the neutrophil activation induced by cellulose contact [17–19].

Although the wide range of Vit.E effects are known, few studies concerning the biocompatibility of Vit.E modified materials have been performed and no report is are available on the effects of Vit.E added UHMWPE (PE Vit.E) on activation of resting granulocytes that represent first line of defense of organism.

The stabilization effect of Vit.E against the oxidation process is already evident at 500 ppm [6] and in this study we use UHMWPE with 5000 ppm to stress the activity of Vit.E. Granulocytes activation has been studied after contact with PE Vit.E using flow cytometry for cell morphological modification and respiratory burst and substrate zymography for matrix metalloproteinase 9 (MMP-9) release and activation.

2. Materials and methods

2.1. Preparation of the UHMWPE samples

Two compression-moulded plates of prosthetic UHMWPE were used. One was the original UHMWPE (Gur 1020 Poly Hi Solidur, Germany) (PE). The second plate was prepared by prosthetic UHMWPE with 5000 ppm of Vit.E (PE Vit.E) according to the following process. Vit.E (5 g) (Ronotec 2001, Roche) was dissolved in 1000 ml of cyclohexane at room temperature. Then, 1000 g of UHMWPE powder was mixed with Vit.E solution. The mixture was shaken for 1 day at room temperature. The cyclohexane was removed under vacuum and the PE-Vit.E was dried under vacuum. With this mixture a plate has been prepared using the same condition applied for the original UHMWPE.

The plates were 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 a cutting speed of 20 mm/s in air at room temperature. Slices of about 3 mm height was recovered. The materials were characterized by differential scanning calorimetry (DSC-Perkin Elmer 6) in inert atmosphere at the heating rate of 10°C/min.

2.2. UHMWPE surface analysis

The surfaces of PE and PE-Vit.E samples were analysed with infrared spectroscopy in the attenuated total reflection (ATR) mode. An FTIR microscope (Perkin-Elmer Autoimage) equipped with an ATR objective (Germanium, angle of incidence of the IR beam 45°, $100 \times 100\,\mu m^2$ nominal surface area) was used. ATR spectra were collected using 64 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.

2.3. Specimen collection

Granulocytes were obtained from peripheral blood essentially by a modification of the method of Boyum [20]. EDTA collected human peripheral venous blood (10 ml) from six healthy donors (three men and three women, average age $\pm$SE = 28 $\pm$ 6) was diluted 1:1 with phosphate buffer solution (PBS, pH 7.4). Diluted blood was layered onto a Ficoll–Hypaque density gradient and centrifuged for 20 min at 2000 rpm to separate mononuclear cells from erythrocytes and granulocytes. The mononuclear fraction was discharged and erythrocytes were then lysed using an ammonium chloride lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4) for 20 min at 4°C. Pellet was centrifuged twice in sterile PBS, cells were counted in optical microscope using trypan blue exclusion test (viability >98%) and suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco) containing penicillin (100 u/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (Sigma) in polypropylene tubes.

2.4. Cell treatment

Granulocytes were seeded onto polystyrene (PS) plates (negative control), UHMWPE (PE) and UHMWPE with 5000 ppm Vit.E (PE Vit.E) after cell loading with dihydrorhodamine 123 (DRH) used for detecting granulocytes respiratory burst [21].

DRH was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 50 mM and stored at −20°C. A fresh working solution (50 mM in serum-free RPMI 1640) has been prepared and immediately used for every experiment. DRH has been added to cell suspension at a final concentration of 50 μM and cells have been incubated for 60 min at 37°C under gentle shaking. At the end of DRH, incubation cells were seeded at a concentration of 1×10^5 cell/cm^2 (0.5 ml cell suspension) for 1 h at 37°C.

Phorbol myristic acid (PMA) treated cells were used as positive control. PMA was dissolved in DMSO at a concentration of 50 μM and added at a final concentration of 50 nM to cells seeded onto PS for 60 min. At the end of incubation cells were detached for cytometric analysis. Serum-free RPMI 1640 containing 10 mM EDTA (1 ml) was added for 5 min to the samples and cells were washed from the samples surface and collected in polypropylene tubes. Few cells were observed on the samples surface after washing (5–10 cells per field when samples were observed at 16× magnification in optical microscopy).

Okadaic acid (Calbiochem-Novabiochem Corporation, San Diego, CA, USA) has been used in some experiments in order to test the involvement of PP2A [22] in the Vit.E observed effects. Briefly, cells were seeded onto various surfaces diluted in RPMI 1640 medium added with Okadaic acid 0.5 nM (final concentration, IC_{50} for PP2A = 0.1 nM) and treated as previously described.

2.5. Flow cytometry analysis

Granulocytes not adherent and detached from plates were collected as previously described and their morphology and activation status were analysed using flow cytometry. All experiments were performed using a FACScan cytometer (Becton-Dickinson) equipped with a 15-mW air-cooled argon ion laser operating at 488 nm. The cytometer was calibrated daily according to the manufacturer's instructions. A minimum of 10,000 events were collected per sample at a low sample flow rate setting (12 μl/min). The forward and side light scatter profiles were adjusted to ensure that granulocytes population was clearly displayed and changes in the light scatter pattern as a result of activation (notably after PMA stimulation). Rhodamine (RD) green fluorescence was measured on the FL1 green channel after a 530 nm band pass filter and displayed on a four-decade log scale. Data were collected using the CellQuest software and their analysis was performed using WinMDI software version 2.8.

2.6. Gelatin zymography

Medium samples were collected after incubation (1 h at 37°C), centrifuged for 10 min at 3000 rpm to eliminate cells and debris and used for measurements of MMP-9 activity by zymography [23]. Latent and active gelatinase B (MMP-9) activity was detected by zimogram analysis using SDS-polyacrylamide gels copolymerized with 0.2% gelatine. In brief, conditioned medium was 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 Zn Cl_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. Positive control for gelatinase A and B (Chemicon International) was used to identify the two enzymes and their activated forms. A densitometric analysis of the bands seen on gels was performed using the NIH Image 1.62 software and results were expressed as arbitrary units of optical density.

2.7. Statistical analysis

Means of different parameters investigated were calculated from single sample data, and group means ±SEM, were then obtained from single samples values. Statistical analysis of variancewas used; the significance of differences between means was assessed by Newman–Keuls multiple range test, taking $p < 0.05$ as the minimum level of significance.

3. Results

3.1. Surface characterisation

The DSC of the two materials shows that the crystallinitiy of PE-Vit.E is slightly lower (48%) compared to the original UHMWPE (53%) (data not shown).

As reported in Fig. 1, the ATR spectra of PE and of PE Vit.E are very similar, both show the presence of ester groups (1740 cm^{-1}) on the surface due to microtoming process. These data indicate that it is evident that the presence of Vit.E cannot inhibit the formation of ester groups [24].

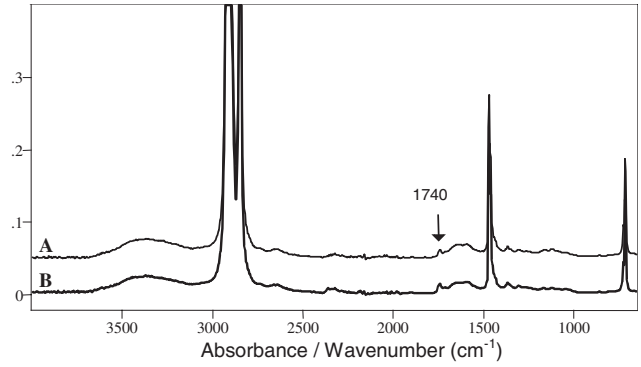

Fig. 1. ATR-FTIR spectra of PE (a) and PEVit.E (b).

3.2. Flow cytometry analysis of granulocytes morphology and respiratory burst

The granulocytes population (88–93% of total events in control samples) was readily identified on the dot plot of forward scatter vs. side scatter (Fig. 2) and fluorescing events in the granulocytes gate (R1) were accumulated and shown as histogram showing the green fluorescence emitted by RD (Fig. 3). In all the experiments performed ($n = 6$), a small population of lymphocytes was observed (2–4% total events in control samples).

The dot plots of forward scatter vs. side scatter showed that PMA dramatically modified the morphology of granulocytes that increased their forward scatter values suggesting an aggregation of neutrophils due to cellular activation as already described [25]. On the contrary, no alteration of cells shape was observed in both PE and PE Vit.E samples.

The percentage ($\pm$SE) of granulocytes present in the gate R1 for cells seeded onto PS was $78.2 \pm 0.9\%$ while, in the PMA samples, the percentage of cells counted in the gate was $58.6 \pm 0.7\%$ ($p < 0.001$) suggesting that activated granulocytes readily become apoptotic.

In fact, a higher presence of cellular debris (events with very low FSC and SSC values) was observed in the scattergrams obtained from those samples compared to PS samples. A similar but less extended phenomenon was observed for PE and PE Vit.E samples where percentage of cells present in the gate R1 was $72.8 \pm 1.1\%$ for PE ($p < 0.01$) and $71.5 \pm 0.2\%$ for PE Vit.E ($p < 0.001$).

The analysis of cell-associated RD fluorescence was performed on the population gated in R1 and assuming that cell culture grade PS was able to induce the lowest granulocytes activation after a 60 min contact at 37°.

As shown in Fig. 3, the mean RD fluorescence ($\pm$SE) in control samples (12.0 ± 0.1) was strongly augmented in the PMA-stimulated samples (208.4 ± 17.4, $p < 0.001$ compared to PS samples), while it was not altered in both PE (13.0 ± 0.4) and PE Vit.E (14.5 ± 0.1).

3.3. MMP-9 release and activity

As shown in Fig. 4A in the conditioned medium obtained by granulocytes seeded onto PS plates, only MMP-9 (gelatinase B) activity was present. MMP-9 was observed both in inactive (pro-MMP-9) and active (MMP-9) forms and in the typical MMP-9 dimers secreted by granulocytes. PMA (50 nM) strongly increased the release of all the three forms of MMP-9 ($205 \pm 47\%$ of the control, $p < 0.001$), while the PE samples showed a MMP-9 release ($116 \pm 19\%$) similar to the PS samples ($100 \pm 5\%$). Surprisingly in the PE Vit.E samples, the MMP-9 release was significantly increased ($215 \pm 16\%$, $p < 0.01$) (Fig. 4A).

In order to explain the mechanism of Vit.E-induced MMP-9 release from granulocytes in the absence of respiratory burst or evident morphological modifications, we added to the cell medium okadaic acid (0.5 nM), a potent PP2A inhibitor. As shown in Fig. 4B, the okadaic acid induced a general reduction of MMP-9 released in all samples even in the PMA stimulated cells ($137 \pm 8\%$ of the control, $p < 0.01$), where a more relevant increase of the gelatinase B release was expected. The MMP-9 release induced by PE samples did not differ from PS samples ($99 \pm 4\%$) while the PE Vit.E samples showed a significant reduction ($88 \pm 4\%$, $p < 0.01$) suggesting a role for PP2A in the Vit.E-induced MMP-9.

4. Discussion

In this paper, we tested the ability of UHMWPE added with Vit.E (5000 ppm) to activate the resting granulocytes from peripheral blood.

After the prosthesis implantation, neutrophil granulocytes adhere along with other leukocytes to polymeric artificial surfaces through the adsorbed plasma protein layer [26,27].

Following adhesion to biomaterial surfaces, granulocytic phagocytosis, respiratory burst, and protease (Gelatinase B or MMP-9 and elastase) release may occur, resulting in injury to surrounding tissue. As granulocytes activation is an early event in the inflammation process, it has been taken as an indicator for risk assessment of biopolymer-mediated inflammation [28].

Neutrophils activation is not an all or nothing phenomenon and each function (calcium influx, F-actin assembly, degranulation, oxygen radical production) has its own threshold for a response [29] in order to protect the host from inappropriate neutrophil-mediated tissue damage.

The neutrophil granulocytes may become activated either directly, through some adhesion receptors, or via platelet-derived mediators [30,31]. In our experimental

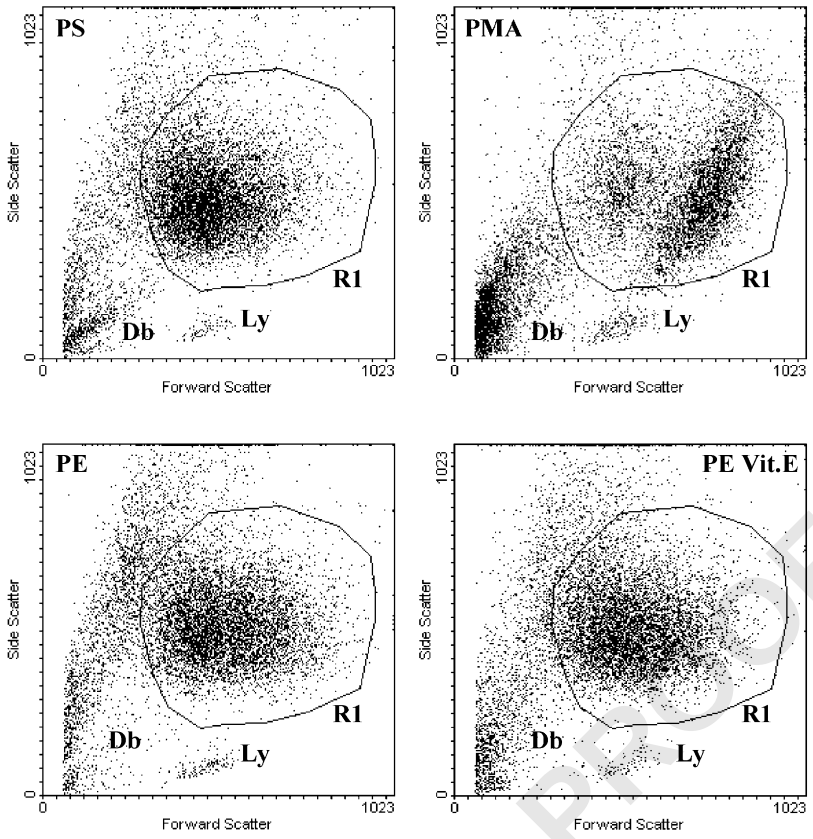

Fig. 2. Flow cytometry analysis of a representative granulocytes population loaded with DHR and seeded onto polystyrene plates without (PS) and in the presence of 50 nM PMA, or onto PE and PE Vit.E disks. In the dot plot of forward scatter vs. side scatter population, white cells are differentiated by their characteristic size (forward scatter) and granularity (side scatter) and granulocytic population (G, mainly neutrophiles) can be easily identified and it has been gated (R1). Ly = lymphocytes, Db = debries.

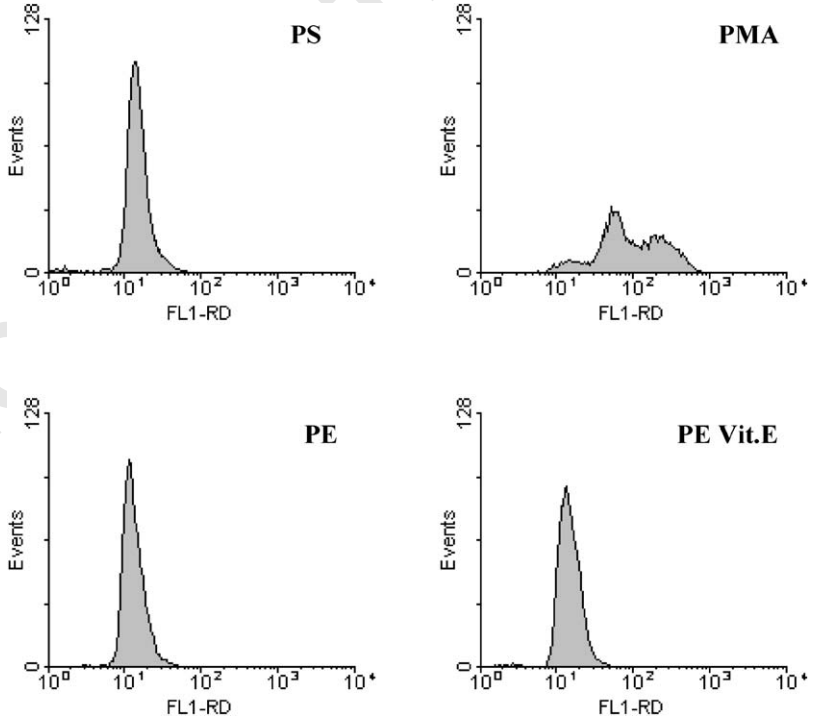

Fig. 3. Representative RD fluorescence (FL1, green fluorescence) emitted by granulocytes present in the gate R1 of Fig. 2 after contact (60 min at 37°C in 5% CO_2) with culture grade polystyrene without (PS) and in the presence of 50 nM PMA, or onto PE and PE Vit.E disks.

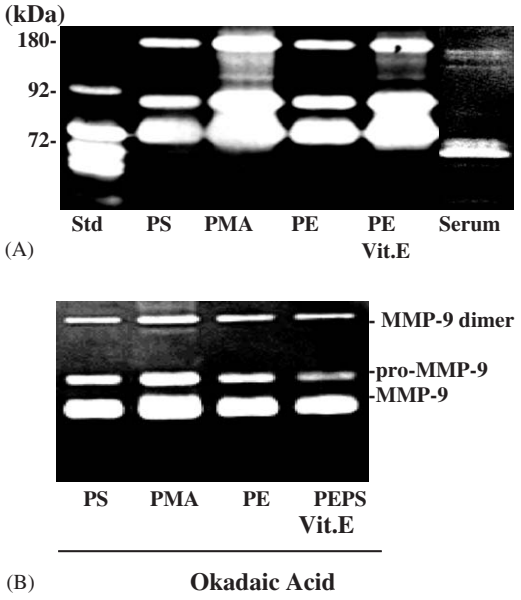

Fig. 4. Representative gelatin zymography for MMP-9 total activity present in conditioned medium obtained from granulocytes seeded onto polystyrene plates without (PS) and in the presence of 50 nM PMA, or onto PE and PE Vit.E disks in the absence (A) and in the presence of okadaic acid (0.5 nM) (B).

model an almost pure granulocytes population, without platelets presence and with a small percentage of lymphocytes, has been used to test the granulocytes (70–80% neutrophils) activation after contact with normal UHMWPE (PE) and PE Vit.E in the presence of 10% FCS. We observed that adhesion onto PE Vit.E increased the granulocytes MMP-9 release and in the absence of an oxidative stress as measured by DRH assay in flow cytometry. The increased MMP-9 release observed could not be due to a change in the surface chemistry as shown in Fig. 1, but to the presence of Vit.E that can alter the protein adsorption. In fact, protein adsorption occurs before cellular adhesion at an implanted surface, and the nature and composition of the first adsorbed protein layer is of major importance for the subsequent granulocytes behaviour via receptor activation [32]. The normal granulocytes adhesion induces MMP-9 release from tertiary granules [33] even if MMP-9 can be released also in response to endotoxin and proinflammatory mediators [34] and by ligation of integrin CD11b/CD18 (macrophage antigen complex; Mac-1) [34].

The interest about granulocyte MMP-9 release arises from the ability of MMP-9 to degrade the major component of endothelial basement membranes and extracellular matrix [36], even if a growing number of studies indicate that MMP-9 acts also as an important tuner and amplifier of immune functions [37–39]. Thus its release and activation could be a sufficient trigger for inflammation.

Initially, we hypothesize that alteration induced by Vit.E in the surface chemistry and the consequent possible different adsorption of proteins from serum could be mainly responsible for MMP-9 release also in the absence of oxidative stress, being the stimulus from the modified surface insufficient to trigger also this part of granulocyte activation or maybe simply thanks to the Vit.E antioxidative activity. However, we observed that okadaic acid, an inhibitor of PP2A, an enzyme directly activated by Vit.E [12], was able to block the MMP-9 secretion from PE Vit.E samples. This phenomenon was not completely surprising due to the fact that PP2A is involved in the granules secretion from mast cells [40] and a similar mechanism could take place also in neutrophil granulocytes. However, it is more plausibly a combined effect between differential protein adsorption and PP2A activation induced by Vit.E.

5. Conclusions

Our data indicate that Vit.E added to UHMWPE was able to induce a PP2A-dependent increase in the MMP-9 release from granulocytes in the absence of the typical oxidative stress that is considered a hallmark of granulocytic activation. These data emphasize the possibility that the use of PE Vit.E could modulate the in situ tissue remodeling and immunitary response through MMP-9 release in absence of other possible pro-inflammatory factors such as superoxide anion radical production.

6. Uncited reference

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