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Polymorphonuclear neutrophil response to hydroxyapatite particles, implication in acute inflammatory reaction

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Abstract

Hydroxyapatite (HA) is widely used as a bone substitute or coating biomaterial in bone diseases or prosthesis metal parts. The release of HA particles induces an inflammatory response and, if uncontrolled, could result in implant loss. Among the hallmarks of such inflammatory response is early recruitment of the polymorphonuclear cells (PMNs). The purpose of this work is to investigate the response of PMNs following exposure to HA in terms of secreted mediators. Our study shows that HA particles increase the release of pro-inflammatory mediators such as interleukin-1α, as well as chemotactic factors such as interleukin-8, macrophage inflammatory protein-1α and macrophage inflammatory protein-1β. HA also induces an increase in matrix metalloproteinase 9 expression. Taken together, our data demonstrate for the first time that HA is capable of activating PMNs, a phenomenon that could potentially contribute to the onset of implant-associated inflammation.
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1. Introduction

Hydroxyapatite (HA) is widely used in orthopaedic surgery because of its biocompatibility and bioactivity. It is used to coat the metal parts of prostheses in order to improve their biocompatibility and as a bone substitute in bone diseases. HA increases the integration of the implant; however, one disadvantage of HA coatings is their tendency to fragment and generate HA particles wear debris [1], which activate leukocytes [2,3].

Study of the tissue/biomaterial interface has shown that there is a recruitment of inflammatory cells, including polymorphonuclear neutrophils (PMNs), in the early stage followed by monocytic cells [4]. It has been demonstrated that, during the chronic stage of inflammation, HA particles can elicit the production and release of inflammatory mediators by monocytes such as proteases and their inhibitors [5], chemokines and cytokines [6–10]. However, little is known about the acute inflammatory stage and the effect of HA debris on PMNs and their response *in vitro* and/or *in vivo*.

A number of studies have reported that osteoblastic cells can release chemotactic factors, such as monocyte chemoattractant protein (MCP-1) and interleukin-8 (IL-8) *in vitro* [11] or *in vivo* during inflammatory diseases [12,13] when in contact with particles [14]. Such chemotactic factors could recruit inflammatory cells to the implant site. The interaction

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of PMNs with microbial or material particles results in cell activation. PMNs are immune cells whose primary function is to protect the host against invading pathogens and foreign materials. However, when uncontrolled, this physiological response can become pathogenic, leading to tissue injury [15]. Activation of PMNs by biomaterial particles has the potential to "prime" the effect of subsequent activation [16] and to increase inflammation. Regulation of the inflammatory mediators can modulate the immune response and increase the lifespan of the prosthesis and its compatibility with the host tissue. The purpose of this study is to investigate the role of hydroxyapatite particles in PMN recruitment and activation.

2. Materials and methods

2.1. Media and reagents

Polymorphprep, used for PMN separation, was from Axis-shield (Oslo, Norway). Dulbecco's phosphate-buffered saline (DPBS) 1× and RPMI 1640 + L-glutamine were from Gibco. Penicillin, streptomycin, RPMI 1640 Glutamax and foetal calf serum (FCS) were purchased from Invitrogen (Cergy Pontoise, France). Phorbol-12-myristate-13-acetate (PMA), gelatin, sodium dodecyl sulfate (SDS), Triton X-100, NaCl and $CaCl_2$ were from Sigma–Aldrich (Saint-Quentin Fallavier, France). Glutaraldehyde and hexamethyldisilazane (HMDS) were from VWR (Fontenay sous Bois, France) and Agar Scientific (Stansted, UK) respectively. Quick May–Grünwald–Giemsa staining was realized with RAL 555 kit from Reactifs RAL (Martignac, France). $Ca(NO_3)_2{\cdot}4H_2O$ and P_2O_5 for the HA powder were from Aldrich, USA, and Avocado Research Chemicals (Heysham, Lancashire, UK).

Cytokines antibody arrays AAH-INF-III were from Raybiotech (Norcross, USA). IL-8 duo-set and recombinant human chemokines IL-8 and RANTES were from R&D systems (Lille, France).

2.2. HA powder

A 2 g quantity of pure sol–gel-derived HA powder was obtained with 4.7 g of $Ca(NO_3)_2{\cdot}4H_2O$ and 0.84 g of P_2O_5 dissolved in ethanol under stirring and under reflux at 85 °C for 24 h. This solution was then cooled and maintained at 55 °C for 24 h to obtain a consistent white gel, which was further heated at 80 °C for 10 h to obtain a white powder. Finally, the powder was heated at 1100 °C for 15 h. Use of such a high temperature allows the synthesis of pure and well-crystallized HA. The powder was characterized as described previously [17]. HA powder was used with a surface area ratio (specific surface of material/specific surface of cell) of 1 as previously described [18]. The particles ranged from 100 nm to 10 μm as judged by scanning electron microscopy (SEM). Importantly, HA was tested and found to be endotoxin free using the E-toxate kit from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

2.3. Collection of blood samples, cell isolation and culture

Venous blood was collected on EDTA (BD Vacutainer® K2E, Franklin Lakes, USA) from healthy donors in accordance with the "Etablissement Français du Sang Nord de France" (March 2007) and with the written informed consent of the donors. Neutrophils were purified from whole human blood using the Polymorphprep™ protocol. Residual erythrocytes were removed by a hypotonic shock. Resulting neutrophils were resuspended in DPBS 1× and represented greater than 97% of the cells.

The PMNs were at least 95% viable. They were cultured in RPMI 1640 + L-glutamine with antibiotics (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) and 2.5% heat-inactivated autologous human serum.

Five million cells in 1 ml were seeded in 24-well FalconTM plates (Becton Dickinson, Le Pont-De-Claix, France) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were cultured for 4 h without stimulation or exposed to hydroxyapatite particles (mixed with culture media before seeding of PMNs) or 1 μM PMA (used as a positive control). Finally, cells were centrifuged at 400*g* for 10 min, supernatants were collected and frozen at −20 °C and cells were frozen at −80 °C. Cytospin samples were prepared with a cell suspension of 10^5 cells in 100 μl of DPBS 1×. Centrifugation was performed in a Shandon cytospin 4 (Thermo Scientific, Cergy Pontoise, France) for 10 min at 500*g*. Then preparations were stained with RAL 555 kit. Differential count was performed based on morphological criteria using a light microscope (Zeiss, Axiovert 200M, Germany).

Monocytes were collected from total blood of healthy volunteers by counter flow elutriation. They were at least 95% viable. Cells were cultured in RPMI 1640 Glutamax with antibiotics (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) and 2.5% heat-inactivated FCS.

2.4. Scanning electron microscopy

Scanning electron microscopy was performed to examine the morphology of PMNs. Cells were fixed with 2.5% glutaraldehyde in DPBS 1× for 1 h at room temperature. After ethanol dehydration, samples were immersed in HMDS for 10 min, air-dried at room temperature and sputtered with thin gold–palladium film under a Jeol ion sputter JFC 1100. Cells were viewed using a LaB6 electron microscope (JEOL JSM-5400 LV, France).

2.5. Antibody cytokine arrays

Cytokines antibody arrays ("Human Inflammation III", Raybiotech) were performed according to the manufacturer's instructions. Briefly, 10-fold diluted culture supernatants or medium alone were incubated on antibody-coated membranes before detection with a streptavidin–horseradish peroxidase biotinylated-antibody complex and chemiluminescence detection on X-ray film for 30 s.

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