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Edouard Jallot, P. Laquerriere, A. Grandjean-Laquerriere, L. Kilian, D. Laurent-Maquin. Characterization of intra-cellular ionic concentrations of monocytes in contact with bioactive glasses and hydroxyapatite particles. A TEM cryo-Xray analysis of diffusible ions. International Symposium on Trace Elements in Human : New Perspectives 4, Oct 2003, Athenes, Greece. pp.1303-1311. in2p3-00014096

HAL Id: in2p3-00014096
https://hal.in2p3.fr/in2p3-00014096

Submitted on 3 Nov 2003

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Characterization of intra-cellular ionic concentrations of monocytes in contact with bioactive glasses and hydroxyapatite particles. A TEM cryo-Xray analysis of diffusible ions.

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Abstract

Cells viability can be altered by the presence of bioactive materials particles and their features. This paper compares the influence of hydroxyapatite particles and bioactive glass particles on intracellular ionic concentrations of the monocytes exposed to them. However, microanalysis of intracellular ions like sodium, potassium, chlorine is a major problem. In order to limit diffusion phenomenon and to preserve the chemical identity of specimens, we used cryomethods (cryo-preparation, cryo-Xray microanalysis) to study cells in contact with bioactive materials particles. We measured intracellular ionic concentrations by scanning transmission electron microscopy (STEM) associated to energy dispersive X-ray spectroscopy (EDXS). Monocytes were exposed to bioactive glass particles and to hydroxyapatite particles during two days. The K/Na ratio was used as a sensitive criteria of viability of cells. Bioactive glass particles and in a less manner hydroxyapatite particles have an effect on intracellular ionic concentrations. These two types of bioactive particles alter cells. However, bioactive glass particles appear to alter cells more than hydroxyapatite particles.

Keywords : bioactive glasses, hydroxyapatite, cytotoxicity, cryo-X-ray microanalysis, diffusible ions, [K]/[Na]

Introduction

Calcium phosphate ceramics (hydroxyapatite) and bioactive glasses are widely used as bone substitutes or to coat metal implants in order to improve their biocompatibility[1,2]. These materials are bioactive materials. They permit an intimate chemical bond with bone tissues through a biologically active apatite layer. This apatite is formed during the material dissolution and through physico-chemical reactions at the particles periphery. However, the dissolution of these materials can lead to the release of particles into the surrounding bone tissues[3,4]. After implantation, the first cells to colonize the biomaterials surface and the released particles are the monocytes and giant cells. They play a major role in the host defensive mechanism by their phagocytic capability[5]. Different authors demonstrated that cells viability can be altered by the presence of particles[6,7]. Moreover, particles features (composition, size, surface area…) can influence cell activity[7,8,9].

The aim of this paper is to compare the influence of two bioactive materials (hydroxyapatite, bioactive glass) on the survival of the monocytes that phagocytose them. In our study, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) particles size is between 150-300 µm and is composed with crystals of the order of 350 nm. Bioactive glass is amorphous and is in the SiO_2-Na_2O-CaO-P_2O_5-K_2O-Al_2O_3-MgO system. Bioactive glass particles size is between 100-300 µm. In order to evaluate the influence of particles on intracellular ionic concentrations and their cytotoxicity on cells, we measure intracellular ionic concentrations by scanning transmission electron microscopy (STEM) associated to energy dispersive X-ray spectroscopy (EDXS).

But, microanalysis of diffusible ions like sodium, potassium, and chlorine is a major problem[10,11]. These elements are not very firmly bound and they can be solubilized during specimen preparation. Thin biological samples for transmission electron microscopy are usually prepared by a process involving fixation, postfixation, dehydration, embedding in

resin and sectioning. During this chemical fixation, most of the diffusible ions are rapidly lost (within minutes) from the samples[11,12]. In order to preserve the chemical identity of specimens we used cryomethods for the preparation of cells in contact with bioactive materials particles. These methods permit retention of all elements of interest at their *in vitro* location close to their native state and allow identification at the level of analytical resolution required. In this work, we paid particular attention to the potassium-sodium ratio which is one of the most sensitive criteria of viability[13,14]. This criteria has already been used to demonstrated cellular alterations induced by biomaterials such as zircon particles[8], hydroxyapatite particles[15] and bioactive glass particles[16].

Materials and Methods

Hydroxyapatite particles

Powder was made of pure crystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with a purity over 99%. The HA particles had indifferent shapes with a distribution size between 150 and 300 µm. They were sintered at 1180°C and their crystal size was around 350 nm.

Bioactive glass particles

The bioactive glass composition is 50% SiO_2, 20% Na_2O, 16% CaO, 6% P_2O_5, 5% K_2O, 2% Al_2O_3 and 1% MgO (% weight). The bioactive glass was obtained by melting the components in a platinum crucible at 1350°C. After the melting process, the glass was cast, crushed, and ground into a powder with grain sizes between 100-300 µm in an alumina ball mill.

Cell cultures

Elutriated monocytes obtained from two healthy volunteer donors were used. They were maintained in RPMI-1640 medium (Gibco, France) supplemented with 10% fetal calf serum (Gibco, France), 2mM glutamine, penicillin (5000 $U.ml^{-1}$) and streptomycin (25 $\mu g.ml^{-1}$)

(called standard medium) at 37°C in a saturated 5% CO_2 and 95% air atmosphere. The cell density was 2.10^5 cells.ml^{-1} in a 5-ml total volume.

It has been shown that there is an effect of surface area of polymethylmethacrylate particles on monocytes activity[17]. The calculation of the surface area ratio (SAR= surface area of cell/surface area of material) demonstrated that cells were sensitive to the surface of material presents in the cell culture. Cellular activity was measured through a range of SAR from 0.1 to 10. The activity increased from a minimum at an SAR of 10 and reached the maximum at an SAR of 1. Below 1, the cellular activity was not affected by the SAR[7]. From all these data, we have chosen a surface area ratio equal to 1.

In this work, three experiments were conducted : the first was a control cells, performed with cells alone in a standard medium. The second experiment consisted of cells exposed to bioactive glass powders with a SAR equal to one. The third experiment consisted of cells exposed to hydroxyapatite powders with a SAR equal to one.

Sample preparation for cryomicroanalysis

After 2 days of culture, the medium of each experiment was removed by centrifugation. Droplets of cells deposited on aluminium specimen holders (0.7 mm diameter made in our laboratory) were cryofixed by plunging into melting ethane cooled by liquid nitrogen (-196°C) with a velocity of 2 m.s^{-1} using a home made cryoplunging system. The frozen specimens were stored in liquid nitrogen before cryosectionning.

Cryosections were cut with a Leica Ultracut E ultramicrotome equipped with an FC-4 cryosectionning attachment (Leica) using a 45° dry glass knife. The specimen temperature was –140°C and knife temperature was –145°C. The cutting speed was 0.5 mm.s^{-1} and the microtome advance 70-90 nm per stoke, giving ultra-thin cryosections. Sections were

deposited onto carbon coated collodion on 200-mesh copper grid, pressed onto the grid and stored in liquid nitrogen.

Instrumentation and analytical conditions

The grids bearing the cryosections were placed into a pre-cooled GATAN cryo-specimen holder and transferred into the microscope chamber. Sections were freeze-dried by warming up them slowly (30 min) from –196°C to –70°C. They were cooled to –170°C for analysis. Analyses described in this paper were carried out using a Scanning Transmission Electron Microscope (STEM) (Philips CM 30). The vacuum in the column was maintained at $< 2.10^{-7}$ Torr. The microscope is fitted with an EDAX 30 mm^2 Si(Li) R-SUTW detector (Mn Kα resolution 140 eV, take-off angle 14.1°, 0.15 sr detection solid angle). Data was acquired with the specimen holder at a 30°-tilt and a microscope accelerating voltage of 100 kV. Beam current was constant at 0.07 nA. The elemental composition of dry specimens was determined by using the normalized Hall continuum method[18-19] and are expressed in $mmol.kg^{-1}$ dry weight. Mean values of elemental concentrations were obtained by scanning the electron probe over a 4 μm^2 sample area during 200 s (which correspond to 24 10^3 $e^-.nm^{-2}$).

In each experiment, ionic concentrations of at least 15 cells from each donor were evaluated. All elemental concentrations and potassium to sodium ratios shown are the means of all data determined in each cell. Statistical significance between results obtained on cells exposed to bioactive glass particles or to hydroxyapatite particles and the control cells was determined by using the Mann-Witney U-test (error probability p; * : $p<0.05$).

Results

Figures 1 represents the elemental concentrations in the cytoplasm of the control cells, of cells exposed to bioactive glass particles and cells exposed to hydroxyapatite particles.

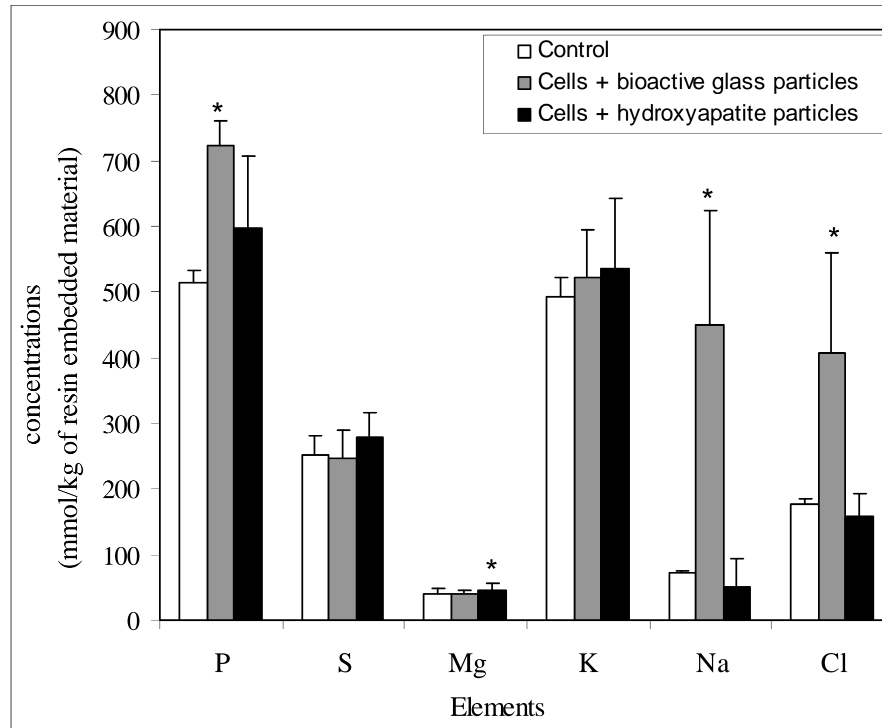

Figure 1 : Cytoplasmic P, S, Mg, K, Na, Cl concentrations in control cells, in cells exposed to bioactive glass particles and in cells exposed to hydroxyapatite particles.

The results show a high increase of phosphorus, sodium and chlorine concentrations when cells are cultured with bioactive glass particles (figure 1). On the other hand, sulphur, potassium and magnesium concentrations are quite the same in the control cells and in cells exposed to bioactive glass particles. Phosphorus, sulphur, potassium, sodium and chlorine stay constant when cells are exposed to hydroxyapatite particles. A slight increase in magnesium concentration is observed.

K/Na ratio

The higher K/Na ratio was measured in the control cells and the lower was obtained in cells exposed to bioactive glass particles (Table 1). The K/Na ratio in cells cultured with hydroxyapatite particles was intermediary.

	K/Na
Control	7.59 ± 0.31
Cells + bioactive glass particles	3.51 ± 3.19
Cells + hydroxyapatite particles	5.16 ± 0.90

Table 1 : Mean value of the potassium:sodium ratio in control cells, in cells exposed to bioactive glass particles and in cells exposed to hydroxyapatite particles.

Discussion

In this paper, we have studied intracellular concentrations of cells after exposure to bioactive glass particles or to hydroxyapatite particles. In order to avoid ions diffusions, conventional fixation method for TEM analysis are not the best choice. Study of intracellular concentrations requires cryotechniques for sample preparation and analysis. We were able to measure P, S, Mg, K, Na and Cl concentrations in cellular compartments. The high standard deviations obtained in the experiments represent inhomogeneities in the cellular populations.

When cells were exposed to bioactive glass, there was a significant increase in phosphorus. Phosphorus reflects the phosphorylation state. This increase of P concentration can signify a phosphorylation state higher than in control cells.

For diffusible ions, fluctuation of potassium, sodium and chlorine concentration might be a consequence of damage to cell membranes because according to the electrochemical gradient, Na and Cl might have been allowed to penetrate into the cell. It seemed to be the case with bioactive glass particles because an increase of Na and Cl is observed. Since generally membrane potential is given by intracellular and extracellular concentrations of Na, Cl and K, modifications in the concentrations of these elements inside the cell cytoplasm raise the possibility of changes in membrane potential. It has been suggested that depolarization could be the result of an increase in the permeability of the plasma membrane of epithelial cells to ions[20], and that fluctuation of membrane fluidity induces modification of membrane permeability to water, ions and solutes[21].

On the other hand, hydroxyapatite particles induced an increase in intracellular magnesium concentration that might be related to an increase in ATP concentration because Mg is known to be bond to ATP[22]. Moreover, magnesium is an important regulator of mitochondrial functions[23], where the ATP formation takes place.

The K/Na ratio permits the evaluation of the toxic effects of bioactive glass particles and hydroxyapatite particles. Our results demonstrated that the bioactive glass particles were more toxic for monocytes compared to hydroxyapatite particles.

Conclusion

In this paper, we study intracellular ionic concentrations in monocytes exposed to bioactive glass particles and to hydroxyapatite particles. Concentrations were determined by cryo-X-ray microanalysis associated to electron probe microscopy. These methods allow us to study diffusible ions and to obtain intracellular concentrations in cells close to the physiological state. Moreover, the K/Na ratio was used as a sensitive criteria of viability of cells.

Bioactive glass particles and in a less manner hydroxyapatite particles have an effect on intracellular ionic concentrations. These two types of bioactive particles alter cells. However, bioactive glass particles appear to alter cells more than hydroxyapatite particles.

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