Evaluation of the effect of three calcium phosphate powders on osteoblast cells

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The aim of the present study was to assess the effect of three calcium phosphate powders entering in the composition of bone substitute materials on osteoblast-cells activity. These powders were hydroxyapatite (HA) widely used as a biomaterial, nanocrystalline carbonate apatite (CA) very close to bone mineral crystals, and an experimental one: calcium phosphate cement-1 (CPC-1) composed of an amorphous Ca-P phase and brushite. The powders were physico-chemically characterized. The very reactive CPC-1 powder became transformed in cell culture medium: recrystallization of amorphous precursors and hydrolysis of brushite into poorly crystalline apatite occurred. Osteoblast-cells activity was evaluated: for low level of calcium phosphates ($>$ 100 μg/ml) CPC-1 enhanced proliferation and, to a lesser degree, differentiation on alkaline phosphatase activity. For 100 μg/ml of powders we observed a great alteration of biological activity of the osteoblasts: evaluation of proliferation indicated an inhibition for all samples, and a decrease of two differentiation markers: alkaline phosphatase activity and osteocalcin release were noticed, suggesting a down regulation due to the presence of large amount of mineral powder.

1. Introduction

Different types of calcium phosphate materials have been proposed as bone substitutes during the last decade. In recent years in particular, several formulae of self-setting bone cements based on Ca-P compounds have been tested. An experimental calcium phosphate cement-1 (CPC-1) composed of an amorphous Ca-P phase (ACP) and dicalcium phosphate dihydrate (DCPD) is proposed for this study in order to check its bone substitute potentiality. It is a self-setting cement leading to interdigitated crystals of carbonate apatite (CA) very analogous to bone crystals. Its physical properties allow its use as an injectable material for hard tissue reconstruction, replacement. In addition, this material could serve for the reinforcenment of mechanically compromised osteoporotic bone. However, complete studies on the biological and physico-chemical properties of this product have not yet been done.

One of the problems related to the use of mineral-containing biomaterials is the release of crystals or agglomerate that can impair cell activity and hinder bone reconstruction. The aim of this paper is to evaluate the effect of particles on osteoblast cell activity and to analyze the physico-chemical modifications of the material put in culture conditions. In addition to CPC-1, two apatite phases have been tested: hydroxyapatite (HA) and CA very close to bone mineral crystals. HA is a heat-stable apatite that can be sintered at high temperature and machined like any other standard ceramic. It can bind firmly to natural bone but appears very different in fact from apatites that develop *in vivo*. Because of its poor mechanical properties compared to bone in a physiological environment, it has only been used as a bone filler or for coating orthopaedic metal prosthesis so its application has been greatly limited. *In vitro* studies have shown an inhibiting effect on cell proliferation, and on alkaline phosphatase activity [1].

CA forms on surfaces of most bioactive biomaterials either *in vivo* or *in vitro* [2]. This nano crystalline compound dissolves more easily than HA due to smaller crystal size and increased lattice disorder. It can be colonized by the cells which mediate the bone healing process. These properties render it ideal for applications aimed at the repair of skeletal defects. However, CA cannot be easily shaped by standard techniques of ceramic making.

Both were tested for their ability to act as growth factor carriers. It has been shown that CA can adsorb more proteins, probably because of a larger surface area and higher reactivity due to labile non-apatitic species [3].

The study of the effect of Ca-P powders on osteoblasts is, however, complicated with CPC-1 due to the transformation of the initial constituents into carbonate apatite with time in the cell culture media. These transformations were evaluated by the study of the evolution of its physico-chemical properties. The

purpose of *in vitro* assays is to provide a functional cell response to different crystals and particularly to CPC-1, a new product. We used osteoblast cells to investigate the ability of these materials to enhance proliferation and/or differentiation of the cells. The first type of osteoblasts: SaOS-2, from a human osteosarcoma cell line, resemble pre-osteoblasts. They present very high levels of alkaline phosphatase activity, but express no osteocalcin or osteopontin and have been used for both proliferation and differentiation studies [4]. For osteocalcin experiment, primary osteoblast-like cells were used for their late osteoblastic differentiation characteristics.

2. Materials and methods

2.1. Materials

Cell culture medium: α-Modified Eagle's Medium (α-MEM), fetal calf serum (FCS), crude bacterial collagenase were purchased from GIBCO, Paris, France. SaOs-2 cells were purchased from ATCC, California, USA.

HA powder was purchased from Merck KgaA, Darmstadt, Germany (ref. 104199).

2.2. Preparation of carbonated apatite

CA was prepared by pouring a solution of calcium nitrate ($Ca(NO_3)_2 4\,H_2O$: 70.8 g in 1 l of distilled water), into concentrated solutions of ammonium dihydrogenophosphate ($(NH_4)H_2PO_4$: 69.6 g in 2 l of distilled water) adjusted to pH 8.36 with ammonia, and sodium bicarbonate ($NaHCO_3$: 80 g). The precipitate was left to mature for 24 h. After filtration through a Büchner funnel, extensive washing with distilled water and lyophilization, the precipitates were characterized by chemical analysis, X-ray powder diffraction patterns and IR spectroscopy.

2.3. Preparation of CPC-1

CPC-1 was a blend of 50% carbonate apatite and 50% brushite. The brushite was prepared as previously described [5]. It has the property to harden in aqueous media. As it evolves with time and cristallizes to give an apatite.

For physico-chemical characterizations, sterilized CPC-1 was tested alone and after incubation time of 1, 3, 6 days in α-MEM, at 37 °C, 5% FCS, 5% CO_2. The samples were microcentrifuged for 5 min, the supernatant was poured off, and the material was then lyophilized.

For the evaluation of the biological response the CPC-1 was freshly put in the same culture medium at different concentrations before testing.

2.4. Physico-chemical characterizations of the powders

X-ray diffraction analysis: the crystal structure and the purity of the samples were investigated by X-ray powder diffraction using monochromatic CoKα radiation with an INEL goniometer (CPS 120).

FTIR (Fourier Transformed Infra-Red): the samples were analyzed on a Perkin Elmer 1700 FTIR spectrometer at 16-scan accumulations and 2 cm^{-1} resolution. The carbonate (ν2 domain) and phosphate bands ($\nu 4PO_4$) were deconvoluted and decomposed using standard Perkin Elmer software or GRAMS 386 (Galactic, NH USA).

Specific surface area was determined by BET (Quantasorb, Quantachrome, Greenvale, NY, USA).

2.5. Evaluation of *in vitro* biological response to the different substrates

2.5.1. Cell culture methods

For primary cell culture, osteoblast-like cells were enzymatically isolated from fetal bovine calvaria as previously described [6]. The cells were grown in α-MEM supplemented with 10% FCS, 50 μg/ml ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma), in 10% CO_2 at 37 °C. The osteoblast-like cells were used at subcultures 3–5.

The cells from the human osteosarcoma cell line SaOs-2 were grown in α-MEM supplemented with 5% FCS in 10% CO_2 at 37 °C.

Two washes with PBS followed by 48 h incubation in α-MEM was standard for all experiments. All powders were added to fresh medium and incubated in their final concentrations.

2.5.2. Proliferation studies

MTT method: the SaOs-2 cells were seeded at a density of $5-7 \times 10^3$/well in 96-well flat-bottomed plates and incubated with α-MEM and 5% FCS for 24 h at 37 °C. The cells were then incubated with serum-free α-MEM for 48 h. The medium was replaced by serum-free α-MEM containing various amount of samples in the form of sterilized powder (0, 0.1, 1, 10, 100 μg/ml of α-MEM). After incubation of cells for 67 h, 20 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) was added to each well, and the plate was re-incubated at 37 °C for 5 h more. Medium was removed and the purple formazan product dissolved in 200 μl of dimethyl-sulfoxide (DMSO), and the spectrophotometric absorbance at 550 nm determined using a Titertek plate reader. Data were derived from triplicate wells for each assay point.

2.5.3. Differentiation studies

For intra-cellular alkaline phosphatase (AP) activity, the SaOs-2 cells were seeded at 3.10^4 per cm^2 into 24-well plates and incubated for 48 h for attachment. At confluence the cells were washed with phosphate balanced salt (PBS), then treated, in triplicate, with sterilized HA, CA, and CPC-1 at 0, 0.1, 1, 10, 100 μg per ml of medium, for 6 days in serum-free medium. The cells were then scraped and sonicated and AP activity determined by an assay based on hydrolysis of p-nitrophenylphosphate to p-nitrophenol [7]. Data are expressed in nanomoles Pi cleaved by the enzyme per minute.

Osteocalcin (OC) secretion was measured by a specific radioimmunoassay using a detection kit (Oris-Industrie). Primary osteoblast-like cells arising from the third

subculture were seeded at 5.10^3 per cm^2 into 6-well plates in α-MEM supplemented with 10% FCS until confluence. The cultures were then maintained in α-MEM without FCS for 24 h the cells grown for the next 24 h in α-MEM containing 5% charcoal-stripped FCS [8]. The cells were treated for 72 h with 100 µg per ml of HA, CA, CPC-1, and the last 3 days with or without 1.25-$(OH)_2D_3$ at 10^{-8} M [9]. The medium was collected and assayed for OC content. The cells were scraped in PBS and sonicated, proteins were precipitated with 50% ammonium sulfate. Data were derived from duplicate wells for each assay point and were expressed as ng osteocalcin released per mg protein. The data are expressed as the mean of three experiments ± SEM. Student's *t*-test was used for statistical analysis.

3. Results

3.1. Physical-chemical characterization of the calcium phosphate materials

The Ca/P atomic ratio presented differences ranging from 1.66 attibuted to HA to 1.44 attributed to CPC-1. When immersed in culture media during various periods of time, CPC-1 powder showed a decrease of Ca/P ratio. The specific surface areas of the materials exhibited marked differences as shown in Table I. It appeared that, at the initial stage, CPC-1 has a specific surface area between those of HA and CA, after 6 days of incubation, it increased following phase transformation. Inversely the amount of carbonate decreased.

With CPC-1 powder in particular a large increase was noticed with time. The amount of carbonate decrease from the powder with time. X-ray diffraction (Fig. 1) indicated that the original mixture composing the CPC-1: DCPD and ACP was altered after incubation in solution. At day one poorly crystalline apatite (PCA) was clearly detected. The dimension of the crystals determined on 310 and 002 lines indicates a close agreement with bone crystals [10]. During this evolution the amount of DCPD decreased but this phase was still present after 6 days. No precise evaluation of the amount of the amorphous phase could be made from the X-ray diffraction data, but it was seen to decrease with time as shown by the diminution of the broad band around 35° assigned to amorphous Ca/P.

The IR study (Fig. 2) essentially confirmed the X-RD observations, and showed a decrease with time of the IR bands characteristic of DCPD. Even after a short contact with the cell culture media, the DCPD bands were already considerably decreased. Similarly, the carbonate bands characteristic of amorphous carbonate at 1480 and 1420 cm^{-1} became transformed into carbonate ion in type B apatitic sites at 1450 and 1414 cm^{-1}. This spectral

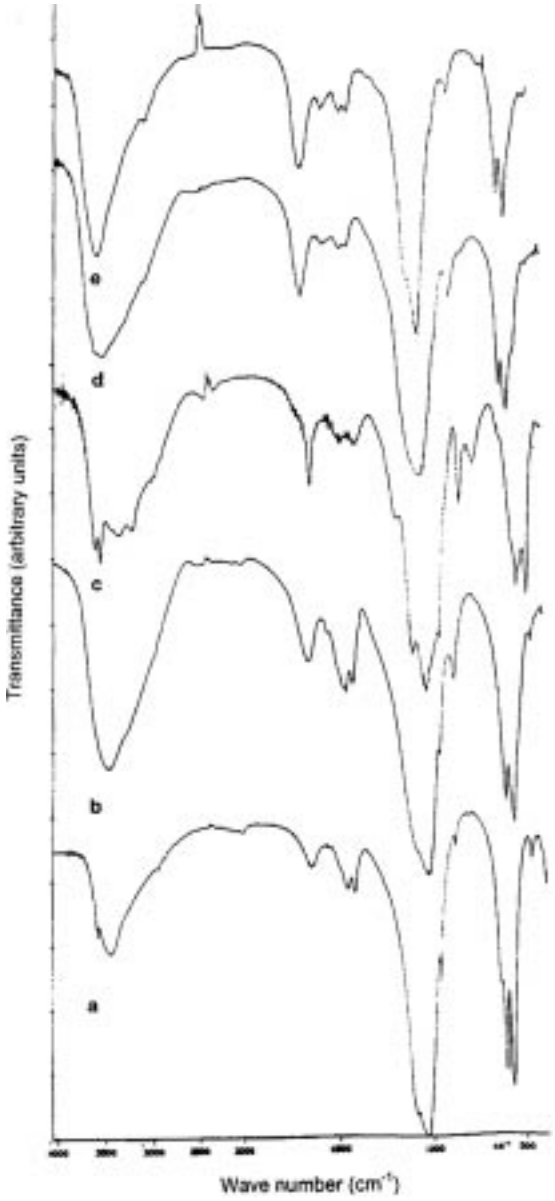

Figure 1 FTIR spectra of (a) hydroxyapatite, (b) carbonate apatite, (c) CPC-1 initial powder, (d) CPC-1 $t = 1$ day and (e) CPC-1 $t = 6$ days incubation in cell culture medium containing 5% dFCS.

evolution indicated the re-crystallization of the initial DCPD and of the amorphous phase into poorly crystalline carbonate apatite.

In a preliminary study, we tested the alterations occurring in CPC-1 in culture medium with 5% or 10% of FCS and no significative changes were observed (data not shown.)

The apparent crystal dimensions are expressed in Table II, and show that CPC-1 powder is transformed

TABLE I Specific surface area, weight percentage of CO_3^{2-} ions, Ca/P molar ratio of the three calcium phosphate cement, at initial state, and after culture medium incubation during 1, 3 or 6 days for CPC-1

Materials	Specific surface area ($m^2\ g^{-1}$)	Weight% of CO_3^{2-} ions	Ca/P
HA	59.42	1.09	1.65
CA	151	5.02	1.63
CPC-1	91	1.65	1.45
CPC-1 ($t = 1$ day)	73.32	1.21	1.28
CPC-1 ($t = 3$ day)	75.92	1.15	1.3
CPC-1 ($t = 6$ day)	96.32	1.1	1.29

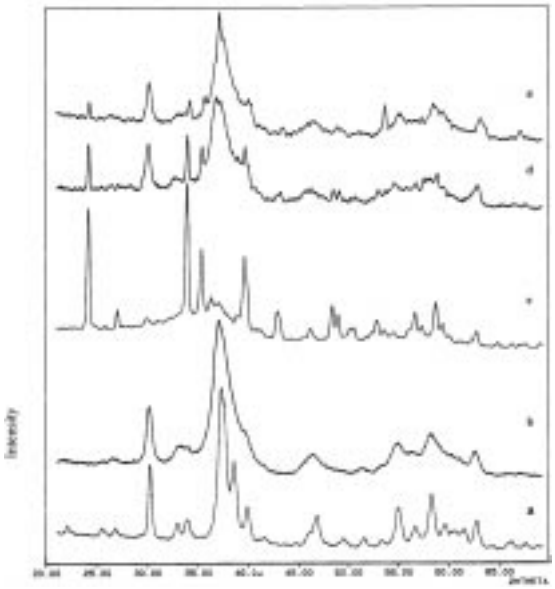

Figure 2 X-ray diffraction analysis for (a) hydroxyapatite, (b) carbonate apatite, (c) CPC-1 initial powder, (d) CPC-1 $t = 1$ day and (e) CPC-1 $t = 6$ days incubation in cell culture medium containing 5% dFCS.

into carbonate apatite crystals of dimensions close to those of bone mineral.

3.2. Proliferation study

The results obtained using MTT assay are shown in Fig. 3. They indicate that the addition of samples as powder to the cultures of osteoblasts, SaOs-2, in particular for 6 days, enhances the dose-dependent response. In the concentration range tested, from 0.1 to 10 μg/ml, all the powders promoted an increase in the proliferative cellular activity: CPC-1 presented the most pronounced effect compared to the control without a test sample (optical density OD = 0.5). When the concentration attained 100 μg/ml, CA and CPC-1 exhibited an inhibition of proliferative activity. This effect was significant, even the curves exibited a complete inversion of the trend. This phenomenon was more acute for CPC-1 compared to CA.

3.3. Differentiation study

Fig. 4 shows the AP activity after 6 days, of SaOs-2 cells in the presence of various amounts of calcium phosphate powders. The AP activity increased for all sustrates. After 6 days of culture it became apparent that the AP activity was significantly higher for CA than for the other materials.

For 1–10 μg/ml the different materials enhanced AP cellular activity by approximatively the same proportion. For 100 μg/ml, CPC-1 exibited a decrease, resulting in an inversion of the slope observed for low amounts; CA and HA on the other hand maintained a parallel increase of their AP activity even AP activity of HA was lower.

Fig. 5 represents OC release in the medium, a later stage differentiation marker, from primary osteoblast-like cell culture. During 3 day experiments, calcium phosphate powders were added at 100 μg/ml. A control with or without $1,25\text{-}(OH)_2D_3$ led to the conclusion that the effect of the calcium phosphates was not additive to that of the vitamin precursor. It appeared that CA and CPC-1 enhanced the release of OC in the medium, approximately 2-fold more than HA.

4. Discussion

Compared to other materials, the evolution of CPC-1 during time, showed strong changes related to the recrystallization of apatite precursors: DCPD and ACP. The very first apatite crystals formed readily and can be considered as the major constituent after a few minutes of contact with cell culture media. The X-RD investigation, more sensitive to well-crystallized phases than IR, indicated however that the brushite crystals persisted in cell culture media even after several days. The chemical evolution is characterized by a decrease of the carbonate content consistent with that of the Ca/P ratio. These events can be interpreted as the crystallization of the amorphous phase (Ca/P = 1.69) into poorly crystalline apatite rich in HPO_4^{2-} ions. It has been shown that the first poorly crystalline apatite crystals precipitated in solution always contain large amounts of HPO_4^{2-} ions [11]. The transformation of the DCPD (Ca/P = 1.00) into apatite on the contrary induces an increase of the Ca/P ratio of the precipitate. The overall evolution of the Ca/P ratio indicates that amorphous phase crystallization is the dominant phenomenon. The small size of the neoformed crystals explains the increase of the specific surface areas and the reactivity of the mineral apatites. These physico-chemical modifications may have an influence on the biological activity of the osteoblastic cells.

Cell differentiation *in vitro* evolves inversely proportionally to the proliferation rate; this general observation seems to be invalidated in this study. Proliferation and differentiation of osteoblasts in the presence of HA, CA and CPC-1 are significantly affected and related to the presence and proportions of mineral powders. For low

TABLE II Apparent crystal dimensions of the three calcium phosphate cement, at initial state, and after culture medium incubation during 1, 3 or 6 days for CPC-1

Composition	L(310) A	L(002) A
HA	158	404
CA	56	169
CPC-1	53	167
CPC-1 ($t = 1$ day)	77	204
CPC-1 ($t = 3$ day)	65	232
CPC-1 ($t = 6$ day)	71	210

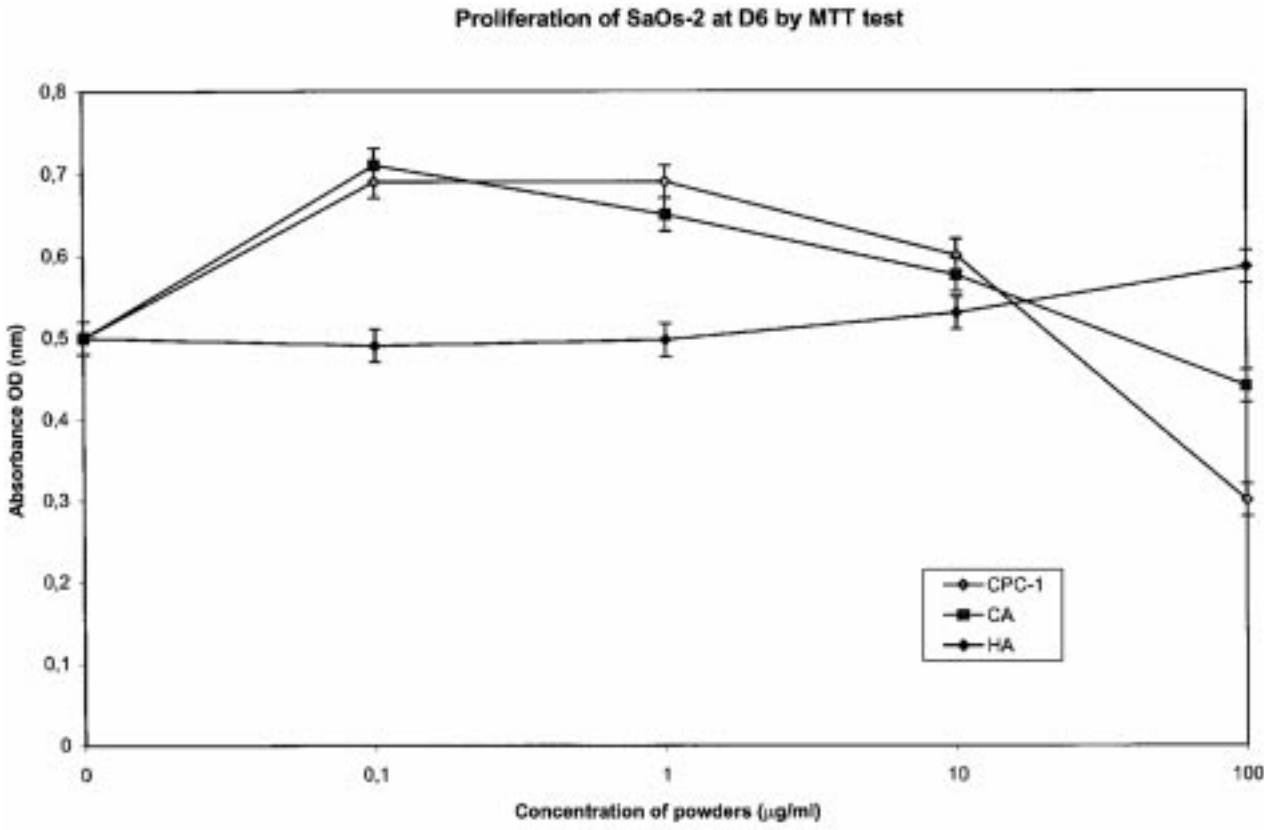

Figure 3 Effect of CA, HA and CPC-1 powders at 0, 0.1, 1, 10, 100 µg/ml on SaOs-2 cell proliferation after 6 days incubation in serum-free medium. The proliferation was assayed using the MTT method and the spectrometric absorbance determined at 550 nm. Data were derived from triplicate wells.

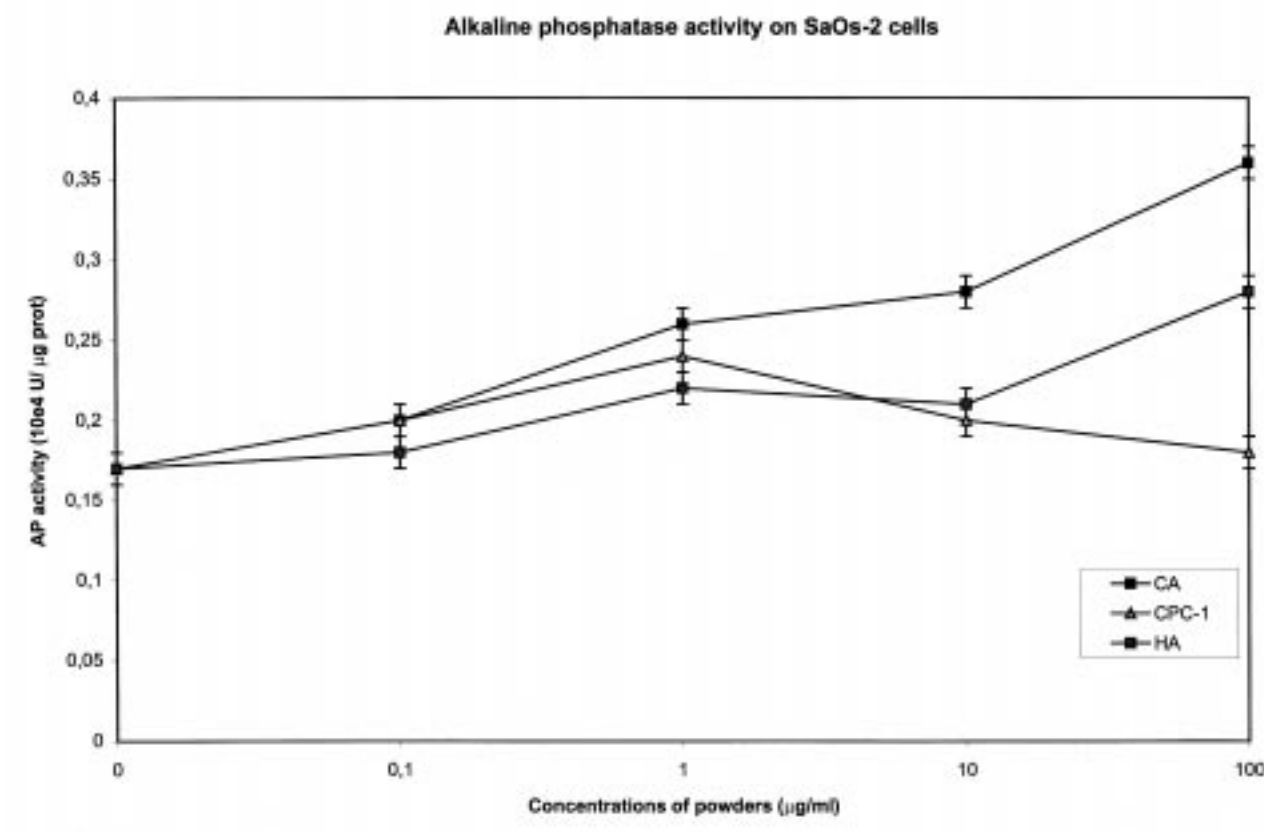

Figure 4 Effect of CA, HA and CPC-1 powders on SaOs-2 cell alkaline phophatase activity. Cells were treated with CA, HA, CPC-1 at 0, 0.1, 1, 10, 100 µg/ml for 6 days in serum-free medium. Data are derived from three measurements.

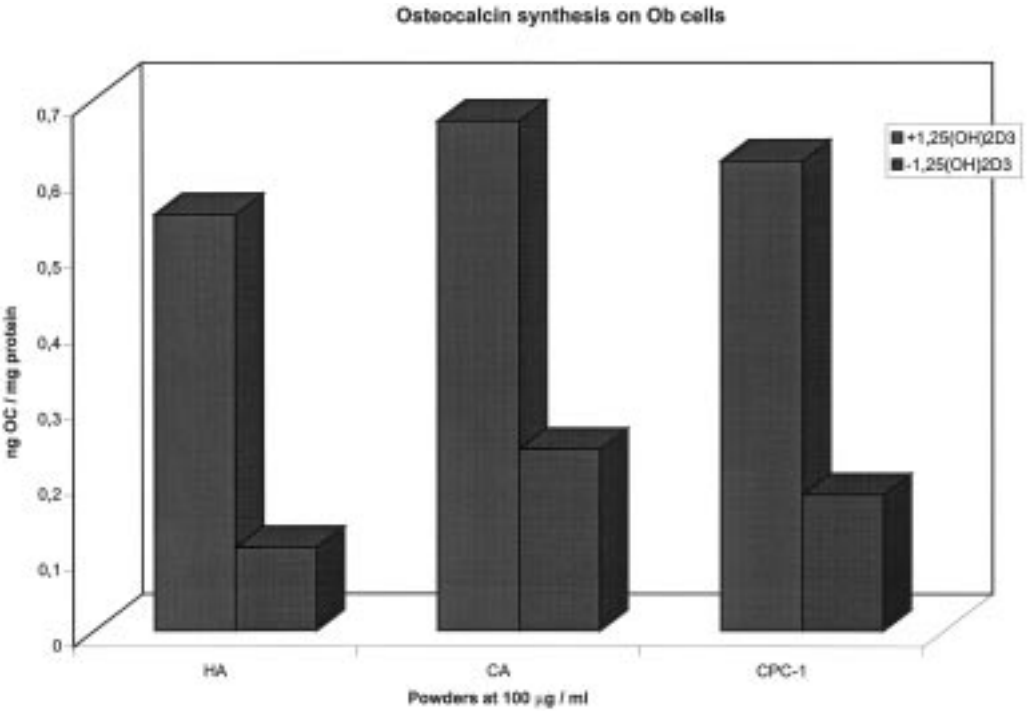

Figure 5 Osteocalcin synthesis and secretion in response to $1,25\text{-}(OH)_2D_3$ 10^{-8} M was measured by radioimmunoassay in the presence of 100 µg/ml of CA, HA and CPC-1 after 6 days incubation, as described in the text. Data were derived from duplicate wells for each point.

levels (under 100 µg/ml), and for all the powders, proliferation and differentiation increased slighly. For all Ca-P samples, and particularly for CPC-1, we found a relatively high level of initial proliferation slowing down after 3 days (data not shown) and a decrease in confluent cells after 6 days. Ca-P particles significantly enhance alkaline phosphatase activity and proliferation compared to other materials. This last result confirms that phenotypic expression of cultured osteoblasts can be delayed due to the effect of the powders. CPC-1 had a significantly lower AP activity in confluent cultures compared to CA. The dose-dependent effects on the AP activity suggest involvement of calcium ions, probably through an increase in cytosolic concentration [12]. AP activity for low powder content remains, however, unexplained.

For high levels of mineral powders ($\geq$100 µg/ml), there is a general inversion of the trend at different rates depending on the type of calcium phosphate powder present.

Concerning the proliferation, the results reported here demonstrate that in the presence of high levels of calcium phosphate particles, the osteoblasts down-regulate their activities. Endocytosis of crystalline particles from HA and their intracellular solubilization, could adversely affect calcium and phosphate homeostatic mechanisms and induce a modification of proliferation regulators. These events could occur even without any cytotoxic effect. HA is considered to be a well tolerated material, however, *in vitro* studies have shown cell death and inhibition of proliferation. The threshold for the toxic effects appears to be a particle diameter of about 5 µm [13]. The particle shape of the calcium phosphates could also be involved. The toxicity of Ca-P particles could be related to the phagocytic activity of the osteoblast-like cells and the related inhibition of DNA synthesis [14]. Large amounts of CPC-1 lead to the lowest proliferation rate and the lowest cell differentiation. An explanation for this cell behavior could reside in the role of calcium in cell biology. The increase in Ca^{2+} concentration inside cell compartments and cell cytoplasm could trigger some Ca-dependent events. Extracellular ionized calcium regulates the levels of intracellular cAMP, intracellular calcium, diacylglycerol, phosphoinositide turnover, protein kinase C activity [15], intracellular pH, hexose monophosphate shunt activity, cell respiration, membrane potential, K^+ channel activity [16], PTH gene expression [17], intracellular degradation of PTH, and probably parathyroid cell proliferation. CPC-1 is composed of a relatively soluble phase easily dissolved inside the acidic environments of secondary lysosomes of macrophages and osteoblasts.

Concerning the AP activity, OC release was inhibited with 100 µg/ml of powder. This amount was chosen for the OC release study to understand the phenomena on more differentiated cells: primary osteoblast-like cells from bovine calvaria. The late-stage marker: osteocalcin is expressed by mature phenotype cells [18]. HA reduces OC level compared to CPC-1 and CA. Osteocalcin synthesis and secretion could be measured at different phenotype maturation times. It may act in limiting the extent of bone mineralization [19] by recruiting osteoclasts to regulate bone resorption. A high level of mineralization has been shown to decrease osteocalcin synthesis. This could be due to a down-regulation mechanism in highly mineralized cell culture where apoptosis is ongoing. Osteocalcin increases transcriptionally during the period of active extracellular matrix mineralization reflecting differentiation of the bone cell phenotype [20]. In a well-mineralized matrix, however, lower bone-related mRNA expression has been observed as the cells became osteocytic [18]. In our experiment,

the high level of Ca-P apatitic compound, could produce similar effects on osteoblasts, acting as a highly mineralized matrix, and explain the decrease of OC release. Regarding the differentiation study, we found significant differences between normal bone cells from bovine calvaria and tumoral cells (SaOs-2). It is not clear if such differences are due to phagocytic response activity or to cell maturation. The difference could be related to the state of differentiation of the cells in the culture.

The present results reflect the *in vitro* biological behavior of the cells in response to Ca-P powders. Other *in vitro* studies, made on bulk solid materials, have shown the adhesion and proliferation of osteoblasts. Cell behavior can be markedly different depending on the cohesiveness of the crystals constituting a material. Individual crystals, in addition to their negative effect at high concentrations on osteoblast cell behavior, may also induce inflammatory responses [21]. Low crystal concentrations on the contrary show a positive effect on cell proliferation and AP activity. Concerning Ca-P cements it seems important to avoid massive dispersion of constitutive powders in or around bone defects. A better understanding of the interactions between cell behavior and physico-chemical properties of the biomaterials may help in the development of more effective orthopaedic and dental implants.

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