Comparative biological properties of HA plasma-sprayed coatings having different crystallinities

P. FRAYSSINET, F. TOURENNE, N. ROUQUET, P. CONTE*, C. DELGA, G. BONEL
Bioland, Biological Department, 132 Route d'Espagne, 31100 Toulouse, France, and also
**Service de Chirurgie Orthopédique, CHU Toulouse Purpan, Toulouse, France*

Plasma-sprayed HA-coatings used for orthopaedic surgery can contain different percentages of crystalline phase. The influence of the amount of crystalline and amorphous phase on the biological properties of coatings is not known. The aim of this study is to determine the effect of this coating characteristic on mammalian cell functions *in vitro*. It appears that 100% crystalline coatings have an inhibiting effect on cell proliferation and on alkaline phosphatase activity. A direct contact of the cells with the material is necessary to obtain this effect. The material evolves during the period of culture. Poorly crystalline coatings dissolve during the period of culture and the roughness parameters change during the same period.

1. Introduction

HA-coatings are used daily in human surgery in order to enhance the biocompatibility and the osteointegration of metallic alloys. These properties are useful for the fixation of cementless hip prostheses in the femoral diaphysis [1]. The specifications of HA-coatings can differ greatly, depending on the plasma-spray parameters and on the specifications of the sprayed powder. The percentage of crystalline HA contained in the coating has an influence on the coating degradation rate. No biological effect is as yet known for this specification. However, synthetic and natural HA phagocytosed particles are known to have an influence on the cell proliferation rate [2, 3]. Moreover, calcium is a second cell messenger [4].

The aim of this study is to show the influence of the crystallinity on the basic bone cell biology. *In vitro* experiments were done to determine the effect of the crystallinity on the basic and specialised cell functions. The evolution of material characteristics during cell culture was evaluated over the same period.

TABLE I Characteristics of the HA powder

Crystalline structure $Ca_{10}(PO_4)_6OH_2$
Powder size 80 μm < 95% < 160 μm
Density $1.2 < d(g l^{-1}) < 1.6$
Tricalcium phosphate < 2%
CaO < 1%
As < 3 p.p.m.
Cd < 5 p.p.m.
Hg < 5 p.p.m.
Pb < 5 p.p.m.
Fe < 5 p.p.m.

TABLE II Percentage of crystalline HA contained in the different groups of coatings

CR1: 38–48%
CR2: 60–62%
CR3: 70–73%
CR4: 100%
CR5: 45–48%
CR6: 100%

2. Materials and methods

2.1. HA-coated specimens

Titanium alloy (Ti6-A4-2V) discs having a diameter of 23 mm and a thickness of 5 mm were plasma-sprayed with a near pure HA-powder (Table I). The plasma-spray parameters (intensity, tension, working distance, powder flow, gas flow, rotational and translational speeds) were fixed in order to obtain a defined percentage of crystalline HA in the coating. Four groups of 24 discs each were determined. Each group had a different crystallinity (Table II). Four different crystallinities were tested in the first experiment, which was the cell proliferation assay. Then, for the other experiments, only the two extreme crystallinities were tested (48% and 100%).

The specifications of the coating were checked on control material made at the same time as the tested material with the same plasma-spray parameters. Coatings of the control material were characterized by quantitative X-ray diffraction and i.r. spectrometry (Perkin Elmer 1600 FTIR). X-ray diffraction patterns were recorded on a Phillips goniometer TW 1050. X-ray emissions were obtained from a cobalt anode ($\lambda = 0.178892$ nm). Crystallinity is measured by the index I_x [I_x = (integrated surface of the crystalline powder spectrum measured between $2\theta = 35–39°$/

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integrated surface of the coating spectrum measured between $2\theta = 35–39°) \times 100$]. Roughness parameters were determined using a microrugosimeter (Mitutoyo 407 microrugosimeter). These characteristics were determined before and after the cell culture test. Trace elements contained in the coatings were measured by an inductive coupled plasma technique (Perkin Elmer, Plasma 40 Emission Spectrometer).

2.2. Cell lines

An established cell line of mouse fibroblasts (L929) was used for testing the influence of coating crystallinity on basic cell functions. They were cultured in a DMEM medium (Gibco, France) supplemented with 10% foetal calf serum (IBF, France), glutamine and antibiotics (Gibco, France). The coating's influence on specialized functions of the cells was established with a chicken osteoblast primary cell line, obtained as described by Syfestad *et al.* [5]. Both cell lines were incubated in a 5% CO_2 and 98% humidity atmosphere at 37 °C.

2.3. Cell culture assay

2.3.1. Cell proliferation

Cell line L929 was suspended in the culture medium after it had been exposed to a trypsin solution (trypsin–EDTA in modified Puck's saline A 1X; Gibco, France). One millilitre of the cell suspension (5×10^5 cells ml^{-1}) was added to each glass culture well containing the coated discs. The recipient diameter was the same as the disc diameter. Three discs for each crystallinity were tested for each period of time. Glass or sand-blasted titanium alloy discs were used as the control materials. Once the culture period was over, the cells grown on the discs were suspended in 1 ml of a trypsin solution (Gibco, France) and were counted in a Malassez's hemocytometer. They were then centrifuged (1500 r.p.m. for 10 min), fixed in a 10% formaldehyde solution and examined using light microscopy.

2.3.2. Neutral red uptake by cells grown in contact with HA-coatings

The disc-containing wells were inoculated with a L929 cell line suspension according to the protocol described above. Only two crystallinities were tested, the highest (100%) and the lowest (48%). Two cell inoculation densities were used in the experiment, 5×10^5 cells ml^{-1} and 10^7 cells ml^{-1}. Once the culture period was over, cells were rinsed and the culture medium was replaced for 3 h at 37 °C by 1 ml of a 0.005% neutral red (Fluka, Switzerland) solution in PBS. Then neutral red was replaced by a formaldehyde–calcium solution (10 ml of a 40% formaldehyde solution, 10 ml of a 10% CaCl solution w/v, 80 ml of distilled water) for 1 min. Wells were rinsed with PBS, then 1 ml of a mixture of ethanol–acetic acid was added to each well. The wells were shaken until a colour homogeneity was obtained. The solution was read in a spectrophotometer at 540 nm.

2.3.3. Membrane alkaline phosphatase activity

Membrane alkaline phosphatase of a chicken osteoblastic cell line grown for 48 h on high and low crystallinity HA-coatings (48% and 100%) was extracted in a solution of PBS with 0.1% Triton X100 and 0.1% NaN_3 and submitted to repetitive freezing–thawing–shaking cycles. The control material used was a glass culture well on which the same cell line was grown. The alkaline phosphatase activity of 0.5 ml of the solution was measured by the hydrolysis of *p*-nitrophenyl phosphate. When made alkaline, *p*-nitrophenol is converted into a yellow complex measured at 400–420 nm. The number of cells grown on both groups of coating during the same period was evaluated with the same cell line grown on identical discs which were not used for the alkaline phosphatase determination.

2.3.4. Cell attachment under static conditions

Comparative cell attachment measurements were made on high and low crystallinity coatings (48% and 100%) during the first 2 h of culture. HA-coated discs were placed in culture wells. They were then inoculated with 2 ml of a L929 cell line suspension in culture medium (5×10^5 cells ml^{-1}). Suspended cells were left to attach to the HA-coatings for from 20 min up to 2 h. At a fixed time the culture medium was aspirated and replaced by a 0.005% neutral red solution in PBS. The number of cells per surface unit which took up the neutral red and which were attached to the coating was counted by reflected light microscopy.

2.3.5. SEM of cells grown on HA-coatings

Cell line L929 was grown on high and low crystallinity coatings (48% and 100%), as described above, for 24 h. The specimens were then rinsed and fixed in Karnovsky's fixative. They were dehydrated in increasing alcohol and acetone, critical point dried and coated with gold–palladium. They were observed in a JEOL JST 200 scanning microscope at 25 kV.

2.3.6. Neutral red uptake by cells separated from the HA-coatings

Cell line L929 was grown on semipermeable membranes (0.45 μm pore size) (Millipore) placed above high and poor crystallinity (100% and 48% crystalline) coated discs. One millilitre of the cell suspension was poured into the semipermeable well above the disc. One millilitre of the culture medium was introduced into the culture well containing the disc. The culture medium bathing the cell line communicated with the culture medium bathing the disc through the semipermeable membrane. Once the culture period was over, cells were rinsed and the culture medium was replaced for 3 h at 37 °C by 1 ml of a 0.005% neutral red solution in PBS. Then neutral red was replaced by a formaldehyde–calcium solution (10 ml

of a 40% formaldehyde solution, 10 ml of a 10% CaCl solution w/v, 80 ml of distilled water) for 1 min. Wells were rinsed with PBS, then 1 ml of a mixture of ethanol–acetic acid was added to each well. The wells were shaken until a colour homogeneity was obtained. The solution was read in a spectrophotometer at 540 nm.

2.3.7. Ca and P concentration in the culture medium

HA-coated discs having four different crystallinities were immersed in 2 ml of a saline medium (DMEM, Gibco-France) and incubated at 37 °C in a 5% CO_2 and 98% humidity atmosphere for a 96 h period. Then culture media were harvested and were slowly evaporated at constant temperature in order to obtain dry samples having a constant weight. Then, they were mineralized at 1000 °C before being solubilized in an acid solution. Samples were diluted to 100 ml and the Ca and P concentrations in the obtained solutions were measured by inductive coupled spectrometry (Perkin Elmer, Plasma 40 Emission spectrometer).

2.3.8. Statistics

The significance of intergroup differences was calculated with the unpaired Student's *t*-test when the variances were equal since the variances were of the same order of magnitude. *P* values < 0.05 were considered statistically different.

3. Results

3.1. Cell adhesion under static conditions

No cells attached to the control material during the first 30 min (Fig. 1). Cell fixation occurred between 60 and 100 min. Then, after 120 min an increase in the number of cells counted on the material was detected and cell clones were identified at the beginning of the growth phase of the fixed cells. Cell fixation on both highly and poorly crystalline coatings occurred earlier, during the first hour (Fig. 1). The lag phase of the cells fixed on the coatings was much longer than that of the cells fixed on the control material. No cell multiplication could be detected during the first 2 h. No statistically significative difference could be shown in cell attachment, either on highly crystalline or on poorly crystalline coating.

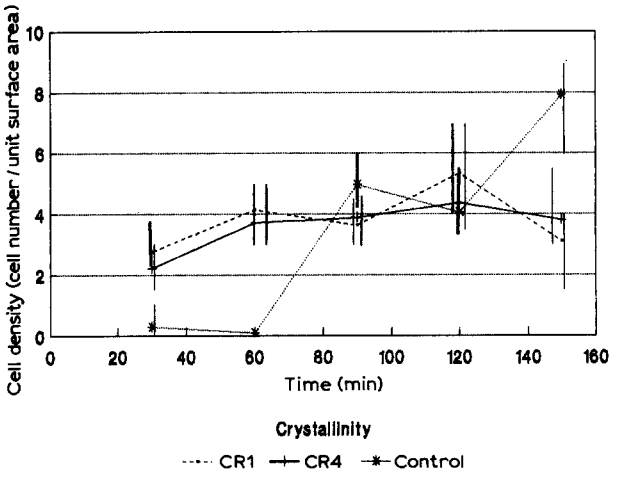


Figure 1 Cell attachment to HA-coatings.

3.2. Cell proliferation on the different coatings

The cell growth curve was typical of the growth curve of an established cell line. No statistical difference could be made between cell proliferation on the control materials (titanium alloy and glass) and CR1, CR2 and CR3 (Fig. 2). However, the growth rate on CR4 (the highly crystalline coating) was found to be much lower than the growth rate on the other specimens.

3.3. Neutral red uptake by cells grown on the coatings

The neutral red uptake by the mouse fibroblastic cells grown at low density on both coatings was inferior to the neutral red uptake of cells grown on the negative control material (Fig. 3). The uptake was much lower on highly crystalline coatings than on poorly crystalline ones. A decrease in the uptake of the cells grown on CR4 was observed between the beginning and the end of the culture.

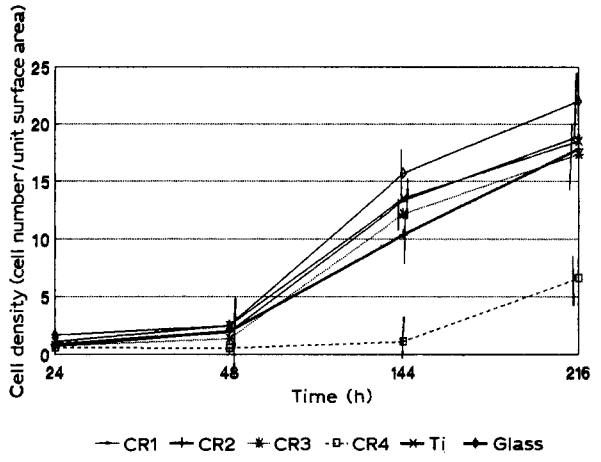
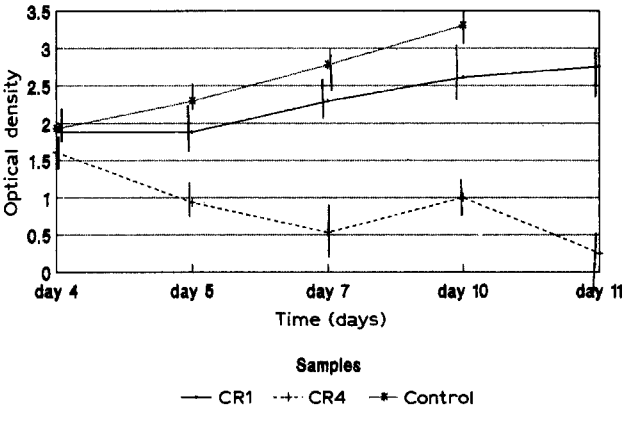


Figure 2 Cell proliferation in contact with HA-coatings.



inoculation at low cell density

Figure 3 Neutral red uptake by fibroblasts grown on HA-coatings: inoculation at low cell density.

The cells grown on the same materials at higher density (Fig. 4) showed a lower alteration of their neutral red uptake, without any significant difference between both coatings.

3.4. Alkaline phosphatase activity of osteoblasts grown on the coatings

Alkaline phosphatase activity (U cells^{-1}) was highest when the osteoblasts were grown for 48 h on the control material (Fig. 5). The membrane enzyme activity decreased when the cells were grown on calcium phosphate coatings. The decrease in activity seemed more significant on the highly crystalline coating than on the poorly crystalline one.

3.5. Particles associated with cells after the culture

Once the cells were suspended in the culture medium and then centrifuged, particles were found associated with cells grown both on the highly and poorly crystalline coatings (Figs 6 and 7). No particles were found associated with cells grown on the control material.

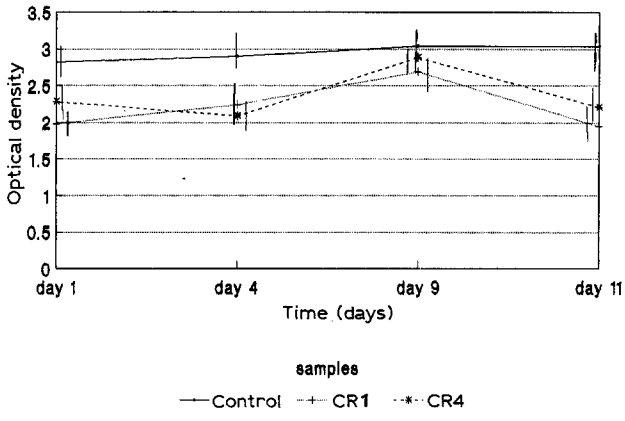


Figure 4 Neutral red uptake by fibroblasts grown on HA-coatings: inoculation at high cell density.

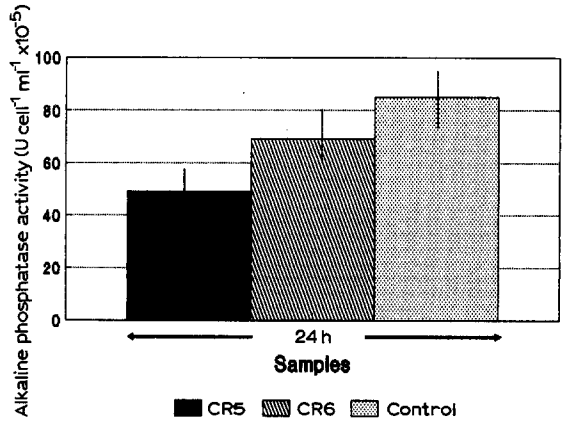


Figure 5 Alkaline phosphatase activity of osteoblasts grown on HA-coatings.

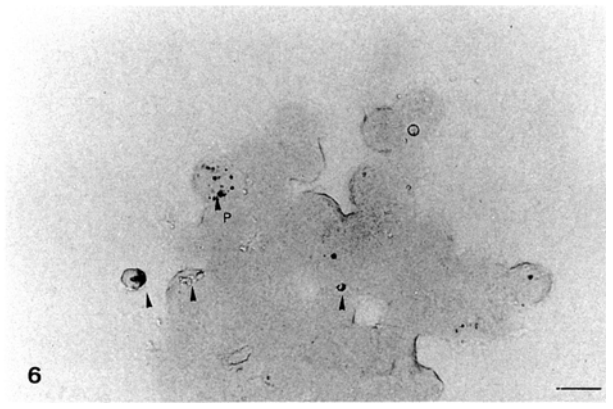


Figure 6 Cells detached from a 48% crystallinity coating then centrifuged, showing calcium phosphate particles (P) at their surface and inside their cytoplasm. Bar 20 μm.

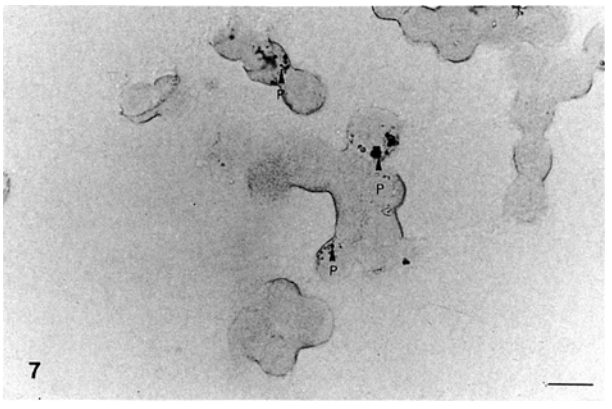


Figure 7 Identical preparation of cells grown on 100% crystalline coating. Bar 20 μm.

3.6. SEM examination of cells grown on the coatings

Cells fixed on the calcium phosphate mineral without morphological abnormalities were seen on both the coatings (Figs 8 and 9). Spindle-shaped cells bridged some coating irregularities and were attached by some focal points to the mineral. Cell expansions were

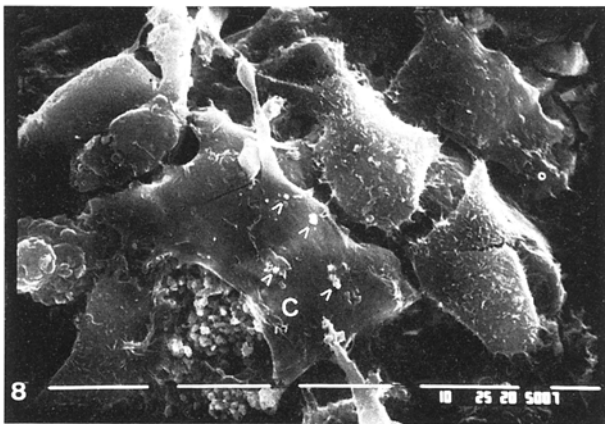


Figure 8 SEM micrograph of a L929 cell line grown on a 48% crystallinity coating. Small HA particles (arrows) are adsorbed on the cell membrane (C). Bar 10 μm.

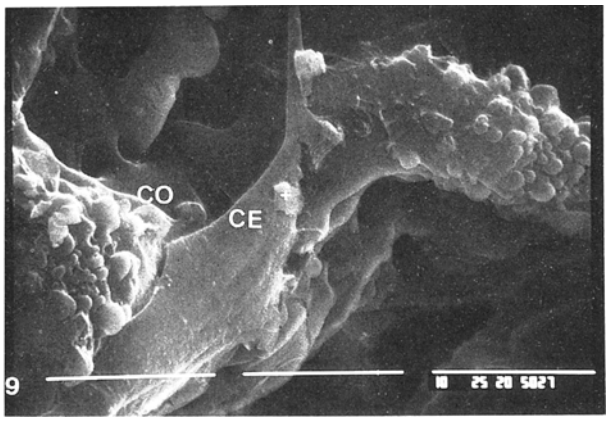


Figure 9 SEM micrograph of a L929 cell line grown on a 100% crystallinity coating. HA particles (+) are adsorbed on the membrane of a cell expansion (CE) in contact with the coating (CO). Bar 10 μm.

penetrating into the coating irregularities. Extracellular matrix proteins were seen adsorbed on the coating. Calcium phosphate particles were found adsorbed on the cell membranes.

3.7. Ca and P measurement in the culture medium

Ca and P concentrations in the culture medium increased dramatically when the culture medium bathed poorly crystalline coatings (Figs 10 and 11), especially CR1, CR2 and CR3. The CR4 coating did not modify the ionic concentration of the culture medium.

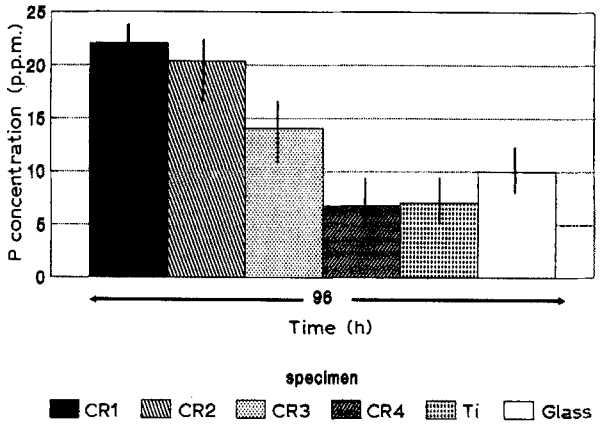


Figure 10 P concentration in the culture medium.

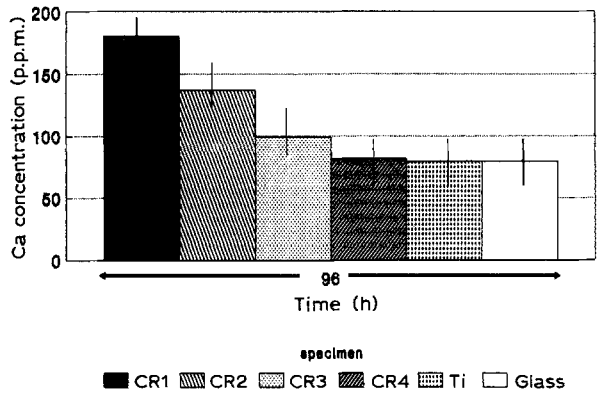


Figure 11 Ca concentration in the culture medium.

3.8. Modification of roughness parameters of the different coatings

The roughness parameters of the coatings were modified whatever the time of culture. R_p, R_a and R_t were found to be higher before culture than after on CR1, CR2 and CR3 coatings. On CR4 coatings, R_p, R_a and R_t were higher after culture than before (Figs 12–14).

3.9. Neutral red uptake by cells separated from the HA-coating

Neutral red uptake by cells grown in the culture medium bathing the highly crystalline HA-coating

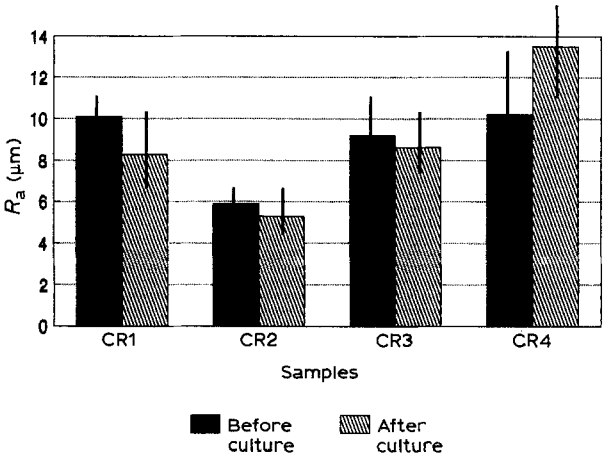


Figure 12 R_a evolution during the period of culture.

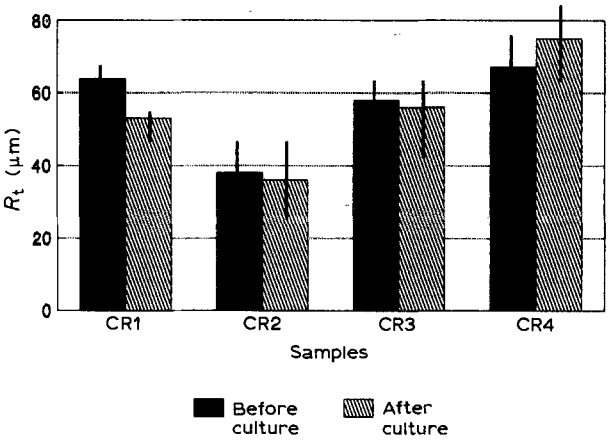


Figure 13 R_t evolution during the period of culture.

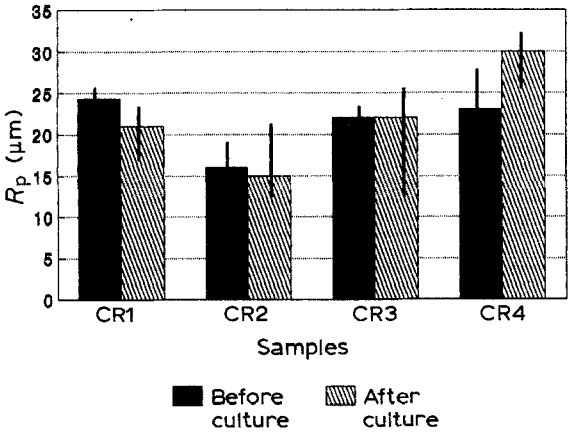


Figure 14 R_p evolution during the period of culture.

was not altered compared to neutral red uptake by cells grown into the medium bathing the poorly crystalline HA-coating and the control material (Fig. 15).

4. Discussion

This experiment shows that the basal functions of cells grown in contact with HA-coatings are affected by the physico-chemical characteristics of the material. The crystallinity of the coatings cannot alter the fixation of the cells on the material, but is able to decrease the cell proliferation rate and neutral red uptake by the cells when it reaches 100%. Specialized cell functions are altered on both highly and poorly crystalline coatings. However, alkaline phosphatase activity is lower in the cell membrane of osteoblasts grown on highly crystalline coatings than in the cell membrane of osteoblasts grown on poorly crystalline coatings. SEM of cells grown on both kinds of coatings does not show morphological abnormalities. This could mean that there is not a general cell alteration but only a modification of some cell functions. Modification of the Ca and P concentrations in the culture medium bathing the poorly crystalline coatings is a sign of the high solubility of coatings containing a greater amount of amorphous phase. This culture medium modification has no influence on cell behaviour. Calcium phosphate debris found inside the cells after their removal from the coating is due to coating degradation or to cleaning of the coating from weakly fixed ceramic grains at the surface of the material. The evolution of the roughness parameters of highly and poorly crystalline coatings in an opposite direction during the culture period could be due to the size of the grains detached during the culture, which are presumably bigger on 100% crystalline coatings than on the others. The change in cellular response to the material characteristics with density of the cell layer is difficult to explain. High density inoculation could lead to a multilayer culture, which has already been described on HA-ceramics [6]. In this case, only the cell layer at the point of contact with the material would be altered. Cell proliferation on the material which does not inhibit cell multiplication (poorly crystalline material) can be decreased by a high cell density [7], even though proliferation on the highly crystalline material would be blocked by the material effect. This could mask a difference in growth rate at the point of contact with the material at high density.

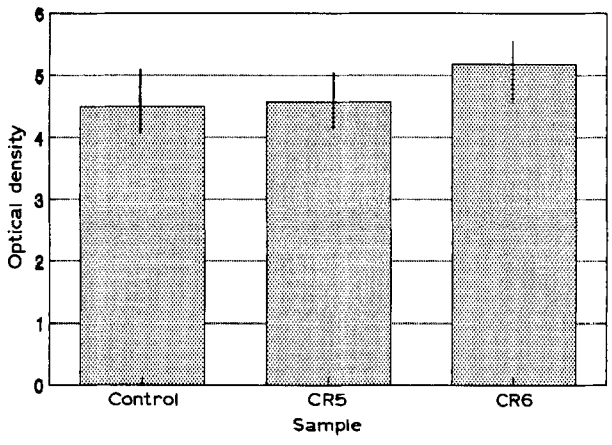


Figure 15 Neutral red uptake by cells separated from the HA-coatings.

Some other results are a little confusing. It is well known that the differentiation of cells *in vitro* evolves in inverse proportion to the proliferation rate [8]. In this study, evolution of the proliferation rate of cells grown on the HA-coatings is not consistent with that. The coatings which allow the lowest proliferation rate also induce the lowest cell differentiation.

An explanation for these dissociated phenomena could reside in the role of calcium in cell biology [4]. Calcium ions are known as a secondary messenger in cell biology [4]. The phagocytosis of calcium phosphate grains increases the Ca^{2+} concentration inside some cell compartments. Hydroxyapatite grains are known to be dissolved inside the acidic environment of secondary lysosomes of macrophages or fibroblasts [9]. The increase in Ca^{2+} concentration inside some cell compartments and cell cytoplasm could trigger some calcium-dependent events which are necessary for cellular metabolism [10]. The action of Ca^{2+}–caldmodulin complex on protein kinase was reported many years ago [11] and the induction of fibroblast proto-oncogens by Ca^{2+} from basic calcium phosphate crystals (hydroxyapatite, calcium pyrophosphate dihydrate) was recently reported by Mitchell *et al.* [12]. Moreover, Ca^{2+} interacts with the metabolism of phosphoinositide-derived messenger molecules. The action of Ca^{2+} on cell proliferation was attributed to such molecular mechanisms. However, we did not find a proliferative effect of calcium on cells grown on HA-coatings, even when they were highly soluble and when they induced a Ca^{2+} concentration increase in the culture medium.

Ca^{2+} is also involved in cytoskeleton changes [13], but there are major difficulties in trying to explain in more than in general terms how the cytoskeletal changes can be mediated by modifications of Ca^{2+} concentration [14].

Therefore, inhibition of cell proliferation shown on the highly crystalline coatings could be suppressed on the poorly crystalline ceramic by the proliferative effect of Ca^{2+} released from the amorphous phase.

However, a direct contact with the highly crystalline coating is necessary for the inhibition of cell proliferation. That could mean that the roughness of the ceramic has a strong influence on the cytocompatibility of the material. It is very difficult to dissociate the roughness parameters from the crystallinity percentage. The modification of plasma-spraying parameters can modify both of them.

5. Conclusions

The crystallinity of HA-coatings has an effect on the biology of the cells immobilized at their surface. This effect is not necessarily related to the well known role of Ca^{2+} in cell biology. It is quite difficult to relate the

effect of crystallinity on cell functions with this specification. There is an interrelation of several specifications due to the plasma-spray process. This could make it possible that the effect of crystallinity on the basal and on the specialized cell functions could be due to changes in the roughness parameters, induced by modification of the plasma-spray parameters carried out in order to change the crystallinity specification.

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