

Research Article

Biological Effect of Calcium Phosphate Bioceramics Microstructure

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Received 11 November 2010; Accepted 2 December 2010

Abstract Understanding of interactions between cells and biomaterials is a huge parameter for improving tissue engineering and regenerative medical fields. Many different materials have already been tested (including calcium phosphate ceramics) and it has been established that surface characteristic is a parameter that influences cell responses. The aim of this work was to characterize calcium phosphate discs containing various ratios of HA/ β -TCP and specific microstructure. First results show that chemical composition and compression parameters modify surface materials. Secondly, cells were cultured (osteoblast-like cells MC3T3-E1) and morphology, viability, and differentiation were studied. SEM observations, mitochondrial (MTS assay), and alkaline phosphatase activity (ALP) measurements showed that osteoblasts have better viability and a higher rate of differentiation when cultured on dense surface compared to porous surface. The aim of this experiment was to contribute to the knowledge of interactions between osteoblast-like cells and microstructured calcium phosphate bioceramics pellets.

Keywords biphasic calcium phosphate ceramic; *in vitro* assay; microstructure; proliferation; differentiation

1 Introduction

Calcium phosphate ceramics biocompatibility results from their chemical composition [7]. The osteoconductive properties of HA and the bioactive properties of β -TCP have been mixed in various ratios to obtain biphasic calcium phosphate (BCP) materials used to provide bone ingrowth. Interactions between cells and materials depend on surface state (topography, chemical composition, and energy surface). Surface state determines biological molecule adsorption and cell behavior [1]. Preparation conditions as sintering temperature, compaction method, or porogens modify calcium phosphate biomaterials. To understand the interactions generated between cells and ceramics, osteoblast-like cells were cultured on calcium phosphate substrates. MC3T3-E1 (a nontransformed cell

line established from newborn mouse calvaria) were used. Furthermore, these cells express osteoblast-specific proteins such as type I collagen, osteocalcin, or alkaline phosphates (ALP). Viability and differentiation were evaluated.

2 Materials and methods

2.1 Calcium phosphate material

CaP discs of 10 mm were composed of hydroxyapatite (HA), beta tricalcium phosphate (β -TCP), and biphasic CaP containing 60% HA and 40% β -TCP, BCP 60/40. They were used with two kinds of density: (a) a high density that increases low microporosity and (b) a low density with high microporosity. Discs were characterized using X-ray diffractometry (XRD), Fourier transformed infra-red spectroscopy (FTIR), and scanning electron microscopy (SEM).

2.2 Cell culture

MC3T3-E1 [10] were cultured in alpha MEM supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% L-Glutamine and seeded at 10,000 cells/cm² on plastic or CaP discs. Before seeding, discs were steam-sterilized and incubated in complete medium for 72 hours. Cell morphology was studied after 2 weeks by fixing them with 4% glutaraldehyde in PBS and dehydrated in graded ethanol and a graded mixture of ethanol/trichlorofluoroethane. Finally, they were coated with gold/palladium and observed with scanning electron microscopy (SEM, LEO 1450 VP Zeiss, Germany) at 15 kV. Cell viability was studied after 4, 7, and 14 days using the mitochondria tetrazolium salt (MTS) test. This colorimetric test measures the ability of living cell mitochondria to oxidize tetrazolium salt in formazan [2]. Results were expressed as relative MTS activity compared to negative control (cells cultured on plastic). After 14 days, alkaline phosphatase activity was evaluated. Cells were lysed and P-Nitrophenol phosphate was used as a colorimetric substrate for ALP and quantified

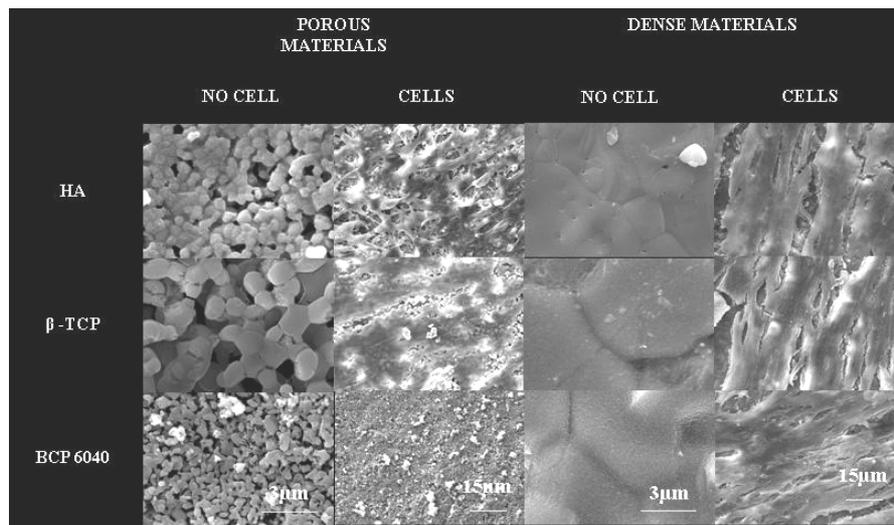


Figure 1: SEM observation of CaP dense and porous materials with or without cells.

with optical density at 405 nm. Total protein content was also measured using the Pierce Coomassie Plus assay reagent (Pierce, Rockford, USA). Results were expressed as relative ALP activity compared with control conditions.

Results are expressed as mean \pm SEM. Comparative studies of means were performed using the ANOVA test. Results were considered to be significant at $p < .05$.

3 Results and discussion

FTIR and XRD analyses have confirmed the crystalline nature of HA, β -TCP, and BCP60/40. Cells and surfaces were observed using SEM; crystal size was measured: 1–2 μm for β -TCP, 0.5–1 μm for HA, and 0.5 μm and less for BCP. Concerning dense materials, no microporosity could be observed on CaP discs surface. Cells morphology showed a correlation with surface properties: the denser is the surface, the more the cells are able to proliferate (Figure 1).

This observation was correlated with cell viability and phosphatase alkaline activity. At day 7, on porous discs, viability was reduced, respectively, of 100% on BCP 60/40, 78% on HA, and 0% on β -TCP. At day 14, viability tends to be close to negative control. Otherwise, cell viability on dense discs was increased up to 40% when compared to plastic control. ALP activity is also improved on dense material compared to porous one: slow decrease from 33 to 50% against 83% to 95% (Figure 2).

In similar works, Suzuki et al. have described MC3T3-E1 behavior cultured on various polished calcium phosphate pellets (HA, β -TCP, and BCP). It was shown at day 6 that cell proliferation increased on all the substrates. At day 14, ALP activity had also increased [8]. Polishing tends to smooth the surface. This smoothing may positively

influence cell viability and osteoblastic differentiation, as suggested by our results. Modification to cell behavior seems to result of the surface change than variations in chemistry. Other complementary studies have been carried out: Hatano et al. have tested roughness effect of MC3T3-E1 on proliferation and differentiation. They described that increasing roughness on polystyrene substrates improved cell proliferation and cell differentiation [3]. Linez-Bataillon et al. also described variations in MC3T3-E1 behavior on TiAl4V pellets with various roughnesses. They deduced from their work that roughness increased cell differentiation and smooth surface increased proliferation [6]. This result is in agreement with the work of Washburn, who tested MC3T3-E1 proliferation on a polylactic acid matrix with various degrees of roughness [9].

All the results confirm that a close relationship exists between viability, differentiation, and surface state. Nevertheless, the studies do not make it possible to determine precisely which surface is favorable to cell viability and differentiation. We must also consider that differences between the studies can be linked with the chemistry of the various materials tested (calcium phosphate, polylactic acid, or titanium). Moreover, surface state variations also depend on micropore size or adsorbance. Lee's team has carried out studies with human MG63 osteoblast-like cells in contact with polycarbonate materials. The materials present surfaces with micropores of different sizes (from 0.2 to 8 μm in diameter). The larger micropores decrease proliferation and increase differentiation [4]. These results can be correlated with Linez-Bataillon's works, even if the chemistry of the material is different. Adsorbance is also an important parameter for cell/material interactions. It will effectively determine cell adsorption as well as protein and cell response. Liao et al. have tested mouse osteoblasts

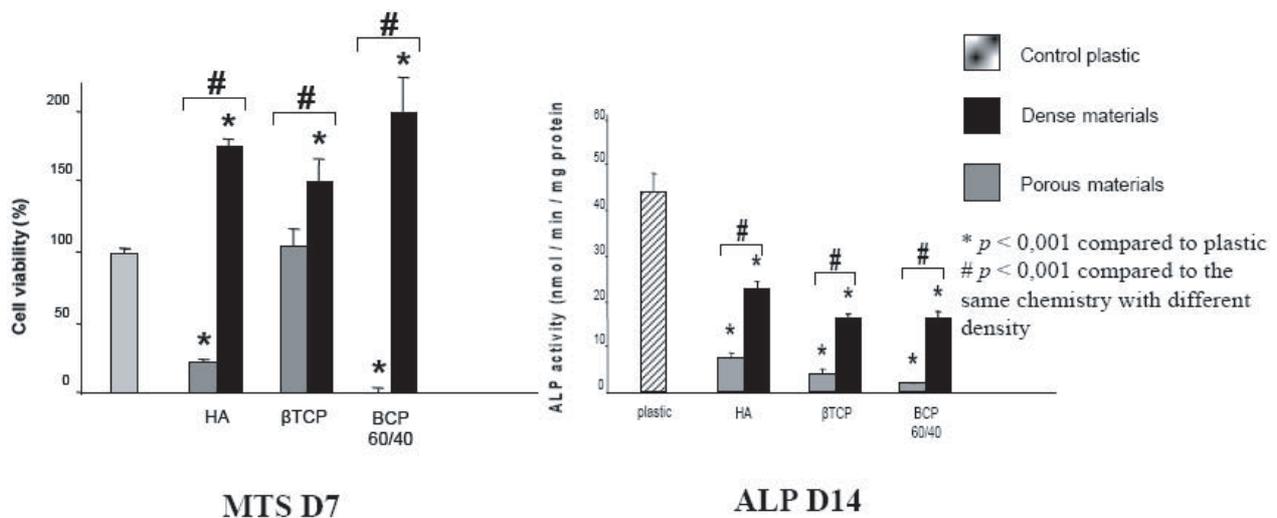


Figure 2: Viability (MTS assay) at day 7 and phosphatase alkaline activity at day 14 of MC3T3-E1 on CaP dense and porous materials.

on polydimethylsiloxane (PDMS) materials. Patterns were drawn on the substrate with hydrophilic and hydrophobic properties. The main result was that hydrophilic patterns increase ALP activity. It seems that hydrophilic patterns also increase cell proliferation [5].

These works demonstrate that nano-microscale surface topography is a main parameter that influences cell responses. Our results show that the chemical composition and micropores contents modify surface topography then cell interactions.

4 Conclusion

This simple *in vitro* study confirmed that smooth and dense surface is more efficient to maintain and promote osteogenic activity. This is in opposition with *in vivo* results that demonstrate osteoinduction and higher osteogenicity for high microporous CaP bone substitutes.

Acknowledgments This work was supported by a Grant ANR BioRimp 2005. The authors would like to thank Françoise Moreau (Biomatlante) and Paul Pilet (SC3M Nantes and Hospital University, Microscopic center) for their helpful technical assistance.

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