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Effect of Stressed and Unstressed Cell Culture Environments on the Survival of MC3T3 Cells Cultured on Calcium Phosphates

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Abstract

The effect of Fetal Bovine Serum (FBS) deprivation on survival and apoptosis of osteoblasts cultured on various calcium phosphates was studied. Test materials were two calcium alkali orthophosphates (materials denominated: GB9 and GB14), which were compared to β-tricalcium phosphate (TCP). Tissue culture polystyrene (PS) served as control. Test materials were characterized by X-Ray Diffraction and scanning electron microscopy. An apoptotic challenge assay entailing serum withdrawal was applied: MC3T3-E1 osteoblasts were cultured for 72h on the test materials in serum containing medium, followed by incubation in serum free medium for another 24h. Serum withdrawal is an apoptotic challenge, which creates a stressed environment. Cells cultured on the test specimens in serum containing medium served as control. The TUNEL Assay was employed to quantify the percentage of apoptotic cells. GB9 and GB14 displayed a significantly lower percentage of apoptotic cells than TCP. TCP had significantly fewer apoptotic cells than PS. The percentage of apoptotic cells on GB9 and GB14 was less than 10%, while the number of apoptotic cells found on the untreated control specimens ranged between 5 and 7%. These findings indicate that GB9 and GB14 endow osteoblasts cultured on them with a decreased sensitivity to apoptosis, which corresponds well with the results of previous in vitro and in vivo studies.

Keywords: Cell culture media; Serum withdrawal; Stressed; Apoptosis; Cell survival; Calcium alkali orthophosphates

Introduction

Although autogenous bone grafts are considered the gold standard for bone reconstruction prior to dental implant placement in oral implantology, bone graft substitutes are extensively studied in order to avoid harvesting autogenous bone. Compared to the bone substitutes, which are currently clinically available, there is a significant need for bone substitutes which degrade more rapidly. As a result, there has been an ongoing search for biodegradable bone substitute materials that degrade rapidly, but still stimulate osteogenesis at the same time, thereby resulting in complete substitution by newly formed functional bone tissue in view of placing dental implants in such augmented sites. This has led to the development of bioactive, rapidly resorbable glassy crystalline calcium alkali orthophosphates [1-8]. These materials have a higher solubility and biodegradability than β-tricalcium phosphate (TCP). Previously we were able to show that some of these calcium alkali orthophosphats had a stimulatory effect on osteoblast differentiation and osteogenesis in vitro [4-7] and in vivo [6,7], thereby displaying excellent bioactive properties. Furthermore, serum protein adsorption has been recognized as an important element of bioactive behavior [9]. Over the last decade a considerable body of knowledge has been generated regarding the mechanisms by which extracellular matrix components induce bone cell apoptosis [10-13]. This has led to the hypothesis that the attachment of bone cells to the calcium alkali orthophosphates mentioned above leads to decreased sensitivity to apoptotic stimuli such as serum withdrawal. Fetal bovine serum (FBS) withdrawal for 24h is a challenge to cells, as it induces cells to undergo apoptosis thereby creating a stressed cell culture environment [14]. This study evaluated the effect of two calcium alkali phosphate graft materials as compared to the currently clinically used material β-tricalcium phosphate (β-TCP) on survival and apoptosis of bone cells after culturing MC3T3-E1 osteoblastic cells on the test materials for 3 days and subsequent exposure to serum withdrawal. As a result, the survival and apoptosis of osteoblastic cells in contact with these synthetic bone graft substitutes under stressed and unstressed conditions was examined.

Materials and Methods

Test materials were two calcium alkali orthophosphates (CAOPs) with the crystalline phase $Ca_2KNa(PO_4)_2$ and with a small amorphous portion containing either magnesium potassium phosphate (material denominated GB14) or silica phosphate (material denominated GB9) [1-8]. These materials were compared to the currently clinically used material β-TCP (Cerasorb®). Specimens were prepared by compress-

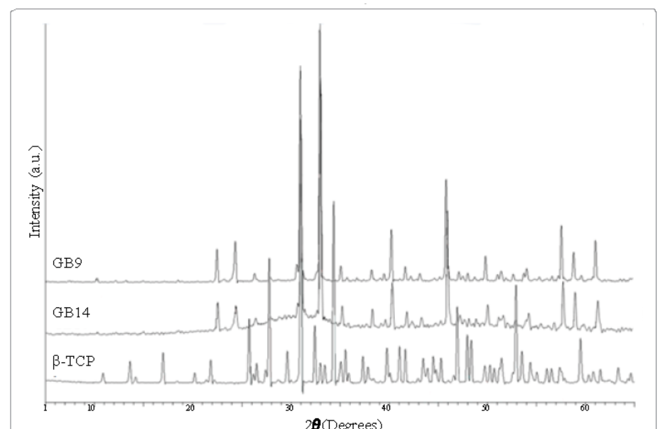

Figure 1: XRD patterns of the different materials used, *i.e.* GB 9, GB14 and β-TCP

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ing granules (grain size 40μm) followed by sintering to form 10-mm diameter discs [3-5]. All test materials were analyzed by X-Ray Diffraction (XRD; Philips, Germany). Scanning Electron Microscopy (SEM, CamScan MaXim.Germany) was used for visualizing the surface morphology of the samples. Prior to cell seeding a, discs were heat-sterilized at 300°C for 3h. The MC3T3-E1, subclone 4, osteoblastic cell line (ATCC, USA) was used in this cell culture study. The cell culture medium was composed of DMEM with 10% FBS and supplemented with 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate, 2 mM L-glutamine and 50 μg/ml penicillin-streptomycin.

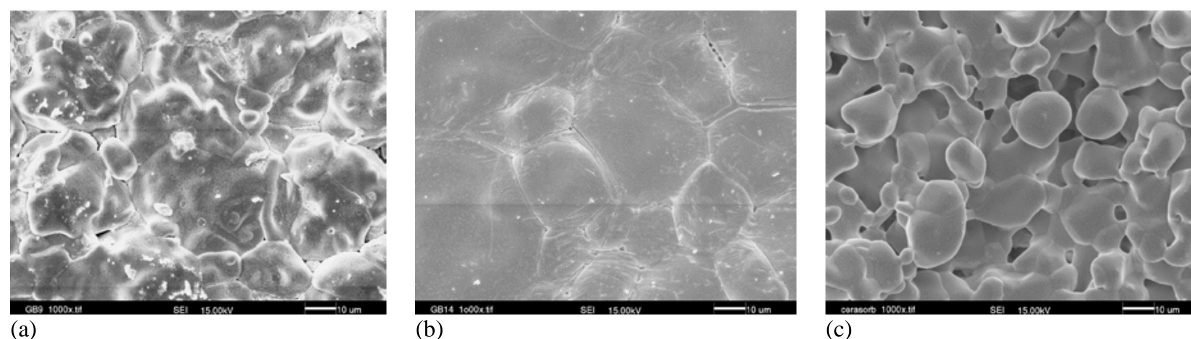

Figure 2: Scanning electron micrographs of the various calcium phosphate specimens after fabrication of the disc-shaped substrata: (a) GB9, (b) GB14, and (c) β-TCP (original magnification 1000x).

Three runs of experiments were performed and an apoptotic challenge assay described by Adams et al. (2001) was applied [11]. 3 discs per materials were placed in wells of proper size in cell culture plates and pre-incubated in fully supplemented medium for 24 hours before use. Tissue culture polystyrene was used as control. After conditioning, medium was removed, and cells were seeded onto the discs in the wells at the density of 7.5 x 10^4 cells per cm^2. Medium exchange was performed after the first day, and cells were cultured in fully supplemented medium for three days. After three days, cells were cultured for 24h in serum free medium and thereby subjected to serum withdrawal. Untreated osteoblasts were used as controls. The TUNEL assay was performed after these 24 hours of serum deprivation to examine the apoptosis of cells, i.e. fragmented chromatin was detected employing the Calbiochem® Klenow FragEL™ DNA Fragmentation Detection Kit (EMD/Merck KgaA, Darmstadt) [11]. The TUNEL assay takes advantage of the fact that during apoptosis, nuclear endonucleases cleave linker DNA into fragments of multiples of ~200 base pairs. This kit allows the recognition of apoptotic nuclei in preparations fixed on biomaterials discs by Fragment End Labeling of DNA. Cells were fixed in 4% formaldehyde in 1x PBS for 10 min. Cells were then treated with proteinase K (20 mg/ml) at room temperature for 15min. Endogenous peroxidase activity was inhibited by exposing cells to 3% H_2O_2 in phosphate-buffered saline. Cells were equilibrated in a transferase buffer for 5-10 min and then incubated in a reaction mixture containing biotin-labeled deoxynucleotides and the Klenow fragment of DNA polymerase at 37 °C. After 60 min, the reaction was stopped, and the biotinylated nucleotides were attached to streptavidin peroxidase. Thus, in this assay Klenow binds to exposed ends of DNA fragments, generated in response to apoptotic signals, and catalyzes the template-dependent addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horse radish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacted with the labelled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. Counterstaining with methyl green aided in the morphological evaluation, characterization and quantification of normal and apoptotic cells. The data of the three experimental runs were pooled. Student's t-test in combination with the Bonferroni-correction was used for statistical analysis. Significance was assumed achieved for $p < 0.05$.

Results and Discussion

XRD (Fig.1) facilitated examining the different compounds and crystallographic organization present with each material. GB9 and GB14 patterns displayed peaks related to the potassium sodium calcium phosphate compound $Ca_2KNa(PO_4)_2$ (Ref. 00-051-0579). β-TCP showed a pattern with the characteristic peak related to β-TCP (Ref. 00- 009-0169) around a 2θ value of 31°. For both GB 9 and GB14 the presence of the same main compound, i.e. crystalline phase, was confirmed. The XRD pattern of GB14 displayed broader and more irregular peaks, which is indicative of a slightly greater amorphous structure. Both the GB9 and GB14 materials displayed a more highly amorphous structure compared to the crystalline structure of β-TCP. The surface morphology of the bioceramics studied is shown in Fig. 2.

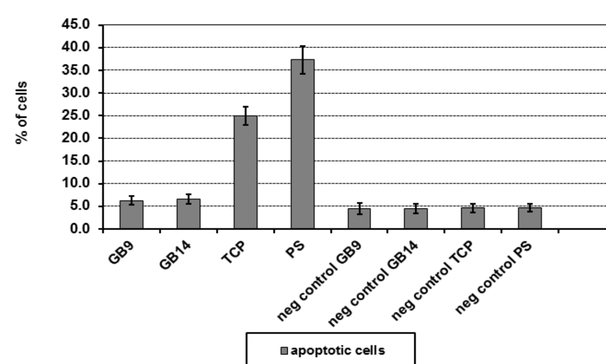

Figure 3: Percentage of apoptotic cells after culturing MC3T3-E1 cells on various test materials for 3 days and subsequent exposure to an apoptotic challenge, i.e. serum withdrawal for 24 h, as compared to unstressed negative controls. All values are mean ± standard deviation of nine measurements.

After 24 h of serum starvation GB9 displayed the lowest percentage of apoptotic cells closely followed by GB14 (Figure 3). Significantly more apoptotic cells were present on TCP and tissue culture polystyrene (PS), with PS displaying the highest percentage of apoptotic cells, while all unstressed negative controls exhibited only a low percentage of apoptotic cells (Figure 3). It is noteworthy that the percentage of apoptotic cells on GB9 and GB14 was less than 10%, i.e. not even twice as high as that on the negative control specimens. On TCP a significantly lower percentage of apoptotic cells were noted compared to the PS controls. These findings indicate that GB9 and

GB14 endow osteoblasts cultured on them with a decreased sensitivity to apoptosis. To a lesser degree this also was true for β-TCP (Figure 3), which exhibited a significantly greater capability to reduce the sensitivity of osteoblasts to apoptosis than the PS controls. The results of the current study correspond well with those of previous in vitro and in vivo studies. Previously, we were able to show that when compared to TCP, bioactive glass 45S5 and other CAOPs the silica containing CAOP GB9 displayed calcium and silicon ion release, and calcium uptake as well as considerable fibronectin and extensive type I collagen serum protein adsorption [8,15,16]. As a result, cell adhesion was mainly mediated through integrin $\alpha_5\beta_1$ and (to a slightly lesser degree) through $\alpha_2\beta_1$, which then in its turn led to simultaneous enhanced activation of key signaling factors of the Erk differentiation pathway, the PI3K/Akt-cell survival pathway, and the alternate p38 pathway [8,15-17]. These studies which showed that GB9 had the greatest stimulatory effect on intracellular signaling events, which upregulate cell differentiation and cell survival, thereby depressing apoptosis, are in good correspondence with the results of the current study. This furthermore is in agreement with the findings of previous studies, in which GB9 had a significantly greater stimulatory effect on osteoblast differentiation *in vitro* [4-7] as well as the best bone-bonding behavior and the greatest stimulatory effect on bone formation and expression of osteogenic markers *in vivo* after implantation in clinically relevant critical-size membrane protected defects in the sheep mandible [6,7], when compared to β-TCP, bioactive glass 45S5, and other CAOP materials. This is in addition to first histologic findings of human biopsies sampled 6 month after sinus floor augmentation with this silica containing CAOP material, which seem to confirm the high capacity of this material to induce enhanced bone matrix mineralization and bone formation at its surface, even at a great distance from the native alveolar crest. The results of the current study are also in good correspondence with findings obtained after in vivo implantation of GB9- and GB14-based cements in paste and putty form, which displayed excellent bone regenerative capacities [7,18]. The findings with respect to TCP correspond well with the well documented osteoconductive properties of this biomaterial [7]. A current study deals with using 3D printed CAOP scaffolds, on which mesenchymal stem cells are cultured dynamically, for generating bone tissue engineered constructs for reconstruction of segmental discontinuity defects.

Conclusions

This study was able to demonstrate the effect of an apoptotic challenge assay involving serum withdrawal on osteoblasts cultured on the calcium alkali orthophosphates GB9 and GB14 as compared to TCP. The results of the TUNEL assay showed that GB9 and GB14 possess the potency to enhance cell survival and depress apoptosis of osteoblasts cultured on them. These findings are in good correspondence with the results of previous in vitro and in vivo studies which showed that when compared to TCP, bioactive glass 45S5 and other CAOPs, GB9 induced enhanced fibronectin and collagen type I adsorption in combination with greatest upregulation of intracellular signaling events which enhance cell differentiation and survival. This was in addition to inducing enhanced osteogenesis *in vivo* in an ovine model, which seems to be further corroborated by first histologic findings in the human case. Consequently, the results of this study further corroborate the excellent osteogenic properties of this silica containing CAOP material, whose angiogenic properties warrant further detailed investigation.

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