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 orthophosphates 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 Ca3KNa(PO4)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-

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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, 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.

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 10⁴ cells per cm². 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 chromatins 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% H₂O₂ 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 evalu-

ion, 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 Ca₃KNa(PO₄)₂ (Ref. 00-051-0579). β-TCP showed a pattern with


![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).](Image)

![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.](Image)
GB14 end 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 protein adsorption [8,15,16]. As a result, cell adhesion was mainly mediated through integrin α5β1, and (to a slightly lesser degree) through αvβ3, which then in its turn led to simultaneous enhanced activation of key signaling factors of the Erk differentiation pathway, the P13K/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 critical-size membrane protected defect s in the sheep mandible [6,7], containing CAOP material, which seem to confirm the high capacity of in vivo osteogenesis 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 those of previous in vitro and in vivo investigations of novel calcium phosphates using osteogenic cultures. Journal of Materials Science, Materials in Medicine; 9:337-345.

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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