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ORIGINAL ARTICLE

Surface- and nonsurface-dependent in vitro effects of bone substitutes on cell viability

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Abstract The aim of the present in vitro study was to evaluate the influence of different bone substitute materials (BSM) on the viability of human primary osteoblasts (PO), bone marrow mesenchymal cells (BMMC), and nonadherent myelomonocytic cells (U937). Six different bone substitute materials were tested: Bio-Oss Spongiosa® (BOS), Tutodent Chips® (TC), PepGen P-15® (P-15), Ostim® (OM), Bio-Base® (BB), and Cerasorb® (CER). Cells were cultivated on comparable volumes of BSM in 96-well plates. Cell culture-treated polystyrol (Nunclon Delta surface; C) served as positive control. After 2 h and 3, 6, 10, and 14 days, viability of cells was evaluated using a standardized ATP viability assay (CellTiter Glo®). Nonsurface-dependent effects of the materials were separately tested using nonadherent U937 suspension cells. For statistical analysis, the Mann–Whitney test was used. Results were considered statistically significant at $P<0.05$. Cell viability of PO increased significantly on TC, C, and CER followed by BB. No changes were found for P-15 and decreasing viability for BOS and OM. BMMC showed similar results on C, TC, CER, and P-15. Lower viability for BB and no viability could be detected for BOS and OM (Mann–Whitney test, respectively). Nonadherent cells displayed increasing viability in presence of CER, BB, and BOS. No changes were observed for TC and P-15, whereas for OM, no viability was detected after a maximum cultivation period of 3 days. It was concluded that granular hydroxyapatite (HA; TC, BOS, P-15) and α- and β-tricalciumphosphate (CER, BB) support, whereas nanosized HA (OM) limit or even inhibit surface- and nonsurface-related cell viability in the in vitro model used.

Keywords Cell viability · Bone substitute material · Osteoblasts · Bone marrow mesenchymal cells · U937 cells

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Introduction

Bone substitutes are commonly used for implant site augmentations. Until now, autogenous bone is recognized as the gold standard. Bone cell precursors in the graft provide osteoinductive properties without adverse immunological response [2, 26, 34]. Nevertheless, autografts increase the morbidity and are limited in availability [10, 21]. These considerations have led to an increased exploration of alternative bone substitute materials (BSM). BSM are supposed to be biocompatible, noninfectious, and nonantigenic. Although most are not considered to be osteoinductive, they should at least be osteoconductive [5, 16].

The common source of xenogenous BSM is bovine bone. Different production methods result in hydroxyapatite (HA) with either residual collagen (Tutodent® Chips, TC) or total removal of all proteins (Bio-Oss® Spongiosa, BOS). Further enhancement of the biochemical properties of hydroxyapatite is intended by addition of a 15-amino acid-long peptide representing the cell-binding domain of collagen I (PepGen P-15®, P-15). α-tricalciumphosphate (TCP; BioBase®, BB) and β-TCP (Cerasorb®, CER) and

nanocrystalline synthetic HA (Ostim®, OM) represent other classes available in the market.

The osseous integration of a BSM depends on the activity of the surrounding bone cells or their precursors. Hereby, migration and proliferation of the osteogenetic cells is mainly influenced by the interaction of the cell membrane with the BSM surface [11, 19]. Since cellular attachment is necessary for proliferation of adherent cell lines, in vitro experiments may be suitable to determine the biocompatibility of a BSM.

Whereas many studies investigated the biocompatibility of different BSM, to the best of our knowledge, no publication exists dividing into surface- and nonsurface-related effects on the viability of cells. It seems to be obvious that a negative effect can either be caused by surface properties or by biochemical releases affecting the cell metabolism. Therefore, the present in vitro study was designed to compare the surface- and nonsurface-dependent influence of various types of BSM on cell viability. Cell lines directly involved in hard tissue healing were represented by primary craniofacial osteoblasts and bone marrow mesenchymal cells. For investigation of nonsurface-dependent aspects, a nonadherent myelomonocytic suspension cell line (U937) was cultivated in the presence of the BSM in order to detect any cytotoxic effects of the BSM, which are independent of cell adherence to the BSM [6, 9].

Materials and methods

Material examined

An overview of the BSM examined is listed in Table 1.

Cell culture

The use of human material for harvesting both bone marrow mesenchymal cells (BMMC) and primary osteoblasts (PO) was approved by the Ethics Committee of the Heinrich Heine University of Duesseldorf, Germany (BMMC No. 2729, PO No. 2505). BMMC were harvested from human iliac crest and generated and expanded according to Kogler et al. [17]. Cells were passaged twice to remove hematopoietic cells. Passage three was used for the experiments.

Primary osteoblasts were harvested from bone chips collected during osteotomies of lower wisdom teeth using a bone chip filter KF-T2 (Schlumbohm, Brokstedt, Germany). Outgrowing cells were characterized as osteoblasts by positive expression of osteocalcin (OC) as controlled by reverse transcriptase polymerase chain reaction. Additionally, OC immunohistochemistry revealed osteocalcin synthesis and a positive alkaline phosphatase (AP) activity [8]. The second passage was used for the experiments. For investigation of BMMC differentiation, cells were seeded onto BSM on culture slides (Lab Tek Chamber Slide, Nunc, Wiesbaden, Germany).

The myelomonocytic suspension cell line U 937 was purchased from the German collection of microorganisms and cell culture (DSMZ, Braunschweig, Germany). The U 937 cells were cultivated without additives (i.e., lipopolysaccharide or phorbolacetate (tetradecanoyl phorbolacetate)) in order to maintain their suspension cell character and to exclude the induction of differentiation towards adherent growing macrophages [23, 28].

All cell types were cultivated in Dulbecco's modified Eagle medium (Gibco®, Invitrogen™ GmbH, Karlsruhe,

Table 1 Overview of different BSM examined

Category	Short name	Product	Material	Consistency of material/particle size examined	Sample weight/ well (mg)
Xenogenous	BOS	Bio-Oss Spongiosa®, Geistlich Biomaterials, Wolhusen, Switzerland	Bovine hydroxyapatite, high temperature HA ceramics, deproteinated	Granular 1,000–2,000 μm	17
	TC	Tutodent® ChipsTutogen Medical, Neunkirchen, Germany	Bovine hydroxyapatite solvent dehydrated natural bone	Granular 1,000–2,000 μm	28
	P-15	PepGen P-15®, Dentsply Friadent, Mannheim, Germany	Bovine hydroxyapatite, high temperature sintered, deproteinated, enhanced with p-15 peptide	Granular 250–420 μm	31
Alloplastic	OM	Ostim®, Heraeus Kulzer, Hanau, Germany	Nanocrystalline hydroxyapatite	Paste	91
	BB	BioBase®, Zimmer Dental Freiburg, Germany	α-tricalciumphosphate	Granular 500–1,400 μm	26
	CER	Cerasorb®, Curasan, Kleinostheim, Germany	β-tricalciumphosphate	Granular 1,000–2,000 μm	50

The wells were filled to approximately 40 μl volume with the listed BSM weights

Germany) with 10% fetal bovine serum (Gibco®), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco®). Incubation was at 5% CO_2 at 37°C. Medium was changed after 24 h to remove unattached cells and then every second day. The pH was controlled with every medium change. No osteogenic factors were added.

Alkaline phosphatase staining

Osseous alkaline phosphatase, a membrane-bound tetrameric enzyme attached to phosphatidyl-inositol moieties located on the outer cell surface was assayed using the release of *p*-nitrophenol from nitrophenolphosphate [27]. After the cultivation period of 14 days, the BMMC were fixed in 4% buffered formaldehyde, incubated for 15 min in an AP staining solution (Sigma Deisenhofen, Germany), and counterstained with 6% hematoxylin (DakoCytomation, Hamburg, Germany). As negative control, the endogenous AP activity was blocked by 0.15 mg/ml levamisole (Sigma) [31].

Cell viability assay

BSM were allocated in 96-well plates (Nunc, Darmstadt, Germany) covering the well bottom (n=6). Respective amounts of the BSM are listed in Table 1. Cells were seeded onto the BSM in a density of 1×10^4 cells per well (in 200 μl volume). As reference surface for optimal cell attachment and proliferation, the cell culture-treated polystyrene Nunclon Delta surface (Nunc) was used [4] and served with cells as positive and without cells as negative control.

After 2 h (baseline) and 3, 6, 10, and 14 days, the ATP content per well was determined using the CellTiter-Glo® luminescent cell viability assay (Promega, Mannheim, Germany). This assay quantifies the ATP present, which signals the presence of metabolically active cells. Arising luminescence, produced by the luciferase-catalyzed reaction of luciferin and ATP, was measured using a counter (Top Count, Canberra-Packard GmbH, Dreieich, Germany). In brief, 100 μl CellTiter-Glo® reagent was added to the well containing cells, BSM, and 100 μl medium supernatant. After an incubation period of 10 min at room temperature, the luminescent signal was recorded in counts per second. Additionally, standard measurements with defined cell numbers (standard curves) were performed with each BSM in order to assure that interferences of the signal with the biomaterial could be excluded. For each cell type and BSM, three independent experiments (n=6 each) were performed.

Statistical analysis

A software package (SPSS 15.0, SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Cell viability factors (CVF) were calculated by dividing the respective ATP signal counts through the ATP signal counts after 2 h incubation period (baseline). After evaluation of means and standard deviations, baseline- and 14-day CVFs were tested for each cell line and BMC for significant changes using the Mann–Whitney test. Results were considered statistically significant at $P<0.05$.

Results

The pH of the medium with bone substitute material ranged between pH 7.2 and 7.4, independent of BSM or incubation time. The BMMC differentiation was investigated qualitatively by histological staining of osseous alkaline phosphatase activity in cells seeded onto BSM on culture slides. A positive signal of AP activity was present on all BSM with increasing BMMC viability: TC, CER, P-15, and BB (Fig. 1).

Adherent cell cultures

The viability of primary osteoblasts expressed as cell viability factor over time is presented in Figs. 2, 3, and 4. Cell viability varied enormously on the different BSM. Increasing PO viability over time was evaluated for TC (17.64), control (15.91), CER (14.7), and BB (6.24), whereas P-15 (0.74), BOS (0.48), and OM (0.01) showed a statistically significant reduction of the signal (Fig. 2) BMMC displayed increasing CVF on C (20.78), TC (16.98), CER (14.06), P-15 (13.42), and BB (2.85) and decreasing factors on OS (0.11) and BOS (0.03; Fig. 3; Mann–Whitney test, respectively; Table 2).

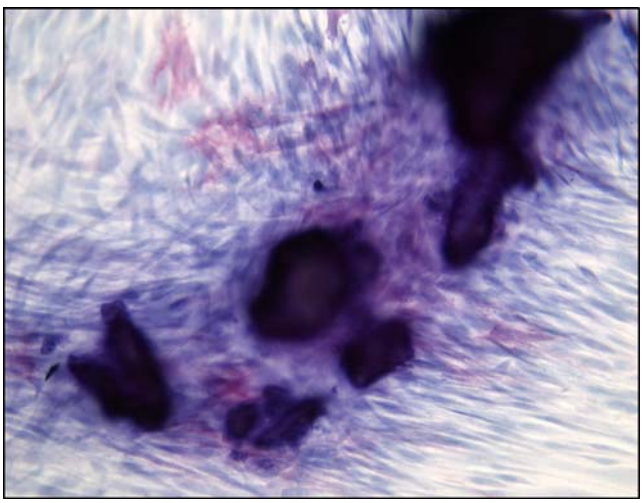

Fig. 1 BMMC on CER, positive AP staining

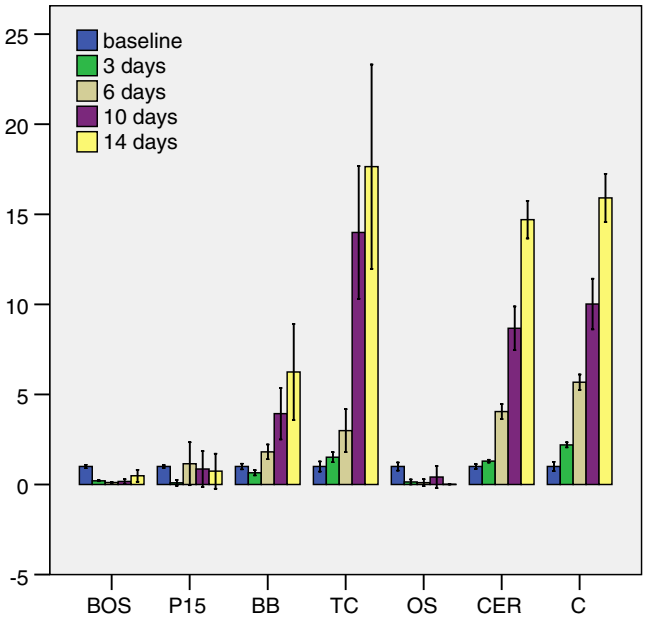

Fig. 2 Cell viability factors of PO on different BSM after 0 (baseline), 3, 6, 10, and 14 days

Nonadherent cell cultures

Regarding nonsurface-dependent effects (Fig. 4), increasing CVF of U937 was found for CER (2.4), BB (2.74), C (2.07), and BOS (1.75). No statistically significant changes were found for TC (0.85) and P-15 (0.77), whereas OM (0.03) showed decreasing cell viability (Mann–Whitney test, respectively; Table 2).

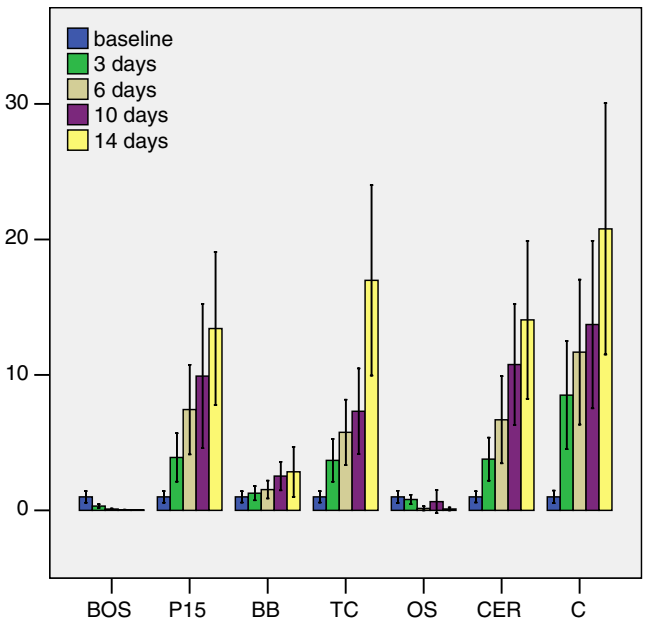

Fig. 3 Cell viability factors of BMMC on different BSM after 0 (baseline), 3, 6, 10, and 14 days

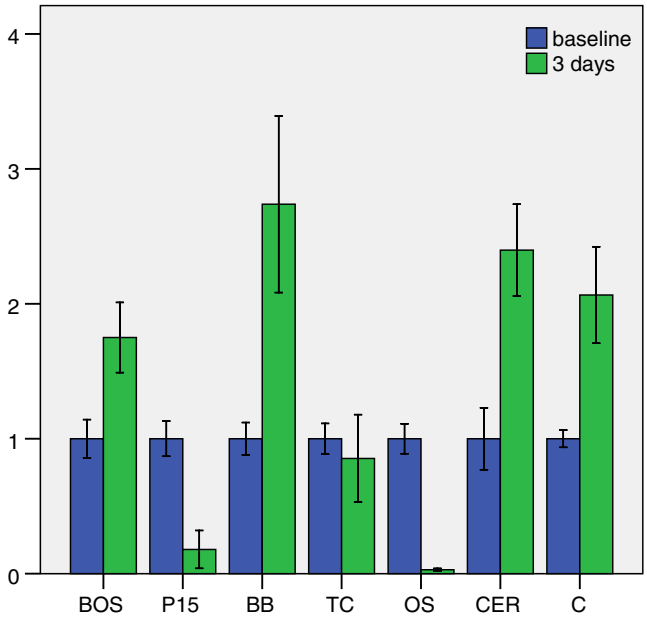

Fig. 4 Cell viability factors of suspension cells U 937 in the presence of BSM after 0 (baseline) and 3 days

Discussion

In this experimental study, the viability of primary osteoblasts and BMMC cultivated on a broad range of BSM was evaluated. To separate nonsurface-dependent effects, further experiments were done with nonadherent cells in the presence of respective amounts of BSM.

Concerning the different hydroxyapatite tested, the highest viability expressed in the cell viability factor could be seen for PO on TC followed by the control, BOS, and P-15. BMMC displayed the highest CVF on TC and P-15, being similar to the control. For OM, neither PO nor BMMC displayed any cell viability after 14 days incubation period. The viability of the suspension cells increased in the presence of BOS and arrested with TC and P-15. Again, no viability could be detected in the presence of OM. These results indicate that granular HA allows for a high cell viability, whereas addition of special peptides (P-15) did not seem to enhance the cell viability significantly. OM seems to display cytotoxic properties in the test system used which might be related to the high amount of free water in the material (65% according to company information). Since the used BSM volume per well of approximately 40 µl corresponded with 91 mg OM (Table 1), an increase of approximately 59 µl free water to a total volume of 160 µl medium per well decreases the osmotic value of the medium enormously resulting in cell death of also non-adherent cells in the presence of this material.

When comparing cell growth on HA with TCPs, it could be shown that both adherent cell types revealed increasing

Table 2 Means, standard deviations, and *P*-values of cell viability factors at baseline and final incubation time (OS—14 days, BMS—14 days, U937—3 days)

	PO			BMMC			U937		
	0	14	*P*-value	0	14	*P*-Value	0	3	*P*-Value
BOS	1.00 (0.09)	0.48 (0.33)	0.009	1.00 (0.43)	0.03 (0.02)	0.002	1.00 (0.14)	1.75 (0.26)	0.002
P-15	1.00 (0.08)	0.74 (0.97)	0.065	1.00 (0.43)	13.42 (5.64)	0.002	1.00 (0.13)	0.77 (1.44)	0.004
BB	1.00 (0.16)	6.24 (2.67)	0.002	1.00 (0.42)	2.85 (1.84)	0.041	1.00 (0.12)	2.74 (0.65)	0.002
TC	1.00 (0.28)	17.64 (5.67)	0.002	1.00 (0.42)	16.98 (7.03)	0.002	1.00 (0.11)	0.85 (0.32)	0.537
OM	1.00 (0.23)	0.01 (0.01)	0.002	1.00 (0.45)	0.11 (0.11)	0.015	1.00 (0.11)	0.03 (0.01)	0.002
CER	1.00 (0.13)	14.70 (1.04)	0.002	1.00 (0.41)	14.06 (5.82)	0.002	1.00 (0.23)	2.40 (0.34)	0.002
C	1.00 (0.26)	15.91 (1.34)	0.002	1.00 (0.47)	20.78 (9.27)	0.002	1.00 (0.06)	2.07 (0.36)	0.002

viability on both CER and BB. Moreover, cell viability testing of U937 cells displayed the highest CVF for CER and BB followed by the control.

The differences in viability of osteoblasts on BOS are in line with the findings of Trentz et al. [30], who investigated the biocompatibility on BOS using a mouse calvarial-derived osteoprogenitor cell line (MC3T3-E1) and human osteoblasts. They could demonstrate that osteoblast proliferation on hydroxyapatite was decreasing after 3 days, whereas the osteoblast-like cell line showed comparable proliferation to the control group. The authors concluded that BOS disturbs the proliferation of osteoblasts.

Wiedmann-Al-Ahmad et al. [32] incubated human osteoblast-like cells on 16 different biomaterials and investigated cell proliferation and cell colonization. In line with our study, BOS showed low proliferation rates. Deligianni et al. [12] found that increased surface roughness of HA improved short- and long-term response of BMMCs in vitro. They suggested a selective adsorption of serum proteins being responsible for this effect. In further experiments, the contribution of fibronectin (FN) preadsorption on osteoblast adhesion on hydroxyapatite substrates was explored [13]. Hereby, two different surface roughness values (rough HA180 and smooth HA1200) were compared. It was found that FN preadsorption and rough HA surface texture synergistically increased in vitro both number and adhesion strength of human osteoblasts.

In contrast to our findings, other reports [1] showed good results for the cultivation of primary osteoblasts on blocks of BO and P-15 for a period of 2, 4, and 6 weeks. The different results could be due to different protocols since (a) the BSM was in blocks and not granular, (b) the cells were seeded in a higher density, (c) the BSM was preocculated with the cells in a smaller volume of medium, and (d) confluence was reached after 4 weeks while our experiments were focused on earlier time points as 3, 6, 10, and 14 days.

In this context, it has to be mentioned that most of the materials evaluated show good clinical results in a high number of clinical studies. Particularly, BOS is well known as BSM showing predictable results and good clinical outcome [15, 24, 33]. A possible explanation to the contrast to the present in vitro experiments for BOS might be that the surface properties of the HA change when in contact with blood proteins and extracellular matrix components [12]. In vitro assays are not without their limitations especially because of loss of influence from the surrounding tissue and the complexity of factors and mechanical forces observed in vivo [22].

For OM recently, several application in reconstructive surgery in human [14], lateral alveolar ridge augmentation in human [29], and in guided bone regeneration animal models [7] were published indicating good clinical results. In opposition, we found that the use of OM in fresh extraction sockets in dogs [25] revealed a remaining gap up to 0.2 mm surrounding some graft areas between the graft and the old bone of the former alveolar wall. The present in vitro investigation may explain the gap as a tissue reaction to the reduced osmotic value around the OM, resulting from the release of free water from the OM paste. In the present results, cell viability of the paste OM was very low, and experiments with nonadherent U-937 cells discovered this being not a surface-related effect but rather a problem of toxicity probably due to a decrease in osmotic value of the medium. Therefore, in this in vitro model, it has to be concluded that OM has a negative effect on cells in vitro.

In contrast to the present study, Kubler et al. [18] found the highest osteoblast proliferation on P-15 compared to the control on polystyrene, followed by BB. However, in this experiment, a much lower density of the bone substitute material (16 mg/cm^2 of P-15 compared to 94 mg/cm^2 P-15 FA in the present study) was used, leaving ample space between the particles. Beside on BSM, cells could adhere on the polystyrene surface as well which might have an positive effect on cell proliferation.

In line with the present TCP results, Aybar et al. [3] found that primary osteoblasts grew equally on CER as on the control. Mayr-Wohlfarth et al. [20] cultivated SaOs-2

osteoblast-like cells on α-TCP. Cells proliferated slightly better on BB than on the polystyrol control.

Within the limits of the present study, it may be concluded that granular hydroxyapatite (TC, BOS, P-15) and α- and β-TCP (CER, BB) provide high cell viability and allow cell proliferation on the surface. Nanosized HA-paste (OM) displayed nonsurface-related negative effects on cell viability in the vitro model used.

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