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β-Tricalcium-phosphate stimulates the differentiation of dental follicle cells

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Abstract The use of dental progenitor cells is a straightforward strategy for regenerative dentistry. For example a cell based therapy with dental follicle cells (DFCs) could be a novel therapeutic strategy for the regeneration of oral tissues in the future. For the regeneration of large bone defects for example dental progenitor cells have to be combined with bone substitutes as scaffolds. This study therefore investigated cell attachment (scanning electron microscopy), cell vitality/proliferation (WST-1 assay) and cell differentiation (under in vitro conditions) of human DFCs on synthetic β-tricalcium phosphate (TCP). DFCs showed considerable cell attachment and proliferation on TCP. Moreover, TCP stimulates osteogenic differentiation in comparison to DFCs with a standard protocol. Here, for example, the osteoblast marker bone sialoprotein (BSP) was highly expressed on TCP, but almost absent in differentiated DFCs without TCP. In conclusion, our study shows that TCP is an excellent scaffold for DFCs for oral tissue regeneration.

1 Introduction

The goal of regenerative dentistry is the regeneration of lost dental tissues such as the periodontium after periodontitis. The periodontium consists of three different tissues: periodontal ligament (PDL), cementum and alveolar bone, which originates from the dental follicle, a tissue of the tooth germ [1, 2]. This tissue contains stem cells and progenitor cells, which could be isolated for example from dental follicles of impacted human third molar teeth or murine tooth germs [3–6]. These dental follicle cells (DFCs) proliferate well under in vitro conditions and they can form periodontal tissues under in vitro and in vivo conditions [3, 7, 8]. Dental mesenchymal cells such as dental pulp stem cells [9] and DFCs are neural crest derived and probably more suitable for dental tissue regeneration than bone marrow derived mesenchymal stem cells. Recently, DFCs in combination with tricalcium-phosphates (TCP) are discussed as novel available therapeutic strategy to restore periodontal tissue [10].

Tissue engineering with dental progenitor cells is a straightforward strategy to obtain periodontal tissues including the alveolar bone. For the engineering of mineralized tissues progenitor cells should be combined with scaffolds that consist of hydroxyl-apatite (HAP) or TCP. While bone substitutes are routinely used, bone augmentation is still problematic step. Dental stem cells in combination with bone substitutes may accelerate the augmentation of alveolar bone. Although dental cells are routinely combined with granular HAP for in vivo differentiation in immunocompromised mice [3, 9], we do not know very much about the adherence and the vitality of dental cells on implant materials. Recently, Kasaj and coworkers investigated the adherence and proliferation of PDL fibroblasts on nanostructured HAP bone replacement grafts in vitro. They demonstrated that PDL fibroblasts attach and proliferate on nanostructured HAP, which were derived from synthetic granular HAP [11]. PDL fibroblasts do also adhere and proliferate on chitosan or a combination

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of chitosan and nanostructured-HAP [12]. Here, the combination of chitosan and nanostructured-HAP was favoured by PDL fibroblasts. The adhesion and proliferation of apical pulp derived cells on HAP was demonstrated recently by Abe et al. [13]. These cells in combination with HAP had an excellent hard tissue regeneration capacity [13]. However, we could show in an unpublished study that DFCs did not adhere or grow on HAP or nanostructured-HAP (unpublished data). Moreover, osteogenic differentiation of DFCs in combination with HAP was only modest under in vivo conditions [3].

Previous studies suggest that DFCs have a limited capacity to differentiate into mineralized tissue cells [14]. However, recent studies have also demonstrated that human DFCs can regenerate bone tissue in a critical size defect animal model [15, 16]. Here, Honda and colleagues used a cell pellet of DFCs without a scaffold. Until today, less is known about the vitality and the capacity of osteogenic differentiation of DFCs on implant materials under in vitro conditions. This study therefore investigated for the first time the adhesion, the proliferation and the differentiation of DFCs on TCP under in vitro conditions.

2 Materials and methods

2.1 Cell culture

Impacted human third molars were surgically removed and collected from patients with informed consent. Dental follicle cells (DFCs) were isolated as described previously [3]. The ethics commission of the University of Regensburg has approved the isolation of DFCs and the experimental design of the project. Isolated DFCs were characterized and compared with dental stem cells for deciduous teeth in a previous study [17]. Briefly, more than 90% of DFCs express Notch-1 and 35% express the mesenchymal stem cell marker Stro-1 [17]. Cells were routinely cultivated in DMEM (PAA) supplemented with 10% fetal bovine serum (PAA) and 100 µg/ml Penicillin/Streptomycin (standard cell culture medium). For experiments DFCs were used at cell passage 6. DFCs were cultivated on standard cell culture plates or cell culture dishes without any treatment of the surface.

2.2 Implant materials

A β-tricalcium-phosphate (TCP) bone substitute was purchased from Curasan (Cerasorb®).

Implant materials were washed with PBS and cell culture medium before use. Experiments with TCP were done with $10 \times 5 \times 5$ mm blocks. 2×10^5 DFCs in 200 µl culture medium were transferred on TCP blocks. Surfaces of cell culture plates, which were used for cultivation of DFCs on TCPs, were pre-treated (1 h) with 1% BSA (Sigma-Aldrich) to reduce unwanted cultivation of DFCs on cell culture plates. TCPs were not treated with BSA. For measurements of alkaline phosphatase (ALP) activity measurements and the isolation of total RNA in DFCs, implant materials with DFCs were transferred in a fresh well or chamber-slide with cell culture medium. DFCs were intensively washed three times with PBS before isolation of total RNA or ALP activity detection. Cell vitality was estimated after 2 and 6 days after seeding on implant materials by WST1 assay (Roche, Mannheim, Germany). WST-1 is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells.

2.3 Osteogenic differentiation

For osteogenic differentiation, DFCs were stimulated with dexamethasone. The dexamethasone standard osteogenic differentiation medium (ODM) comprised DMEM (PAA) supplemented with 10% fetal bovine serum (PAA), 100 µmol/l ascorbic acid 2-phosphate, 10 mmol/l KH_2PO_4, 1×10^{-7} mol/l dexamethasone sodium phosphate (Sigma-Aldrich), HEPES (20 mmol/l) and 100 µg/ml Penicillin/Streptomycin. For control, long-term cultures of DFCs were carried out in DMEM, 10% FBS and 100 µg/ml Penicillin/Streptomycin (DMEM).

2.4 Alkaline phosphatase (ALP) activity

For quantitative evaluation of alkaline phosphatase activity the phosphatase assay kit (Jena Biosciences) was used. The DNA concentration of each sample was estimated with Quant-iT^{TM} PicoGreen ® Kit (Invitrogen) according to the manufacture's protocol. The specific ALP activity of each sample was calculated by the alkaline phosphatase activity and the DNA concentration. The relative ALP activity was based on the specific ALP activity of the control (100%).

2.5 Scanning electron microscopy (SEM)

For SEM, the implant materials with DFCs (constructs) were washed in PBS and fixed in 2.5% glutaraldehyde in PBS. After fixation, constructs were washed in PBS and examined in low-vacuum modus with a scanning electron microscope (Fei Quanta 400 FEG, FEI Europe B.V., Eindhoven, Holland).

2.6 Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cell with NucleoSpin® RNA II. In order to digest contaminating genomic DNA, RNA

was treated with DNAse I. The cDNA synthesis was performed using 400 ng total RNA and ReverAid™ M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany). Quantitative PCR was performed with LightCycler SybrGreen Master kit (Roche Applied Science). Sequences for primers are listed in Table 1 (supplemental materials). Quantitative RT-PCR (qRT-PCR) was performed with the Light Cycler 2.0 (Roche). The Light Cycler software was used for estimation of threshold cycles (Cp-value). *RPS18* gene expression was used for normalization of each sample (housekeeper gene). Quantification was done with the delta/delta calculation method. A total RNA sample from DFCs in standard medium on standard cell culture dishes was used for calibration (relative gene expression = 1).

3 Results

DFCs were seeded on TCP materials for the analysis of cell adhesion. To verify the presence of viable cell on TCP a cellular metabolic activity (WST1-assay) was estimated after 2 and 6 days (Fig. 1a). The vitality (cell number) increased after 6 days of cultivation. DFCs on implant materials and implant materials without cells were analysed with SEM after 6 days of incubation in standard cell

Table 1 Primers for PCR analysis

Gene	Foreward 5′ → 3′	Reverse 3′ → 5′
Cementum protein 23 (*CP23*)	CTAGCCCTGTGGACCAACC	CCAGTCCAGAGCTGGTGAG
Fibronectin (*FN1*)	AGGCTCAGCAAATGGTTCAG	TCTGATAGTGTTTTCCATTGTCATAA
Osteopontin (*SPP1*)	GAATCTCCTAGCCCCACAGAATGC	TTCGGTTGCTGGCAGGTCCG
RUNX2	GTGCCTAGGCGCATTTCA	GCTCTTCTTACTGAGAGTGGAAGG
Bone sialoprotein (*BSP*)	CAATCTGTGCCACTCACTGC	TCATTTTGGTGATTGCTTCCT
RPS18	ACCTCACCAAGAGGGCGGGAG	CACGAAGGCCCCAGAAGTGACG

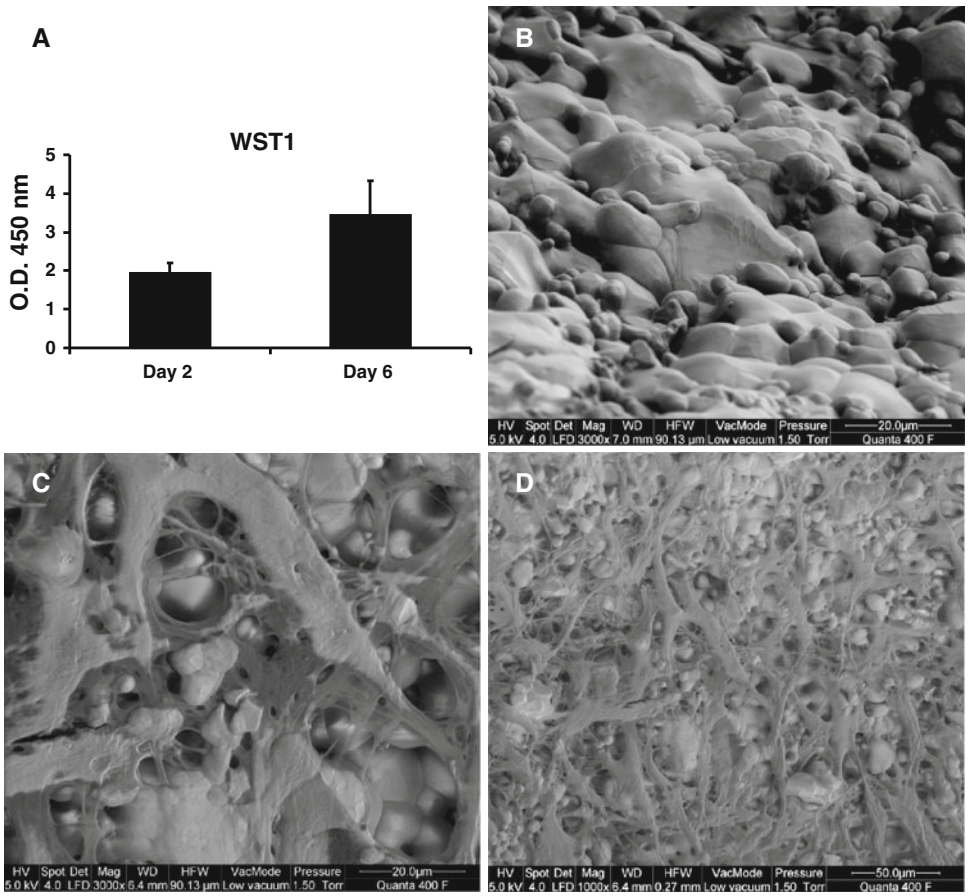

Fig. 1 **a** WST1 assay with DFCs cultivated on TCP for 2 and 6 days. Values are averages ± SD of three biological replicates. SEM pictures of TCP of our study without **b** and with DFCs **c**, **d** cultivated in standard cell culture medium for 6 days

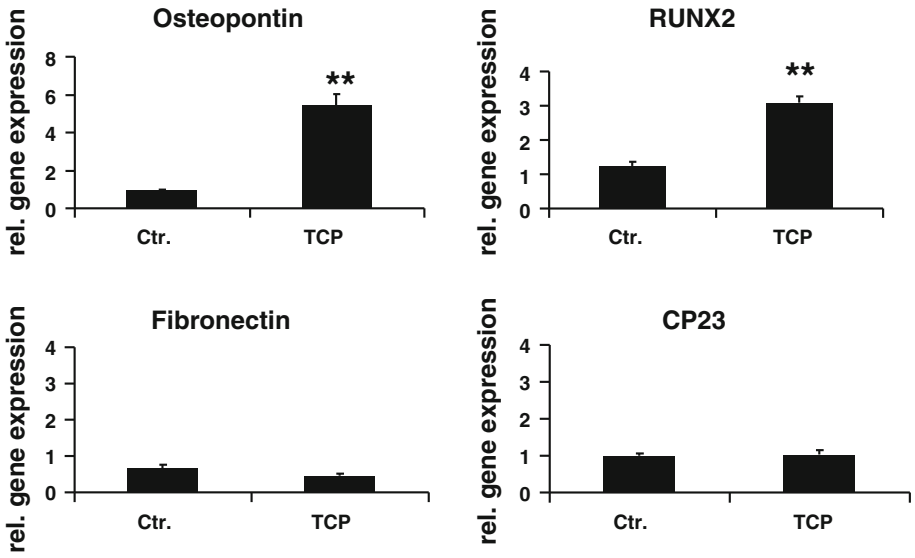

Fig. 2 Relative gene expression of ostegenic markers *osteopontin*, *RUNX2*, *fibronectin* and the cementoblast marker *CP23* in DFCs on TCP and in standard cell culture dishes (control; ctr.). All values are averages ± SE of three biological replicates. For statistics a Student's *t* test was applied between DFCs cultivated in standard cell culture dishes (control) and on TCP (*$P < 0.05$; **$P < 0.01$)

culture medium. The TCP material of our study consists on rounded shaped structures of various sizes (1 µm–more than 20 µm) with a smooth surface. DFCs on TCP had a typical flattened-shaped morphology with close contacts to the bone substitute (Fig. 1c, d).

Real-time RT-PCR analyses were carried out to investigate the expression of osteogenic differentiation markers on TCP after 2 days. Here, osteogenic markers such as osteopontin or RUNX2 were increased on TCP (Fig. 2). In contrast, the gene expression of fibronectin and the cementoblast marker CP23 was not up-regulated on TCP (Fig. 2).

DFCs were cultivated in differentiation medium for 7 days to evaluate osteogenic differentiation on TCP. The ALP activity was increased on TCP (Student's *t* test, $P = 0.09$) in differentiation medium (Fig. 3). Gene expression of osteogenic differentiation markers was increased in differentiated DFCs on TCP in comparison to osteogenic differentiated DFCs on cell culture dishes (Fig. 4). Here, the expression of the osteoblast marker bone sialoprotein (BSP) was significantly increased after differentiation on TCP.

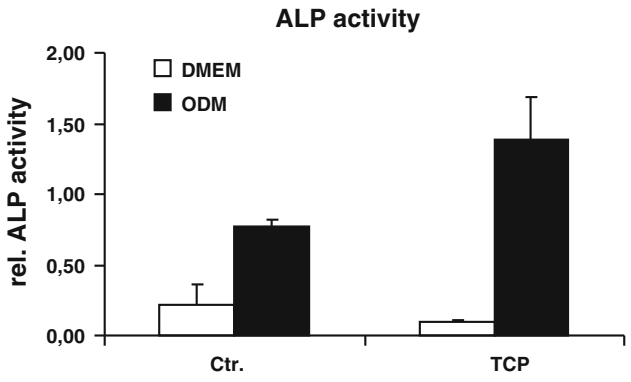

Fig. 3 Estimation of the specific ALP activity of DFCs after cultivation in standard cell culture dishes and on TCP. DFCs were cultivated in standard cell culture medium (DMEM) and differentiation medium with dexamethasone (ODM). All values are averages plus SE of three biological replicates

4 Discussion

This study investigated for the first time the cell attachment, cell proliferation and cell differentiation of DFCs in combination with a commercially available TCP, which has a considerable market share in the clinics. In a previous study, we had used HAP particles as a scaffold for the in vivo differentiation of DFCs [3]. Here we found only a modest osteogenic differentiation of DFCs. Moreover, we did not find cell attachment and survival of DFCs on HAP implant materials under in vitro conditions (unpublished data). This study evaluates TCP as a scaffold for DFCs under in vitro conditions. The assessment of the biocompatibility for a specific cell type such as DFCs is a crucial step for the initialization of additional experiments such as tissue engineering.

DFCs showed attachment and survival on TCP of our study, which is a suitable material for the filling of bone defects in the alveolar region [18]. DFCs proliferate on TCP for at least 6 days of cultivation and cell numbers increased. However, previous studies revealed a reduced number of pre-differentiated human cord blood stem cells after long term cultures on TCP [19]. Although cell numbers decreased between days 1 and 7 in this previous study, the gene expression of osteogenic cell differentiation markers was increased on TCP, if gene expression was compared to cells on an insoluble collagenous bone matrix or on Bio Oss® [19]. In contrast, Marino et al.

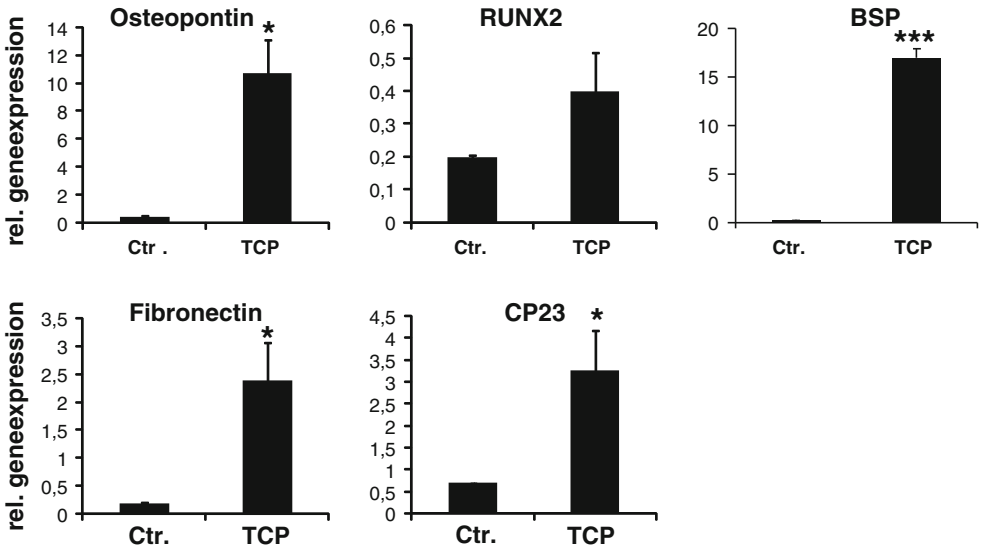

Fig. 4 Relative gene expression of ostegenic markers *osteopontin*, *RUNX2*, *fibronectin*, *bone sialoprotein* (*BSP*), the cementoblast marker *CP23* in DFCs on standard cell culture dishes and on TCP after 7 days in osteogenic differentiation medium. All values are averages ± SE of three biological replicates. (Student's *t* test $*P < 0.05$; Student's *t* test $***P < 0.001$)

demonstrated that TCP scaffolds promote cell proliferation and osteogenic differentiation of human adipose stem cells [20]. In our study, the expression of osteogenic cell markers in DFCs on TCP was up-regulated and the ALP activity in differentiated DFCs on TCP was increased, when compared to differentiated DFCs under standard cell differentiation conditions. Although cells of different studies are heterogenic (adipose derived stem cells, cord blood stem cells and DFCs) results of our and previous studies suggest that TCP support the differentiation of undifferentiated precursor cells. However, Seebach et al. [21] showed that TCP products from different suppliers differ substantially in their morphology and that surface or porous structure seems to be of importance for the cell seeding. Moreover, modifications of implant materials such as fibronectin coating of TCP or composites with a combination of polymer of poly glycolic-lactic acid (PGLA) with TCP may also influence cell attachment and proliferation of seeded cells [21, 22]. Although cell vitality of DFCs was high on TCP with standard cell culture conditions, more sophisticated protocols are recommended to improve the versatility of TCP for tissue engineering. An optional use of cell culture reactors may also increase the number of viable cells on TCPs or other implant materials [23]. This will be our next step.

It is recommended that bone substitutes should be tested individually with precursor cells before more sophisticated studies such as animal testings can be performed. Our study reveals that DFCs attach, proliferate and differentiate on TCP (Cerasorb). In comparison to a standard protocol on ordinary cell culture dishes, TCP improves the osteogenic differentiation of DFCs. Our study showed that TCP is an excellent scaffold for DFCs and should be used for dental tissue engineering with this cell type.

Conflict of interest None.

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