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The poly (l-lactid-co-glycolide; PLGA) fiber component of brushite-forming calcium phosphate cement induces the osteogenic differentiation of human adipose tissue-derived stem cells

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Abstract

A brushite-forming calcium phosphate cement (CPC) was mechanically stabilized by addition of poly (l-lactid-co-glycolide; PLGA) fibers (≤10% w/w). It proved highly biocompatible and its fiber component enhanced bone formation in a sheep lumbar vertebroplasty model. However, possible effects on the osteogenic differentiation of resident mesenchymal stem cells (MSCs) remained unexplored. The present study used a novel approach, simultaneously analyzing the influence of a solid CPC scaffold and its relatively low PLGA proportion (a mimicry of natural bone) on osteogenic, chondrogenic, and adipogenic differentiation, as well as the pluripotency of human adipose tissue-derived mesenchymal stem cells (hASCs). hASCs were cultured on CPC discs with/without PLGA fibers (5% and 10%) in the absence of osteogenic medium for 3, 7, and 14 d. Gene expression of osteogenic markers (Runx2, osterix, alkaline phosphatase, collagen I, osteonectin, osteopontin, osteocalcin), chondrogenic markers (collagen II, Sox9, aggrecan), adipogenic markers (PPARG, Leptin, and FABP4), and pluripotency markers (Nanog, Tert, Rex) was analyzed by RT-PCR. The ability of hASCs to synthesize alkaline phosphatase was also evaluated. Cell number and viability were determined by fluorescein diacetate/propidium iodide staining. Compared to pure CPC, cultivation of hASCs on fiber-reinforced CPC transiently induced the gene expression of Runx2 and osterix (day 3), and long-lastingly augmented the expression of alkaline phosphatase (and its enzyme activity), collagen I, and osteonectin (until day 14). In contrast, augmented expression of all chondrogenic, adipogenic, and pluripotency markers was limited to day 3, followed by significant downregulation. Cultivation of hASCs on fiber-reinforced CPC reduced the cell number, but not the proportion of viable cells (viability > 95%). The PLGA component of fiber-reinforced, brushite-forming CPC supports long-lasting osteogenic differentiation of hASCs, whereas chondrogenesis, adipo genesis, and pluripotency are initially augmented, but subsequently suppressed. In view of parallel animal results, PLGA fibers may represent an interesting clinical target for future improvement of CPC-based bone regeneration.

Introduction

Osteoporosis is a systematic skeletal disease characterized by reduced bone strength and increased susceptibility to fractures [1]. Fractures mostly occur in the proximal femur, vertebral bodies, and distal radius. Minimally-invasive methods (e.g. vertebroplasty and kyphoplasty) are increasingly used for the treatment of vertebral body fractures [2-4]. However, the currently used poly(methyl methacrylate) (PMMA) cements show several disadvantages [5], e.g. monomer toxicity, extravasation [5, 6], lack of bioactivity and
biodegradability, and a higher loading resulting in the induction of new fractures in the neighboring vertebral bodies [7, 8]. Injectable calcium phosphate cements (CPCs) are a promising alternative material due to their excellent biocompatibility, bioactivity, and resorbability [9–11]. However, their low tensile, shear, and mechanical strength limits their use in load-bearing bone areas, e.g. vertebral bodies [12–14]. One possibility to improve the mechanical strength of the CPC is the addition of reinforcing fibers [15, 16]. Recently, a poly(lactide-co-glycolide) (PLGA) fiber-reinforced CPC with an increased strength and stiffness showed good biocompatibility and improved bone regeneration [17–19].

Mesenchymal stem cells (MSCs) are an attractive cell population for tissue engineering, because of their ability to differentiate into several mesenchymal lineages including bone, cartilage, adipose, and muscle tissues under the appropriate culture conditions. The most commonly used source for MSCs is the bone marrow. However, MSCs can be isolated from several other tissues, such as adipose tissue, umbilical cord blood, and fetal tissues [20, 21]. Especially human adipose tissue-derived mesenchymal stem cells (hASCs) are regarded as an alternative for bone marrow-derived MSCs, due to their relatively easy isolation and high cell yield from adipose liposuction tissue [22]. Their capability to differentiate into the osteogenic, chondrogenic, and adipogenic lineages has been extensively characterized using lineage-specific markers [23–28]. Such osteogenic differentiation of hASCs has been shown on a variety of matrices, e.g. PLGA, different calcium phosphates, and collagenses [29–33].

MSCs, either as resident precursor cells at the site of fractures/bone defects or therapeutically combined with osteoconductive materials and osteoinductive growth factors to support bone formation [34, 35], are likely an important component of local bone defect regeneration and may also have contributed to the enhancement of bone formation by the PLGA fiber component of a PLGA fiber-reinforced CPC in a sheep lumbar vertebroplasty model [19].

The present study thus aimed at analyzing the influence of a PLGA fiber-reinforced, brushite-forming CPC on the osteogenic, chondrogenic, and adipogenic differentiation and pluripotency of human adipose tissue-derived mesenchymal stem cells (hASCs).

Materials and methods

Fabrication of PLGA fibers and PLGA fiber-reinforced cement

PLGA fibers and fiber-reinforced cement were prepared as previously described [17]. PLGA fibers (diameter: 25 μm) were cut to 1 mm length with a cutting mill (PULVERISETTE 19; FRITSCH GmbH, Idar-Oberstein, Germany).

The CPC powder contained 98.5% (w/w) β-tricalcium phosphate [β-TCP] and 1.5% (w/w) tetrasodiumpyrophosphate, the liquid consisted of 3.0 M phosphoric acid, 0.1 M sulfuric acid, and 0.1 M citric acid (all Sigma-Aldrich, St. Louis, MO, USA). CPC powders with different fiber content [0, 5 or 10% (w/w)] were fabricated, autoclaved, and mixed with the liquid in a powder-to-liquid ratio of 2.2.

For gene expression analysis, 200 μl of CPC paste with/without fibers were added to each well of a 12-well plate under sterile conditions and allowed to set for 24 h. For viability testing, CPC discs with the 3 different fiber concentrations were instead cast in silicon forms (diameter: 8.0 mm; height: 1.0 mm). The cement paste was thoroughly mixed and then transferred to the forms for setting. The next day, the CPC discs underwent sterilization by autoclaving at 131 °C and 210 kPa for the 20 min. CPC discs were pre-washed for a total of 24 h with repeated changes of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 × 2 H2O, 2 mM KH2PO4; pH 7.4) to remove harmful components/additives, as previously published [36], and then used without delay.

Isolation of hASCs

hASCs for the current study were isolated using a well-established method described previously [37, 38]. For cell isolation, subcutaneous adipose tissue was collected from both male and female subjects (n = 6, mean age 45.2 ± 4.8 years). The study was approved by the ethics committee of the University Hospital Jena (registration number: 3331-21/11) and all donors gave written consent prior to the procedure. The tissue was washed three to four times with an equal volume of pre-warmed PBS for 5 min to remove blood components. An equal volume of pre-warmed collagenase solution [0.1% type I collagenase (Roche, Mannheim, Germany) and 1% bovine serum albumin dissolved in PBS supplemented with 2 mM calcium chloride] was then added to the tissue samples and incubated at 37 °C for 60 min. After the collagenase digestion, the sample was spun down at 300 × g at room temperature (RT) for 5 min. For the disaggregation of stromal cells from primary adipocytes, the sample was shaken vigorously to disrupt the pellet and to mix the cells. Thereafter, the sample was spun down again at 300 × g at RT for 5 min. The top layer of fat, oil, and primary adipocytes and the underlying collagenase solution was carefully removed. The hASC pellet was re-suspended in PBS and spun down again at 300 × g at RT for 5 min. The supernatant was removed, the cells were suspended in DMEM/F12 with 10% FCS, 1% Gentamycin (10 mg ml−1), and 1% Penicillin/Streptomycin (10,000 Units ml−1, 10 mg ml−1, respectively; all Invitrogen, Darmstadt, Germany) and cultured in 225 cm² flasks for 7 d (1 × 10⁶ cells/flask; medium change every 2 d). Thereafter, hASCs were trypsinized, characterized by flow cytometry, and
subjected to anti-CD34 negative purification to remove CD34-positive hematopoietic progenitor cells.

Characterization of hASCs by flow cytometry
For characterization of hASCs by flow cytometry, the appropriate cell number (2 × 10^5 cells/FACS tube) was spun down at 290 g for 3 min, the supernatant was removed, and pre-diluted antibodies were added. Fluorochrome-labeled antibodies were purchased from BD Pharmingen, Heidelberg, Germany (CD73, CD90, CD31), Beckmann Coulter, Marseille, France (CD14, CD34, CD45) or Ancell, Bayport, MN, USA (CD29, CD44, CD105). Cells were incubated for 30 min at 4°C, followed by washing thrice with PBS/1% FCS. Surface antigen expression was analyzed using a FACScan and the Cell Quest software (both BD Bioscience, San Jose, CA, USA). Forward and side scatter gates were set to include all viable cells. For quantification of the percentage of cells positive for each surface antigen, a gate was set to exclude 99% of the cells stained with isotype control IgG.

Anti-CD34 negative purification of hASCs
Anti-CD34 negative purification was performed as previously described for anti-CD14 negative purification of synovial fibroblasts [39]. Briefly, trypsinized hASCs from primary culture (10^7 cells ml^-1) were washed twice with PBS/2% FCS and then incubated with 4 × 10^5/ml Dynabeads® CD34 (Invitrogen) in PBS/2% FCS at 4°C for 1 h with bidirectional rotation. Nine milliliters of PBS/2% FCS were added and the conjugated cells removed with a Dynal magnetic particle concentrator®. Unconjugated cells were harvested, washed 2× in PBS, counted, and frozen in FCS/10% DMSO in liquid nitrogen until further use.

Cell differentiation assays
Dissociation of hASCs into the osteogenic, chondrogenic, or adipogenic lineage was performed as previously described [40]. For osteogenic differentiation, confluent hASCs were cultured for 21 d on plastic cell culture dishes with low-glucose DMEM (1000 mg l^-1 D-glucose) containing 5% human serum and osteogenic supplements (0.1 mM dexamethasone, 50 mM L-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate; all Sigma-Aldrich). Medium was changed every second day. Osteogenic differentiation was assessed by staining the calcium component of calcium phosphate deposits with alizarin red.

Chondrogenic differentiation of hASCs was achieved in high-density pellet cultures (2.5 × 10^5 cells/pellet) under serum-free conditions. Chondrogenesis was induced by the addition of 10 ng ml^-1 TGF-β (Peprotech, Hamburg, Germany) and cells were cultured for 28 days (medium change every 2 to 3 d). Chondrogenic differentiation was analyzed by embedding micro-masses in OCT compound, freezing, and cryosectioning (6 mm thickness). Sections were stained (Alcian blue; Roth, Karlsruhe, Germany) to visualize the deposition of charged extracellular matrix molecules, e.g. proteoglycans.

For adipogenic differentiation, hASCs were plated on culture dishes at a density of 1 × 10^5 cells cm^-2 and, 3 d after reaching confluence, stimulated for 15 d with high-glucose DMEM (Invitrogen Corporation; 4500 mg l^-1 D-glucose) containing 5% human serum and the adipogenic supplements 1 mM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methyloxanthine, and 10 mg ml^-1 insulin (all Sigma-Aldrich). Intracellular lipid droplets in adipogenic cultures were visualized using oil red O staining (Sigma-Aldrich).

Controls for the 3 differentiation studies were done by omitting the respective osteogenic, chondrogenic, or adipogenic supplements.

Cell viability assay
The cell viability assay was performed by seeding 4 × 10^5 cells on CPC discs with/without fibers in 48-well plates and cultivation for 3, 7, or 14 d. Viability was then evaluated using fluorescein diacetate/propidium iodide life/dead staining. According to the protocol, cells were washed with Dulbecco’s PBS (DPBS; 137.93 mM NaCl, 2.67 mM KCl, 0.90 mM CaCl_2, 0.49 mM MgCl_2, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and incubated with 6 μM fluorescein diacetate in DPBS for 2 min at 37°C, followed by staining with propidium iodide for 1 min at RT (0.2 μg ml^-1 DPBS). Shortly after staining, four images were obtained from each disc with a fluorescence microscope Axiovert200M, an AxioCam MRm camera, and the AxioVision Rel4.8 program (all Zeiss, Oberkochen, Germany). Viable and dead cells were counted using the cellprofiler software [www.cellprofiler.org; [41]], and used to calculate the mean percentage of viable cells.

RNA isolation, cDNA synthesis, and RT-PCR
For gene expression analysis, 2 × 10^5 cells were seeded on CPC with/without fibers in 12-well plates and cultured for 3, 7, or 14 d. Thereafter, total RNA was isolated from the hASCs by adding the lysis buffer component of a commercially available RNA isolation kit (Macherey & Nagel, Düren, Germany) directly to the CPC discs and then reverse-transcribed as previously described [42]. mRNA expression of the osteogenic markers Runt-related transcription factor 2 (Runx2), osterix, alkaline phosphatase, type I collagen, osteonectin, osteopontin, and osteocalcin, the chondrogenic markers Sox9, type II collagen, and aggrecan, the adipogenic markers peroxisome proliferator-activated receptor gamma (PPARG), leptin, and fatty acid binding protein 4 (FABP4), as well as the pluripotency markers Nanog, Rex1, and Telomerase reverse transcriptase 2 (Tert2), and the house-keeping
gene aldolase was analyzed by real-time PCR using a RealPlex® PCR machine (Eppendorf, Hamburg, Germany). Primer pairs and PCR conditions are shown in table 1. The relative mRNA concentrations of the analyzed genes in each sample were calculated by the RealPlex® software using an external standard curve. The specificity of the real-time PCR was confirmed by: (i) melting curve analysis; (ii) agarose gel electrophoresis; and (iii) initial cycle sequencing of the PCR products.

**Alkaline phosphatase activity assay**

Alkaline phosphatase activity was assessed by conversion of para-nitrophenyl phosphate to para-nitrophenol. Four \(4 \times 10^5\) hASCs were seeded on CPC with/without fibers in 48-well plates. After 1, 2 and 3 d the cells were washed once with PBS followed by incubation in alkaline phosphatase lysis buffer for 1 h at 37 °C (100 mM glycine, 1 mM MgCl₂, and 1% Nonidet P40 (Roche Diagnostics, Penzberg, Germany)). These early time points were chosen, since subsequently the constitutive alkaline phosphatase activity considerably increases. For alkaline phosphatase activity determination, 50 μl of the cell lysates were incubated with 50 μl alkaline phosphatase reagent consisting of 2× diethanolamine buffer containing p-nitrophenylphosphate (0.0148 mg PNPP/1 ml buffer; both Pierce, Rockford, IL, USA). After an incubation time of 30 min at 37 °C, the absorbance was measured at 405 nm in an ELISA-reader (Fluostar, BMG Labtech, Ortenberg, Germany). The concentration of 4-nitrophenol was calculated using a standard curve (ranging from 200 to 3.13 μM) and the resulting concentrations were normalized to the cell number (number of viable cells in the cell viability assays).

**Table 1.** Primer sequences, annealing (\(T_{\text{annealing}}\)) and melting (\(T_{\text{melting}}\)) temperatures, and gene accession numbers applied in RT-PCR.

| Gene                  | Forward/reverse primers                                                                 | \(T_{\text{annealing}}\) °C | \(T_{\text{melting}}\) °C | Accession number |
|-----------------------|----------------------------------------------------------------------------------------|----------------------------|---------------------------|-----------------|
| Runx2                 | FW: gccttcaggggtgtagccc Rev: cgttaaggcagctgacata                                     | 60                         | 79                        | NM_001024630    |
| Runt-related transcription factor 2 | Rev: tgcctgaggaggaagtccc | 60                         | 78                        | NM_001173467    |
| Osterix               | Rev: aggcctccgacacagtagta                                                          | 60                         | 79                        | NM_000478       |
| Alkaline phosphatase | Rev: agacccatctcctctc                                                            | 60                         | 85                        | NM_000088       |
| Collagen I            | Rev: ccaagcagccgagcagtagt                                                         | 60                         | 79                        | NM_003118       |
| Osteopontin (OPN)     | Rev: attgtacgggagcctactc                                                          | 60                         | 82                        | NM_001040058    |
| Osteocalcin           | Rev: attgtacgggagcctactc                                                          | 60                         | 79                        | NM_199173       |
| Sox9                  | Rev: ttgagcagccgagctgtagt                                                          | 60                         | 85                        | NM_000346       |
| Collagen II           | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_0011844      |
| Aggrelna              | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_0001135      |
| PPARG                 | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_0153869      |
| Peroxisome proliferator -activated receptor gamma                  | Rev: attgtacgggagcctactc                                                          | 60                         | 76                        | NM_000230       |
| Leptin                | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_0011442      |
| FABP4                 | Rev: attgtacgggagcctactc                                                          | 60                         | 85                        | NM_024865       |
| Nanog                 | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_174900       |
| Rex1                  | Rev: attgtacgggagcctactc                                                          | 60                         | 78                        | NM_198253       |
| Tert2                 | Rev: attgtacgggagcctactc                                                          | 58                         | 82                        | NM_000034       |

Statistical analysis

The results were presented as means ± SEM. The non-parametric Kruskal–Wallis and Mann–Whitney U tests and the IBM SPSS Statistics 21 program were used to assess statistical significance (\(p < 0.05\)).

**Results**

**Characterization of hASCs**

hASCs expressed surface mesenchymal stem cell markers after 7 d of culture (CD29: 85.4% ± 3.5%;
CD44: 86.5% ± 2.3%; CD73: 57.8% ± 6.2%; CD90: 94.7% ± 1.2%; CD105: 77.2% ± 9.0% (figures 1(A) and (B)). Only few cells carried markers of monocytes, leukocytes, and endothelial cells (CD14: 2.6% ± 0.4%; CD45: 11.0% ± 2.9%; CD31: 8.1% ± 1.5%, respectively). However, 30.9% ± 7.4% of the cells expressed the hematopoietic progenitor cell antigen CD34 on their surface. Anti-CD34 negative purification reduced the proportion of CD34-positive cells to 2.9% ± 1.3% (figures 1(A) and (B)).

Multi-lineage differentiation assay
In comparison to the respective culture in control medium (figures 2A, C, E, and G), purified hASCs stimulated with differentiation media showed the capacity for osteogenic differentiation (as assessed by detecting calcium phosphate deposits by phase contrast microscopy or by staining with alizarin red; figures 2B and D, respectively), chondrogenic differentiation (as shown by the deposition of charged extracellular matrix molecules such as proteoglycans; figure 2F), and adipogenic differentiation (as visualized by intracellular lipid droplet formation; figure 2H).

Influence of PLGA fiber reinforcement on viability and number of hASCs
hASCs cultivated on CPC with/without fibers showed the typical vital morphology of MSCs with a spread-out cytoplasm and long cytoplasmatic processes (figure 3).
There was no influence of PLGA fiber reinforcement on the viability of hASCs (viability > 95% for all groups; figures 3 and 4(A)), excluding any toxic effects of the fibers.
On the other hand, the number of hASCs cultivated on PLGA fiber-reinforced CPC (5% and 10%) significantly decreased from day 3 to days 7 and/or 14 (figures 3 and 4(B)).
For these parameters, there were no significant differences between pure CPC and fiber-reinforced CPC, or between CPC with 5% and 10% PLGA fibers.

Influence of PLGA fiber-reinforced CPC on mRNA expression of osteogenic markers and alkaline phosphatase activity
Compared to pure CPC, the mRNA expression of the osteogenic transcription factors Runx2 and osterix in hASCs cultivated on PLGA fiber-reinforced CPC was upregulated on day 3 (Runx2: 5% fibers: numerical; 10% fibers: significant upregulation; osterix: both fibers
concentrations numerical upregulation; figures 5(A) and (B)). On days 7 and 14, however, PLGA fiber-reinforced CPC numerically or significantly downregulated the expression of these genes, resulting in several significant differences between day 3 and the subsequent time points (figures 5(A) and (B)).

In contrast, PLGA fiber reinforcement numerically or significantly induced the expression of the osteogenic markers alkaline phosphatase, collagen I, and osteonectin at all analyzed time points, with a general decrease of the levels from day 7 to day 14 (figures 5(C)–(E)).

PLGA fiber reinforcement also transiently upregulated the gene expression of osteopontin and osteocalcin on days 3 and 7, with a subsequent decrease on day 14 (figures 5(F) and (G)).

The differences between CPC with 5% and 10% PLGA fibers were not significant for any of the analyzed osteogenic markers.

The levels of alkaline phosphatase enzyme activity were augmented by the presence of 10% PLGA fibers in the CPC at all time points (days 1, 2 and 3; \( p \leq 0.05 \) versus pure CPC on day 2), but not by 5% PLGA (figure 6).

Influence of PLGA fiber-reinforced CPC on the mRNA expression of chondrogenic, adipogenic, and pluripotency markers

The mRNA expression of the chondrogenic markers Sox9, collagen II, and aggregan was transiently upregulated by PLGA fiber reinforcement on day 3, but then strongly downregulated on days 14 and/or 7 (figures 7(A)–(C); please note numerous significant differences between day 3 and the subsequent time points).

Very similar results were observed for the adipogenic markers PPARG, Leptin, and FABP4 (figures 7(D)–(F)) and the pluripotency markers
Nanog, Rex1, and Tert2 (figures 7(G)–(I)), indicating an only transient activation of chondrogenesis, adipogenesis, and pluripotency by PLGA fiber-reinforced CPC, but a long-lasting induction of osteogenesis (figure 5).

As in the case of the osteogenic markers, the differences between CPC with 5% and 10% PLGA fibers were not significant.

**Discussion**

The main focus of the study was the influence of a newly developed PLGA fiber-reinforced, brushite-forming CPC on the osteogenic differentiation of hASCs (as a model primary MSC population). The results showed the following aspects: (i) PLGA fiber-reinforced CPC supported the osteogenic differentiation of hASCs, as demonstrated by an upregulation of several osteogenic markers (Runx2, osterix, alkaline phosphatase, collagen I, osteonectin, and osteopontin); (ii) the osteogenic differentiation coincided with or followed an early chondrogenic differentiation on day 3; (iii) in parallel, PLGA fiber-reinforced CPC initially augmented, but subsequently suppressed the adipogenic differentiation and pluripotency of hASCs. In view of the parallel augmentation of bone formation by the fiber component of a PLGA fiber-reinforced CPC in a sheep lumbar vertebroplasty model [19], PLGA fibers may thus be an interesting target for an improved clinical effect of CPC-based bone replacement materials.

**MSC features of hASCs**

In agreement with previous reports, the hASCs used in the present study carried CD29, CD44, CD73, CD90, and CD105, and were mainly negative for CD14, CD45, CD31 [21, 22, 24, 25, 43], identifying these cells as mesodermal/mesenchymal precursors. However, a substantial percentage of these cells also expressed CD34 on their surface (approx. 31%). A wide range of percentages has been reported for CD34-positive cells in hASC cultures (ranging from about 4% to 60% [23–25, 43]), likely reflecting differences in tissue source, isolation protocol, and passage of the cells [43, 44]. Since CD34-negativity is regarded as one of the minimal criteria for the definition of multipotent mesenchymal stromal cells [45, 46], the hASC preparations in the present study were negatively purified (<3% positive cells after anti-CD34 depletion) and thereafter used for the experiments. As further support of their primary MSC character, the hASCs were able to differentiate into the osteogenic, chondrogenic, and adipogenic lineages (figure 2; [22, 44]).
In observation period number and viability of hASCs affecting the proportion of viable cells scaffolds of hASCs on different calcium phosphate-containing studies observed a continuous increase in proliferation the expansion of hASC are controversial. Several effects of calcium phosphate-containing scaffolds on differentiation of cells reduction in the proliferation may also point toward a osteogenic potential, but lower proliferation than the neous human bone marrow-derived MSC population, titanium surfaces induced apoptosis in a heteroge- proliferation of MSCs by the dissolution of these scaffolds inhibits the PLA component, respectively. The lactic acid released only a slight increase higher osteogenic potential under the appropriate et al [32, 33, 47] in analogy to differentiation process [57].

Effects of PLGA fiber-reinforced CPC on mRNA expression of osteogenic markers in hASCs and alkaline phosphatase activity

Cultivation of hASCs on PLGA fiber-reinforced CPC induced an early upregulation (day 3) of the transcription factors Runx2 and osterix. Both transcription factors play a pivotal role in the differentiation of pre-osteoblasts to osteoblasts by the upregulation of osteoblast-specific genes [38, 39]. Indeed, a concomitant upregulation of the expression of several osteoblast- specific markers in hASCs by PLGA fiber-containing CPC was observed in the current study, namely alkaline phosphatase, collagen I, osteonectin, and osteopontin. For alkaline phosphatase, collagen I, and osteonectin, the upregulation persisted over the whole observation period, indicating a (partial) osteogenic differentiation of hASCs under the influence of PLGA-reinforced CPC. The osteogenic effects of the fibers were further confirmed at the protein/enzyme level by an increased activity of alkaline phosphatase. This osteogenic differentiation may contribute to the augmented bone regeneration induced by this novel PLGA fiber-containing CPC in sheep vertebroplasty [19].

A direct comparison of the present results with previously published data is hampered by differences in the scaffold composition. Whereas in the current study the CPC was fiber-reinforced to improve its biomechanical properties while retaining its injectability [17], other studies used polymer scaffolds (PLGA, PLA, or PCL) with different calcium phosphate coatings (β-TCP or hydroxyapatite [HA]; 5%–33%) to improve the osteogenic differentiation of hASCs [29, 47, 49, 60]. Despite these differences, interestingly, both types of studies showed a maximal increase of alkaline phosphatase, collagen I, and osteonectin on day 7 [47, 60] and a decreased expression of osteocalcin on day 14 [47], indicating a similar time course of osteogenic differentiation under considerably different experimental conditions.

Influence of PLGA fiber-reinforced CPC on the mRNA expression of chondrogenic, adipogenic, and pluripotency markers in hASCs

PLGA fiber reinforcement transiently upregulated the expression of chondrogenic, adipogenic, and pluripotency markers in hASCs on day 3, but substantially downregulated these markers thereafter. This indicates that PLGA fiber-reinforced CPC initially leads to a broad activation of numerous potential differentiation pathways (in analogy to differentiation

Influence of PLGA fiber reinforcement on cell number and viability of hASCs

Cultivation of hASCs on PLGA fiber-reinforced and also pure CPC reduced the cell number over the whole observation period (14 d; 1.8-fold reduction) without affecting the proportion of viable cells (>95%). The effects of calcium phosphate-containing scaffolds on the expansion of hASC are controversial. Several studies observed a continuous increase in proliferation of hASCs on different calcium phosphate-containing scaffolds [32, 33, 47], whereas others reported no or only a slight increase [48, 49]. These differences may be explained by differences in the scaffold composition. The scaffolds in the studies of Buschmann et al and Haimi et al, for example, contained a PLGA or PLA component, respectively. The lactic acid released by the dissolution of these scaffolds inhibits the proliferation of MSCs [50]. On the other hand, a reduction in the proliferation may also point toward a differentiation of cells [51, 52]. Indeed, hydrophilic titanium surfaces induced apoptosis in a heterogeneous human bone marrow-derived MSC population, resulting in an enrichment of stem cells with a higher osteogenic potential, but lower proliferation than the parental population [53]. This selection of cells with a higher osteogenic potential under the appropriate conditions may be based on different subpopulations in the parental MSCs [54–56]. In addition, an expression shift from proliferative genes to apoptotic genes, and the suppression of cell cycle-associated genes, may contribute to a lower cell number during the differentiation process [57].
experiments without biomaterials; [57]), but then strongly and selectively favors osteogenic differentiation. To our knowledge, this is the first study addressing the induction of osteogenic markers such as alkaline phosphatase, collagen I, and osteonectin in parallel to the effects on other lineages in a scaffold content. Other studies have previously demonstrated that the differentiation into one lineage suppresses differentiation into other lineages (e.g. osteogenesis suppresses adipogenesis [61]; chondrogenesis suppresses osteogenesis [62]), but these effects have not yet been investigated in the context of bone replacement materials.

Mechanistic hypotheses

Several features of biomaterials, such as the current PLGA fiber-reinforced CPC, are involved in providing multidimensional, 3D support for bone regeneration, including the promotion of migration, attachment, anchorage, proliferation and/or differentiation, and function of MSC [63, 64]. These features include both solution-mediated effects (i.e. factors released from PLGA fibers, CPC, or adherent MSC [36, 65], and surface-mediated effects resulting from the direct contact of the MSC with the biomaterial [18, 66, 67]). Specific features of the current, PLGA fiber-reinforced CPC possibly supporting osteogenic differentiation of the hASCs include favorable pore size [17, 66] and increased tensile and/or flexural strength of the CPC [17, 68], increased stiffness of fibers or CPC [17, 66], suitable fiber diameter and anisotropy [17, 66, 67], augmented coating of the fibers with calcium phosphates [18, 69], and autocrine or paracrine effects of soluble mediators released from adjacent or remote hASCs [65].

Novel aspects of the present study

Most previous studies reporting on the matrix-driven osteogenic differentiation of stem cells have utilized polymer/CPC composites based on PLA, PLGA or polycaprolactone fibers(67%–95%) coated with a calcium phosphate (β-TCP or HA; 5%–33%; [29, 47, 49, 60]). In contrast, the present study shows an induction of osteogenic differentiation of hASCs by the PLGA fiber component (10%) in a solid CPC, i.e. a system intended to mimic natural bone consisting of HA and collagen fibers. In addition, the induction of osteogenesis was observed in the absence of osteogenic medium or growth factors such as BMPs, further stressing the osteogenic potential of a relatively low proportion of the PLGA component. These effects may thus contribute to the augmented bone regeneration induced in vivo by this PLGA fiber-containing CPC in sheep vertebroplasty [19].

As already mentioned above, another novel aspect is the simultaneous analysis of the influence of this solid CPC scaffold on the hASC differentiation into different lineages.
Limitations of the present study

In vivo, bone healing and concurrent differentiation of MSCs is a complex cascade of a tightly regulated spatio-temporal release of cytokines and growth factors [70], also including a permanent supply of MSCs from the blood stream. These cascades cannot be completely mimicked by in vitro systems. However, direct in vivo studies with large animals raise ethical concerns and generally require demanding surgical and technical designs, in particular when addressing the local release of CPC components and the differentiation of MSCs directly at the bone implant interface. Despite its limitations, therefore, the present simplified in vitro model of co-cultivation of MSCs...
with CPC provides data that may be very useful for a meaningful design of future in vitro and in vivo studies on these issues.

Conclusion

The current study demonstrates that a newly developed, PLGA fiber-containing, brushite-forming CPC favors the osteogenic differentiation of MSCs, namely hASCs, as seen by an early upregulation of osteoblast-specific transcription factors (Runx2 and osterix) and a long-lasting expression of the osteoblast-specific markers alkaline phosphatase, collagen I, and osteonectin. In contrast, there was only a transient upregulation of chondrogenic, adipogenic, and pluripotency markers. Of note, the osteoblast-specific genes in hASCs were induced in the absence of osteogenic medium.

The induction of osteogenic differentiation by PLGA fiber reinforcement may partially explain the augmentation of bone regeneration by the PLGA-containing CPC in a sheep model of vertebroplasty. Therefore, a PLGA fiber reinforcement of CPC may be suitable to further augment the bone regeneration in clinical use.

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