In vitro osteogenic potential of human bone marrow stromal cells cultivated in porous scaffolds from mineralized collagen

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Abstract: Porous 3D structures from mineralized collagen were fabricated applying a procedure in which collagen fibril reassembly and precipitation of nanocrystalline hydroxyapatite (HA) occur simultaneously. The resulting matrices were evaluated in vitro with respect to their suitability as scaffolds for bone tissue engineering. We found a high capacity of the material to bind serum proteins as well as to absorb Ca$^{2+}$ ions, which could be advantageous to promote cell attachment, growth, and differentiation. Human bone marrow stromal cells (hBMSCs) were seeded onto the 3D scaffolds and cultivated for 4 weeks in the presence and absence of osteogenic supplements. We studied viability, proliferation, and osteogenic differentiation in terms of total lactate dehydrogenase (LDH) activity, DNA content, and alkaline phosphatase (ALP) activity. Furthermore, the expression for bone-related genes (ALP, bone sialo protein II (BSP II), and osteocalcin) was analyzed. In our investigation we found a 2.5-fold to 5-fold raise in DNA content and an increase of ALP activity for osteogenic induced hBMSC on collagen HA scaffolds. The expression of ALP and BSP II in these cells was also stimulated in the course of cultivation; however, we did not detect an upregulation of osteocalcin gene expression. These data suggest, that porous collagen HA scaffolds are suitable for the expansion and osteogenic differentiation of hBMSC and are therefore promising candidates for application as bone grafts. © 2008 Wiley Periodicals, Inc.

Key words: mineralized collagen; marrow stromal cells; alkaline phosphatase; osteogenic gene expression; 3D scaffolds

INTRODUCTION

The extracellular matrix of natural bone consists of two main components, collagen type I and nanocrystalline hydroxyapatite (HA), which form together a highly organized nanocomposite. In the field of bone engineering many attempts aim in mimicking the composition of natural bone matrix in order to obtain scaffold materials with properties close to those of the tissue. The high demand for bone replacement materials, which should be bioresorbable, osteoconductive, and osteoinductive led to various strategies which combine collagen I with HA. Collagen-HA composites were and are investigated in numerous compositions and manufacturing methods (recently reviewed by Wahl and Czernuszka). A high number of these efforts involves sintered HA which is known for its poor resorbability. The generation of biomimetic collagen-HA composites which are produced under nearly physiological conditions and therefore reach good resorbability is highly advantageous. A promising strategy is the synchronous mineralization during collagen fibril reassembly, leading to a homogenous nanocomposite material, developed in our group. This composite (“mineralized collagen”) was used to create several biomaterials like a membrane with pores only in the micrometer range (“tape”). In vitro studies with this collagen-HA tape demonstrated the positive impact of the composite material on osteogenic differentiation of bone marrow stromal cells. Furthermore, we investigated osteoclastogenesis of human monocytes on the tapes and could show that osteoclasts are capable of resorbing the material in vitro. By cocultivating osteoblasts with osteoclasts on the collagen-HA tapes—which can be seen as an artificial extra-
cellular bone matrix—we are trying to establish an in vitro model for the processes of bone remodeling.\(^6\)

Since three-dimensional matrices are more suitable as bone grafts and bigger pores favor the in growth of osteogenic cells and allow fast vascularisation, we developed sponge-like porous scaffolds from the biomimetically mineralized collagen.\(^7\) A first in vivo study revealed good biocompatibility and faster resorption compared to other HA-collagen composites.\(^8\) Combining the 3D scaffolds made of mineralized collagen with (nonmineralized) collagen hyaluronic acid composites we recently presented biphasic, but monolithic scaffolds for the therapy of osteochondral defects.\(^9\) In the present study we performed first in vitro experiments to evaluate the influence of the three-dimensional porous scaffolds from mineralized collagen on proliferation and osteogenic differentiation of human bone marrow stromal cells (hBMSCs). Among the stromal cells in mammalian bone marrow there exists a multipotent population of cells which are referred to as mesenchymal stem cells or marrow stromal cells (MSC). Because of their capability for self replication and differentiation along various cell lineages,\(^10–12\) MSC are potentially useful for tissue engineering applications. To analyze how appropriate the 3D collagen-HA scaffolds would be for a prospective use in human bone regeneration, the interaction between osteogenic cells and the porous mineralized collagen matrix was investigated. We studied the proliferation and osteogenic differentiation of hBMSCs, seeded on 3D collagen-HA. Hereby osteogenic differentiation was evaluated in terms of alkaline phosphatase (ALP) activity and expression analysis of bone-related genes.

**MATERIALS AND METHODS**

**Preparation of 3D scaffolds from mineralized collagen**

Mineralized collagen was prepared using a biomimetic method developed in our lab.\(^3\) Using this nanocomposite material, porous three-dimensional scaffolds were generated by a freeze-drying process followed by chemical crosslinking of the collagen. The procedure was described in detail previously.\(^7\) Briefly, acid-soluble collagen I (isolated from bovine tendon and kindly provided by Syntacoll (Saal/Donau, Germany) was dissolved in 10 mM hydrochloric acid and mixed with a calcium-chloride solution. The pH was adjusted to 7 by addition of TRIS and phosphate buffer and the mixture was warmed to 37°C for 12 h. Under these conditions the collagen fibril reassembly and formation of nanocrystalline HA occurs simultaneously. The reaction was performed under air, so carbonated HA was formed. The product—a nanocomposite consisting of homogeneously mineralized collagen fibrils—was collected by centrifugation as a wax-like, colorless material. To prepare the 3D porous scaffolds used in this study, the mineralized collagen was mixed with the mother liquid and stirred up until a just-castable suspension was formed, which was used to fill in the cavities of a 48-well plate and frozen at −25°C. The samples were freeze-dried and then crosslinked with a 1% solution of N-(3-dimethylamino propyl)-N′-ethyldiamine (EDC) hydrochloride in 80% (vol %) ethanol for 1 h. Finally, the scaffolds were rinsed thoroughly in distilled water, in 1% glycine solution, once again in water, and finally freeze dried. The samples for use in the study were sterilized with gamma irradiation.

**Absorption of serum proteins and Ca\(^{2+}\)**

Scaffolds from mineralized collagen (10 mm diameter, 5 mm height) were incubated in 2 mL as well as 5 mL DMEM, supplemented with 10% FCS (Biochrom, Berlin, Germany) at 37°C in a humidified, 7% CO\(_2\)/93% air incubator. Medium was changed every three to five days. The Ca\(^{2+}\) concentration of the removed medium was analyzed using a pHOX analyzer (NOVA Biomedical GmbH, Rodearmark, Germany). The total protein content of the medium samples was determined using Coomassie protein assay reagent (Sigma, Taufkirchen, Germany) with bovine serum albumin (Sigma) as standard. The absorbed amounts of Ca\(^{2+}\) and protein was calculated by subtracting the Ca\(^{2+}\) and protein amounts left in the media after incubation from the Ca\(^{2+}\) and protein amount of control media under the same incubation conditions.

**Human bone MSCs**

hBMSCs, isolated from bone marrow aspirate of two healthy male donors (I: 33 years old, II: 31 years old) were kindly provided by Sabine Boxberger, Medical Clinic I, Dresden University Hospital. Expansion of the cells was performed in Dulbecco’s Modified Eagles Medium (DMEM) low glucose (Biochrom, Berlin, Germany), containing 10% FCS, 10 U/mL penicillin, and 100 μg/mL streptomycin (Biochrom) at 37°C in a humidified, 7% CO\(_2\)/93% air incubator. Cells of the fifth passage were used for the seeding of scaffolds. Each passage corresponds to two population doublings.

**Cell seeding and cultivation on 3D mineralized collagen scaffolds**

For the present study we used cylindrical samples from mineralized collagen with 10 mm diameter and 5 mm height. Prior to cell seeding scaffolds were preincubated with cell culture medium for 24 h. For cell seeding equilibration medium was removed and samples were placed on sterile filter paper to remove excess liquid from the pores. Samples were placed in 24-well polystyrene culture dishes and 2 × 10\(^5\) cells were given within 60 l of medium to the top of each cylindrical sample. After 1 h of initial adhesion 1 mL cell culture medium was added to each sample. After 24 h cell seeded samples were transferred to fresh culture dishes. Cell culture medium of half of the samples was supplemented with 10\(^−7\)M dexamethasone.
were also taken on day 1, 7, 14, 21, and 28 of culture.

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thought the scaffolds by suction and these lysates were pushed on filter paper until no liquid leaked and frozen at −80°C until further analysis. For RNA isolation 2 × 700 µL of RLT buffer (Qiagen, Hilden, Germany) were pushed thought the scaffolds by suction and these lysates were stored at −80°C until RNA isolation. These samples (n = 3) were also taken on day 1, 7, 14, 21, and 28 of culture.

Biochemical analysis of ALP activity, DNA content, and total LDH activity

Frozen cell-seeded scaffolds were cut into pieces with a razor blade and transferred each into a 2-mL tube containing six beads of stainless steel (Peqlab, Erlangen, Germany). A total of 900-µL of ice-cold PBS were added and the samples were homogenized using a Precellys24 apparatus (Peqlab). Homogenization process was operated twice 10 s with 5800 rpm. After removing the beads, 100 µL of 10% Triton X-100 were added, the mixture was shaken shortly and then incubated on ice for 50 min. An aliquot of 200 µL of each supernatant was transferred to another tube for subsequent biochemical analysis.

One aliquot of the cell lysate was added to ALP reaction buffer, containing 1 mg/mL p-nitrophenyl phosphate (Sigma), 0.1 M diethanolamine, 1% Triton X-100 (pH 9.8), 1 mM MgCl₂ and the mixture was incubated at 37°C for 30 min. Finally, 1 M NaOH was added to stop the enzymatic reaction. After centrifugation at 16,000g for 10 min the supernatant was transferred to a microtiter plate and the absorbance was read at 405 nm with a multifunction microplate reader (Spectra fluor plus, Tecan, Crailsheim, Germany). A calibration line was constructed from different dilutions of 1 mM p-nitrophenol stock solution.

Another aliquot of the same cell lysate was mixed with LDH reaction buffer (Cytotox96 kit, Promega, Madison). After incubation for 30 min at room temperature 1N acetic acid was added to stop the enzymatic reaction. Absorbance was measured at 492 nm.

A third aliquot of the same cell lysate was mixed with Pico-green ds DNA quantification reagent (Molecular Probes) diluted 1:379 in TE buffer (=10 mM TRIS and 1 mM EDTA) and incubated for 5 min in the dark. The intensity of fluorescence was measured with a multifunction microplate reader (Spectra fluor plus, Tecan) at an excitation and emission wavelength of 485/535 nm. Relative fluorescence units were correlated with the cell number using a calibration line.

Microscopic evaluation

SEM

After 28 days of culture, cell-seeded scaffolds were washed twice with warm PBS and fixed for 30 min with 3.7% formaldehyde in PBS, washed with distilled water, and dehydrated using a gradation series of ethanol/distilled water solutions. Critical point drying was performed with a CPD 030 apparatus (BAL-TEC AG, Liechtenstein). Sections of the samples were created using a razor blade, coated with gold, and imaged using a Philips XL 30/ESEM with FEG (field emission gun), operating in SEM mode.

cLSM

After 2 and 28 days of culture, cell-seeded scaffolds were washed twice with warm PBS and fixed for 30 min with 3.7% formaldehyde in PBS. For staining cells were permeabilized using 0.2% Triton in PBS, followed by five washes with PBS and blocking of materials autofluorescence with 3% bovine serum albumin (Sigma) in PBS. Cytoskeleton of the cells was stained using 50 µg/mL FITC phalloidin (Sigma), nuclei were stained using 0.3 µM DAPI (Sigma). Prior to microscopic investigation scaffolds were cut with a razor blade into sections.

Confocal LSM studies were performed using a cLSM 510 meta (Zeiss, Jena, Germany) mounted on an upright Axioskope 2 FS mot and equipped with an additional NIR-femtosecond-2-photons excitation unit (coherent Mira 900F). Excitation of DAPI was carried out with NIR-fs-laser at 770 nm (emission 376–494 nm), FITC phalloidin was excited with an Ar⁺-ion laser at 488 nm.

Real-time PCR

RNA containing lysates were purified using RNeasy Mini Kit (Qiagen). During this procedure residual DNA was digested with Rnase free DNase I (Qiagen), according to manufacturer’s instructions. cDNA was synthesized from 1 µg of total RNA per 20 µL reaction mix containing 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM of each dATP, dGTP, dCTP, and dTTP, 500 ng of oligo(dT)₁₅ primer (Promega, Mannheim, Germany) and 200 U of Super-Script™ III RNase H⁻ reverse transcriptase (Invitrogen, Karlsruhe, Germany) in 50 mM Tris-HCl, pH 8.3. Real time PCR reactions were performed using RotorGene FG-3000 PCR
machine (Corbett, Wasserburg, Germany). For PCR experiments, 1 μL of total cDNA was amplified in a 20 μL reaction mix containing 20 pmol of forward and reverse primer and 10 μL of QuantiTect® SYBR green mixture (Qiagen). After initial activation step at 95°C for 15 min, PCR cycles were run for denaturation at 94°C for 30 s, annealing (for temperatures, see Table I) for 30 s, and for synthesis at 72°C for 45 s. Primers were constructed by use of Primer Quest program (http://biotools.idtdna.com/biotools/primerquest/primer_quest.asp) (for primer sequences, see Table I). Identity of the PCR products was verified by sequence analysis. The same single stranded cDNA was used to analyse the expression of all genes described. The relative expression values are counted using the comparative quantification method of the RotorGene software release 6.0.

Statistical analysis

All results are shown as mean ± standard deviation. Statistical analysis was performed using a two-tailed, unpaired Student’s *t*-test. Significant differences were assumed at *p* < 0.05.

RESULTS

Absorption of serum proteins and Ca\(^{2+}\) onto the scaffolds

We found a high capacity of porous 3D mineralized collagen scaffolds to bind serum proteins as well as to absorb Ca\(^{2+}\) ions from the medium. Incubation of mineralized collagen scaffolds with cell culture medium containing 10% FCS led to a decrease of protein concentration in the regularly changed medium over the whole incubation time of 42 days. No repletion of the scaffold was observed within this period. The final uptake of protein was influenced by the volume of the incubating medium and found to be around 7.2 mg per scaffold, when incubated in 2 mL medium (Fig. 1).

In the case of Ca\(^{2+}\) accumulation a similar behavior was observed. Over the whole investigation period, a permanent decrease of Ca\(^{2+}\) content in the regularly changed medium was detected. The Ca\(^{2+}\) uptake also depended on the volume of incubation medium. When incubating scaffolds in 1 mL medium, the Ca\(^{2+}\) concentration in the surrounding medium decreased under the detectable level (data not shown). After 42 days the final uptake of Ca\(^{2+}\) was determined as 50 μmol/scaffold when incubated with 5 mL cell culture medium, and 24 μmol/scaffold when incubated with 2 mL cell culture medium (Fig. 1).

Histological evaluation of cell penetration

To evaluate the efficiency of the applied cell seeding procedure, slices taken orthogonal to the seeded

<table>
<thead>
<tr>
<th>Primer Pairs Used for Real-Time RT-PCR</th>
<th>Forward Primer Sequence (5’ – 3’)</th>
<th>Reverse Primer Sequence (5’ – 3’)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
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<tr>
<td>Actin</td>
<td>AAC GGC CCC GCC GGC TTT GAT TGC TTA GGA TGG CAA GGG ACT TCC TGT</td>
<td>AAT GTG GCC GAG GAC TTT GAT TGC TTA GGA TGG CAA GGG ACT TCC TGT</td>
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<tr>
<td>ALP</td>
<td>AAG CAC TCC CAC TTC ATC TGG A TCC GTC ACG TTG TTC CTG TT</td>
<td>AAG CAC TCC CAC TTC ATC TGG A TCC GTC ACG TTG TTC CTG TT</td>
<td>65</td>
</tr>
<tr>
<td>BSPII</td>
<td>AAG CAT GCC TAC TTT TAT CCT CAT T CAT TCG ATT CTT CAT TGT TTT CTC C</td>
<td>AAG CAT GCC TAC TTT TAT CCT CAT T CAT TCG ATT CTT CAT TGT TTT CTC C</td>
<td>58</td>
</tr>
<tr>
<td>OC</td>
<td>CCA GCT CTG CTT GAA CCT ATT TTA ACT GTT TAT ACC CTC TGG GCT GT</td>
<td>CCA GCT CTG CTT GAA CCT ATT TTA ACT GTT TAT ACC CTC TGG GCT GT</td>
<td>55</td>
</tr>
</tbody>
</table>

**TABLE I**

ALP, alkaline phosphatase; BSPII, bone sialoprotein II; OC, osteocalcin.
surface of the scaffolds were analyzed using cLSM after fluorescent staining of actin and nuclei 2 days after cell seeding. Cells were detected in all areas of the scaffolds, though showing a higher concentration in areas near the surface compared to the bottom. Figure 2 shows a representative section covering an area near the cell-seeded surface.

Figure 1. Adsorption characteristics of collagen-HA scaffolds incubated in different volumina of complete medium containing 10% FCS as a function of time. (a) protein adsorption, (b) Ca<sup>2+</sup> adsorption.

Figure 2. cLSM image, showing a cross section of cell seeded collagen-HA scaffold after cultivation for 2 days. [Color figure can be viewed in the online issue, which is available at wwwinterscience.wiley.com.]

Figure 3. hBMSC on collagen-HA scaffolds, cultivated with osteogenic supplements (OS+) and without (OS-) over 28 days. Proliferation, evaluated by determination of the DNA content, n = 3 (±standard deviation of the mean). Cell number was calculated from DNA content using a calibration line of known cell numbers. Viability, evaluated by determination of total LDH activity, n = 3 (±standard deviation of the mean). Significant differences from day 1 *p < 0.05, **p < 0.01, ***p < 0.001.
Recovery of active LDH and ALP, as well as DNA from cell lysates after incubation with homogenized 3D mineralized collagen

Recovery experiments were carried out with both entire scaffolds and homogenized scaffolds. We found a nearly complete recovery of active LDH and ALP as well as DNA after incubation with undamaged scaffolds (data not shown). In the case of homogenized samples, the recovery of LDH and ALP activity as well as DNA content was not complete. We found 79% of the LDH activity, 75% of the ALP activity, and 53% of the DNA content.

Proliferation of hBMSC on 3D mineralized collagen

hBMSC and osteogenically induced hBMSC showed good proliferation rates within the three dimensional scaffolds. Determination of DNA content revealed for both donors a significant increase of cell number for osteogenic induced as well as non-induced hBMSC from day 1 to days 7, 14, 21, and 28. (Fig. 3). Coincidental to these findings we furthermore detected a significant increase of total LDH activity from day 1 to day 7 for osteogenic induced hBMSC, as well as a significant increase for osteogenic induced and non-induced hBMSC up to days 14, 21, and 28 of cultivation. These findings imply a boost of viable cells within the cultivation period (Fig. 3). Microscopic evaluation of osteogenic induced hBMSC using both SEM and cLSM revealed a thick cell layer covering the pores of the scaffold after 28 days of cultivation (Fig. 4).

Specific ALP activity

hBMSC of both donors, seeded on 3D mineralized collagen scaffolds showed a significant increase of specific ALP activity after stimulation of osteogenic differentiation. We found a varying behaviour for the cells of different donors. For cells of donor I the highest specific ALP activity was detected on day 28, whereas for donor II the maximum ALP activity was determined at day 21. The same progression of specific ALP activity was found for osteogenically stimulated hBMSC seeded on cell culture polystyrene, which served as control. The specific ALP activity of the non-induced hBMSC on mineralized collagen scaffolds was not raised during the whole cultivation time. The level of specific ALP activity for cells cultivated on 3D scaffolds was comparable with the ALP activity level of cells cultured on polystyrene. (Fig. 5).

Quantitative real-time PCR analysis

The ALP expression pattern of osteogenic induced hBMSC was similar to the pattern of the detected ALP activity. The increase of relative ALP expression from day 1 to days 21 and 28 was significant for the cells of donor I. Significant raised ALP expression was found at day 14 for donor II. Non-induced hBMSC showed no increase in ALP expression over the whole cultivation period. The ALP expression for cells cultivated on 3D scaffolds was somewhat lower compared to the ALP expression of cells cultured on polystyrene. (Fig. 6).
BSP II expression of osteogenic induced hBMSC of both donors seeded on 3D HA collagen scaffolds showed a strong increase at day 28 of cultivation. In contrast BSP II expression of osteogenic induced hBMSC on control polystyrene raised earlier, showing elevated levels from d7 to d28 of cultivation (Fig. 7).

Figure 5. Specific ALP activity of induced (OS+) and non-induced (OS-) hBMSC on collagen-HA scaffolds and cell culture polystyrene over 28 days. n = 3 (± standard deviation of the mean). Significant differences for OS+ from day 1 *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6. ALP expression of induced (OS+) and non-induced (OS-) hBMSC on collagen-HA scaffolds and cell-culture polystyrene over 28 days, n = 3 (± standard deviation of the mean). Significant differences for OS+ from day 1 *p < 0.05, **p < 0.01, ***p < 0.001.
We found no increase of osteocalcin expression for osteogenic induced hBMSC of the examined donors, both when seeded in 3D HA collagen scaffolds and in monolayer culture on polystyrene dishes (Fig. 8).

**DISCUSSION**

The focus of this study was on the impact of porous three dimensional scaffolds made of mineralized collagen on growth and osteogenic differentiation of hBMSC. The porous structure of the material allowed the penetration of cells into the inner parts of the scaffold, as revealed by cLSM investigation of sections. The deep penetration of cells into the inner parts of the scaffold was further demonstrated by MTT staining. However, cells in the interior of the construct are not exposed for extraction of DNA and proteins when applying standard isolation protocols. Consequently, after cell lysis without scaffold

**Figure 7.** BSP II expression of induced (OS+) and non-induced (OS−) hBMSC on collagen-HA scaffolds and cell culture polystyrene over 28 days, n = 3 (± standard deviation of the mean). Significant differences for OS+ from day 1 *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 8.** Osteocalcin expression of induced (OS+) and non-induced (OS−) hBMSC on collagen-HA scaffolds and on cell culture polystyrene over 28 days, two donors in triplicate.
homogenisation we detected only small amounts of DNA, suggesting a low number of attached cells. On the other hand we demonstrated a high seeding efficiency of hBMSC on collagen-HA scaffolds. Therefore we applied homogenisation of the cell-seeded scaffolds to enhance the DNA yield as well as the levels of detected ALP and LDH activity. One disadvantage of the homogenization is the potential loss of DNA and protein due to binding to the scaffold mineral particles. A high correlation between specific surface area of HA particles and the absorbed amount of protein was demonstrated by different studies. Thus, after incubation of cell lysates with homogenized collagen HA scaffolds we recovered only 75% of the initially applied ALP activity and 53% of the DNA content, whereas after incubation with the entire scaffolds ALP activity and DNA content were nearly completely recovered. Despite these finding we applied homogenization of the cell-seeded scaffolds because of the high increase of ALP activity and DNA content in the respective lysates, which multiply exceeds the loss of DNA and protein through binding to the HA particles.

As outlined above, the entire mineralized collagen scaffolds did not bind ALP and LDH during short-time incubation under lysis conditions. In contrast we found a considerable capability to bind serum proteins during long-time incubation of the collagen HA scaffolds with cell-culture medium, supplemented with 10% FCS under cell-culture conditions. The potential of HA-containing materials to bind proteins was demonstrated by several studies. It is anticipated, that the absorption of serum proteins, among them adhesion promoting proteins like fibronectin and vitronectin, promotes cell adhesion to the respective scaffolds. Furthermore the possible binding of growth factors as well as bone-related proteins like osteocalcin to the surface of the HA collagen scaffold could establish a microenvironment with positive impact on osteogenesis. For scaffolds of mineralized collagen we detected continuous protein absorption over 45 days without repletion. These data are in coincidence with a study of Zhao et al. who found a continuous protein uptake of HA/chitosan-gelatin scaffolds at least over 15 days of incubation. In this context preincubation of the HA collagen scaffolds with serum containing medium before cell seeding could be particularly advantageous to promote cell attachment, growth, and differentiation. Moreover, incubation of mineralized collagen scaffolds with cell-culture medium led to a decrease of calcium concentration suggesting that Ca$^{2+}$ ions are absorbed by the material. This behavior of nanocrystalline HA surfaces was reviewed by Cazalbou et al. who propose, that the hydrated surface layer of nanocrystalline HA can trap several ions such as Ca$^{2+}$, Mg$^{2+}$, and HPO$_4^{2-}$. Calcium uptake of HA-containing materials was also shown for HA/$\beta$-TCP ceramics, for HA-coated titanium, and for HA/chitosan-gelatin scaffolds. Ramires et al. demonstrated for HA-coated titanium that the decrease of calcium concentration in the medium is accompanied by the appearance of needle-like calcium and phosphorus containing structures on the materials surface, indicating the precipitation of a calcium phosphate mineral phase. Some studies suggest, that the Ca$^{2+}$ uptake of HA scaffolds is caused by the enhanced absorption of proteins which in turn bind Ca$^{2+}$, acting as nucleating agents of mineralisation. On the other hand several HA-containing ceramics are reported to release Ca$^{2+}$ ions. This varying behavior could be addressed to differences in physicochemical properties of HA like crystallinity and particle size. To examine cell response to the HA collagen surface of the porous 3D scaffolds, enriching with serum proteins and calcium over the cell-cultivation period, we investigated the proliferation of osteogenic induced as well as non-induced hBMSC on these scaffolds. During cultivation over 28 days cell number increased continuously as revealed by determination of DNA content, leading to an 2.5-fold to 5-fold raise of cell number. These findings are supported by the simultaneous augmentation of total LDH activity, which indicates a raise of viable cells over the whole period of cultivation. Interestingly, the LDH activity of osteogenically differentiated hBMSC was higher compared to non-induced cells, whereas the DNA content of induced and non-induced cells was comparable. A possible higher metabolic activity of osteogenic induced hBMSC could be an explanation for this difference. The observed proliferation rates are in a similar range compared to other studies involving MSC and 3D HA/collagen composites. Liu et al. reported the cultivation of rat MSC in 3D collagen/sintered HA composites and found a doubling of cell number after 7 days of cultivation. In coincidence Niemeyer et al. showed a doubling of cell number within 8 days when cultivating hBMSC on collagen sponges coated with sintered HA. However, the authors describe an arrest of proliferation between days 8 and 24, whereas in our study cell number increased consistently over the whole cell culture period. We conclude from our results, that the examined scaffolds from biomimetically mineralized collagen are appropriate to support proliferation as well as viability of hBMSC.

Furthermore we demonstrated a marked increase of specific ALP activity for osteogenically stimulated hBMSC in porous collagen HA scaffolds. Hereby we found differences between cells of donor I and II concerning the extent of stimulation and the time period required to achieve the maximum ALP activity. These findings are consistent with earlier studies on donor variations of the osteogenic potential of...
MScs. Noninduced hBMSC did not show an increase of ALP activity during the investigated period of cultivation, suggesting that the mineralized collagen matrix itself has no stimulating effect on osteogenic differentiation and dexamethasone is required to differentiate the hBMSC towards the osteoblastic lineage. To further evaluate the osteogenic differentiation of hBMSC in HA collagen scaffolds we determined the expression of genes, which are involved in osteogenesis, such as ALP, BSP II, and osteocalcin (OC). The progress of ALP expression was in coincidence with the detected ALP activity. Cells of donor I showed higher ALP activity as well as ALP expression compared to cells of donor II. The osteogenic differentiation of hBMSC was furthermore confirmed by the upregulation of BSP II expression at later time points of cultivation. Here, too, cells of donor I showed higher BSP II expression compared to cells of donor II. Phinney et al. studied donor dependent variations in hBMSC populations and found dramatic differences in the levels of bone-specific gene induction. They furthermore demonstrated that levels of ALP activity are strongly correlated with the ability of a cell population to upregulate the expression of bone specific genes, such as ALP and bone sialoprotein.

We were not able to detect upregulation of OC mRNA in osteogenic induced hBMSC seeded on collagen HA scaffolds as well as on cell-culture polystyrene. There is some evidence that the expression of OC may be suppressed in cultures of hBMSC due to treatment with dexamethasone. Jaiswal et al. reported, that OC mRNA was not detectable in hBMSC cultures in the presence of osteogenic supplements, including 10^{-7}M dexamethasone. Cheng et al. could also not detect OC secretion in dexamethasone treated hBMSC cultures. Furthermore it shown, that dexamethasone attenuates the increased OC expression induced by vitamin D3. A study of Meyer et al. demonstrated, that the human OC gene is transcriptionally repressed by glucocorticoids, which could be an explanation for the low osteocalcin expression in the presence of dexamethasone. In contrast, several studies revealed an increase of OC expression in dexamethasone-treated hBMSC cultures, even in the absence of vitamin D3. Possibly the interaction of dexamethasone with OC expression in hBMSC is influenced by additional components of the medium, culturing substrate, origin of the MSC population and other factors.

CONCLUSIONS

Porous 3D scaffolds from biomimetically mineralized collagen I are suitable for the expansion and osteogenic differentiation of hBMSC. Their absorption properties for serum proteins as well as Ca^{2+} provide a microenvironment which may promote cell adhesion, viability, and osteogenic differentiation. Because of its similarity to extracellular bone matrix this biomaterial is a promising matrix for bone tissue engineering applications.

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