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Mineralised collagen—an artificial, extracellular bone matrix—improves osteogenic differentiation of bone marrow stromal cells

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Abstract In the field of bone tissue engineering there is a high demand on bone graft materials which promote bone formation. By combination of collagen type I with nanocrystalline hydroxyapatite (HA) we generated a resorbable material which structure and composition is close to those of the extracellular bone matrix. This nanocomposit material was produced in a biomimetic process in which collagen fibril assembly and mineralisation with hydroxyapatite occur simultaneously. In this study the proliferation and osteogenic differentiation of human bone marrow-derived stromal cells (hBMSC) on membranes of biomimetically mineralised collagen type I was investigated. To this end, we optimised biochemical assays for determination of cell number and alkaline phosphatase activity corresponding to the special properties of this biomaterial. For cell experiments hBMSC were seeded on the mineralised collagen membranes and cultivated over 35 days, both in static and perfusion culture, in the presence and absence of dexamethasone, β-glycerophosphate and ascorbate. Compared to cells grown on tissue culture polystyrene we found attenuated proliferation rates, but markedly increased activity of alkaline phosphatase on the mineralised collagen indicating its promoting effect on the osteogenic differentiation of hBMSC. Therefore this bone-like material may act as a suitable artificial extracellular matrix for bone tissue engineering. Perfusion of the 2D cell matrix constructs with cell culture medium did not improve proliferation and osteogenic differentiation of the hBMSC.

Introduction

Autologous bone graft is highly regarded for its osteoinductivity and immunological compatibility and is therefore still the gold standard for many clinical purposes. But this material is limited due to the availability of a sufficient amount of tissue to harvest and morbidity on the harvest site. Therefore a variety of synthetic materials with osteoconductive properties and high biocompatibility are proposed as substitutes [1]. We developed a synthetic material that mimics the composition and structure of the extracellular bone matrix, which mainly consists of collagen type I fibrils, mineralised with hydroxyapatite (HA) nanocrystals, as an alternative to the use of autologous grafts [2–4]. The membrane like material ("tape") exhibits pores only in the micrometer range and might therefore be useful for the separation of tissues. It has already been tested in two in vivo studies [5, 6]. The potential use of this composite for human bone regeneration and tissue engineering demands in addition the thorough investigation of cellular interactions with the material. First in vitro studies involved the generation of osteoclasts from human monozytes on the HA collagen tape [7].

In this study we present first investigations on the proliferation and osteogenic differentiation of human bone marrow stromal cells (hBMSC) on tapes made of mineralised collagen.

Cultures of hBMSCs contain several populations of progenitor cells that retain multipotentiality. Thus, they are

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capable to differentiate into cells of mesenchymal tissues like osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts [8, 9]. The differentiation along the osteoblastic lineage has been studied extensively [10–12].

We seeded hBMSC on the material and cultivated these constructs for 35 days both in static and perfusion culture. Osteogenic differentiation of hBMSC was quantified via measurement of alkaline phosphatase (ALP) activity, which is a marker of osteoblastic cells.

Experimental procedure

Preparation of membranes from mineralised collagen

Membranes from mineralised collagen I ("tapes") [3] were prepared by synchronous mineralisation of a collagen type I solution according to a method developed by Bradt et al. in our group [2] (Fig. 1). The procedure was published in detail elsewhere [4].

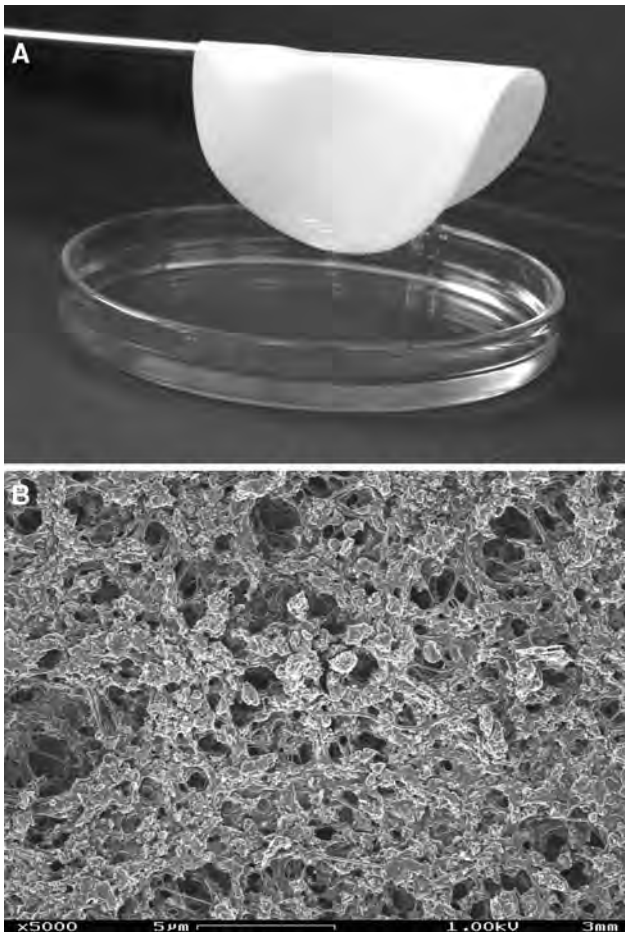


Fig. 1 (**A**) Membrane from biomimetically mineralised collagen I, 7 cm diameter. (**B**) SEM micrograph of tape surface, magnification 5,000×

To describe the procedure of collagen mineralization and tape preparation briefly, acid-soluble collagen type I isolated from calf skin (Collaplex 1.0, GfN, Wald-Michelbach, 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 product—homogeneously mineralised collagen fibrils—was finally collected by centrifugation. The material consists of about 30 wt% collagen and 70 wt% hydroxyapatite; the latter forms needles with a length of 70-80 nm, closely bound to the surface of reconstituted collagen fibrils which was shown in TEM investigations. The calcium phosphate phase is calcium deficient, carbonated HAP which was proven by EDX, XRD and FT-IR (data not published yet).

To prepare the membrane-like tapes, thoroughly resuspended mineralised collagen was condensed by vacuum filtration using a porous G4 glass filter frit (Schott, Germany) and then cross-linked with an aqueous solution of 1% *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC; Merck, Germany) for 1 h. Finally, the membranes were rinsed thoroughly in distilled water, in 1% glycine solution, once again in water, and freeze dried. For the present study circle-shaped samples with a diameter of 12 mm were cut from the material. Prior to use in the cell culture experiments the samples were sterilised by gamma irradiation.

Cell isolation and culture

Human bone marrow stromal cells were isolated from bone marrow aspirate of healthy donors (age 30–39) after obtaining informed consent as described previously [13]. Cells were tested negative for haematopoetic surface markers (CD 34, CD 45) and positive for CD 105 and CD 166. Expansion of the cells was performed in Dulbeccos Modified Eagle's Medium low glucose (Biochrom, Berlin, Germany), containing 10% foetal calf serum (Biochrom, Berlin, Germany), 10 U/mL penicillin and 100 µg/mL streptomycin (Biochrom) at 37 °C in a humidified, 7% CO_2/93% air incubator.

Cell seeding and culture on tapes

Prior to cell seeding the tapes were preincubated with cell culture medium for 24 h and fixed in tissue carriers ("Minusheets", Minucells, Bad Abbach, Germany). These carriers are necessary for perfusion culture. However, we also used tissue carriers for the static culture to get comparable seeding conditions and a comparable effective seeding area. For cell seeding the carriers were placed in

culture dishes and $1.5 \cdot 10^4$ cells were given within a droplet of medium to the surface of each support. After 1 h of initial adhesion medium was refilled to 1 mL per sample. For a control cells were seeded on 24-well polystyrene culture dishes ($1 \cdot 10^4$ per well). After 24 h mineralised collagen samples for static culture were transferred to fresh culture dishes, whereas the remaining samples were transferred to perfusion containers (Minucells GmbH). From the seventh day of culture, cell culture medium of half of the samples was supplemented with 10^{-7} M dexamethasone (Sigma-Aldrich, Taufkirchen Germany), 10 mM β-glycerophosphate (Sigma) and 0.05 mM ascorbic acid 2-phosphate (Sigma) (osteogenic supplements = OS+). Medium was changed twice weekly in the case of static culture. The perfusion culture was carried out with a medium flow of 1 mL/h. Samples (n = 2) were taken on day 1, 5, 7, 14, 21, 28 and 35 of culture. The tapes were removed from the tissue carriers, washed twice with warm PBS and frozen at –80 °C until further analysis.

Determination of ALP and LDH activity and protein content

Frozen cell samples were thawed for 20 min on ice, followed by cell lysis with PBS containing 1% Triton X-100 for 50 min on ice. During cell lysis each sample was sonicated for 1 min at 80 W with an ultrasonic processor UP 100H (Dr. Hielscher GmbH, Teltow, Germany). One aliquot of the cell suspension was added to ALP reaction buffer, containing 1 mg/mL p-nitrophenylphosphate (Sigma), 0.1 M diethanoamine (pH 9.8), 1% Triton X-100 and the mixture was incubated at 37 °C for 30 min. 1 M NaOH was added to stop the enzymatic reaction. After centrifugation at 16,000g for 30 min the supernatants were transferred to a microtiter plate and the absorbance was read at 405 nm with a multifunction microplate reader (Spectra fluor plus, Tecan, Crailsheim, Germany).

Another aliquot of the same cell suspension was mixed with LDH reaction buffer (Cytotox96 kit, Promega, Madison, USA). After incubation for 30 min at room temperature 1 N acetic acid was added to stop the enzymatic reaction. Absorbance was measured at 492 nm. The LDH activity was correlated with the cell number using a calibration line. Protein concentration of lysates was determined using Coomassie protein assay reagent (Sigma) with bovine serum albumin (Sigma) as standard.

SEM

After 1, 6 and 28 days of culture, cell seeded samples of mineralised collagen were washed twice with warm PBS, fixed for 10 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, Liechtenstein). Samples were coated with gold and imaged using IDSM 982 Gemini (Zeiss, Jena, Germany).

Results

Optimisation of biochemical assays in the presence of mineralised collagen

An acknowledged method for the evaluation of osteogenic differentiation is the measurement of the ALP activity. When osteogenic cells grow on a material different from cell culture polystyrene, some additional experiments have to be done. First, we could show, that ALP does not bind in considerable amounts to the mineralised collagen, when released after cell lysis (Fig. 2). Furthermore we applied freezing and thawing cycles to the cell seeded samples, followed by treatment with ultrasonic waves, according to Sabokbar et al. [14] and found a remarkable increase of detected ALP activity using this method.

To evaluate the progress of ALP activity during the experiment the detected activity has to be normalised to the cell number of the respective sample. A commonly used method for determination of the cell number is the measurement of the protein content of a cell lysate. This method is not suitable for cells grown on mineralised collagen. Incubation of the material with serum containing medium

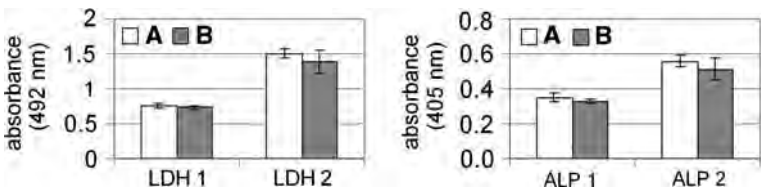


Fig. 2 Recovery of active LDH and ALP from cell lysates after incubation with mineralised collagen. hBMSC were seeded on 24-well polystyrene culture dishes ($1 \cdot 10^4$), and cultivated for 21 days. From the seventh day of culture osteogenic supplements were added to the cell culture medium (see experimental procedure). Samples were taken, washed, frozen and lysed as described. Cell lysates of six wells were pooled and equal amounts (non-diluted and diluted 1:1 with lysis buffer) were incubated with tapes from mineralised collagen (n = 3) for 50 min on ice, to simulate the lysis procedure. ALP and LDH activity was determined before (**A**) and after (**B**) incubation as described

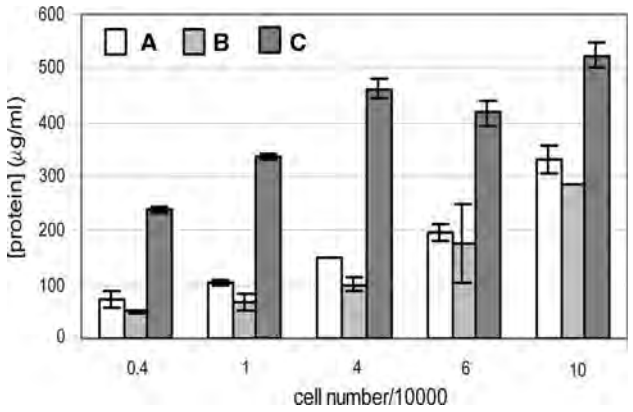


Fig. 3 Adsorption of protein to tapes made of mineralised collagen. hBMSC were seeded on 24-well polystyrene culture dishes in different amounts ($4{\cdot}10^3$–$1{\cdot}10^5$ cells). After complete adherence, the cell layer was washed, frozen and lysed as described. (**A**) Original protein content of the lysates, (**B**) protein content of lysates after 50 min incubation with tapes which were equilibrated in PBS for 24 h before, (**C**) protein content of lysates after 50 min incubation with tapes which were equilibrated in DMEM + 10% fetal calf serum for 24 h before

leads to an accumulation of protein on the surface of the sample. During cell lysis these proteins are released too and falsify the detected protein content (Fig. 3). The application of metabolic assays, which generate a dye in the presence of the material (for instance MTT, MTS, Alamar Blue) are also not applicable, because a considerable amount of the dye binds to the mineralised collagen and is not available for quantification (data not shown). Thus, we chose the measurement of LDH activity for determination of the cell number. In this metabolic assay cell lysis and dye formation are spatially separated. Furthermore we demonstrated that LDH does not bind in appreciable amount to the tapes (Fig. 2). Since we improved the conditions for the measurement of ALP activity, we could change to a lysis buffer with neutral pH, owing the possibility to determine ALP and LDH activity in the same lysate.

Proliferation of hBMSC on mineralised collagen

Human bone marrow stromal cells adhered on the tape material and showed good proliferation rates. After 28 days the whole surface of the tapes was covered by a thick cell layer, showed by SEM (Fig. 4). Compared to the polystyrene control the proliferation rate on the tape was much lower (Fig. 5). Interestingly, the cells which were not treated with osteogenic supplements showed higher proliferation rates compared to the osteogenic induced cells. In contrast, proliferation rates of osteogenically induced cells on polystyrene are higher compared to the non induced cells. The culture of cell seeded tapes in perfusion containers did not improve the proliferation rate compared to the static cultivation (Fig. 6).

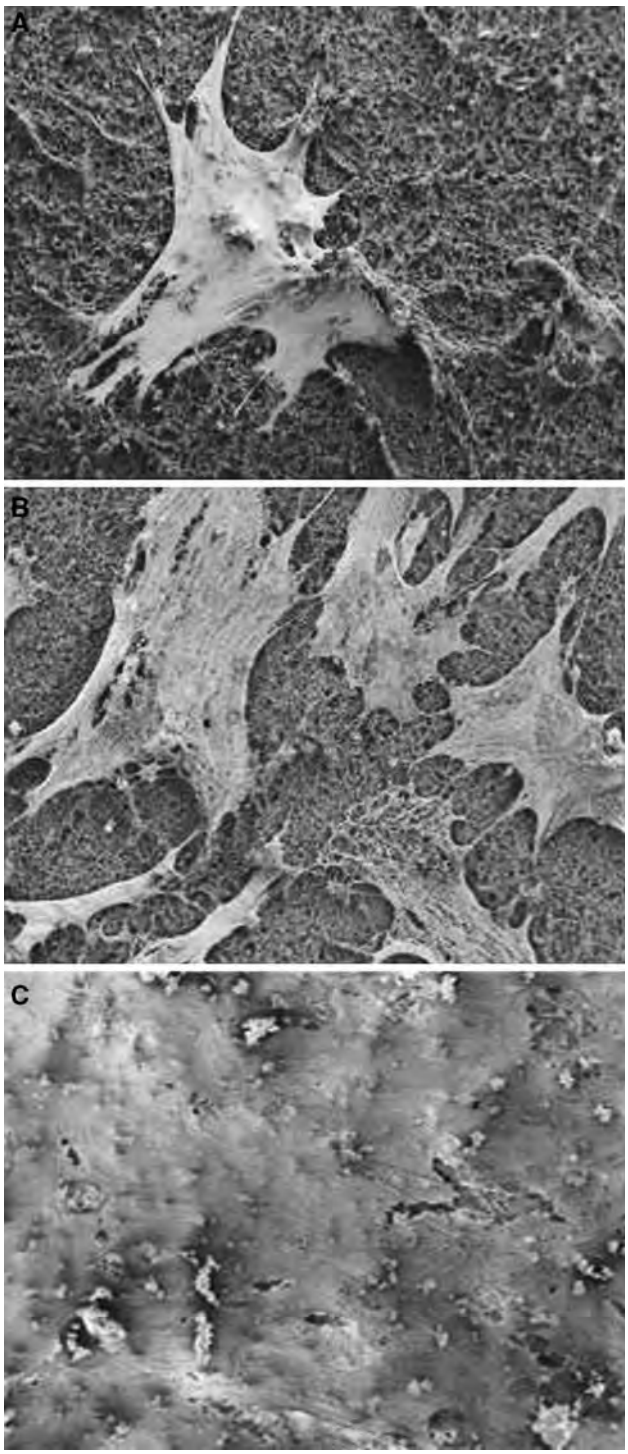


Fig. 4 SEM micrographs of hBMSC on tapes made of mineralised collagen, cultivated for 1 day (**A**), 6 days (**B**) and 28 days (**C**). Magnification 500×

Osteogenic differentiation of hBMSC on mineralised collagen (ALP activity)

We found a marked increase of specific ALP activity for cells seeded on tapes and cultivated with osteogenic sup-

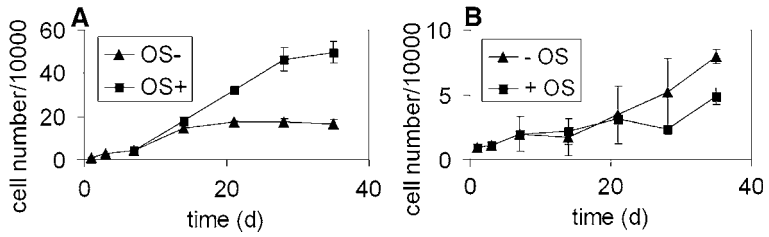


Fig. 5 Proliferation of induced (OS+) and non induced (OS–) hBMSC over 35 days. (**A**) On polystyrene culture dishes, (**B**) on tapes made of mineralised collagen (static culture). Cell number was calculated from LDH activity; $n = 2$ (+/– standard deviation of the mean)

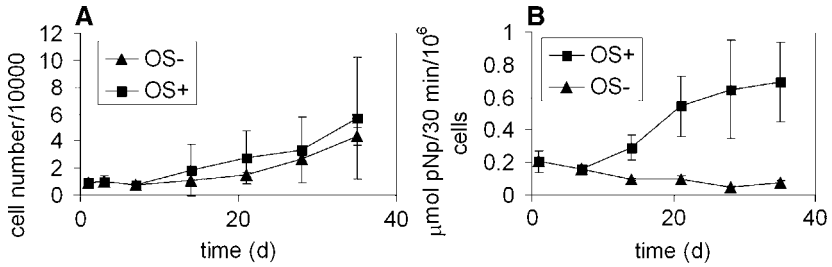
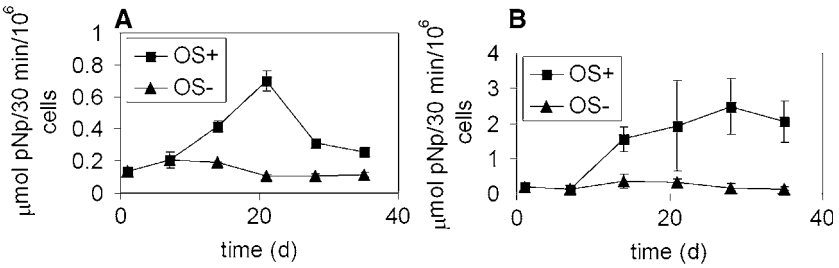


Fig. 6 Perfusion culture. (**A**) Proliferation of induced (OS+) and non induced (OS–) hBMSC on tapes from mineralised collagen over 35 days, $n = 2$ (+/– standard deviation of the mean), (**B**) ALP activity of induced (OS+) and non induced (OS–) hBMSC on tapes prepared of mineralised collagen over 35 days (perfusion culture); $n = 2$ (+/– standard deviation of the mean)

Fig. 7 ALP-activity of induced (OS+) and non induced (OS–) hBMSC over 35 days. (**A**) On polystyrene culture dishes, (**B**) on tapes made of mineralised collagen (static culture); $n = 2$ (+/– standard deviation of the mean)



plements (OS+) (Fig. 7). The highest ALP activity was detected on day 28 of cultivation (corresponding to day 21 after osteogenic induction) both in static and perfusion culture. However, the specific ALP activity on the statically cultured cell matrix constructs was nearly 4-fold higher compared to the perfused samples (Fig. 6).

In the polystyrene control we found the maximum of ALP activity for OS+ cells at day 21 (corresponding to day 14 after osteogenic induction). Comparing the ALP activity of cells cultured on polystyrene with that cultured on tape (both at the maximum) shows, that the ALP activity on the material was 4-fold higher.

Discussion

In this preliminary study we obtained some fundamental information about the effect of biomimetically mineralised collagen on the growth and osteogenic differentiation of bone marrow stromal cells. We investigated the proliferation of hBMSC on the material and detected a 5–8-fold increase of cell number on the tape material after 35 days of cultivation. These proliferation rates are comparable with growth of hBMSC on other collagen based biomaterials. Hattori et al. report an only 2-fold increase of DNA-content between 1 and 4 weeks cultivation for hBMSC seeded on atelocollagen scaffolds, and the cell number in the scaffolds increased at a significantly slower rate when compared to the polystyrene control [15]. Niemeyer et al. found for hBMSC on mineralised collagen sponges approximately a doubling of the cell number between day 1 and day 8 and no further increase of cell number at later time points [16].

The decreased proliferation rates of osteogenically induced cells compared to non induced hBMSC on the mineralised collagen tapes may have been caused by the fact, that a high amount of these cells started differentiating towards the osteoblastic lineage [17]. Jager et al. cultivated

hBMSC on collagen I/III membranes and found also a significantly inhibited proliferation for osteogenically induced hBMSC compared to non induced cells [18].

In contrast, we detected increased proliferation rates for osteogenically induced hBMSC on polystyrene when compared to non induced cells (Fig. 5). These findings are supported by studies which suggest a proliferation stimulating effect of dexamethasone—at least on polystyrene surfaces [11, 19]. Furthermore an enhancement of cell growth through ascorbic acid was reported [12]. To understand these altered proliferation patterns of osteogenically induced BMSC it should be taken into consideration, that marrow stromal cell cultures are not homogenous but will contain cells at different points in the lineage. As has been shown by Atkins et al. [20] for bone derived cells and Walsh et al. [21] for MSCs there are sub-populations of cells within these cultures that may respond differently to the prevailing conditions. Therefore some cells may continue to proliferate and a number may express alkaline phosphatase and stop proliferating. Possibly, the effect of osteogenic supplements on cell proliferation depends on the material on which the cells are cultivated. If the osteogenic differentiation is supported by the implant material, a higher amount of cells starts differentiating and the cell number will be raised slowlier compared to cultures of non induced cells. These suggestions are supported by the study of Jager et al. [17], who investigated proliferation and osteogenic differentiation of hBMSC on different materials: On collagen I/III, which was shown to promote the osteogenic differentiation of hBMSC (as revealed by determination of Ca enrichment) the proliferation of osteogenically induced cells was inhibited, whereas on PLLA, which promoted the osteogenic differentiation to a lesser extend, the proliferation of osteogenically induced cells was higher.

Surprisingly, the proliferation of cells, cultivated in perfusion culture on the tapes, was not increased compared to those under static culture conditions. There are some studies, reporting an increase of proliferation for cells cultivated in three-dimensional matrices under medium perfusion [22, 23]. Possibly, the effect of perfusion on cell proliferation is limited to three-dimensional constructs, where the nutrition of cells inside the pores is remarkably improved. In contrast, Wiedmann-Al-Ahmad et al. could improve the (very low) proliferation of human osteoblast-like cells on collagen membranes with perfusion [24]. The specific ALP activity of cells, cultivated in perfusion culture on the tapes was also decreased compared to static culture conditions (Fig. 6). While the enhancement of osteogenic differentiation through perfusion for three-dimensional cell/matrix constructs is well documented [25–27], there are only a few reports on perfusion culture with 2D materials. Fuss et al. found no morphological differences for osteoblasts on collagen membranes when comparing cultivation in perfusion culture to cultivation in standard dishes [28].

The specific ALP activity of osteogenically induced hBMSC grown on tapes of biomimetically mineralised collagen is considerably increased compared to the polystyrene control. There is some evidence, that hydroxyapatite may promote the osteogenic differentiation of cells. Thus, the specific ALP activity of rat BMSC on crystalline apatite surfaces is 2-fold higher compared to polystyrene [29]. MC3T3-E1 preosteoblasts showed an 2.3-fold increase of ALP activity on hydroxyapatite compared to culture plastic [30]. The ALP activity of osteogenically induced rat marrow stromal cells on a hydroxyapatite based microporous composite was about double that of control culture dishes [31]. For osteogenically induced hBMSC on calcium phosphate cement a significant increase of specific ALP activity compared to polystyrene was reported [32]. On the other hand there are reports on negative effects of hydroxyapatite on the osteogenic differentiation of cells. Deligianni et al. found a slight decrease of specific ALP activity of osteogenically induced hBMSC on discs of sintered HA compared to cell culture plastic [33]. ALP expression of MC3T3-E1 preosteoblasts was significant decreased on apatite coated PLGA surfaces compared to non coated PLGA [34]. The effect of hydroxyapatite based scaffolds on osteogenic differentiation depends on their physical and chemical characteristics, like crystallinity, particle size and surface topography [33]. These properties have various influences on cell response. For instance, rat calvaria osteoblasts showed higher expression of osteogenic markers on high crystalline HA coatings compared to low crystalline ones [35]. The absorption of a high quantity of serum proteins to HA based materials (Fig. 3) [36] may also play a role in affecting cell differentiation. Furthermore the collagen type I component of our tape material could contribute to the enhanced ALP activity of the osteogenically induced hBMSC. Salasnyk et al. cultivated hBMSC on collagen type I coated dishes and found an increased ALP-activity compared to hBMSC on glass [37]. Mizuno et al. demonstrated significantly raised ALP activities for rat BMSC cultivated on collagen type I compared to conventional culture dishes [38]. A study of Yang et al. indicates that the cell binding domain of collagen type I (P15 peptide) immobilized on HA, increases the ALP activity of attached hBMSC [39]. Georges et al. [40] found significantly increased ALP activity for osteogenically induced hBMSC on honeycomb shaped atelocollagen compared to polystyrene control.

Our data suggest, that the biomimetically generated material made of collagen I and nanocrystalline HA, applied in this study, seems to have optimal physicochemical properties to support osteogenic differentiation. Therefore mineralised collagen may act as a suitable artificial extracellular matrix for bone tissue engineering.

Conclusion and outlook

A synthetic material that mimics the composition and structure of the extracellular bone matrix, which mainly consists of collagen type I fibrils, mineralised with HA nanocrystals, was applied in this study. The membrane like tapes from biomimetically mineralised collagen type I show a substantial influence on the osteogenic differentiation of hBMSCs. The bone-like composition of the material combined with its stimulating effect on the osteogenic differentiation of hBMSC makes it appropriate for human bone regeneration.

In further studies we will extend our investigations on the proliferation and osteogenic differentiation of hBMSC in three-dimensional scaffolds made from biomimetically mineralized collagen with an interconnecting pore structure.

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