Cultivation of human bone marrow stromal cells on three-dimensional scaffolds of mineralized collagen: Influence of seeding density on colonization, proliferation and osteogenic di...

ARTICLE *in* JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE · OCTOBER 2008

Impact Factor: 5.2 · DOI: 10.1002/term.110 · Source: PubMed

3 AUTHORS:

Anja Lode
Technische Universität Dresden
56 PUBLICATIONS **733** CITATIONS
SEE PROFILE

Anne Bernhardt
Technische Universität Dresden
46 PUBLICATIONS **925** CITATIONS
SEE PROFILE

Michael Gelinsky
Technische Universität Dresden
165 PUBLICATIONS **2,172** CITATIONS
SEE PROFILE

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2008; **2**: 400–407.
Published online 29 August 2008 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.110

RESEARCH ARTICLE

Cultivation of human bone marrow stromal cells on three-dimensional scaffolds of mineralized collagen: influence of seeding density on colonization, proliferation and osteogenic differentiation

Anja Lode[#]*, Anne Bernhardt[#] and Michael Gelinsky
Max Bergmann Center for Biomaterials, Technische Universität Dresden, Institute of Materials Science, Budapester Strasse 27, D-01069 Dresden, Germany

Abstract

In this study human bone marrow stromal cells (hBMSCs) were cultured on three-dimensional porous scaffolds of biomimetically mineralized collagen type I developed for bone engineering. Three different cell numbers were used for seeding of the nanocomposites, and the impact of the seeding density on proliferation and osteogenic differentiation of hBMSCs was investigated. In addition, the effect of the seeding cell number on seeding efficiency and distribution of the cells within the scaffolds was studied. Our data revealed that the open and interconnecting porosity of the mineralized collagen scaffolds allows a very efficient seeding for all seeding densities tested. Although penetration of the cells into the interior of the scaffolds was demonstrated for all seeding densities, the application of higher cell numbers resulted in a better colonization also of the deeper scaffold regions. A substantial influence of the seeding density was observed on proliferation and osteogenic differentiation of hBMSCs. Thus, the highest proliferation rate and specific alkaline phosphatase activity was found for the cell matrix constructs seeded with the lowest density. RT–PCR analyses revealed a higher expression of alkaline phosphatase and bone sialoprotein II at lower seeding densities; however, expression of osteopontin was unaffected by the seeding cell number. Our results demonstrated that the seeding density might be an important factor for the development of optimal cell matrix constructs for bone tissue engineering. Copyright © 2008 John Wiley & Sons, Ltd.

Received 4 June 2008; Accepted 5 June 2008

Keywords mineralized collagen; mesenchymal stem cells; bone marrow stromal cells; proliferation; osteogenic differentiation; alkaline phosphatase; seeding density; reverse transcriptase PCR

1. Introduction

Bone substitutes for the treatment of osseous defects are in high demand. The use of autologous bone graft, the current gold standard as osteogenic bone replacement material, is restricted by the limited amount of bone which can be harvested and considerable donor site morbidity. Therefore, a variety of synthetic bone graft materials have been developed. However, for the repair of large defects, an osteogenic potential of the bone graft substitute is required which will be provided only by viable cells. The combination of artificial biodegradable matrices with autologous cells according to the tissue engineering (TE) concept is a promising strategy to overcome the limitations of autologous bone graft. For this approach,

*Correspondence to: Anja Lode, Max Bergmann Centre for Biomaterials, Technische Universität Dresden, Institute of Materials Science, Budapester Strasse 27, D-01069 Dresden, Germany. E-mail: anja.lode@nano.tu-dresden.de
[#] These authors contributed equally to this paper.

cells are isolated from the patient, expanded *in vitro* and seeded into an appropriate three-dimensional (3D) scaffold. After implantation, the cell matrix construct should be remodelled into native tissue.

Bone marrow is the source of a rare cell population with stem cell characteristics, referred to as mesenchymal stem cells or marrow stromal cells (MSCs). MSCs are able to differentiate along the osteogenic and various other cell lineages (Owen, 1988; Beresford, 1989; Caplan, 1991; Pittenger *et al*., 1999). The capability of MSCs for self-replication allows their extensive *in vitro* expansion (Bruder *et al*., 1997). These properties as well as the fact that they can be easily enriched from bone marrow aspirates make MSCs ideal candidates for bone TE. The potential of the MSC-based TE approach for the healing of bone defects has been demonstrated in several *in vivo* models (Krebsbach *et al*., 1997; Bruder *et al*., 1998; Kon *et al*., 2000).

Critical for the success of bone TE is the selection of the scaffold for physical support of the cells. The scaffold has to fulfil a number of requirements, including biocompatibility, interconnective porosity, adequate mechanical strength and bioresorbability (Logeart-Avramoglou *et al*., 2005; Ikada, 2006). The major function of a scaffold is similar to that of the natural extracellular matrix that assists proliferation, differentiation and biosynthesis of cells (Ikada, 2006; Badylak, 2007). In the present study, human bone marrow stromal cells (hBMSCs) were cultured on a resorbable material that mimics the composition and structure of the extracellular bone matrix, which mainly consists of collagen type I fibrils mineralized with hydroxyapatite (HA) nanocrystals. This nanocomposite material was produced in a biomimetic process in which collagen fibril assembly and mineralization with HA occur simultaneously (Bradt *et al*., 1999). Starting from the suspension of mineralized collagen, either membrane-like composites with pores only in the μm range (Burth *et al*., 1999) or 3D scaffolds with a porosity of 72% and a mean pore diameter of 180 μm (Gelinsky *et al*., 2004, 2008) can be created. The latter were used for the experiments described in this paper. Recently, we performed *in vitro* studies with both the membrane-like and the 3D composites which revealed a positive impact of the biomimetic mineralized collagen on osteogenic differentiation of hBMSCs (Bernhardt *et al*., 2008a, 2008b).

Cell seeding is of critical importance for the generation of 3D cell matrix constructs suitable for tissue regeneration. Besides the seeding technique, the initial cell number is a crucial factor to achieve an uniform distribution of a number of cells which is optimal for new tissue formation. In the present study, the influence of the initial seeding density on proliferation and osteogenic differentiation of hBMSCs seeded into porous composites was investigated. To this end, cell matrix constructs were cultivated in the presence and absence of osteogenic supplements for 15, 21 and 42 days. Moreover, seeding efficiency, as well as the distribution of the cells within the porous scaffolds depending on the seeding density, were analysed.

2. Materials and methods

2.1. Scaffold preparation

The preparation of the porous scaffolds has been described in detail elsewhere (Gelinsky *et al*., 2004; Gelinsky *et al*., 2004, 2008). Briefly, acid-soluble collagen I (isolated from bovine tendon and kindly provided by Syntacoll, Saal/Donau, Germany) was dissolved in 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, collagen fibril reassembly and nanocrystalline HA precipitation occur simultaneously, which results in the formation of homogeneously mineralized collagen fibrils. These can be collected by centrifugation as a wax-like, colourless material. Resuspended mineralized collagen was put into wells of a 48-well plate, frozen at −20 °C, freeze-dried and then crosslinked with a 1 wt% solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) hydrochloride in 80 vol% ethanol for 1 h. Finally, the sponge-like scaffolds were thoroughly rinsed in distilled water, 1% glycine solution, once again in water and finally freeze-dried. For this study, cylindrical samples with a diameter of 10 mm and heights of 4 and 7 mm, respectively, were prepared. Prior to use for cell culture experiments, the scaffolds were sterilized by γ-radiation at 31 kGy.

2.2. Cell isolation and culture

hBMSCs, isolated from bone marrow aspirate as described previously (Oswald *et al*., 2004), were kindly provided by Sabine Boxberger, Medical Clinic I, Dresden University Hospital. The cells were expanded in Dulbecco's modified Eagle's medium, low glucose (DMEM; Biochrom, Berlin, Germany) containing 10% fetal calf serum (Biochrom), 10 U/ml penicillin and 100 μg/ml streptomycin (Biochrom) at 37 °C and 7% CO_2. Cells in passage 5 were used for the experiments.

2.3. Cell seeding and culture of the scaffolds

Three different cell numbers were chosen for seeding of the scaffolds: 5×10^4, 2×10^5 and 1×10^6. Prior to seeding, the scaffolds were pre-incubated with cell culture medium (DMEM containing 10% fetal calf serum, 10 U/ml penicillin and 100 μg/ml streptomycin) for 24 h. Thereafter they were placed on filter paper for approximately 1 min. This procedure removes excess medium from the pores and therefore improves penetration of cells into the porous structure. The scaffolds were then transferred to a polystyrene cell culture plate and the cell suspension was pipetted onto the top side of each scaffold. After 1 h of initial adhesion, 1 ml cell culture medium was added to each sample.

DOI: 10.1002/term

To estimate the seeding efficiency, 12 cell-seeded scaffolds (diameter 10 mm × height 4 mm) of each seeding density were transferred to a new polystyrene cell culture plate 3 h after seeding. To determine the number of cells which had not adhered to these scaffolds, seeding well plates and pelleted cells from the remaining medium were frozen at −80 °C until the analysis of their DNA content (see next section).

For biochemical investigation of proliferation and osteogenic differentiation, cell-seeded scaffolds (diameter 10 mm × height 4 mm) were transferred to new polystyrene cell culture plates 24 h after seeding. Freshly added cell culture medium contained osteogenic supplements (+OS) for half of the samples (10^{-7} M dexamethasone, 3.5 mM β-glycerophosphate and 0.05 mM ascorbic acid 2-phosphate; all from Sigma-Aldrich, Taufkirchen, Germany). The medium was changed twice weekly. Samples were taken on days 1, 15 and 21 or on day 42 of culture. After removing the medium, cell-seeded scaffolds were placed on filter paper, washed twice with warm PBS, placed again on filter paper and frozen at −80 °C until further analysis (see next section).

For analysis of gene expression by reverse transcriptase-PCR (RT–PCR), cell-seeded scaffolds (diameter 10 mm × height 4 mm) were transferred to new polystyrene cell culture plates 24 h after seeding and cell culture medium containing osteogenic supplements (+OS) was added. The medium was changed twice weekly. After 21 days of cultivation, the samples were washed twice with warm PBS, placed on filter paper and immediately used for RNA preparation (see Section 2.5.).

2.4. Determination of DNA content and alkaline phosphatase (ALP) activity

For estimation of the seeding efficiency, cells attached to the seeding wells and cells pelleted from the remaining medium were incubated with lysis buffer (1% Triton X-100/PBS) for 50 min on ice. Cell lysis was supported by sonication for 10 min in an ice-cooled ultrasonic bath. DNA content in the lysates was determined using the Picogreen® dsDNA Quantitation reagent (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions, and correlated with the cell number using a calibration line.

For analysis of proliferation and osteogenic differentiation, frozen samples were each transferred into a 2 ml tube containing six beads of stainless steel (peqlab, Erlangen, Germany). 900 µl ice-cold PBS were added and the samples were homogenized using the Precellys® 24 system (peqlab). The homogenization process was operated twice for 10 s with 5800 r.p.m. After removing the beads, 100 µl 10% Triton X-100 were added and the homogenates were incubated on ice for 50 min. During this time, the pulverized scaffold material sedimented and the supernatants were transfered to fresh 1.5 ml reaction tubes. For each sample, one aliquot of the supernatant was used to determine the DNA content with the Picogreen® dsDNA quantitation reagent according to the manufacturer's instructions. DNA content was correlated with the cell number using a calibration line. Another aliquot of the lysates was added to ALP reaction buffer [1 mg/ml *p*-nitrophenylphosphate (Sigma-Aldrich), 0.1 M diethanolamine, 1% Triton X-100, 1 mM $MgCl_2$ pH 9.8] and incubated at 37 °C for 30 min. The enzymatic reaction was stopped by addition of 1 N NaOH. After centrifugation at 16 000 × *g* for 10 min, the supernatants were transferred to a microtiter plate and absorbance was measured at 405 nm. A calibration line was constructed from different dilutions of 1 mM *p*-nitrophenol stock solution.

2.5. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Preparation of RNA from the cell-seeded scaffolds was performed using the peqGOLD MicroSpin Total RNA Kit (peqlab) according to the manufacturer's instructions. cDNA was transcribed from 250 ng total RNA in a 20 µl reaction mix containing 200 U Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs (Invitrogen), 12.5 ng/µl random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U of RNase inhibitor RNase OUT (Invitrogen). For PCR experiments, 1 µl cDNA was amplified in a 20 µl reaction mix containing specific primer pairs to detect transcripts of ALPL, BSPII, osteopontin (OPN) and the housekeeping gene GAPDH, respectively, for each sample. The primers (MWG Biotech) for each gene are summarized in Table 1. After the initial activation step at 95 °C for 4 min, 30 PCR cycles were run, each with a denaturation step at 95 °C for 45 s, an annealing step at 55 °C (in the cases of ALP, BSPII and GAPDH) or 57 °C (in the case of OPN) for 45 s, and a synthesis step at 72 °C for 1 min followed by a final synthesis step at 72 °C for 10 min in a Thermocycler (peqlab). The same single-stranded cDNA was used to investigate the expression of all genes described. The resulting PCR products were analysed using the FlashGel™ Dock system (Cambrex Bio Science, Rockland, USA).

2.6. MTT staining

After cultivation of cell-seeded scaffolds (diameter 10 mm × height 7 mm) for 2 days, the culture medium was

Table 1. Primers for RT–PCR

Gene	Forward primer	Reverse primer
GAPDH	5′-GGTGAAGGTCGGA-GTCAACGG-3′	5′-GGTCATGAGTCCT-TCCACGAT-3′
ALPL	5′-ACCATTCCCACGT-CTTCACATTTG-3′	5′-ATTCTCTCGTTCA-CCGCCCAC-3′
BSPII	5′-AATGAAAACGAAG-AAAGCGAAG-3′	5′-ATCATAGCCATCG-TAGCCTTGT-3′
OPN	5′-GTCTCAGGCCAGT-TGCAGCC-3′	5′-GCCATGTGGCCAC-AGCATCTG-3′

DOI: 10.1002/term

supplemented with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich), followed by further incubation at 37 °C for 4 h. Staining of the scaffold by development of a dark blue formazan derivative was documented photographically.

2.7. Scanning electron microscopy (SEM)

Two days after seeding, scaffolds (diameter 10 mm × height 7 mm) seeded with 2×10^5 cells were washed with PBS and fixed with 3.7% formaldehyde in PBS. The samples were washed with distilled water and dehydrated using a graded series of ethanol : distilled water mixtures. 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.

2.8. Statistical analysis

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

3. Results

3.1. Colonization of the porous scaffolds

The porous scaffolds were seeded with three different cell densities. After 3 h the number of cells not adhering to the matrix was determined to evaluate the seeding efficiency for each scaffold. We found a very high efficiency for all three seeding densities. In the supernatant, <0.1% of the initial cell number was detected in all cases. Interestingly, the number of cells on the polystyrene surface was similar for the three different seeding densities: on average 1.7×10^3 cells were found attached to the polystyrene surface of the seeding wells. This cell number is equivalent to $3.4 \pm 0.8\%$ for scaffolds seeded with 5×10^4, $0.8 \pm 0.4\%$ for those seeded with 2×10^5, and $0.2 \pm 0.1\%$ for those seeded with 1×10^6 cells.

The penetration of the cells into the porous structure was studied by means of both a macroscopic and a microscopic approach. First, scaffolds were seeded with three different cell numbers and 2 days after seeding viable cells were visualized by MTT staining. MTT is incorporated by viable cells and intracellularly converted into an insoluble dark blue formazan derivative. Therefore the MTT staining is applicable to visualize the distribution of viable cells macroscopically (Hofmann *et al.*, 2003; Mauney *et al.*, 2004).

An influence of the seeding density on cell penetration could be shown (Figure 1A). In the case of the lowest seeding cell number (5×10^4), only a weak staining of the upper quarter of the scaffold was observed. In contrast, the increase of the seeding cell number to 2×10^5 resulted in a more intense staining of about two-thirds of the scaffold. Using a cell number of 1×10^6 for seeding, a strong staining of approximately three-quarters of the scaffold was detected. Longitudinal sections through the scaffolds clearly demonstrated a colonization of the interior of the scaffolds (Figure 1B).

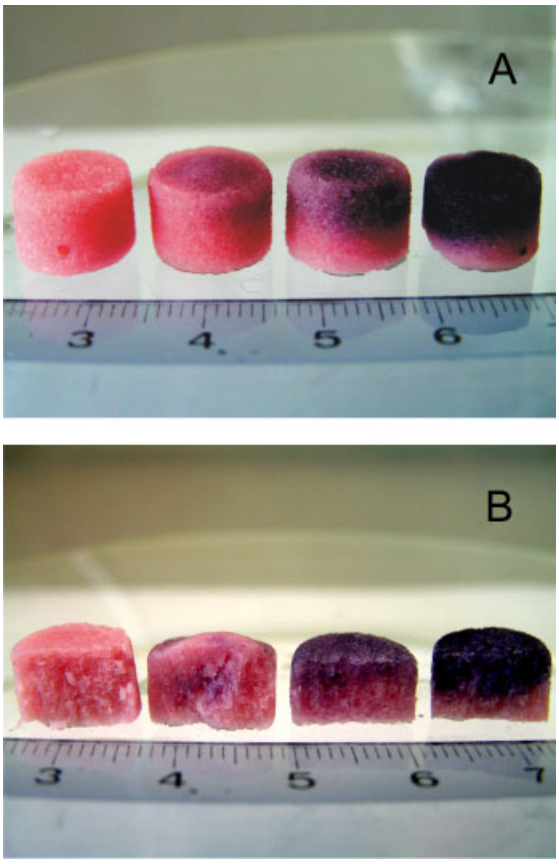

Figure 1. MTT staining of scaffolds after 2 days of culture: (A) whole scaffolds; (B) longitudinal sections through the scaffolds. From left to right: scaffold without cells (control); scaffold seeded with 5×10^4, 2×10^5 and 1×10^6 cells, respectively

Next, different sections of scaffolds seeded with 2×10^5 cells were analysed by SEM 2 days after seeding. Cells were detected in the upper part and in the middle of the scaffolds (Figure 2A, B). However, at clearly reduced concentrations cells could also be found in the lower part of the sample, which means about 5 mm below the front surface at the point where the cell suspension was pipetted (Figure 2C).

3.2. Proliferation

Proliferation of the cells seeded onto the porous scaffolds was investigated by quantification of the DNA content on days 1, 15, and 21 of culture, with and without osteogenic supplements. An increase of the number of both osteogenically-induced and non-induced hBMSCs was

DOI: 10.1002/term

Figure 2. SEM analysis of scaffolds seeded with 2 x 10^5 cells 2 days after seeding. (A) SEM picture from the centre of a longitudinal section through the upper half of the cylindrical scaffold. (B) SEM picture of a horizontal cross-section through the centre of the scaffold parallel to the top side. (C) SEM picture of the centre of a longitudinal section through the lower half of the cylindrical scaffold

observed for samples of all three seeding densities, but to different degrees (Figure 3A–C). Thereby, proliferation rates were inversely proportional to the seeding density (Figure 3D). Samples seeded with 1×10^6 cells showed only a minor increase of cell number from day 1 to day 21. In contrast, the highest proliferation rates were detected for samples seeded with 5×10^4 cells. Experiments with hBMSCs from a second donor gave similar results (data not shown).

An additional experiment was performed to study the alteration of the cell numbers during a longer cultivation period. To this end, samples of the three seeding densities were cultivated for 6 weeks with and without osteogenic supplements and the cell numbers were quantified by determination of the DNA content. Interestingly, we found considerable differences between induced and non-induced cells. Whereas a clear gradation in the number of osteogenically induced cells subject to the seeding density was observed (Figure 4A), the number of the non-induced cells of all samples is in the same range after 6 weeks of cultivation, independently of the initially seeded cell number (Figure 4B).

3.3. Osteogenic differentiation

Specific ALP activity of osteogenically-induced hBMSCs increased over the cultivation period of 21 days, with a maximum on day 15 in the case of scaffolds seeded with 1×10^6 cells, as well as on day 21 in the case of scaffolds seeded with 5×10^4 and 2×10^5 (Figure 5A). In contrast, ALP activity of non-induced cells was not increased during cultivation time (data not shown).

Obviously, an influence of the seeding density on the specific ALP activity of osteogenically induced cells could be observed (Figure 5A). As for cell proliferation, the highest values were detected for samples seeded with 5×10^4 cells. Specific ALP activity of samples seeded with 1×10^6 cells was significantly lower compared to those of samples seeded with 5×10^4 and 2×10^5 cells, respectively. Experiments with hBMSCs from a second donor gave similar results (data not shown). Overall ALP activity per scaffold showed comparable values for scaffolds of the three different seeding densities, with

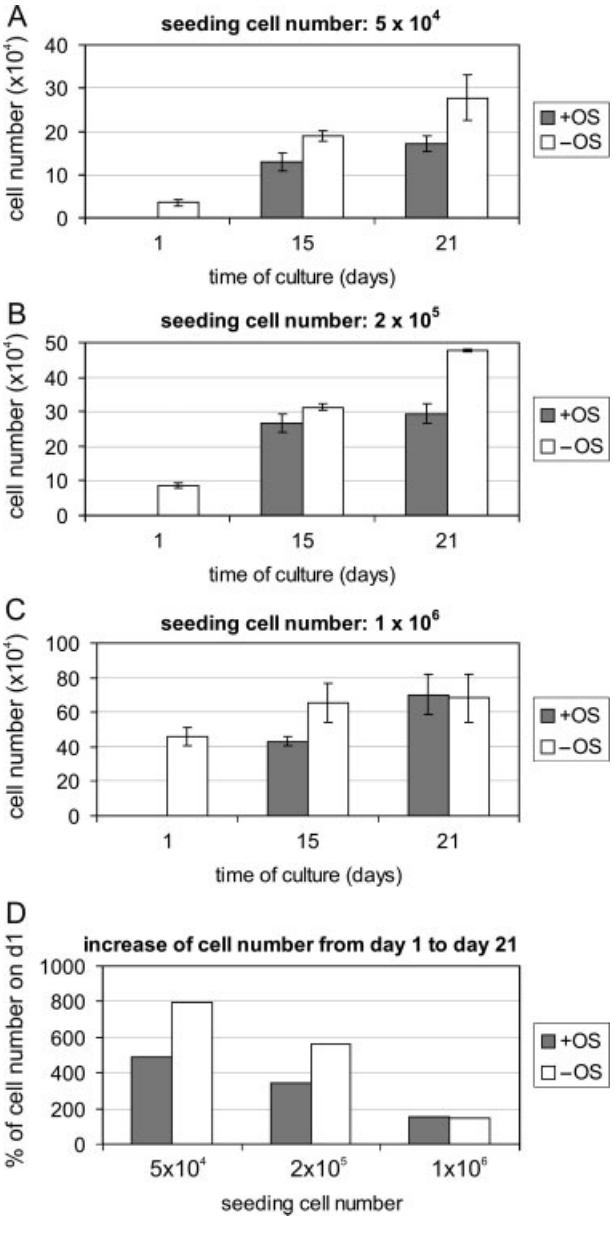

Figure 3. Proliferation of osteogenically induced (+OS) and non-induced (−OS) hBMSCs cultured on porous scaffolds of mineralized collagen over 21 days. Cell numbers were determined by measurement of the DNA content. $n = 3$ (± SD of the mean)

DOI: 10.1002/term

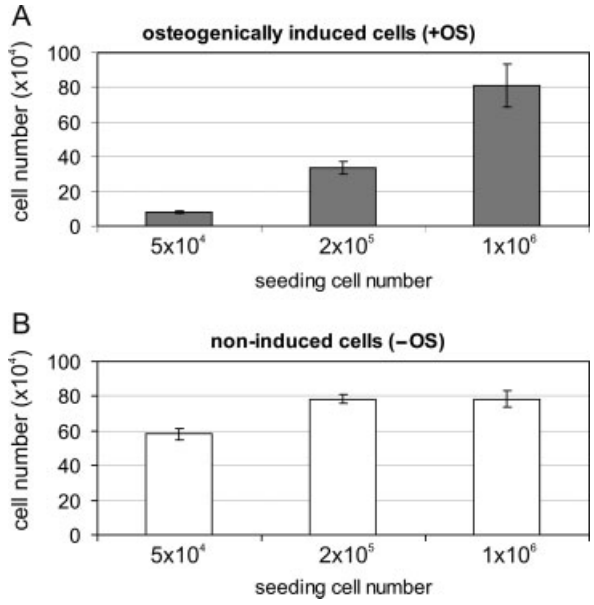

Figure 4. Cell numbers of osteogenically induced (A) and non-induced (B) hBMSCs cultured on porous scaffolds of mineralized collagen for 42 days. Cell numbers were determined by measurement of the DNA content. $n = 3$ (± SD of the mean)

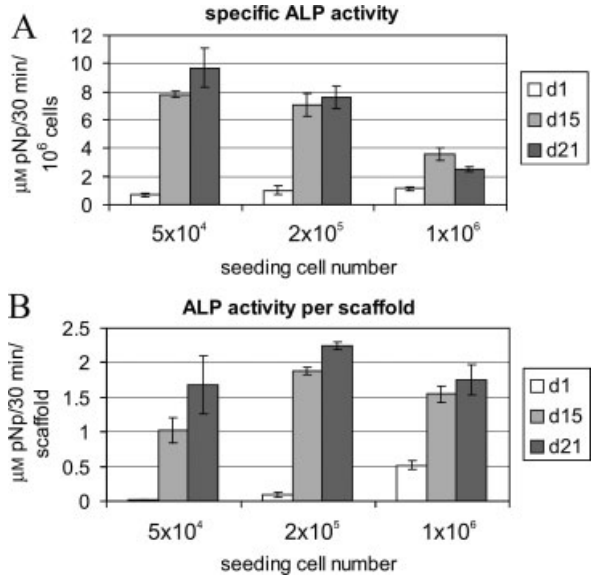

Figure 5. (A) Specific ALP activity of osteogenically induced hBMSCs cultured on scaffolds of mineralized collagen and (B) ALP activity per scaffold. $n = 3$ (± SD of the mean). **$p < 0.001$: $5 \times 10^4 \leftrightarrow 1 \times 10^6$ (d14 and d21); $2 \times 10^5 \leftrightarrow 1 \times 10^6$ (d21); *$p < 0.005$: $2 \times 10^5 \leftrightarrow 1 \times 10^6$ (d14)

a little preference for the seeding density of 2×10^5 (Figure 5B).

Expression analysis of osteogenically induced hBMSCs on day 21 revealed an ALP transcript level that was highest on samples seeded with 5×10^4. Compared to that, ALP expression was slightly reduced on samples seeded with 2×10^5 cells and strongly reduced on those seeded with 1×10^6 cells (Figure 6). A higher expression on samples seeded with the lowest cell number was also detected for BSPII. However, the transcript

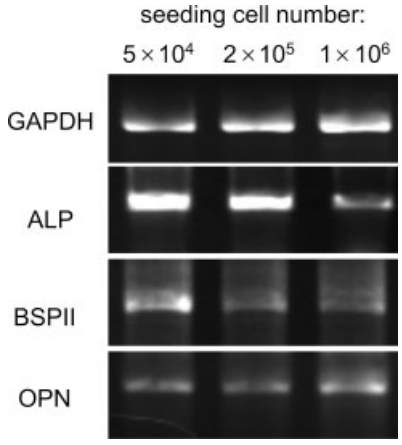

Figure 6. RT–PCR expression analysis of osteogenically induced hBMSCs cultured on scaffolds of mineralized collagen for 21 days. For each seeding density, RNA was prepared from three scaffolds and pooled for further analysis. ALP, BSPII and OPN were used to characterize osteogenic differentiation and GAPDH was used as an internal control

level of OPN was unaffected by the seeding density (Figure 6).

4. Discussion

The effect of the seeding density has been attended in a number of *in vitro* and *in vivo* studies for different cell types and biomaterials (Ishaug *et al*., 1997, 1998; Mendes *et al*., 1998; Holy *et al*., 2000; Dar *et al*., 2002; Wilson *et al*., 2002; Dvir-Ginzberg *et al*., 2003; van den Dolder *et al*., 2003; Shearn *et al*., 2005; Zhou *et al*., 2006). Among them, there are several reports demonstrating that higher seeding densities do not inevitably produce better results concerning seeding efficiency, proliferation and differentiation. Hence, it seems worthwhile to estimate the optimal seeding density for each cell sort and scaffold type. The use of cells for TE applications requires normally their *ex vivo* expansion in the highly artificial environment of a two-dimensional plastic culture system, which is associated with a number of drawbacks. For instance, it was shown that extensive expansion of MSCs *in vitro* led to a progressive decrease of their proliferation rate, loss of their multilineage differentiation potential and finally to senescence (for review, see Derubeis and Cancedda, 2004). The estimation of an optimal seeding density might also minimize the number of cells required and thus the *in vitro* cell expansion step.

The data from the present study reveal that the open and interconnecting porosity of the mineralized collagen scaffold and the seeding method deploying the capillary forces of this porous matrix allow a very efficient seeding with a negligible loss of cells, independently of the seeding density. Zhou *et al*. (2006) reported recently on the seeding of different densities of human alveolar osteoblasts into porous polycaprolactone/tricalcium phosphate (PCL/TCP) scaffolds. They found seeding efficiencies of 80–90% which were also independent from the

DOI: 10.1002/term

initial seeding density. In contrast, other groups observed a considerable decrease of the seeding efficiency at higher seeding densities in their systems (Holy *et al.*, 2000; Dar *et al.*, 2002).

By means of SEM as well as staining of living cells with MTT, a penetration of the cells into the interior of the scaffold was demonstrated. However, in contrast to the seeding efficiency, an influence of the seeding density on the distribution of the cells in the scaffold was observed (Figure 1). For the sample size used in these experiments, the higher seeding densities are more efficient to colonize the whole scaffold. That the effectual penetration of cells into 3D biomaterials could be problematic – even if the pore size is sufficient – was demonstrated by Niemeyer *et al.* (2003, 2004), who investigated a comparable material consisting of cross-linked collagen type I fibres coated with sintered HA particles. This Healos® matrix has a porosity of more than 95% and the diameters of the pores are in the range 4–200 μm. Coincident with our study, the authors found >95% of the seeded cells adhered to the scaffold 24 h after seeding. However, cells could be detected in sections from 500 μm below the surface in only three of 10 cases (Niemeyer *et al.*, 2003, 2004).

During cultivation over 21 days, we observed a considerable influence of the initial seeding density on the proliferation of the cells inside the scaffolds (Figure 3). The highest proliferation rate was found for cell matrix constructs with the lowest seeding density. For the seeding cell number of 1×10^6, only a 1.5-fold increase was detected within 21 days. This observation hinted at a saturation phenomenon: a certain number of cells in the scaffolds cannot be exceeded and, irrespective of the number of seeded cells, all scaffolds might contain comparable amounts of cells after prolonged cultivation. This assumption was supported by data of Zhou *et al.* (2006), who observed similar cell numbers for five different seeding densities after cultivation of human alveolar osteoblasts in PCL/TCP scaffolds for 28 days. Ishaug *et al.* (1998) compared two different seeding densities of neonatal rat calvarial osteoblasts on porous poly(DL-lactic-*co*-glycolic acid; PLGA) foam scaffolds and found also similar cell numbers after 56 days of culture. Therefore, we performed a further experiment with a cultivation period of 6 weeks. In the case of the non-induced cells, we indeed found cell numbers in the same range for all seeding densities: after 42 days, the cell number in the scaffolds seeded with 2×10^5 and 1×10^6 cells were comparable and the cell number of samples seeded with 5×10^4 corresponded to approximately three-quarters of those determined for the samples seeded with 2×10^5 and 1×10^6 cells (Figure 4B). Interestingly, this phenomenon was not observed for the osteogenically-induced cells: in contrast to the non-induced cells, clear differences corresponding to the initial seeding cell number were found (Figure 4A). Since the determination of the ALP activity on days 15 and 21 also revealed an inverse relationship between specific ALP activity of induced cells and seeding density (Figure 5A), one could imagine that the stronger proliferation in the case of lower seeding densities is superposed by an also stronger differentiation rate.

Wilson *et al.* (2002) investigated different seeding densities of goat bone marrow cells which were cultured on HA scaffolds for 7 days. They found cell proliferation in the scaffolds only for the middle seeding densities but for neither seeding cell numbers under a certain threshold nor an extremely high seeding density. Similarly to our assumption, the authors conclude from their data that there is a maximum number of viable cells that can be maintained on these scaffolds. A limited cell number in a 3D scaffold might rather be caused by limitations in the transport of nutrients, oxygen and waste under static culture conditions, as suggested by Zhou *et al.* (2006) and Ishaug *et al.* (1998), than by spatial restrictions. Perfusion culture of cell matrix constructs could offer a possibility to enhance the maximum cell number.

As mentioned above, an influence of the seeding density could be also observed on the osteogenic differentiation, as indicated by ALP activity. In the case of the lower seeding densities, the smaller cell numbers in the scaffold were compensated by a higher specific ALP activity (Figure 5). Thus, the differences between the ALP activities per scaffold are not substantial. With respect to ALP activity per scaffold, the seeding density of 2×10^5 was apparently optimal. Concordantly with our results, Zhou *et al.* (2006) also observed an inverse relationship between seeding density and level of ALP activity at days 7 and 14 for alveolar osteoblasts cultured on PCL/TCP scaffolds. Furthermore, Ishaug *et al.* (1998) reported on a greater specific ALP activity for the PLGA foams seeded with the lower osteoblast seeding density.

The data concerning the ALP activity (Figure 5) are supported by results of RT–PCR analysis, which indicate a higher expression level for ALP in samples seeded with lower cell numbers (Figure 6). Moreover, BSPII, a specific bone marker protein which is expressed in osteogenically induced but not in non-induced hBMSCs, showed a similar pattern. In contrast, the expression of the bone matrix protein OPN is not influenced by the seeding density. This might be explained by the observation that OPN is less specific for osteoblastic cells and can be detected in osteogenically-induced as well as in non-induced hBMSCs (data not shown).

The effect of the initial seeding density on bone formation was studied *in vitro* by Holy *et al.* (2000). The authors cultivated rat bone marrow-derived cells on PLGA scaffolds and investigated cell differentiation by osteocalcin staining and mineralized matrix production after 3 and 6 weeks. The data from that study revealed that the initial seeding density did not significantly affect tissue formation. Lowering seeding densities only delayed the formation of mineralized tissue and after 6 weeks tissue formation was independent of initial seeding density (Holy *et al.*, 2000). The observation of the present study, that cell matrix constructs seeded with 1×10^6 cells had an earlier maximum of ALP activity than those seeded

DOI: 10.1002/term

with 2×10^5 and 5×10^4 cells (Figure 5A), could be in line with these findings.

5. Conclusion

The substantial influence of the seeding density on cellular functions observed in our study points to the necessity to consider the seeding cell number in the *in vitro* evaluation of cell matrix constructs. Especially for comparison of biomaterials which differ in their porosity and pore structure, optimized seeding densities should be used. Moreover, the seeding density might be an important factor for the development of optimal cell matrix constructs for bone TE. For the porous scaffolds of mineralized collagen investigated in this study, we favour a seeding cell number of 2×10^5 hBMSCs/scaffold. Seeding with the lower cell number of 5×10^4 led to a diminished penetration of the cells into the matrix. In contrast, for samples seeded with 1×10^6 cells, a reduced proliferation rate as well as a lower specific ALP activity was found, but a more extensive *in vitro* expansion was required.

Acknowledgements

The authors thank Sabine Boxberger, Medical Clinic I, University Hospital Carl Gustav Carus, Dresden, for providing hBMSCs and Syntacoll (Saal/Donau, Germany) for a generous supply of bovine collagen. We are grateful to Ortrud Zieschang for scaffold preparation and Cornelia Wentzel and Stefanie Zumpe for technical assistance with the biochemical analyses.

References

Badylak SF. 2007; The extracellular matrix as a biologic scaffold material. *Biomaterials* **28**: 3587–3593.

Beresford JN. 1989; Osteogenic stem cells and the stromal system of bone and marrow. *Clin Orthop Relat Res* **240**: 270–280.

Bernhardt A, Lode A, Boxberger S, *et al*. 2008a; Mineralised collagen – an artificial, extracellular bone matrix – improves osteogenic differentiation of bone marrow stromal cells. *J Mater Sci Mater Med* **19**: 269–275.

Bernhardt A, Lode A, Mietrach C, *et al*. 2008b; Biomimetic porous scaffolds from mineralised collagen support growth and osteogenic differentiation of human bone marrow stromal cells. *J Biomed Mater Res A* (in press) DOI: 10.1002/jbm.a.32144.

Bradt J, Mertig M, Teresiak A, *et al*. 1999; Biomimetic mineralization of collagen by combined fibril assembly and calcium phosphate formation. *Chem Mater* **11**: 2694–2701.

Bruder SP, Jaiswal N, Haynesworth SE. 1997; Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64**(2): 278–294.

Bruder SP, Kraus KH, Goldberg VM, *et al*. 1998; The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* **80**(7): 985–996.

Burth R, Gelinsky M, Pompe W. 1999; Collagen-hydroxyapatite tapes – a new implant material. *Tech Textile* **8**: 20–21.

Caplan AL. 1991; Mesenchymal stem cells. *J Orthop Res* **9**(5): 641–650.

Dar A, Shachar M, Leor J, *et al*. 2002; Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. *Biotechnol Bioeng* **80**(3): 305–312.

Derubeis AR, Cancedda R. 2004; Bone marrow stromal cells (BMSCss) in bone engineering: limitations and recent advances. *Ann Biomed Eng* **32**: 160–165.

Dvir-Ginzberg M, Gamlieli-Bonshtein I, Agbaria R, *et al*. 2003; Liver tissue engineering within alginate scaffolds: effects of cell-seeding density on hepatocyte viability, morphology, and function. *Tissue Eng* **9**(4): 757–766.

Gelinsky M, König U, Sewing A, *et al*. 2004; Porous scaffolds made from mineralised collagen – a biomimetic bone graft material. *Mat-wiss Werkstofftech* **35**(4): 229–233 [in German].

Gelinsky M, Welzel PB, Simon P, *et al*. 2008; Porous three-dimensional scaffolds made of mineralised collagen: preparation and properties of a biomimetic nanocomposite material for tissue engineering of bone. *Chem Eng J* **137**: 84–96.

Hofmann A, Konrad L, Gotzen L, *et al*. 2003; Bioengineered human bone tissue using autogenous osteoblasts cultured on different biomatrices. *J Biomed Mater Res A* **67**(1): 191–199.

Holy CE, Shoichet MS, Davies JE. 2000; Engineering three-dimensional bone tissue *in vitro* using biodegradable scaffolds: investigating initial cell-seeding density and culture period. *J Biomed Mater Res* **51**(3): 376–382.

Ikada Y. 2006; Challenges in tissue engineering. *J R Soc Interface* **3**(10): 589–601.

Ishaug SL, Crane GM, Miller MJ, *et al*. 1997; Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J Biomed Mater Res* **36**(1): 17–28.

Ishaug-Riley SL, Crane-Kruger GM, Yaszemski MJ, *et al*. 1998; Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**: 1405–1412.

Kon E, Muraglia A, Corsi A, *et al*. 2000; Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res* **49**(3): 328–337.

Krebsbach PH, Kuznetsov SA, Satomura K, *et al*. 1997; Bone formation *in vivo*: comparison of osteogenesis by transplantated mouse and human marrow stromal fibroblasts. *Transplantation* **63**: 1059–1069.

Logeart-Avramoglou D, Anagnostou F, Bizios R, *et al*. 2005; Engineering bone: challenges and obstacles. *J Cell Mol Med* **9**: 72–84.

Mauney JR, Blumberg J, Pirun M, *et al*. 2004; Osteogenic differentiation of human bone marrow stromal cells on partially demineralized bone scaffolds *in vitro*. *Tissue Eng* **10**(1–2): 81–92.

Mendes SC, van den Brink I, de Bruijn D, *et al*. 1998; *In vivo* bone formation by human bone marrow cells: effect of osteogenic culture supplements and cell densities. *J Mater Sci Mater Med* **9**(12): 855–858.

Niemeyer P, Krause U, Punzel M, *et al*. 2003; Mesenchymal stem cells for tissue engineering of bone: 3D-cultivation and osteogenic differentiation on mineralized collagen. *Z Orthop* **141**: 712–717 [in German].

Niemeyer P, Krause U, Fellenberg J, *et al*. 2004; Evaluation of mineralized collagen and α-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. *Cells Tissues Organs* **177**(2): 68–78.

Oswald J, Boxberger S, Jorgensen B, *et al*. 2004; Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* **22**(3): 377–384.

Owen M. 1988; Marrow stromal stem cells. *J Cell Sci Suppl* **10**: 63–76.

Pittenger MF, Mackay AM, Beck SC, *et al*. 1999; Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.

Shearn J, Hellmann L, Boivin G. 2005; Effect of initial cell-seeding density on postoperative cell number and dispersion. *Tissue Eng* **11**(11–12): 1898–1904.

van den Dolder J, Spauwen PHM, Jansen JA. 2003; Evaluation of various seeding techniques for culturing osteogenic cells on titanium fiber mesh. *Tissue Eng* **9**(2): 315–325.

Wilson CE, Dhert WJA, van Blitterswijk CA, *et al*. 2002; Evaluating 3D bone tissue engineered constructs with different seeding densities using the alamarBlue™ assay and the effect on *in vivo* bone formation. *J Mater Sci Mater Med* **13**: 1265–1269.

Zhou YF, Sae-Lim V, Chou AM, *et al*. 2006; Does seeding density affect *in vitro* mineral nodules formation in novel composite scaffolds? *J Biomed Mater Res* **78A**: 183–193.