Cultivating Cells of Different Origin for 3d Bone Constructs Considering Physiological Conditions

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Abstract: One major objective in bone tissue engineering is the construction and application of precise matrices in order to support and guide cell growth and differentiation. These matrices are supposed to replace the extracellular matrix (ECM), which naturally provides cells with a supportive framework of structural proteins, carbohydrates and signaling molecules. The ideal matrix has to mimic these ECM characteristics and should form the desired structure with similar physical properties as bone tissue. Moreover it is known that mechanical strain is an essentiell stimulus for proper function of tissue like bone and cartilage. The mechanical strain mimics the physiological environment and thus supports the differentiation process. In this work MC3T3-E1 cells (mouse) were seeded onto macroporous ceramic scaffolds and cultured with and without differentiation conditions. Cell viability was monitored via MTT test. The differentiation status of MC3T3-E1 cells was investigated using the alkaline phosphatase assay and von Kossa staining of the extracellular matrix. Furthermore BMSCs were subjected to mechanical strain. Strained cells were compared to non strained cells with regard to proliferation, differentiation and repair mechanism by performing standard BrdU assay and collagen I and III specific radio immuno assays (RIA).

Key words: bone tissue engineering, Sponceram®, Biostat® RBS, rotating bed system, bioreactor, differentiation, mineralisation, BMP-2, MC3T3-E1 cells, BMSCs, mechanical strain, alkaline phosphatase, collagen I, collagen III, von Kossa, MTT assay

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1. INTRODUCTION

One approach in tissue engineering is to isolate cells or a tissue from a patient and to generate a construct of matrix and cells *ex vivo*, before reimplantation into the patient.

These matrices have to be biocompatible, should support cell attachment, growth and differentiation towards the desired phenotype.

The matrix for bone engineering has to fulfil requirements concerning their mechanical stability, biodegradability and porosity. Currently, the most frequently used materials for bone tissue engineering are ceramics like calciumphosphates, hydroxyapatites, degradable polymers such as poly (glycolic acid)/poly(lactid acid) or composite materials (Burg *et al*., 2000, Hutmacher, 2000, Logeart-Avramoglou *et al*., 2005). Also zirconium oxide has been investigated as a possible suitable implant material (Hentrich *et al*., 1971). Zirconia is often applied in composite materials to increase the strength of ceramics (Lee *et al*., 2004). In this study we used Sponceram® as biomaterial for bone engineering. It is a highly porous ceramic compound, consisting of doped zirconium oxide ($Zr0_2$). Moreover, special proteins, such as growth factors play an important role in tissue engineering. Bone morphogenetic proteins are the main inductors for bone and cartilage formation (Reddi 1992). They belong to the TGF-β superfamily (Sebald *et al*., 2004). Besides BMP-7, BMP-2 is the most prominent used cytokine for the differentiation process into bone tissue (Hollinger *et al*., 1998, Yamaguchi *et al*., 2000).

Recently, the field of tissue engineering has utilized mechanical stimulation *in vitro* as a tool for promoting the development of a number of tissue types including bone (Wakitani *et al*., 1994; Yang *et al*., 2002), cartilage (Davisson *et al*., 2002), ligament (Altman *et al*., 2002), skeletal muscle, and cardiac muscle (Kim & Mooney 2000; Zimmermann *et al*., 2002).

For bone tissue, according to the general physiological strain *in vivo,* the most frequently used systems elongate, compress or deflect cells grown on different substrates (Neidlinger-Wilke *et al*., 1994; Ngan *et al*., 1988; Pender & McCulloch 1991; Schaffer *et al*., 1994). While in circular devices cells are strained radially which is adequate for cells from dermal tissues or muscle cells, bone cells should better be strained longitudinally on rectangular substrates, where a homogenous strain distribution is given. Such a strain mimics a physiologic strain as present in bone tissue *in vivo*.

Cellular reaction to mechanical strain *in vitro* is dependent on substrate material and strain parameters like elongation amplitude, frequency, or strain duration. In this study, we chose rectangular elastic silicone dishes which are simple to deal with and do not influence cell morphology, total protein content and alkaline phosphatase activity (Neidlinger-Wilke *et al*., 1994). A frequency of 1 Hz was obtained as optimum for the proliferation of human

osteoblastic cells on silicon dishes (Kaspar *et al*., 2002). In contrast to the frequency, for elongation amplitude and duration, no optimal values seem to exist yet. Therefore, different strain durations were tested in this study.

2. MATERIALS AND METHODS

2.1 Scaffold production

Under *static conditions* Sponceram® (Zellwerk, Germany) matrices (3 x 4 mm) were incubated for 24 h in standard medium (DMEM, 10% FCS, antibiotics) at 37°C, 5% CO_2 in 96-well dishes.

1.5 x 10^4 MC3T3-E1 cells were seeded on each matrix in 96-well dishes for 30 min at gentle stirring at 37°C. Non attached cells were removed and the wells were filled up with 200 µl medium: 1. standard medium; 2. differentiation medium (1 µM dexamethasone, 10 mM β-glycerolphosphate, 50 µg/ml ascorbic acid); 3. BMP-2 medium (differentiation medium + 10 ng/ml BMP-2) and for each of the following tests the matrices were placed into a new 96-well dish. Scaffolds were cultured for up to 20 days. Since it was not possible to estimate the number of attached cells, the first measurements were performed directly after cell seeding.

Cell viability was assayed using MTT test (Sigma, Germany). DNA concentration was measured using the Pico Green assay (Molecular Probes, USA). Alkaline phosphatase (ALP) was determined by an assay based on the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol.

Under *rotating conditions*, MC3T3-E1 cells were injected by a sterile canula onto dry Sponceram® disc for 30 min. Two different types of cultivation were performed in the Biostat® RBS (rotating bed system) bioreactor (Sartorius BBI Systems, Germany). One cultivation was performed for 28 days under standard medium conditions (seeding cell number: 25 x 10^6/disc). The second one was cultivated for 10 days under standard medium followed by 11 days cultivation under differentiation medium + 10 ng/ml BMP-2 (seeding cell number: 55 x 10^6/disc).

At the end of the cultivation period, the cells were briefly washed with PBS and fixed in ice-cold 100% ethanol for 20 min at room temperature.

For von Kossa staining, fixed cells were washed with deionized water and incubated for 30 min in 5% $AgNO_3$ in the dark, washed with deionized water and exposed to ultraviolet light for 2 min.

Scaffolds for scanning electron microscopy were fixed in Karnovsky´s buffer (Ito and Karnovsky 1968) and subsequently dried in increasing acteone solutions.

2.2 Mechanical strain experiments

For the strain experiments, stem cells obtained from bone marrow were used. Human bone marrow aspirates were obtained during routine orthopaedic surgical procedures needing exposure of the iliac crest. Bone Marrow derived Stromal Cells (BMSCs) were isolated using Percoll density gradient centrifugation. Isolated cells were subsequently transferred to T-75 cm^2 flasks in DMEM containing 10% FCS and antibiotics. Adhering cells are believed to be BMSC. Cells in suspension were discarded. For strain experiments, 1.5×10^5 second passage BMSCs were seeded onto silicone dishes (11.5 cm^2) prepared of a two component silicone resin. Serum concentration was reduced to 1% for 24 h to align the cells into the G0 cell cycle phase. The cells on the silicone dishes were strained longitudinally with a frequency of 1 Hz, an amplitude of 5% for 15 min and 60 min, respectively. In order to investigate the proliferation characteristics of the cells, BrdU was added to the culture medium two hours before strain initiation. BrdU, being a thymidin analogue, is incorporated in the DNA of proliferating cells during the S-phase of the mitosis cycle.

For proliferation assay, cells were fixed with 70% ethanol in 0.5 M HCl at 20°C for 30 min and washed three times with DMEM. Cell proliferation was performed with a standard BrdU assay (Hoffmann La Roche, Germany).

Collagen I and III production was determined with a competitive Radio Immuno Assay (RIA) in the supernatant against the C-terminal propeptide of collagen I and the N-terminal propeptide of collagen III using ^{125}I radiolabeled antigens (DPC Biermann, Germany). A standard curve was used to determine the exact concentrations.

3. RESULTS AND DISCUSSION

3.1 Scaffold production under controlled conditions

To test the applicability of Sponceram® for the cultivation of MC3T3-E1 cells, a first screening in cell culture dishes under static conditions was performed. The cells were seeded in 96-well plate onto the matrices and cultured for up to 20 days. Several analyses for cell proliferation and differentiation into bone were carried out.

Cultivation of MC3T3-E1 cells in BMP-2 medium showed that the cells grew well inside the macroporous structure of Sponceram® with a typical flat morphology of osteoblatic like cells (Fig. 1A). The presence of single cells is rare. The cells grow as an interconnecting network having intercellular contacts to the surrounding cells (Fig. 1B).

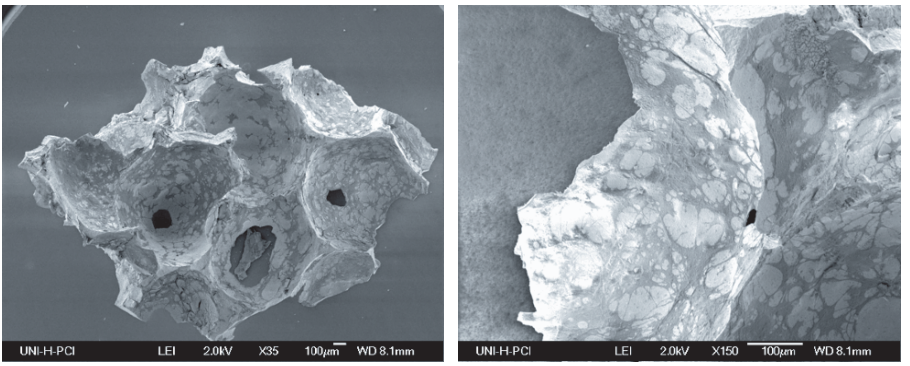

Figure 1. Scanning electron microscopie image of MC3T3-E1 cells cultured for 8 days in BMP-2 medium on Sponceram®.

3.1.1 Cell proliferation and alkaline phosphatase activity

Cell viability of MC3T3-E1 cells cultured under static conditions was analysed with standard MTT assay. Cultured MC3T3-E1 cells on Sponceram® in standard medium, differentiation medium and BMP-2 medium showed a fast cell growth during the first ten days. Due to high confluence on the matrix, the proliferation decreased after 10 days of culture (Fig. 2, left). To evaluate the differentiation process of MC3T3-E1 cells cultured on Sponceram®, the activity of the early osteogenic marker alkaline phosphatase was determined. Cells were seeded onto Sponceram® matrices in 96-well dishes and cultured in standard medium, differentiation medium and BMP-2 containing differentiation medium. Due to the differentiation induction of BMP-2 the alkaline phosphatase activity had a maximum at day 5 of cultured MC3T3-E1 cells in the cytokine containing medium. These results clearly indicate the beginning differentiation process into bone tissue on the matrix (Fig. 2, right).

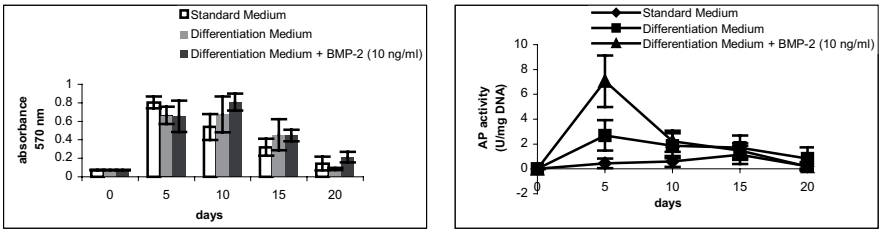

Figure 2. Cell viability (left) and alkaline phosphatase activity (right) of MC3T3-E1 cells cultured on Sponceram® over a time period of 20 days. Values represent the mean of 5 samples of cultured scaffolds +/- SD.

3.1.2 Mineralisation of ECM

MC3T3-E1 cells were cultured for 4 weeks in the Biostat® RBS bioreactor. One cultivation was performed in standard DMEM medium, in an additional cultivation, cells were cultured for two weeks in standard medium followed by two weeks in BMP-2 medium.

The production of calcified extracellular matrix (ECM) by MC3T3-E1 cells cultured under described conditions in the Biostat® RBS was qualitatively determined by histochemical staining with von Kossa.

Cells cultivated under differentiation conditions with BMP-2 showed a more intense von Kossa black positive staining, which indicates a higher mineralisation (Fig. 3, upper right). The same results were obtained by Alizarin red staining (data not shown).

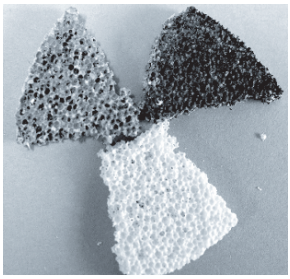

Figure 3. Results of mineralisation after cultivation in the Biostat® RBS. Von Kossa staining of non seeded Sponceram® (middle), cultured in standard medium (upper left) and in differentiation medium (upper right).

However, also MC3T3-E1 cells cultured under standard conditions showed mineralisation of the ECM, which means, that the Sponceram® matrix itself induced the bone differentiation process (Fig. 3, upper left). Thus differentiation medium with BMP-2 potentiate this to a large extent.

3.2 From genotype to phenotype by mechanical straining

3.2.1 Proliferation

BMSCs subjected to 15 minutes of longitudinal mechanical strain showed a two-fold increase in proliferation 6 hours after cessation of strain application. After 12 hours, this increase did not induce changes in proliferation during the entire observation period.

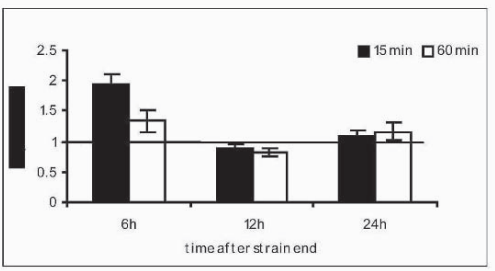

Figure 4. Cell proliferation measured by BrdU incorporation. Values represent the mean of 10 patients +/- SD. The baseline at 1 shows the control of non strained cells.

3.2.2 Differentiation and repair mechanism

15 minutes duration of strain application resulted in an increase of collagen typ I production. The maximum production was observed 12 hours after cessation of strain application (Fig. 5, left). This strain duration did not result in any change in collagen typ III production. Conversely, 60 minutes of strain induced increases of collagen III already 6 hours after termination of strain application (Fig. 5, right). This increased production remained until the end of the observation period. No significant deviation from baseline values were observed for the collagen I production using this strain duration.

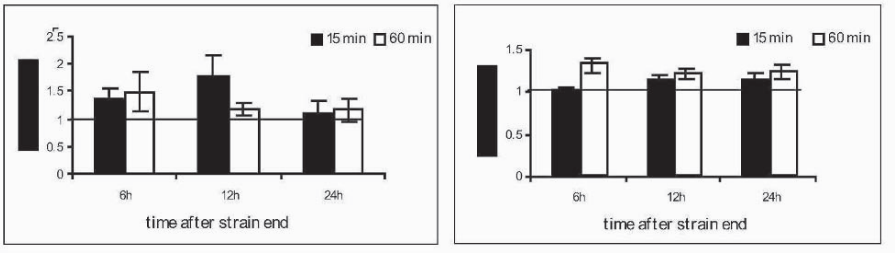

Figure 5. Collagen I (left) and III (right) production measured with a RIA. Values represent the mean of 10 patients +/- SD. The baseline at 1 shows the control of non strained cells.

4. CONCLUSION AND OUTLOOK

The results of cultivating MC3T3-E1 cells on macroporous Sponceram® showed, that it represents a suitable matrix for bone engineering. For a more controlled cultivation of functional bone tissue the Biostat® RBS was successfully applied. Our mechanical strain experiments showed that a longitudinal strain accelerates the differentiation of human bone marrow stromal cells towards bone tissue. Moreover, choosing an optimal duration

leads to physiologic extracellular matrix composition (like collagen I) and increases proliferation.

In further experiments the differentiation status will be analyzed in more detail by self developed DNA microarrays. The mechanical strain experiments and the scaffold production will be combined by cultivation of preconditions cells (BMSCs, MC3T3-E1) on Sponceram® matrices.

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