Bettina Hiemer*, Kathleen Achenbach, Juliane Pasold, Andreas Wree and Rainer Bader

Transient supplementation of growth factor TGF-β1 effectively initiates chondrogenic redifferentiation of human chondrocytes

Abstract: Cartilage tissue is avascular with less regeneration potential and therefore, cartilage regeneration is still a major challenge for therapeutic approaches. Commonly used treatment strategies involve the transplantation of autologous chondrocytes into the defect. Before that, it is required to increase the cell number *in vitro* resulting in unwanted chondrocyte dedifferentiation. This could impair subsequent tissue regeneration. Both growth factors TGF-ß1 and IGF-1 are used as strong inducer of chondrogenic redifferentiation, however, a controlled application of TGF-ß1 is essential to avoid adverse effects. Therefore, in the present study, we investigated the time-dependent influence of TGF-ß1 administration on chondrocyte redifferentiation.

Human chondrocytes were embedded in alginate and cultured in serum-free DMEM containing ascorbic acid, dexamethasone, ITS^{TM} and IGF-1. TGF-β1 was supplemented for 3, 7 and 21 days. Afterwards, cell viability and synthesis of extracellular matrix (ECM) proteins was analyzed by histological staining.

Live/dead staining of chondrocytes incubated with TGF-β1 for 21 days displayed an enhanced proliferation and formation of cell clusters resulting in excessive outgrowth of fibroblastic-like cells. However, exposure to TGF-β1 over only 7 days caused also cell clustering with moderate cell proliferation. Additionally, after 21 days of cultivation proteoglycan synthesis was identified by alcian blue staining after both TGF-β1 supplementation for 21 and also 7 days. Aggrecan was also detected in the periphery of the cell clusters after TGF-β1 incubation for only 7 days. Chondrocytes lacked proteoglycan expression after three-day TGF-β1 administration.

We could show, that prolonged administration of TGF-β1 results in massive proliferation of chondrocytes which is accompanied by cell outgrowth. We found that TGF-ß1 exposure for seven days is sufficient for achievement of cell clustering without excessive cell proliferation, which is important for inducing subsequent chondrogenic differentiation. Results indicate that even an initial TGF-β1 administration could be sufficient for inducing chondrocyte proliferation and differentiation *in vitro*.

Keywords: chondrocytes, chondrogenic redifferentiation, growth factors, aliginate matrix

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1 Introduction

The self-healing capacity of cartilage tissue is limited and, therefore, cartilage healing after lesion is restricted. A wide range of treatment strategies were developed involving replacement of injured tissue by osteochondral graft (autologous, allogenous or synthetic), marrow stimulation and transplantation of autologous chondrocytes into the defect [1, 2]. For the latter cell-based regeneration technique autologous chondrocytes were harvested from articular cartilage obtained from non-weight bearing region of the joint and cultured *in vitro* to increase cell number. Subsequent transplantation of these cells with or without combination with a three-dimensional scaffold into the defect side supports cartilage healing. However, during cell expansion using monolayer cultivation chondrocytes dedifferentiate, characterized by loss of chondrogenic phenotype and decreased type II collagen synthesis with simultaneous enhanced type I collagen production [3]. The implantation of dedifferentiated chondrocytes could cause production of inferior cartilaginous repair tissue *in vivo*,

***Corresponding author: Bettina Hiemer:** Rostock University Medical Centre, Department of Orthopaedics, Doberaner Str. 142, 18057 Rostock, Germany, e-mail: bettina.hiemer@med.uni-rostock.de

Kathleen Achenbach, Juliane Pasold, Rainer Bader: Rostock University Medical Centre, Department of Orthopaedics, Doberaner Str. 142, 18057 Rostock, Germany

Andreas Wree: Rostock University Medical Centre, Department of Anatomy, Gertrudenstraße 9, 18057 Rostock, Germany

whereby, the fibrocartilage is not able to withstand biomechanical forces in the knee joint [4]. In order to prevent this, three-dimensional cell culture systems are used for cultivation after cell expansion and the addition of several growth factors [5]. The most investigated molecules that stimulate the anabolic activity in cartilage include transforming growth factor (TGF)-β 1, 2, 3 and insulin-like growth factor (IGF) 1 [5, 6]. Both growth factors TGF-ß1 and IGF-1 are used as strong inducer of chondrogenic redifferentiation. Hence, in the present study we investigated the time-dependent effect of the administration of TGF-ß1 in addition to continuously added IGF-1, with the aim to promote proliferation of human chondrocytes at the beginning and then to facilitate the differentiation phase. Therefore, we embedded human chondrocytes in alginate, added TGF-β1 over different periods of time (3, 7 or 21 days) and analyzed cell proliferation and expression of cartilage ECM proteins.

2 Material and methods

2.1 Chondrocyte isolation and culture

Human chondrocytes were isolated from articular knee cartilage obtained from patients (n=4; male: 64 years ± 8 years; female: 76 years) undergoing primary knee replacement. The samples were collected after patient's agreement and approval by the Local Ethical Committee (registration number: A2009 17). The cartilage tissue was washed with phosphate buffered saline (PBS, PAA, Coelbe, Germany), minced and enzymatically digested using 1 % trypsin/EDTA (Gibco® Invitrogen, Darmstadt, Germany) for 20 minutes at 37 °C prior treatment with 0.2 % collagenase A (Roche, Mannheim, Germany) for three hours at 37 °C. The isolated chondrocytes were cultured in cell culture medium DMEM (Dulbecco's Modified Eagle Medium) containing 10 % foetal calf serum (FCS), 1 % amphotericin B and 1 % penicillin-streptomycin (all from Fisher Scientific, Darmstadt, Germany) supplemented with ascorbic acid (50 µg/mL, Sigma, Seelze, Germany) up to passage two [7].

2.2 Chondrogenic differentiation

In passage three, human chondrocytes were embedded in alginate to form three-dimensional constructs. The chondrocytes were mixed with 1.2 % sodium alginate ($1x10^6$ cells/mL, Sigma, Seelze, Germany) and dropped evenly into a calcium chloride solution (150 mM) to create beads. Alginate beads were cultured in serum-free DMEM containing 1 % ITS™ (Becton Dickinson, Heidelberg, Germany), ascorbic acid (50 µg/mL), dexamethasone (100 nM, Sigma, Seelze, Germany) and IGF-1 (50 ng/mL, RD Systems, Wiesbaden, Germany) further mentioned as chondrogenic medium. The growth factor TGF-β1 (50 ng/mL, tebu-bio, Offenbach, Germany) was supplemented for 3, 7 or 21 days of cultivation or cells were cultured without addition of TGF-β1. Chondrocytes embedded in alginate were cultured over 21 and 42 days under standard cell culture conditions (5 % CO_2 and 37 °C).

2.3 Cell viability

The metabolic cell activity was measured by WST-1 test (Roche, Berlin, Germany). This colorimetric assay measured enzymatic activity enabling quantification of metabolic activity. The viability of human chondrocytes was assessed using a LIVE/DEAD© assay kit (Life Technologies, Carlsbad, USA). The two-colour assay discriminates live from dead cells by simultaneously staining with green fluorescent (494-517 nm) calcein-acetoxymethyl (calcein-AM), indicating intracellular esterase activity and red-fluorescent (528-617 nm) ethidium homodimer-1, indicating the loss of plasma membrane integrity. The assay was performed as recommended by the manufacturer. Images of the cells were acquired using the confocal laser scanning microscope LSM 780 and evaluated with ZEN Imaging Software 2.0 (both from Carl Zeiss, Jena, Germany).

2.4 Histology

For investigating chondrocytes redifferentiation, histological and immunhistochemical analyses were performed identifying production of extracellular matrix (ECM) proteins. After harvesting the alginate beads were fixed in 4 % paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (pH 7.4; Roth, Karlsruhe, Germany) containing 10 mM $CaCl_2$ at room temperature for 4 h. Subsequently, beads were washed overnight at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 50 mM $BaCl_2$ and embedded in paraffin prior sectioned into 5 µm slices.

To display sulphated glycosaminoglycans of ECM the alginate beads were stained using alcian blue. After deparaffinising the samples were briefly incubated with 3 % acetic acid and stained with 1 % alcian blue 8GS (Chroma, Gujarat, India) in 0.9% NaCl solution containing 0.19 M $MgCl_2$ for 1.5 hours. Beads were carefully rinsed in 3 %

acetic acid and distilled water prior cell counterstaining with 0.1 % eosin for 5 min.

For aggrecan visualization deparaffinised beads were treated with chondroitinase (0.1 U/ml chondroitinase ABC in 50 mM TRIS) and Endo-β -galactosidase (5 U/ml in 20 mM TRIS) at 37 °C for 30 min, respectively. After blocking unspecific bindings samples were incubated with the primary monoclonal antibody anti-human Aggrecan G1-IGD-G2 domains (1:200, #MAB1220 R&D Systems, Minneapolis, USA) overnight at 4 °C. Secondary antibody used was Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:200, #A-11029 Invitrogen Molecular Probes, Eugene, USA) at room temperature in the dark for 1 hour followed by an incubation with Hoechst 33342 (5 µg/ml, Enzo Life Science, Loerrach, Germany) in order to visualize cell nuclei.

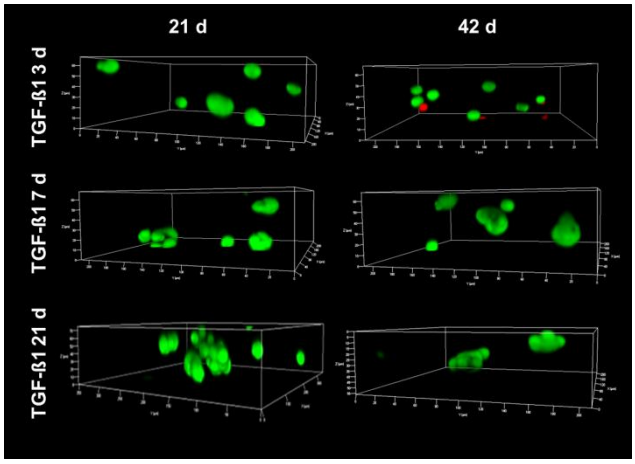

Figure 1: Human chondrocytes embedded in alginate were cultured in chondrogenic medium exposed to TGF-β1 for 3, 7 and 21 days over 21 or 42 days. Afterwards, cells were stained using live/dead staining to examine cell viability and investigated using confocal laser scanning microscopy. Living cells were stained green and dead stained red (n = 4).

3 Results

3.1 Influence of transient TGF-β1 supplementation on cell viability

Investigations showed that TGF-β1 addition for 7 and 21 days resulted in an enhanced metabolic cell activity compared to chondrocytes cultured for 3 days with TGF-β1 (data not shown), whereby only TGF-β1 exposure for 21 days reached level of significance (p=0.001, one-way ANOVA LSD posthoc). Live/dead staining of the whole bead was investigated by confocal laser scanning microscopy in order to analyse cell morphology and arrangement. Human chondrocytes cultured in alginate supplemented with TGF-β1 for 7 days indicated an increased stimulation of cell proliferation so that cell clustering was detected even after 42 days (**fig. 1**). Although the addition of TGF-β1 for only 3 days caused an initial cell clustering, after 21 days only single cells could be detected. The cultivation of cells in culture medium supplemented with TGF-β1 for 21 days displayed also an enhanced formation of cell clusters after 21 and 42 days. However, the superior stimulation of cell proliferation resulted in unwanted cell outgrowth from the alginate matrix.

3.2 Influence of transient TGF-β1 supplementation on ECM synthesis

Histological and immunhistochemical staining allowed the examination of proteoglycans as a marker for chondrogenic redifferentiation (**fig. 2**). Human chondrocytes embedded in alginate and cultured in chondrogenic medium without the addition of TGF-β1 exhibited low cell number and rarely synthesized proteoglycans after 21 days of cultivation. Alcian blue staining revealed low cell number and proteoglycan deposition for cultivation with TGF-β1 over 3 days as well, however, immunhistological staining showed a narrow aggrecan edge around chondrocytes. Histological investigation confirmed observation that the longer TGF-β1 application the stronger the formation of cell clusters within the alginate matrix. Even if TGF-β1 was applied for only 7 days during 21 days of cultivation, it resulted in enhanced cell number and deposition of proteoglycans. Although aggrecan staining displayed slightly higher amount for chondrocytes cultured in TGF-β1 for the whole 21-day cultivation, the TGF-β1 exposure for only 7 days was suitable for formation of neocartilage-like ECM in human chondrocytes embedded in alginate.

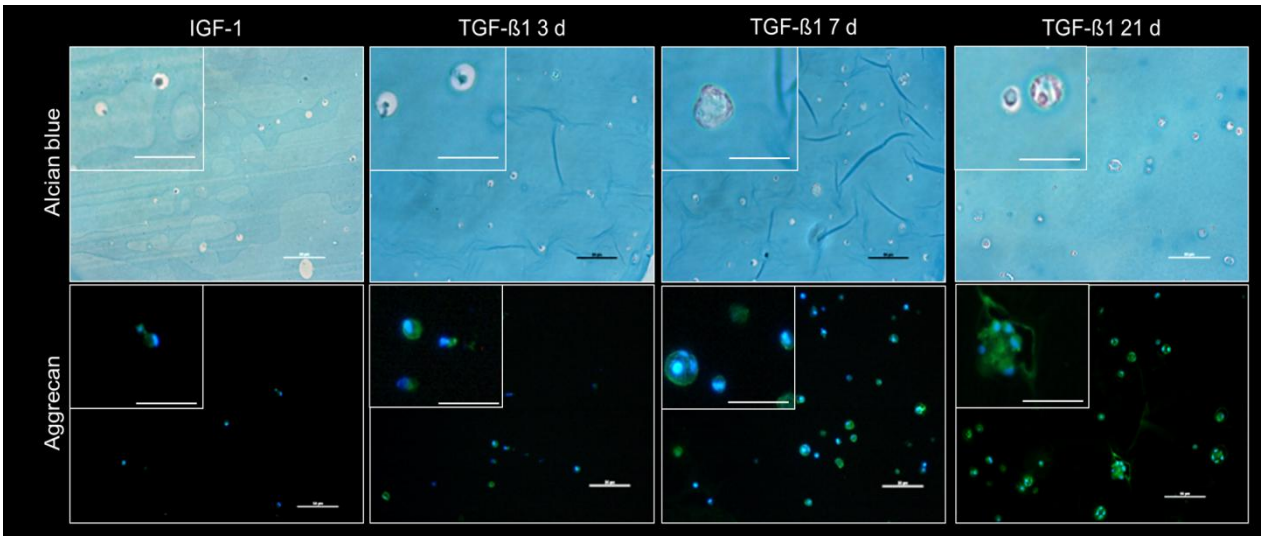

Figure 2: Human chondrocytes embedded in alginate were cultured in chondrogenic medium supplemented without or with TGF-β1 for 3, 7 and 21 days. Histological and immunhistochemical images of representative samples after 21 days of cultivation are presented. Using alcian blue (upper panel) proteoglycans were visualized by blue staining and chondrocytes were counter stained by eosin (pink). Immunhistochemical staining using anti-aggrecan antibody (lower panel) presented green fluorescent aggrecan depositions and hoechst-stained cells fluorescenting violett (n = 4).

4 Discussion

Improving cell-based therapies for the treatment of cartilage lesions the understanding of chondrocytes fate during *in vitro* cultivation and influence of different growth factors on chondrogenic redifferentiation is required.

Using alginate as matrix for embedding human chondrocytes to form a three-dimensional construct, the physiological environment of cartilage tissue is imitated well [7,8]. Images of live/dead staining displayed round-shaped phenotype of chondrocytes encapsulated within alginate matrix as single cells or a cell couple indicating cartilage-like arrangement as clusters. TGF-β1 supplementation influenced the cell clustering required for chondrogenic differentiation. Although the addition of TGF-β1 over 21 days of cultivation resulted in superior cell cluster formation, surface outgrowth of fibroblastic-like cells was detected demonstrating high potential of TGF-β1 for induction of proliferation [9,10]. However, the formation of cell clusters initiating chondrogenic differentiation was also observed after TGF-β1 exposure for only seven days. Our findings are in agreement with studies of Wolter et al. [9] showing that a sequential addition of TGF-β1 and IGF-1 increased the proteoglycan synthesis in chondrocytes. Human chondrocytes embedded in alginate and cultured in chondrogenic medium supplemented with IGF-1 only and without TGF-β1 displayed inferior chondrogenic differentiation demonstrating that TGF-β1 is essential for inducing chondrogenic redifferenetiation [10]. Chondrocytes exposed to TGF-β1 for only seven days showed proteoglycan deposition even after 21 days of cultivation. This indicated that an initial TGF-β1 administration period of seven days can be beneficial for inducing chondrocyte proliferation and differentiation *in vitro* and that IGF-1 may be used for maintaining ECM expression while preventing massive chondrogenic cell proliferation.

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Author's Statement

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has been approved by the authors' institutional review board or equivalent committee (registration number: A2009 17).

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