Dermatan sulphate in methoxy polyethylene glycol-poly lactide-co-glycolic acid scaffolds upregulates fibronectin gene expression but has no effect on in vivo osteochondral repair

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Abstract

Purpose The purpose of the study was to investigate the effect of dermatan sulphate (DS) addition to biodegradable methoxy polyethylene glycol (MPEG) substituted polylactide-co-glycolic acid (PLGA) scaffolds for cartilage repair in vitro and in vivo.

Methods Human chondrocytes from eight patients undergoing anterior cruciate ligament reconstruction were isolated and cultured in 5% oxygen on MPEG-PLGA scaffolds $\pm$ DS for one, three, seven and 14 days. Analyses were performed using quantitative gene expression analysis for chondrogenic and cell attachment markers. An osteochondral drill hole defect was created in the intertrochlear groove of the distal femur in 20 New Zealand white rabbits (defects $n=20$). When bleeding was observed, the defects were treated with MPEG-PLGA scaffolds $\pm$ DS. Twelve weeks after

surgery the rabbits were sacrificed and the defects were analysed using histological grading with O'Driscoll scoring. **Results** DS addition to MPEG-PLGA scaffolds resulted in a significant upregulation of fibronectin gene expression on day 1. No differences were observed in chondrogenic gene expression. There were no differences between the two groups in histological grading (+DS 10.3 and -DS 9.6). **Conclusions** Upregulation of fibronectin in vitro indicating early cell-scaffold interaction and attachment did not result in improved cartilage repair in an osteochondral defect model in rabbits.

Introduction

The limited potential of spontaneous self-repair of articular cartilage defects has prompted several investigations of surgical repair strategies. Insufficient regeneration might over time lead to early development of osteoarthritis [1]. Arthroscopic microfracture or bone marrow stimulation with penetration of the subchondral bone is a technically feasible procedure for treatment of cartilage defects at a relatively low cost [2]. Despite good short-term results Kreuz et al. [3] demonstrated a decrease in cartilage quality after 36 months [3]. Similar results were found by Mithoefer et al. [4], who showed that the initial good clinical outcome deteriorated over time [4]. A microfracture technique has been suggested for smaller cartilage defects leaving larger defects to open surgery two-step techniques such as autologous chondrocyte implantation (ACI) and matrix-supported chondrocyte implantation and associated techniques [5, 6]. More recent approaches aim at stabilising

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the formed blood clot and providing a scaffold for cell attachment by the addition of a supporting membrane to the microfracture technique [7].

ASEED™ is a methoxy polyethylene glycol (MPEG) substituted polylactide-co-glycolide (PLGA) scaffold, consisting of a hydrophilic part, MPEG, and a hydrophobic part, PLGA. ASEED™ seeded with autologous chondrocytes has demonstrated good results in vitro and significantly better cartilage repair than empty defects, osteochondral drilling alone and fibrin hydrogel with chondrocytes in a goat model [8, 9]. Dermatan sulphate (DS), formerly known as chondroitin sulphate B, is one of several glycosaminoglycans (GAGs) in articular cartilage and is a major component of connective tissue matrix and cell surface and basement membranes [10, 11]. These are attached to a protein core producing proteoglycans. DS is synthesised as chondroitin sulphate in which some of the glucuronate residues undergo epimerisation to form iduronate, which alters the sugar chain configuration and thus its properties [12]. It has been shown that exposing synovio-cytes to sulphated GAGs such as DS can increase the quantity and improve the quality of hyaluronic acid produced by these cells [13, 14]. Munteanu et al. showed that adding highly sulphated GAGs to cartilage explants decreased the aggrecanase-induced degradation of aggrecan [15]. In addition, DS triggers maturation of mesenchymal stem cells, the cells believed to be the primary cell source in cartilage repair by the microfracture technique [16].

Based on these properties of DS, the aim of this study was to test the effect of DS addition to MPEG-PLGA scaffolds in in vitro and in vivo cartilage repair. We hypothesised that addition of DS to MPEG-PLGA scaffolds would improve the cartilage repair relevant gene expression in chondrocytes and cartilage repair in an osteochondral drill hole defect in a rabbit model.

Materials and methods

Scaffold fabrication

The scaffold was produced by dissolving methoxy polyethylene glycol-block-co-poly(lactide-co-glycolide) (MPEG-PLGA) (50:50 LA:GA) in 100 ml 1,4-dioxane overnight at 50°C. For the DS-containing scaffolds DS powder was suspended in a concentration of 4 w/w% in this polymer solution the following day. Seven millilitres of polymer/DS suspension was poured into a precooled 7.3 × 7.3 cm² aluminium form and placed in a freeze dryer at −20°C and one hour at +30°C. After overnight drying at room temperature in a vacuum desiccator the scaffolds were sterilised in 100% C₂H₅OH, dried and packed into aluminised polyethylene terephthalate (PET) bags. The average porosity of the scaffolds was above 90% and the thickness 2.0 mm. Using

dermal biopsy punches (Miltex Inc., York, PA, USA) scaffolds with a diameter of 4.0 mm (for in vitro) and 6.0 mm (for in vivo) were created for the experiments.

Human chondrocyte harvest and culture

After obtaining written consent from four healthy patients undergoing anterior cruciate ligament reconstruction, full-thickness cartilage biopsies were collected from the intercondylar groove in the distal femur at the site of the anticipated tunnel for the newly reconstructed ligament. The protocol was approved by the local Ethics Committee under the Danish National Committee on Research Ethics, #M-2008-008. After transportation of the biopsies to the laboratory the chondrocytes were isolated and cultured by a method previously described [17]. Briefly, the cells were isolated by enzymatic digestion cultured separately for each patient in 21% oxygen until confluence and then divided into two groups for culture on MPEG-PLGA scaffolds (ASEED™, Coloplast A/S Incubation, Humlebæk, Denmark) with or without DS. After seeding the cells onto the scaffold they were moved to a pre-balanced hypoxic workstation (Xvivo System, BioSpherix, Lacona, NY, USA) for culture in 5% oxygen. The oxygen tension was chosen to resemble an approximation of in vivo conditions of chondrocytes in the superficial to middle part of the articular cartilage layer [18, 19]. We have previously shown that the cells remain viable and experience the hypoxic challenge to as low as 1% oxygen [9]. Oxygen and carbon dioxide tensions and temperature were measured throughout the experiment. RNA extractions from these subcultures of four cell lines (*n*=4) were performed one, three, seven and 14 days after the baseline time point, respectively (passage 2).

Quantitative gene expression analyses

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as described elsewhere [17]. Briefly, the total RNA was extracted from vortexed scaffolds. The concentration was assessed spectromorphometrically and RNA quality analysis was performed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). qRT-PCR was performed using commercially available TaqMan® Gene Expression Assays (Applied Biosystems) for collagen type 1 alpha 1 (COL1A1) Hs00164004_m1, collagen type 2 alpha 1 (COL2A1) Hs00264051_m1, collagen type 10 Hs00166657_m1 (COL10A), aggrecan (AGC) Hs00153936_m1, SOX9 Hs00165814_m1, vinculin (VCL) Hs01009035_m1, fibronectin (Fn) Hs01565277_m1 and β-actin (ACTB) Hs99999903_m1. Standard enzyme and cycling conditions for the 7500 Fast System were used. In order to maximise amplification efficiency, amplicon size for all primer

sets was <180 bp. Values were normalised to ribosomal protein L13a (RPL13A) Hs03043885_g1 and β_2 -microglobulin (B2M) Hs99999907_m1 [17]. Each biological sample was run in technical duplicates for each gene.

In vivo design

Twenty mature New Zealand white rabbits weighing 3.5 kg (SD 0.27 kg) were used in the study. The animals received a bilateral osteochondral drill hole defect (5 mm diameter, 2 mm depth) in the femoral part of each knee joint, in which only one knee on each rabbit was used in this study. The intervention in the contralateral knee did not conflict with this study. They were divided into two treatment groups of ten defects each: (1) MPEG-PLGA scaffold (ASEED™) ($n=10$) and (2) DS-containing MPEG-PLGA scaffold ($n=10$). The implanted scaffolds were 2 mm in height and 6 mm in diameter. All experiments complied with the Danish Law on Animal Experimentation and were approved by the Danish Ministry of Justice Ethics Committee, J.nr. 2008-561-1459.

Anaesthesia and surgery

The animals received an intramuscular injection of 10 mg/kg xylazine (Rompun vet, Bayer HealthCare, Leverkusen, Germany) and 50 mg/kg S-ketamine (Pfizer, Ballerup, Denmark). After the knee joint was shaved, the rabbit was placed in a supine position and prepared for a sterile procedure. The knee joint was reached through an anteromedial incision of 2.5–3.0 cm after location and lateral retraction of the patella. The intertrochlear groove was located and a K-wire 1.5 mm in diameter was drilled in 1.0 cm proximal from this. Using this guide, a defect 5.0 mm in diameter with a depth of 2.0 mm was made. When bleeding from the subchondral bone was observed the defect was either left empty or an MPEG-PLGA scaffold (6.0 mm in diameter, 2.0 mm thickness) was press-fitted into the defect. The scaffolds were fixed with fibrin glue (Tisseel® Duo Quick, Baxter AG, Vienna, Austria). The joint capsule was closed with continuous Polysorb 3-0, and the skin was closed with single Surgipro 4-0 stitches, which were removed 14 days post-operatively.

Follow-up and termination

The animals were housed individually with free access to water in a designated and approved facility for 12 weeks and were sacrificed under general anaesthesia using an intracardiac injection of pentobarbitone (100 mg/kg).

Histology

The distal femur was resected and stripped of soft tissue followed by dehydration in a graded series of ethanol (70–96%), clearing in xylene and embedding in methyl methacrylate (MMA). Three slices with a thickness of 7 μ m were made through the centre of the defect on a microtome (Polycut E, Reichert-Jung, Heidelberg, Germany) in a random direction orthogonal to the surface plane. The samples were stained with safranin O, haematoxylin and eosin or collagen 2 antibody staining. Scoring of cartilage quality was performed using the O'Driscoll semi-quantitative scoring system [20].

Statistics

Normalised data from qRT-PCR were ln-transformed and the residuals were checked for normal distribution using QQ plots. The data from the individual scaffolds were analysed using two-way analysis of variance (ANOVA) with repeated measurements (scaffold*time). The non-parametric data from histological evaluation were analysed using the χ^2 test; p values less than 0.05 were considered significant.

Results

The gene expression for fibronectin was significantly higher on day 1 in the DS-containing scaffolds ($p<0.01$), while no difference was observed at the other time points. The gene coding for β -actin (ACTB), the major cytoskeletal protein, showed increased expression in the DS-containing scaffolds at all time points. However, this was not significant ($p=0.07$). No differences in the VCL gene expression were observed. No significant differences in gene expression for the genes coding for collagen type 1, 2, 10, aggrecan and SOX9 were observed. The results from quantitative gene expression analyses for ACTB and fibronectin are depicted in Fig. 1.

Representative histological images are shown in Fig. 2 and the results from the semi-quantitative histological O'Driscoll score are shown in Fig. 3. The mean total score from O'Driscoll evaluation was 10.3 (SD 5.0) for the DS-containing scaffolds and 9.6 (SD 3.9) for the MPEG-PLGA scaffolds without DS. Although the safranin O staining sub-score was 1.2 in the DS-containing scaffolds compared to 0.7 in the group without DS indicating increased proteoglycan content, no significant differences were observed. No significant differences were observed in any sub-scores for nature of predominant tissue, degenerative changes or structural parameters (Fig. 3).

Fig. 1 Gene expression of ACTB (*left*) and fibronectin (*right*) relative to the housekeeping genes RPL13A and B2M. MPEG-PLGA (*white*) and MPEG-PLGA + DS (*grey*) are presented for each time point. Data

are presented ln-transformed as mean, lower and higher quartile (*box*) and lowest and highest observation (*whiskers*). At day 1 fibronectin expression was significantly higher in DS-containing scaffolds

Discussion

This study demonstrated that addition of DS to MPEG-PLGA scaffolds led to a significant upregulation of fibronectin expression in human chondrocytes, but that this difference did not affect the cartilage repair in an osteochondral defect in vivo. Fibronectin is a glycoprotein that mediates a wide variety of cellular interactions with the extracellular matrix and plays an important role in cell adhesion, migration, growth and differentiation [21, 22]. Several binding sites for the fibronectin protein have been identified and binding to these various domains leads to the downstream events mentioned above such as cell attachment and cell migration (C-terminal domain) [23, 24]. In tissue engineering early cell attachment to the scaffold or implant and the migration into the material are important factors for the biological and functional outcome of the regenerative tissue,

as cells do not start producing extracellular matrix before they are firmly attached. We suspected that early attachment and trapping of the cells within the scaffold would lead to enhanced cell migration in the scaffold and an increase in matrix synthesis resulting in a subsequent improvement in cartilage repair in vivo.

In this study we found no significant effect of adding DS to the scaffold in the semi-quantitative histological scoring. This suggests that when considering the complexity of cartilage repair, the limited positive effect of DS as shown in vitro was not sufficient to increase the outcome in vivo. Articular cartilage repair and certainly regeneration are recognised as challenging tasks [25]. In an osteochondral defect model as used in this study, mesenchymal stromal cells (MSCs) are the cell source for repair response. When using MSCs as the cell source for cartilage repair, these cells are expected to differentiate into chondrocytes without

Fig. 2 Representative histological sections of the repair tissue stained with safranin O. In the best sections, *red stain* representing GAG content fills up the defect. The average repair also representing the

bulk of the sections only shows GAG staining on the edges. The worst repair tissue is mainly fibrous tissue with irregular surface structure. There were no differences between the two types of scaffolds

Fig. 3 Semi-quantitative histological score from O'Driscoll evaluation. No significant differences were observed

undergoing hypertrophy, which would lead to an increase in collagen type X and vascular endothelial growth factor (VEGF) expression. Hence, one of the major challenges using MSCs is adverse angiogenesis in the regenerating tissue, which is also a factor in osteoarthritic cartilage degeneration [26]. In articular cartilage, being an avascular tissue, the extracellular matrix synthesis in the regenerating environment leads to increasing hypoxia and adverse blood vessel recruitment. The increase in oxygen tension initiates dedifferentiation of differentiated chondrocytes and limits the differentiation stimuli of hypoxia towards chondrogenesis [27, 28]. The impact of these factors is among those aspects not dealt with by adding DS and they might overshadow a possible positive effect of DS in cartilage repair.

Cartilage repair has been one of the most intensively investigated areas in orthopaedics for the past two decades.

Several different biomaterials have been applied such as synthetic polymer scaffolds and injectable gels as well as different tissue engineering approaches using different cell sources such as stem and progenitor cells from different origins [29]. Despite the tremendous effort, repair of articular cartilage defects continues to be a challenging task [30]. This study illustrates that optimising in vitro properties of repair scaffolds can be insufficient to gain an improved repair response in vivo. Small changes in vitro might easily be overridden in vivo by much more potent factors present in the challenging environment in which cartilage repair is expected to take place such as hypoxia and mechanical loading of the repair area [31, 32], which might be dealt with by improving the mechanical stability of the scaffolds and controlling the intended hypoxic environment [33, 34].

The repair model being an osteochondral drill hole defect has limitations in comparison to an isolated cartilage defect where the subchondral bone plate is left intact. The regeneration and remodelling of the subchondral bone was not investigated. Also in the *in vivo* study we only investigated the effect of the two scaffolds as a support for marrow-stimulated treatment. While our *in vitro* model investigated the maintenance state of fully differentiated chondrocytes, the *in vivo* model investigated the regenerative capacity and initiation of differentiation of MSCs from the bone marrow, which yielded an equally insufficient outcome in the two groups. Hence, the results from human chondrocytes *in vitro* could not be translated to the response of rabbit bone marrow and MSCs. Finally, we did not investigate the release profile of DS from the MPEG-PLGA scaffold. The lack of improvement in cartilage repair might not be a lack of function of DS itself, but rather a limited bioavailability at the time of implantation and during the regenerative period.

Conclusion

Our study showed that adding DS to biodegradable MPEG-PLGA scaffolds upregulates the gene expression of fibronectin *in vitro* in human chondrocytes. However, the effect of DS addition to a MPEG-PLGA scaffold in an osteochondral drill hole defect in rabbits did not show significant differences between the treatment groups. This suggests that the potential positive effect of DS alone was not sufficient to enhance cartilage repair in an osteochondral defect model.

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Conflict of interest The authors declare that they have no conflict of interest.

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