Polyethylene terephthalate (PET) enhances chondrogenic differentiation of ovine meniscocytes in a hyaluronic acid/polycaprolactone scaffold in vitro

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Abstract

Purpose The purpose of this study was to assess the effect of polyethylene terephthalate (PET) on proliferation, differentiation, and attachment of ovine meniscocytes seeded in a hyaluronic acid/polycaprolactone biomaterial (BF-1)

Methods BF-1 (30 % hyaluronic acid and 70 % polycaprolactone) cylinders with PET (CO-PET) or without PET, were seeded with 2×10^6 ovine meniscus cells. The specimens were harvested in triplets at 12 hours, seven, 14, 21 and 28 days. DNA content was measured to test proliferation, histological analysis for cell morphology, and biochemical assessment of glycosaminoglycan content and RT-PCR for type I and II

collagen were used to assess differentiation, with immunohistochemistry as post-translational control. Attachment was evaluated by electronic microscopy at 28 days.

Results DNA content was consistent and equal across groups, suggesting no effect of PET on cell proliferation. However, the BF-1 CO-PET showed a higher percentage of cells with spherical morphology which is typical for a chondrocytic phenotype. This biomaterial with PET also showed a higher type II collagen mRNA expression and an eightfold higher GAG-content than the material without PET. Small amounts of type I collagen mRNA expression were present on both materials at all time points. PCR results were confirmed by immunohistochemistry.

Conclusion Addition of PET to a hyaluronic acid/polycaprolactone biomaterial enhances a cartilaginous phenotype, increased type II collagen mRNA expression and a higher GAG production in ovine meniscocytes.

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Introduction

The menisci play a critical role in load bearing, stability, shock absorption, nutrition and lubrication of the knee joint [1–3]. Loss or removal of the meniscus is both a major risk factor for osteoarthritis and a painful and troublesome condition per se [4–6]. Unfortunately, the potential for intrinsic healing of the menisci is restricted to the outer two thirds and current treatment options are limited [7]. Hence, the development of new treatments [8] with the help of tissue engineering techniques, based on a combination of cells, growth factors, genetically modified cells [9] and biomaterials [10, 11], has become a focus of research.

Such a biomaterial should be biodegradable, biocompatible, and should allow unlimited diffusion of nutrients [12]. It has to

withstand mechanical stress and stimulation of integration into the adjacent host tissue. Lastly, the biomaterial should support meniscocyte growth and differentiation. Different types of biomaterials made from natural polymers such as collagen, or synthetic polymers including polyglycolide, polylactides [13] and polycaprolactone [14] have been developed. Particularly good results were seen with BF-1 CO-PET, which is a biomaterial consisting of fast degrading hyaluronic acid (30 %) and slower degrading poly- ϵ -caprolactone (70 %) augmented with polyethyleneterephthalate (PET) fibers [11, 12]. The addition of PET is usually done to augment the biomechanical capabilities of the scaffold [12, 13], yet recent research has shown a beneficial effect of PET on the differentiation of cells of the mesenchymal lineage other than meniscocytes. If such effects also existed for meniscocytes they might have a profound effect on *in vivo* results of meniscus repair with a PET augmented biomaterial [13–15].

Therefore, the objective of this study was to assess the effect of PET on proliferation, differentiation, and attachment of meniscocytes seeded in a hyaluronic acid/polycaprolactone biomaterial. Such a material has been successfully used for meniscus repair in large animal studies and has been proposed for clinical use [13–15].

Materials and methods

Biomaterial and cells

Meniscus cells were obtained from sheep undergoing knee surgery for an IACUC approved study. Cells were isolated by enzymatic digestion from a sheep meniscus using a collagenase protocol and cultured with Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) containing 10 % foetal calf serum and 0.025 % ascorbate in a sterile incubator at 37 °C, 5 % CO₂, and 95 % rH [11, 16]. Medium was changed every three days. Once they reached 90 % confluence, cells were split in a 1:2 ratio using a Trypsin protocol [11, 16]. Cells from second passage were used for all experiments.

We used a PCL/HYAFF-11 biomaterial p75 HE 70/30 w/w with a pore size of 150 μ m to 200 μ m (BF-1) with or without addition of non-degradable polyethyleneterephthalate (PET) fibres (Fidia Advanced Biopolymers, Abano Terme, Italy). For each group 47 cylinders were punched out with a round biopsy punch ($\varnothing$ 6mm), and nine were used as unseeded, empty controls. The cylinders were hydrated 24 hours prior to cell seeding. A total of 2×10^6 cells were suspended in 50 μ l of medium and seeded in dropwise fashion. The cylinders were kept in 24 well-plates at 37 °C, 5 % CO₂, and 95 % rH for one hour before 2 ml of medium were carefully added. The medium was changed every other day. Sample triplets were obtained at 12 hours, seven, 14, 21 and 28 days for DNA and GAG measurement,

PCR, and histology and immunohistochemistry. Additional samples for electron microscopy were obtained at 28 days.

Biochemical assessment of DNA and GAG content

For DNA measurement the Quant-iT™ PicoGreen® dsDNA Assay Kit (P7589, Molecular Probes, Eugene, USA) was used. The GAG-content of the seeded cylinders was measured using the 1.9-dimethyl-methylen-blue (DMMB) dye assay (Aldrich, Sheboygan, WI, USA) described in Farndale et al. [15]. GAG content was normalised by DNA content to account for differences in cell density across samples. Unseeded cylinders acted as empty controls.

Histological and immunohistochemical analysis

All microscopic assessments were done by a blinded investigator. The harvested triplets were cut into halves, embedded and cut with a microtome perpendicular to the horizontal plane in three series of 5- μ m thick slices. Between-series distance was 500 μ m. Haematoxylin and eosin was used to evaluate cell density and morphology using a grid eyepiece ("Net" 5 mm, Olympus, Precision Instrument Division). For each sample five randomly chosen areas were assessed in 40x magnification. Cell morphology was graded as spherical (length:width ratio smaller 2:1), elongated (length:width ratio 2:1), or unassigned, as previously described [16–18]. The spherical shape is associated with chondrocytic differentiation; the elongated shape is associated with fibroblastic differentiation. The histological assessment was carried out by one of the authors (UK).

Type I and type II collagen production was assessed in immunohistochemistry done using earlier published protocols [11, 16]. The antigen antibody reaction was graded from "0" to "3", where "0" represents no visible antigen antibody reaction and "3" is clear visible antigen antibody reaction.

Transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed for type I and type II collagen mRNA. Frozen matrix cylinders were cut with a micropistill and suspended with 1 ml TRI® reagent (Molecular Research Center Inc, MRC, USA). After incubation for five minutes at room temperature 100 μ l of 1-Bromo-3-Chloropropane (BCP, MRC, USA) was added to the homogenate, shaken for 15 seconds and incubated for five minutes at room temperature. Then it was centrifuged for 15 minutes at 14,000 rpm at +4 °C. The aqueous supernatant was transferred, RNA was precipitated with 500 μ l isopropanol (Sigma, USA) for ten minutes, pelleted for eight minutes at 13,000 rpm at RT, washed and resuspended with 1 ml 75 % alcohol. Using an UV-spectrophotometer, concentrations of total RNA solutions were analysed at wavelengths of 260 nm and 280 nm respectively. One μ g of total RNA was submitted to cDNA synthesis using a

First Strand cDNA-Synthesis Kit containing an AMV (avian myeloblastosis virus) reverse transcriptase (Roche Diagnostics Corp., IN, USA). To specifically transcribe mRNA into template cDNA for further amplification, Oligo-p(dT)₁₅ primers were used. For the PCR 3 μ l of the cDNA were taken to detect the synthesis of Collagen Typ1 (COL1A1) and Typ2 (COL2A + B), using beta-actin for house keeping. The used primers are given in Table 1. Immunohistochemistry for type I and II collagen was used as post-translational control.

Electron microscopy

The specimens were fixed in glutaraldehyde 2.5 %, dehydrated in a graded series of ethanol and critical point dried. Before the samples were mounted on stubs they were sputtered with gold. Both materials were examined in a JEOL (JSM 6310, Tokyo, Japan) scanning electron microscope and were scanned at 10 kv. Only samples from 28 days were used for electron microscopy.

Statistical methods

Sample triplicates were used and averaged for all tests. Differences between groups were tested with multivariate ANOVA with Bonferroni correction for post hoc testing. The change in GAG per weight, DNA per weight, and GAG/DNA between materials was calculated with linear regression and adjusted for time in culture. All values are presented as mean $\pm$ standard deviation. An alpha of 5 % was considered significant. All calculations were done with intercooled Stata 10 (StataCorp LP, College Station, TX).

Results

DNA assay

We found no evidence for differences in DNA content (per mg of sample) across groups ($p=0.1510$) or over time ($p=0.1428$). The DNA assay showed that on the BF-1 the DNA content decreased from time point 12 hours to time point day 28 from $100 \% \pm 14.7 \%$ to $82.5 \% \pm 13.7 \%$. The DNA content on the

Table 1 RT-PCR primers

Sequence	PCR products	Forward and reverse primer
COL1A1	138 bp	F: 5'-AGGGCCAAGACGAAGACATC-3' R: 5'-AGATCACGTCATCGCACAACA-3'
COL2A + B	106 bp	F: 5'-CAACACTGCCAACGTCAGAT-3' R: 5'-CTGCTTCGTCCAGATAGGCAAT-3'
β -Actin	532 bp	F: 5'-GATTCCTATGTGGGCGACGAG-3' R: 5'-CCATCTCTTGCTCGAAGTCC-3'

Fig. 1 Results of the DNA assay on the BF-1 and the BF-1 CO-PET

BF-1 CO-PET slightly decreased from $100 \% \pm 2.2 \%$ to $82.5 \% \pm 9.5 \%$ on day 14. At time point day 21 a DNA content

Fig. 2 Results of the GAG assay on the BF-1 and the BF-1 CO-PET

of $87.7 \% \pm 3$ that slightly decreased to $83.6 \% \pm 13.4$ on day 28 was detected (Fig. 1).

GAG assay

We found a significant overall increase in GAG per weight over time for all samples ($p < 0.001$). On the BF-1 CO-PET GAG/DNA increased to $820.3 \% \pm 7.4 \%$ over 28 days, but on BF-1 without PET only to $157.5 \% \pm 13.7 \%$, which is consistent with a significant difference ($p < 0.001$) (Fig. 2).

Histological results

The results of cell morphology distribution in the BF-1 showed that the cell morphology continuously changed from a round/spherical type to elongated but mostly not assignable cell morphology (Fig. 3). Within the first two weeks the same trend of dedifferentiation was visible on the BF1 CO-PET (Fig. 3). However, after day 14 the cells regained their round/spherical morphology, leading to a significantly larger percentage of round cells in BF1 CO-PET ($21.1 \% \pm 0.9 \%$)

compared to BF-1 ($3.4 \% \pm 4.9 \%$) at 28 days ($p = 0.008$). This difference resulted from a difference in non-assignable cells across groups ($p = 0.0002$) rather than elongated cells ($p = 0.1297$) (Fig. 4a–f).

Immunohistochemistry on BF-1 for collagen type I was negative during the first 12 hours and seven days. Day 14 was slightly positive, day 21 negative and day 28 again slightly positive. Staining for collagen type II was clearly positive after 12 hours, decreased until day seven and revealed a constant staining until day 28 (Table 2). For BF-1 CO-PET immunohistochemical staining for type I collagen was negative after 12 hours, slightly positive on day seven, again negative after two weeks and weakly positive after three and four weeks. Staining for collagen type II was clearly positive after 12 hours, increased till day seven and revealed a constantly weak staining until day 28 (Table 2).

RT-PCR

The type I collagen mRNA expression on the BF-1 decreased from 12 hours to 28 days, but remained constant

Fig. 3 Cell morphology distribution on the BF-1 and on the BF1 CO-PET

Fig. 4 The meniscus cells on the BF-1 after 12 hours (a), 14 days (b), 28 days (c). Meniscus cells on the BF-1 CO-PET after 12 hours (d), 14 days (e), 28 days (f). Sections were stained haematoxylin & eosin, original magnification 20×, bar =100 μm

in BF-1 CO-PET. The type II collagen mRNA expression was decreased in BF-1 from seven days to day 28, but increased in BF-1 CO-PET throughout the experiment.

Electron microscopy

The scanning electron microscopy of both materials demonstrated that the cells were attached to the surface of the scaffolds during the four weeks of the experiment (Fig. 5a–f).

Discussion

In this study we investigated whether the addition of polyethylene terephthalate (PET) to a hyaluronic acid/polycaprolactone scaffold (BF-1) had an effect on meniscocyte proliferation differentiation and attachment in vitro and found a more cartilaginous phenotype, with a higher proportion of spherical cells, an increased collagen type II mRNA expression and a higher GAG content in the PET group.

Given the high incidence of meniscus lesions and deleterious effects of even limited, partial meniscectomy there is an obvious need for additional meniscus therapies. Meniscal

repair is only amenable in a small subpopulation of tears and patients, and meniscus transplantation is severely burdened by scarce allograft availability. The larger public would benefit from a meniscus implant with off-shelf availability, but current grafts are affected by issues of cellular compatibility and biomechanical resilience. In this study we were able to show that the addition of PET, as a mechanical reinforcement of an artificial meniscus, does not exert a detrimental effect on meniscocytes but rather stimulates their bioactivity and sustains their chondrocytic differentiation. The clinical impact of such a meniscus implant, that can be shaped to a desired size and that supports cellular behaviour, could be substantial.

Table 2 Immunohistochemical staining of Coll I and Coll II for the BF-1 and BF1 CO-PET

Type	12 h	7 days	14 days	21 days	28 days
BF-1 Coll I	0±0	0±0	0.2±0.4	0±0	0.3±0.4
BF-1 Coll II	2.3±0.5	1±0	1±0	1±0	1±0
BF-1 CO-PET Coll I	0±0	0.1±0.3	0±0	0.3±0.5	0.2±0.4
BF-1 CO-PET Coll II	1±0	1.1±0.6	0.6±0.5	0.9±0.3	0.3±0.3

Fig. 5 Scanning electron micrograph of sections, showing the cell attachment on the BF-1 (a–c) and the BF-1 CO-PET (d–f) after four weeks. **a** A dense layer of cells covering the BF-1 indicated by the white arrow (original magnification 300×, bar 100 μm). **b,c** Cells fixed in the biomaterials poor (**b**: original magnification 350×, bar 100 μm; **c**: original magnification 650×, bar 10 μm). **d,e** Matrix deposition on the BF-1 CO-PET (**d**: original magnification 100×, bar 100 μm; **e**: original magnification 200×, bar 100 μm). **f** A homogenous cell distribution and matrix deposition is visible after four weeks

This study has potential shortcomings. First, while the inner and outer meniscus reveal characteristics of two somewhat distinct cell types, we used a heterogeneous mix of cells from the inner and middle third of the meniscus. We wanted to produce results that are representative of the situation *in vivo*, where the types of migrating cells cannot be selected, rather than a controlled laboratory environment, which is of more basic scientific interest, but limited clinical relevance. Second, we followed the cell culture only for 28 days, despite the fact that the biomaterial will be present for months *in vivo*; but since meniscocytes are finally differentiated cells we did not expect substantive changes in cellular behaviour after 28 days.

Hyaluronic acid based polymers have been evaluated for tissue engineering of cartilage and meniscus [19–23]. Nakata et al. [24] investigated human meniscus cells and found enhanced proliferation of cells in culture but no effect on GAG production. The BF-1 CO-PET is a hyaluronate scaffold that originally was augmented with polyethylene-teraphtalate (PET) fibres for mechanical reasons. In an *in vitro* study and a subsequent ovine *in vivo* model, Chiari et al. [22] showed excellent properties of this material concerning tissue in-growth and mechanical stability. Since

then recent studies have shown that addition of PET to biodegradable materials enhances mesenchymal stem cell proliferation and differentiation [13, 15]. While it is not clear whether this is a chemical or physical effect of PET, the question remains whether such potentially clinically meaningful effects of PET exist for meniscocytes too.

DNA measurements were used to assess cell proliferation but no statistically significant effect of the addition of PET to a BF-1 scaffold was seen. However, we did not find a time trend either, suggesting that the meniscocyte populations in both groups were stable throughout the experiment. Given the fact that these cells are terminally differentiated mesenchymal cells and show very little proliferative activity, our findings align with our expectations.

The effect of addition of PET to BF-1 on meniscocyte differentiation was our second endpoint and we could see that the addition of PET influenced cellular phenotypes. Our results revealed that on the BF-1 CO-PET cells expressed a chondrocytic cell morphology, but on the BF-1 the cells consistently changed to a non assignable cell shape, suggesting that PET addition enhances a chondrocytic phenotype. This interpretation of our histological findings is supported by the PCR results. The mRNA expression of type II

collagen on the BF-1 CO-PET increased after day 14 concurrent with the re-expression of the chondrocytic cell morphology. The mRNA expression of type II collagen decreased on the BF-1, parallel to the dedifferentiation of the non assignable cell shape. The mRNA expression of type I collagen was not conclusive in either of the two biomaterials. The immunohistochemical findings corroborate these PCR results on the post-translational level. The other biosynthetic product of differentiated fibrochondrocytes is glycosaminoglycan. Over 28 days, the GAG content detected in the BF-1 without PET increased to 157.5 % of the amount at 12 hours, whereas in the BF-1 with PET GAG increased up to 820.2 % in the same period of time. This eightfold increasing GAG content provides further, biochemical evidence for a stronger expression of the chondrocytic phenotype in the BF-1 CO-PET biomaterial.

While these findings from PCR, histology, immunohistochemistry, and biochemistry consistently show a stimulating effect of PET on the expression of a chondrocytic phenotype, it is unclear what the underlying mechanisms are. It might be possible that the PET augmentation provides a more stable environment for the cells that favours their biosynthesis compared to the non-PET-augmented scaffold. Other studies investigating the effect of pore size diameter and geometry on chondrocytic differentiation and PET might affect pore size and shape while the biomaterial is being remodelled. Concerning the desirability of the observed effect of PET on meniscocytes, the question of the optimal cell differentiation for meniscus repair has to be addressed. As mentioned above, meniscocytes have two different appearances, but it is unknown what the implied functional capabilities are and whether one of these two forms would produce better results in a clinical scenario. An argument can be made that a chondrocytic differentiation is beneficial, because of its higher level of specialisation and the known poor performance of fibroblastic scars in cartilaginous tissues.

Lastly, we assessed cell attachment at 28 days by scanning electron microscopy and found good cell cover of both biomaterials. PET is used as a coating material on a number of devices, partly for its hydrophobic characteristics and subsequent low cell adhesion. While these characteristics might reduce cell adhesion to BF-1 CO-PET, we found no evidence for reduced cell attachment compared to BF-1 without PET.

Our study produced strong evidence for a beneficial effect of PET on the chondrocytic differentiation of ovine meniscocytes seeded in a biodegradable scaffold of hyaluronic acid/polycaprolactone. The cells seeded in scaffolds containing PET showed a more cartilaginous phenotype, as spherical morphology with an increased collagen type II mRNA expression and a higher GAG content. While the mode of action is still elusive our findings promote the BF-1 CO-PET for further investigations as an implant for the regeneration of meniscus in vivo.

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

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