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Effect of transforming growth factor-beta 1 (TGF-ß1) released from a scaffold on chondrogenesis in an osteochondral defect model in the rabbit

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Abstract: Articular cartilage repair might be stimulated by the controlled delivery of therapeutic factors. We tested the hypotheses whether TGF-ß1 can be released from a polymeric scaffold over a prolonged period of time *in vitro* and whether its transplantation modulates cartilage repair *in vivo*. Unloaded control or TGF-ß1 poly(ether-ester) copolymeric scaffolds were applied to osteochondral defects in the knee joints of rabbits. *In vitro*, a cumulative dose of 9 ng TGF-ß1 was released over 4 weeks. *In vivo*, there were no adverse effects on the synovial membrane. Defects treated with TGF-ß1 scaffolds showed no significant difference in individual parameters of chondrogenesis and in the average cartilage repair score after 3 weeks. There was a trend towards a smaller area (42.5 %) of the repair tissue that stained positive for safranin O in defects receiving TGF-ß1 scaffolds. The data indicate that TGF-ß1 is released from emulsion-coated scaffolds over a prolonged period of time *in vitro* and that application of these scaffolds does not significantly modulate cartilage repair after 3 weeks *in vivo*. Future studies need to address the importance of TGF-ß1 dose and release rate to modulate chondrogenesis.

Keywords: Cartilage defects, TGF-ß1, cartilage repair, scaffold, transplantation, rabbit

1 Introduction

Traumatic lesions of adult articular cartilage do not regenerate [1, 2]. Cartilage defects that penetrate the underlying subchondral bone are repopulated with mesenchymal cells from the bone marrow, which differentiate into chondrocytes, deposit a cartilaginous matrix, and form a fibrocartilaginous repair tissue [3]. This repair tissue has inferior structural characteristics and degenerates over the course of several months [2]. A variety of approaches is currently used to clinically treat articular cartilage defects, including marrow-stimulating techniques, the transplantation of isolated and expanded autologous chondrocytes in the absence or presence of supportive biodegradable matrices or the transplantation of cylindrical osteochondral autografts to sites of articular cartilage damage. However, even such highly sophisticated procedures do not predictably lead to the formation of articular cartilage that is identical in its structure to the normal cartilage and capable of withstanding mechanical stresses over time [4]. The regeneration of the original hyaline articular cartilage, therefore, remains a great challenge for orthopaedic researchers and clinicians.

The process of chondrogenesis within a healing articular cartilage lesion can be enhanced by regenerative signals provided to the site of articular cartilage repair [5]. Transforming growth factor-beta (TGF-ß) plays an important role in the growth and differentiation of articular cartilage and promotes chondrogenic differentiation of mesenchymal cells [6]. However, TGF-ß1, the predominant isoform of TGF-ß in articular cartilage, has a very short half-life [7]. Recently, an emulsion-coating method was successfully used to associate TGF-ß1 to porous poly(ether-ester) scaffolds and release the bioactive protein in a controlled fashion [8]. These biodegradable hydrogels are based on poly(ethylene glycol)-terephtalate and poly(butylene terephtalate) i.e., PEGT/PBT, and poly(ethylene glycol)-succinate and poly(butylene succinate) i.e., PEGS/PBS and have been successfully used to release different proteins [9]. Although PEGT/PBT porous scaffolds have been previously used in tissue engineering applications [10] it remains unknown if transplantation of TGF-ß1 scaffolds to sites of articular cartilage damage would modulate the chondrogenesis and cartilage repair *in vivo*. In the present study, we tested the hypothesis that TGF-ß1 can be released from a biodegradable polymeric scaffold over a prolonged period of time *in vitro*. We further investigated whether transplantation of these scaffolds in osteochondral defects modulates articular cartilage repair *in vivo*. To determine the effects of TGF-ß1 at the onset of chondrogenesis [3], 3 weeks after transplantation was chosen as the optimal time for evaluation.

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2 Experimental procedures

2.1 Materials

PEGT/PBT and PEGS/PBS multiblock copolymers were obtained from OctoPlus, Leiden, The Netherlands, and were used as received. Vitamin B_{12}, bovine serum albumin (BSA), and recombinant human TGF-ß1 were obtained from R&D Systems (Minneapolis, USA). Roswell Park Memorial Institute medium (RPMI 1640) was purchased from Cambrex (East Rutherford, USA). Chloroform, obtained from Fluka (Buchs, Switzerland), was of analytical grade.

2.2 Preparation of TGF-ß1-loaded polymeric scaffolds

The TGF-ß1 protein was associated to the porous scaffolds by means of a water-in-oil emulsion method previously described [11]. TGF-ß1 in a 4-mM HCL aqueous solution containing 1 mg/ml BSA (according to the supplier's protocol) was emulsified with PEGS/PBS copolymer solution in chloroform, using an Ultra-Turrax (T25 Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 30 s at 19 krpm. The TGF-ß1 concentration of the aqueous solution was set at 19.8 μg/ml and the volume of the aqueous phase was set to 0.5 ml. The copolymer solution was obtained by dissolving 0.5 g of copolymer in 3 ml of chloroform. The copolymer compositions used had a PEGS content of 70 weight % and a PEG molecular weight of 1,000 g/mol.

Compression molded/salt leached scaffolds were obtained by applying pressure (10,000 PSI during 10 min) and heat (240 °C) to a homogeneous mix of NaCl salt crystals and copolymer powder in a mold. The volume fraction of salt in the mixture was adjusted to 75 %. After cooling of the resultant dense block, the salt was extracted by successive immersions in RX-water (water conductivity less than 25 μS). Subsequently, the porous blocks were dried in ambient air for at least 24 h, and then placed in a vacuum oven (50 °C) for a minimum of 12 h. The PEGT/PBT copolymer used to prepare the scaffold had a PEGT content of 55 weight % and a PEG molecular weight of 300 g/mol. The salt crystals were sieved between 400 and 600 μm. Coated scaffolds were prepared by forcing the TGF-ß1-containing emulsion through a prefabricated cylindrical porous scaffold (1 cm in diameter and 1 cm in length) with the use of a vacuum (300 mBars). This vacuum was applied for at least 5 min, in order to evaporate as much chloroform as possible from the emulsion, thereby creating a polymeric coating on the scaffold. The resulting TGF-ß1-coated scaffolds (termed TGF-ß1 scaffolds) were frozen in liquid nitrogen, and freeze-dried at room temperature for 24 h. Blank scaffolds (termed control scaffolds) were prepared by using a TGF-ß1-free 4-mM HCL solution (with 1 mg/ml BSA) and the same conditions as TGF-ß-containing scaffolds. For *in vivo* implantation and *in vitro* release determination, 4-mm cylinders were aseptically cored from the coated scaffolds prepared as described above. Each cylinder was subsequently cut to a length of 4 mm.

2.3 Scanning electron microscopy

A Philips XL 30 ESEM-FEG was used to evaluate the internal morphology of the scaffolds. The internal porous structure was observed by cutting the scaffolds along the longitudinal axis with a razor blade. All samples were gold sputter-coated using a Cressington 108 auto apparatus before analysis.

2.4 Characterization of scaffold porosity and interconnection

The average porosity (%) of the scaffolds was evaluated from their dry weight, dry volume, and density of the PEGT/PBT copolymer (density = 1.2 g/ml) according to the following equation:

$$p = 1 - \frac{\text{sample weight}}{\text{sample volume} \times 1.2} \quad (1)$$

Three scaffold pieces were used to determine the porosity of a specific emulsion-coated scaffold. The scaffold pore interconnection was quantified using a method that applies Darcy's law, as described elsewhere [11]. Briefly, water is forced through the porous samples by applying a constant pressure and the flow rate is measured, from which the sample permeability (κ, 10^{-12} m^2) can be calculated. This parameter reflects the sample porosity and pore interconnection.

2.5 In vitro release of TGF-ß1 protein

TGF-ß1-loaded scaffolds (4×4-mm cylinders of approximately 19 mg) were incubated in 1 ml RPMI 1640 medium at 37 ^{o}C, in polypropylene tubes. All samples were kept under constant agitation (25 rpm). The release medium was entirely refreshed at various time points, immediately frozen in liquid nitrogen, and conserved at -20 °C until quantification. TGF-ß1 concentration was quantified using an enzyme-linked immunosorbent assay (ELISA) with a detection limit of 76 pg/ml (Quantikine human TGF-ß1 immunoassay, R&D Systems). The TGF-ß1 used for the standards and the preparation of the releasing scaffolds originated from the same batch. Aliquots of different volumes were frozen in liquid nitrogen immediately after reconstituting the protein solution and stored at −20 °C. They were thawed immediately prior to use for scaffold preparation or as standards.

To determine the quantity of emulsion effectively coated on the porous scaffold and establish the amount of protein present, coated scaffolds were prepared in the same conditions using a polymer emulsion containing 10 mg of vitamin B_{12} per g of polymer. The size of this molecule allows a complete release within 3 days when entrapped in the copolymers used in this study. The quantity of vitamin released is correlated to the amount of polymer coated onto a given scaffold, as the vitamin is homogeneously distributed in the emulsion. In turn, the amount of polymer coated can be related to the amount of protein associated with the scaffold. The amount of vitamin released was calculated using a standard curve of vitamin B_{12} in phosphate buffered saline and a spectrophotometer

(El 312e, BioTek Instruments) at 380 nm. This indirect detection method was proven to be accurate for lysozyme [11].

The amount of emulsion effectively coated onto the scaffolds was 47 % of the emulsion applied, while the weight of the coated scaffolds was close to 1 g. Therefore, the amount of TGF-$ß_1$ incorporated in a scaffold piece of 19 mg was about 85 ng.

To determine the stability of TGF-ß1 in the release medium, the absolute concentration decrease of the growth factor was measured by ELISA. Fresh TGF-ß was added to the release medium (1 ml) containing control scaffolds at a concentration of 3 and 7 ng/ml. After 1 day, the medium was collected in triplicate and assayed for concentration.

2.6 Transplantation of PEGT/PBT porous scaffolds to osteochondral cartilage defects *in vivo*

The transplantation of PEGT/PBT porous scaffolds to articular cartilage defects *in vivo* was performed in a previously described animal model [**?**]. Animal procedures were approved by the Saarland Governmental Animal Care Committee. Chinchilla bastard rabbits (Charles River, Sulzfeld, Germany) were kept in air-conditioned rooms at constant temperature and a regular light:dark cycle. They were fed a standard diet and received water *ad libitum*. Six female rabbits (mean weight: 3.1±0.2 kg; six animals per group) were anesthetized by intramuscular injection of Rompun (0.2 ml/kg of body weight; Bayer, Leverkusen, Germany) and Ketavet (0.75 mg/kg of body weight; Pharmacia & Upjohn, Erlangen, Germany). The knee joint was entered through a medial parapatellar approach; the patella was dislocated laterally and the knee flexed to 90°. Using a manual cannulated burr (3.5 mm in diameter; Synthes, Umkirch, Germany), a cylindrical osteochondral defect was created in the patellar groove of each knee (n = 12 defects). All defects were washed with PBS to remove debris and blotted dry. PEGT/PBT porous scaffolds were press-fit into the defects. The right and left knees alternately received control or TGF-ß1 scaffolds. After reducing the patella, the knee was put through a range of motion to assure the stability of the scaffolds. Incisions were closed in layers. Immediately postoperatively, animals were allowed full weight bearing without any immobilization.

2.7 Histological and immunohistochemical analysis

Three weeks after implantation, rabbits were euthanized with Pentobarbital (150 mg/kg body weight; Merial, Hallbergmoos, Germany) and the knee joints were rinsed with 1 ml PBS, exposed and examined for synovitis, osteophytes, or other reactions. The appearance of the defect (color, integrity, contour) and the articulating surfaces were documented. Distal femurs were retrieved, fixed in 4 % phosphate-buffered formalin, trimmed, and decalcified. Paraffin-embedded frontal sections (5 μm) were stained with safranin O, fast green, hematoxylin, and eosin according to routine histological protocols [15].

The synovial membrane was evaluated using a previously published scoring system [16, 17]. The categories of the score included villus thickening (fibrosis), villus architecture (blunting), and the presence of inflammatory cell infiltrates. For type-II collagen and type-I collagen immunostaining, deparaffinized sections were submerged for 30 min in 0.3 % hydrogen peroxide. After washing with PBS and incubation in 0.1 % trypsin for 30 min, sections were washed with PBS and blocked with 3 % bovine serum albumin in PBS (blocking buffer) for 30 min. Sections were then incubated with a 1:50 dilution of a monoclonal mouse anti-type-II or type-I collagen IgG (Acris Antibodies, Hiddenhausen, Germany) in blocking buffer for 24 h at 4°C, washed, and exposed to a 1:500 dilution of a biotinylated anti-mouse antibody (Vector Laboratories, Grünberg, Germany) for 1 h at room temperature. After washing with PBS, the sections were incubated for 30 min with the avidin-biotin-peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories), washed, and exposed to diaminobenzidine (Vector Laboratories). To control for secondary immunoglobulins, sections were processed as above, excepting the secondary antibody. Immunoreactivity to type-II collagen in the repair tissue was compared to that of the adjacent normal articular cartilage, which served as a positive internal control. Immunoreactivity to type-I collagen in the repair tissue was compared to that of the subchondral bone adjacent to the normal articular cartilage, which served as a positive internal control. A score was given to each knee: – no immunoreactivity; + weaker immunoreactivity; ++ similar immunoreactivity; and +++ stronger immunoreactivity.

For the quantitative assessment of the repair tissue, serial histological sections of the distal femora were taken at 200-μm intervals. Safranin O-stained sections within approximately 1.0 mm from the center of the defect (n = 9-10 per defect) were analyzed using the articular cartilage repair scoring system described by Sellers and colleagues [18, 19]. Specific parameters that were evaluated include filling of the defect, integration of the new cartilage, safranin O staining, cell morphology, architecture within the defect and of its surface, restoration of the subchondral bone, and tidemark. Scores were combined and resulted in an average total score. Values ranged from 31 (empty defect without repair tissue) to 0 points (normal articular cartilage, complete regeneration). A total of 110 sections were independently scored by two individuals without knowledge of the treatment groups. The safranin O-positive area in the new tissue filling the defects was measured on serial histological sections of the distal femora that were taken within approximately 0.6 mm from the center of the defects at 200-μm intervals (n = 3-6 per defect). Low magnification images of the cartilage defects were acquired by a solid-state CCD camera mounted on a BX-45 microscope (Olympus, Hamburg, Germany). The image on the monitor was digitalized and the safranin O-positive area was manually outlined by a blinded observer. The safranin O-positive area was calculated using the analySIS program (Soft Imaging System, Münster, Germany). Collagen fibrils were evaluated using polarized light microscopy (Olympus).

2.8 Statistical analysis

In vitro data were evaluated using ANOVA and expressed as mean±SD. *In vivo*, points for each category and total score were compared between the two groups using a mixed general linear model with repeated measures (knees nested within the same animals). All continuous variables were tested for normality using the Kolmogorov-Smirnov goodness-of-fit method and no significant skewness or kurtosis was detected. Therefore, continuous data are expressed as mean ±95 % confidence interval. A two-tailed $P<0.05$ was considered statistically significant. Statistical analysis of the data was performed using the SPSS software package (version 12.0, SPSS Inc., Chicago, USA).

3 Results

3.1 Human TGF-β1 is efficiently released from poly(ether-ester) copolymeric scaffolds over at least 4 weeks *in vitro*

The ability of the scaffold to release TGF-ß1 over a prolonged period of time is a prerequisite for the localized delivery of the protein to cartilage defects *in vivo*. Accordingly, we prepared scaffolds coated with TGF-ß1 using an emulsion-coating method. The morphology of these scaffolds prior to implantation, evaluated by Scanning Electron Microscopy (SEM), is presented in Figure 1. The average porosity of the control and TGF-ß1 scaffolds was 65±4 %. Pores were of various sizes and shapes, ranging from 80 to 650 μm, and were visually interconnected. The interpore connection of the scaffolds was reflected by their high permeability to water (κ), which averaged $78 \pm 14 \times 10^{-12}$ m^2.

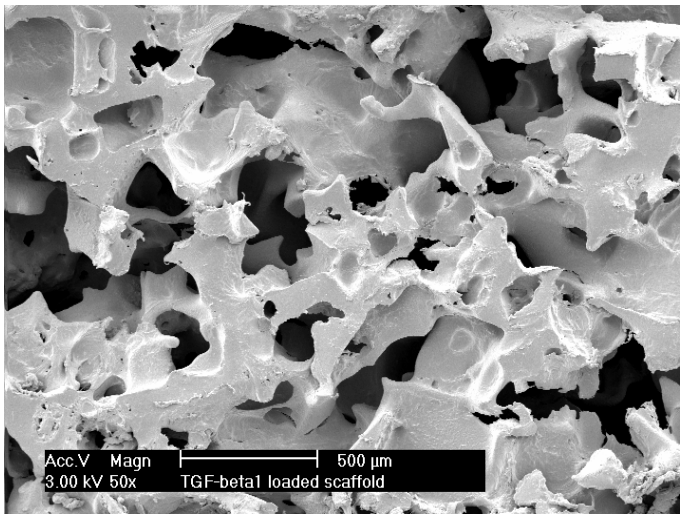

Fig. 1 Cross sections of a TGF-β1-loaded porous scaffold examined by scanning electron microscopy.

The *in vitro* release of TGF-ß1 from the porous scaffolds is depicted in Figure 2. A fast release was observed during the first 8 days, followed by a slow and linear release over the

following 3 weeks. The total cumulative amount of growth factor released, as measured by ELISA, was on average 7.5 ng after 8 days and 9 ng after 28 days. Remarkably, the amount of TGF-ß1 released over 28 days only reached 9 % of the amount effectively entrapped in the scaffold (85 ng).

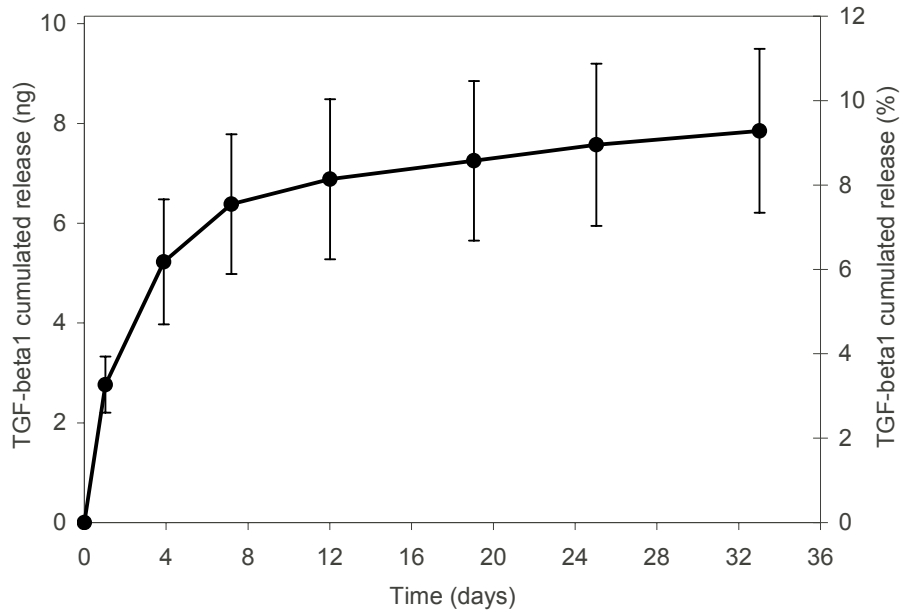

Fig. 2 Cumulated release of TGF-β1 from 4×4 mm cylindrical porous polymeric scaffolds. At each time point, the release medium was collected and the amount of growth factor released quantified by ELISA. (n = 3 ± S.D.).

3.2 Spatial delivery of human TGF-ß1 does not induce a synovial inflammatory response *in vivo*

Three weeks following implantation in osteochondral defects in each patellar groove of rabbits, there were no macroscopic signs of inflammation or hematoma. The new tissue in these defects had a white color and was still distinguishable from the neighboring normal articular cartilage (Figure 3).

TGF-ß1 concentrations were monitored in the lavage fluid of knees. After 3 weeks, TGF-ß1 concentrations were below the detection limit of the assay in knees receiving control or TGF-ß1 scaffolds (n = 6). We then investigated whether the released TGF-ß1 had an effect on the synovial membrane. Analysis of the thickness, architecture of synovial villi, and presence of inflammatory cell infiltrates was performed using a previously published score [17]. The data revealed no significant differences between knees receiving control or TGF-ß1 scaffolds at 3 weeks ($P > 0.05$, n = 6; Table 1).

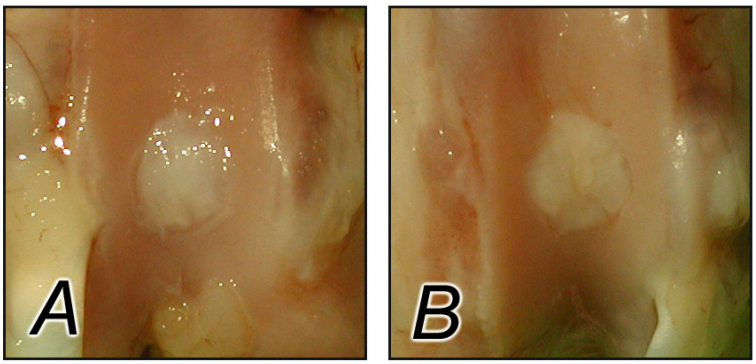

Fig. 3 Macroscopic view of femoral condyles receiving a control scaffold (*left*; A) or a TGF-ß1 scaffold (*right*; B). Defects in both groups are filled with a new white tissue that is distinguishable from the neighboring normal articular cartilage.

Category	Control Scaffolds	TGF-ß1 Scaffolds	*P* value
Villus thickening	0.45 ± 0.42	0.44 ± 0.54	> 0.05
Villus architecture	0.29 ± 0.43	0.27 ± 0.42	> 0.05
Inflammatory cell infiltrate	0.12 ± 0.37	0.13 ± 0.41	> 0.05
Average total score	**0.86 ± 0.17**	**0.84 ± 0.18**	**> 0.05**

Effects of TGF-ß1 scaffolds on the histological grading of the synovium at 3 weeks.

Table 1 Histological grading of the synovium at 3 weeks *in vivo*.

3.3 Poly(ether-ester) copolymeric scaffolds remain present in the osteochondral defect for at least 3 weeks *in vivo* and allow cartilage formation

After 3 weeks *in vivo*, the scaffolds remained in a subchondral location (Figure 4A, B). Both control and TGF-ß1 scaffolds were filled with a tissue consisting of undifferentiated mesenchymal cells, histiocytic cells, and new bone, its trabeculae surrounding the biomaterial (Figure 5A-D). Polarized microscopy confirmed the presence of trabecular bone (data not shown). There were few signs of scaffold degradation.

3.4 Effect of TGF-ß1 released from copolymeric scaffolds on chondrogenesis *in vivo*

The new tissue in the cartilage defects was analyzed by immunohistochemistry for the presence of type-II collagen, a major component of the extracellular matrix of hyaline articular cartilage (Figure 4E, F; Table 2). Three weeks after transplantation, immunoreactivity to type-II collagen was more pronounced in defects receiving TGF-β1 scaffolds than in defects receiving control scaffolds.

Type-I collagen is mainly expressed in fibrocartilage and in the subchondral bone.

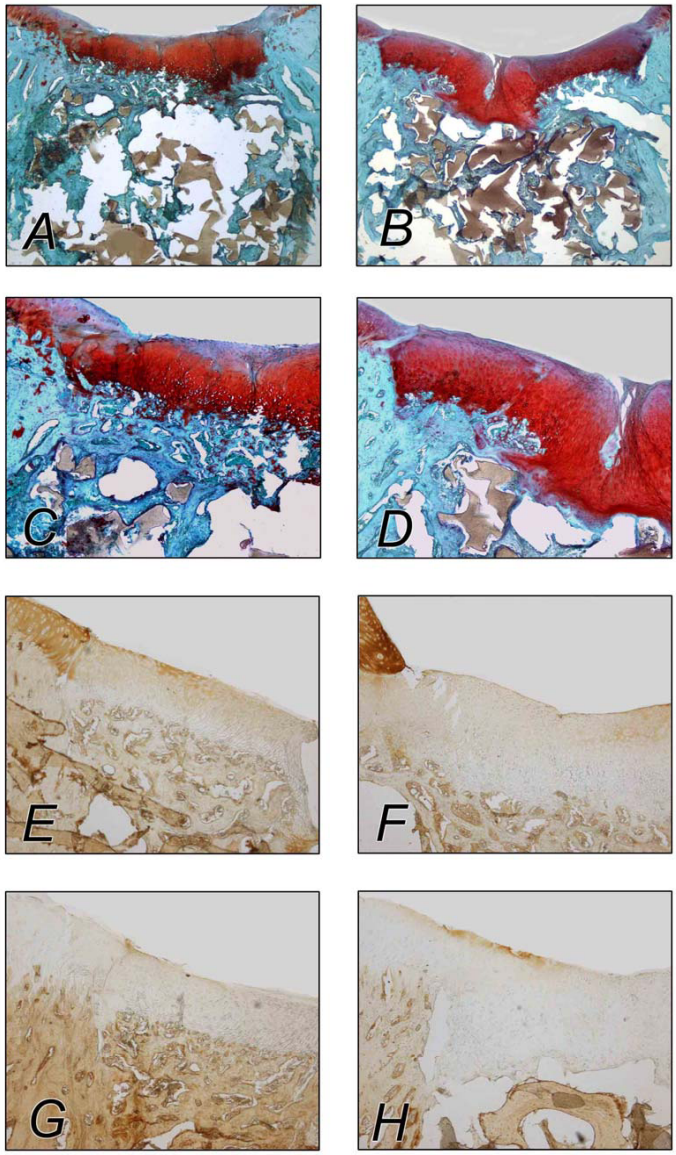

Fig. 4 Effect of TGF-ß1 scaffolds on chondrogenesis in articular cartilage defects 3 weeks after transplantation *in vivo*. Histological appearance of osteochondral defects following implantation of a single control scaffold (*left*; A, C, E, G) or a TGF-ß1 scaffold (*right*; B, D, F, H) stained with safranin O – fast green (A - D), a monoclonal mouse anti-human type-II collagen IgG (E, F) or a monoclonal mouse anti-human type-I collagen IgG (G, H).Images C and D are magnified views of the left side of images A and B. Normal articular cartilage can be identified on the far left side of Images (A – H) including the area of integration between the repair tissue (right side of each picture) with the adjacent normal articular cartilage (left side of each picture). The scaffolds remain in a subchondral location and can be identified by their brown color (A, B). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×10 (A, B), ×40 (C - H).

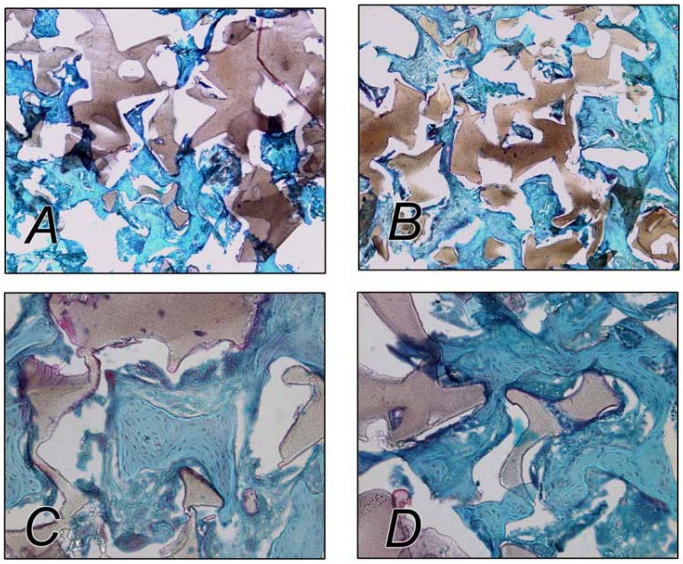

Fig. 5 Effect of TGF-ß1 scaffolds on new bone formation in the subchondral space 3 weeks after transplantation *in vivo*. Histological appearance of the subchondral bone below the osteochondral defects that has been filled with a single control scaffold (*left*; A, C) or a TGF-ß1 scaffold (*right*; B, D) stained with safranin O – fast green (A - D). New subchondral bone has formed within the pores of the scaffold, its trabeculae surrounding the biomaterial (C, D). Photomicrographs were obtained using standardized photographic parameters, including light intensity. Original magnifications ×40 (A, B), ×100 (C, D).

Animal number	Control Scaffolds	TGF-ß1 Scaffolds
1	+	+
2	++	+++
3	+++	++
4	+	++
5	+	++
6	++	++
Range	**+ to +++**	**+ to +++**

Type-II collagen immunoreactivity in the repair tissue of the defect was compared to that of the normal articular cartilage adjacent to the defect, used as a positive internal control.

Type-II collagen immunoreactivity was scored as follows:
−−, no immunoreactivity;
+, weaker immunoreactivity;
++, similar immunoreactivity;
+++, stronger immunoreactivity compared to the normal articular cartilage.

Table 2 Semiquantitative analysis of type-II collagen immunoreactivity in the repair tissue after 3 weeks *in vivo*.

Immunohistochemical analysis of the repair tissue revealed more type-I collagen in the repair tissue of defects receiving TGF-β1 scaffolds than in control defects (Figure 4G, H;

Table 3).

Animal number	Control Scaffolds	TGF-ß1 Scaffolds
1	+	+
2	+	++
3	+	+
4	++	+++
5	-	+
6	++	++
Range	**- to ++**	**+ to +++**

Type-I collagen immunoreactivity in the repair tissue of the defect was compared to that of the subchondral bone adjacent to the normal articular cartilage, used as a positive internal control.

Type-I collagen immunoreactivity was scored as follows:
−−, no immunoreactivity;
+, weaker immunoreactivity;
++, similar immunoreactivity;
+++, stronger immunoreactivity compared to the normal articular cartilage.

Table 3 Semiquantitative analysis of type-I collagen immunoreactivity in the repair tissue after 3 weeks *in vivo*.

To study the effects of TGF-ß1 on articular cartilage repair *in vivo*, the newly formed repair tissue within the defect was evaluated using a previously published grading system [18] that consists of eight individual parameters. When combined, values ranged from 31 (indicating an empty defect without repair tissue) to 0 points (indicating the complete regeneration of normal articular cartilage). No complete articular cartilage regeneration (0 points) was achieved at 3 weeks for defects treated with control or TGF-ß1 scaffolds. Interestingly, nearly all individual categories of defects treated with TGF-ß1 scaffolds received higher score values than defects treated with control scaffolds, indicative of a lesser grade of cartilage repair than the control group. For example, the scores for the filling of the defect and for the staining of the new tissue with safranin O, an indicator of proteoglycans, were 2.6-fold worse in defects treated with TGF-ß1 scaffolds than in the control group (Table 4). Although the average total score of TGF-β1-treated defects was 12 % higher (worse) than the average total score of control defects, statistical significance was not reached (Table 4).

The area that was safranin O-positive was $1,137,686 \pm 140,494$ μm^2 in defects receiving control scaffolds and $798,229 \pm 687,286$ μm^2, 42.5 % more than in defects receiving TGF-β1 scaffolds ($P > 0.005$).

4 Discussion

The poor accessibility of the cartilage defect to agents that modulate chondrogenesis is a major obstacle in the development of strategies to regenerate articular cartilage

Category	Control Scaffolds Mean (95 % CI)	TGF-β Scaffolds Mean (95 % CI)	P value
Filling of the defect	0.55 (0.08 – 1.02)	1.41 (0.01 – 3.33)	0.34
Integration	1.56 (1.07 – 2.05)	1.87 (1.24 – 2.50)	0.34
Matrix staining	0.36 (0.11 – 0.61)	0.94 (0.00 – 2.28)	0.33
Cell morphology	2.83 (2.14 – 3.51)	2.91 (1.55 – 4.26)	0.85
Architecture of defect	3.09 (1.93 – 4.23)	3.08 (2.14 – 4.03)	0.99
Architecture of surface	2.05 (1.76 – 2.33)	2.21 (1.85 – 2.57)	0.47
New subchondral bone	2.86 (1.91 – 3.81)	3.18 (2.83 – 3.52)	0.34
Tidemark	3.98 (3.90 – 4.04)	3.79 (3.37 – 4.20)	0.31
Average total score	**17.3 (14.6 – 19.8)**	**19.4 (13.5 – 25.3)**	**0.42**

Each category and total score is based on the average of 2 independent evaluators. Means indicate estimated scores in points for each category (lower scores indicate better healing). Comparisons were made by repeated-measures ANOVA (knees within the same animal). CI = confidence interval. No statistically significant differences were observed for any category (all P > 0.30).

Table 4 Effects of TGF-ß1 on the histological grading of the repair tissue after three weeks.

lesions. With a view to delivering a chondrogenic growth factor to an articular cartilage defect, we developed a system that allowed for a controlled release of TGF-ß1 from porous supportive structures. We tested the hypothesis that TGF-ß1 can be released from these polymeric scaffolds over a prolonged period of time *in vitro*. We further evaluated whether transplantation of such TGF-ß1 scaffolds into osteochondral defects in the patellar groove of rabbits modulates articular cartilage repair *in vivo*. The data indicate that human TGF-ß1 is efficiently released from poly(ether-ester) copolymeric scaffolds over a minimum of 4 weeks *in vitro*. The data further suggest that delivery of a cumulative dose of approximately 9 ng TGF-ß1 in 4 weeks via these scaffolds is not sufficient to modulate articular cartilage repair *in vivo*.

The release of TGF-ß1 over a prolonged period of time is a prerequisite for the safe and localized delivery of the protein to cartilage defects *in vivo*. The scaffolds employed in the present study released a total cumulative amount of 7.5 ng TGF-ß1 after 8 days and 9 ng TGF-ß1 after 28 days, confirming the suitability of poly(ether-ester) copolymer to release proteins [20, 21]. The mechanism of release from these hydrogel copolymers is a combination of diffusion and matrix degradation. During the first 8 days, the release is likely to be mainly driven by diffusion mechanisms as the degradation of the coated copolymer is minimal after 1 week [9]. The cumulative amount of TGF-ß1 released over this period of time was relatively low when compared to the total amount entrapped in the scaffolds. This reduced recovery may be linked to the denaturation of the protein during the scaffold preparation or during release. It was previously demonstrated that TGF-ß1 and lysozyme are not denaturated during the emulsion coating process and that different release rates can be obtained with the same scaffold.[8, 11] Therefore, the amount

of TGF-ß1 released is possibly linked to the release profile of the scaffold and the intrinsic stability of the protein in the release medium between sampling. TGF-ß1 is an unstable protein and its half-life *in vivo* is less than 30 min when in its active form [7]. In our experiment, the absolute depletion of TGF-ß1 over 1 day in the release medium (RPMI 1640) was 93±2 %. Consequently, the cumulative amount of TGF-ß1 measured by ELISA may reflect only a part of the TGF-ß1 that was effectively released. Nevertheless, the amounts of TGF-ß1 released in the present study are about 50-fold lower than applied by Abe and colleagues in liposomes via intraarticular injection [22] and about 5- to 50-fold lower than applied by Mierisch encapsulated in alginate spheres into articular cartilage defects [23]. Additional studies need to test higher doses of TGF-ß1 using these scaffolds. We designed the slow release system employed in the present study to maximize the duration of defect's exposure to the growth factor while avoiding undesirable intraarticular side effects. For example, when 500 ng TGF-ß1 encapsulated in liposomes were applied by intraarticular injection, extensive fibroblastic hyperplasia was seen [22]. Injection of high doses of TGF-ß2 led to synovial hyperplasia and cartilage loss [24]. When an adenoviral vector carrying a TGF-ß1 cDNA was injected intraarticularly to naive and arthritic rabbit knee joints, a dose-dependent stimulation of glycosaminoglycan release and nitric oxide production, and induction of fibrogenesis and muscle edema were observed [25]. In addition, chondrogenesis within the synovial lining was induced [25]. These results suggest that TGF-ß1 may stimulate cartilage degradation and may serve to caution the intraarticular application of high doses. The absence of elevated TGF-ß1 levels in the synovial fluid in the present study is probably due to the containment of the protein within the new tissue. Such a lack of elevated intraarticular TGF-ß1 and of inflammatory changes of the synovial membrane may be advantageous in a clinical setting, to avoid undesirable effects of the therapeutic factor.

After 3 weeks *in vivo*, the scaffolds remained underneath the original articular cartilage without any signs of adverse reactions. They allowed cartilage formation and the formation of new subchondral trabecular bone. These features are important as the scaffold integrated with the bony compartment. As the implanted scaffolds were devoid of cells, the permeability towards fluids is important to allow the progenitor cells present in the bone marrow to reach the cartilage zone and to allow tissue ingrowth after implantation in osteochondral defects. This was effectively seen *in vivo*, as the scaffolds (either control or TGF-ß1-scaffolds) showed tissue growth in the pores present in the bone area, while cartilage was formed on top of the scaffolds. Scaffold fragments could be seen in all animals indicating that the scaffolds dissolve very slowly thereby acting as a valuable support for new bone and articular cartilage. These features of poly(ether-ester) copolymeric scaffolds may be desirable in tissue engineering and in clinical applications, as some biomaterials have been shown to induce undesired effects when applied intraarticularly [26].

No difference in articular cartilage repair was seen in the present study following transplantation of TGF-ß1 scaffolds compared to control scaffolds. This was somewhat surprising, as previous studies reported improved cartilage repair following application

of TGF-ß1 [22, 23]. In defects receiving TGF-ß1 scaffolds, trends towards an inhibition of chondrogenesis were observed without reaching significance. In particular, categories indicative of proteoglycan synthesis, such as matrix staining and the area that stained positive for safranin O, were inferior to defects treated with control scaffolds. When TGF-ß1 encapsulated in liposomes was intraarticularly injected into the knee joints of rabbits 1 week after creation of osteochondral cartilage defects, the early repair of these defects was accelerated [22]. Similarly, when alginate spheres containing 50 or 500 ng TGF-ß1 (per sphere) were applied to osteochondral defects in the trochlear grooves of rabbits, better repair compared with controls was seen [23]. Others reported a trend towards improvement of articular cartilage repair without statistical significance when polymer oligo[poly(ethylene glycol) fumarate] scaffolds containing TGF-ß1 were applied to osteochondral defects in rabbits [27]. Earlier experiments using the scaffold applied in the present study demonstrated that the TGF-ß released from the scaffold is bioactive and is capable of modulating chondrogenesis *in vitro* [8]. One of the reasons for the lack of a significant effect may be that the concentration of TGF-ß1 was 5- to 50-fold lower than in other reports [22, 23]. It is also possible but unlikely that the effect of TGF-ß1 treatment would have become more evident at later time points. Using gene-based treatments, significant improvements in articular cartilage repair were already present after 3 weeks *in vivo* [12–14] in an identical animal model.

Although mainly considered as a stimulator of articular cartilage repair, TGF-ß1 has also been implicated in inhibiting chondrocyte maturation *in vitro* and in inducing alterations of skeletal morphogenesis *in vivo* [28]. Galera and colleagues described differential effects of TGF-ß1 on major components of extracellular matrix, collagen, and proteoglycans, depending on the differentiation state of the cells [29]. Recently, it was reported that TGF-ß1 can inhibit type-II collagen biosynthesis in primary articular chondrocytes at transcriptional levels [30]. In a study by Hunziker and Rosenberg, application of 6 ng/ml TGF-ß1 in a fibrin matrix to partial-thickness (chondral) defects in Yucatan minipigs did not lead to cartilage formation [31].

In summary, the results of the present study demonstrate that TGF-ß1 is released from emulsion-coated scaffolds over a prolonged period of time *in vitro*. Scaffolds releasing a cumulative dose of 9 ng TGF-ß1 that are applied to osteochondral defects in the knee joints of rabbits do not significantly modulate articular cartilage repair at 3 weeks *in vivo*. In future, it will be important to address the relative importance of TGF-ß1 dose and release kinetics of the scaffold employed. It will be essential to better characterize the effect of TGF-ß1 on chondrogenesis, perhaps in cartilage defects in a larger, clinically more relevant animal model over an extended period of time. We are currently investigating these possibilities. In addition, it will be critical to test other therapeutic factors involved in enhancing articular cartilage repair. A better understanding of the role of TGF-ß1 within the context of a cartilage lesion will lead to improved strategies for articular cartilage defects.

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