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Gelatin-based haemostyptic Spongostan as a possible three-dimensional scaffold for a chondrocyte matrix?

AN EXPERIMENTAL STUDY WITH BOVINE CHONDROCYTES

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The gelatin-based haemostyptic compound Spongostan was tested as a three-dimensional (3D) chondrocyte matrix in an in vitro model for autologous chondrocyte transplantation using cells harvested from bovine knees. In a control experiment of monolayer cultures, the proliferation or de-differentiation of bovine chondrocytes was either not or only marginally influenced by the presence of Spongostan (0.3 mg/ml).

In monolayers and 3-D Minusheet culture chambers, the cartilage-specific differentiation markers aggrecan and type-II collagen were ubiquitously present in a cell-associated fashion and in the pericellular matrix. The Minusheet cultures usually showed a markedly higher mRNA expression than monolayer cultures irrespective of whether Spongostan had been present or not during culture. Although the de-differentiation marker type-I collagen was also present, the ratio of type-I to type-II collagen or aggrecan to type-I collagen remained higher in Minusheet 3-D cultures than in monolayer cultures irrespective of whether Spongostan had been included in or excluded from the monolayer cultures. The concentration of GAG in Minusheet cultures reached its maximum after 14 days with a mean of 0.83 ± 0.8 µg/10^6 cells; mean ±, SEM, but remained considerably lower than in monolayer cultures with/without Spongostan.

Our results suggest that Spongostan is in principle suitable as a 3-D chondrocyte matrix, as demonstrated in Minusheet chambers, in particular for a culture period of 14 days. Clinically, differentiating effects on chondrocytes, simple handling and optimal formability may render Spongostan an attractive 3-D scaffold for autologous chondrocyte transplantation.

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The treatment of full-thickness cartilage defects (chondromalacia grade 4[1]) by autologous chondrocyte transplantation (ACT) has limitations in regard to the thickness and volume of the transplant. New procedures using three-dimensional (3D) matrices and chondrocytes as bioactive elements have only partially overcome these deficits. According to recent studies, chondrocytes are responsible for the fixation of the transplant on to the subchondral bone and for lateral bonding.[2,3] We have investigated the use of a biodegradable gelatine matrix (Spongostan, Johnson & Johnson, Norderstedt, Germany, Fig. 1) as a carrier and analysed its interaction with bovine chondrocytes. Preliminary results of such interactions have been recently published by other authors[4] showing that there was no de-differentiation of chondrocytes in Spongostan sponges modified with hyaluronic acid, a finding confirmed using a different type-I collagen hydrogel.[5] Additional studies have shown that implantation of chondrocytes into a 3D matrix consisting of fibrin and polyglactin/polydioxanon for one week partially reversed the de-differentiation observed in a monolayer culture (ie suppression of collagen type-II/aggrecan and the induction of collagen types I and III).[6,7] These results indicate that chondrogenic differentiation is strongly supported by the embedding of the chondrocytes in 3D carrier systems.[8-10] This 3D culture is considered to be preferable to direct re-injection of the isolated chondrocytes without further cultivation, because it favours adaptation of the cells to the final scaffold/matrix conditions and the beginning of the formation of an extracellular matrix.

In our study, therefore, we embedded bovine chondrocytes in a Spongostan scaffold consisting of the easily shapeable Spongostan powder in a culture medium and subsequently cultured in a 3D Minusheet chamber[11,12] without prior expansion in a monolayer. As pre-requisites for future clinical application, the density of chondrocytes in normal cartilage[13] was approxi-

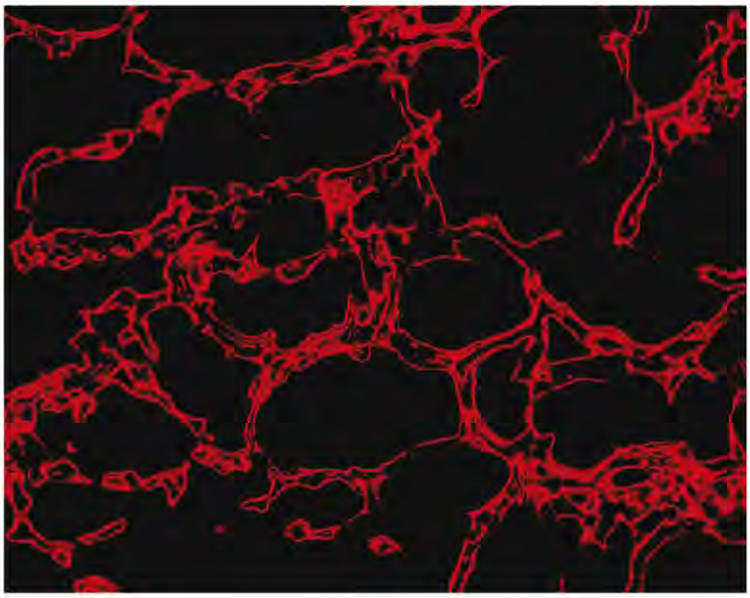

Fig. 1

Photomicrograph of cell-free, shock-frozen Spongostan (red, polarised light × 40).

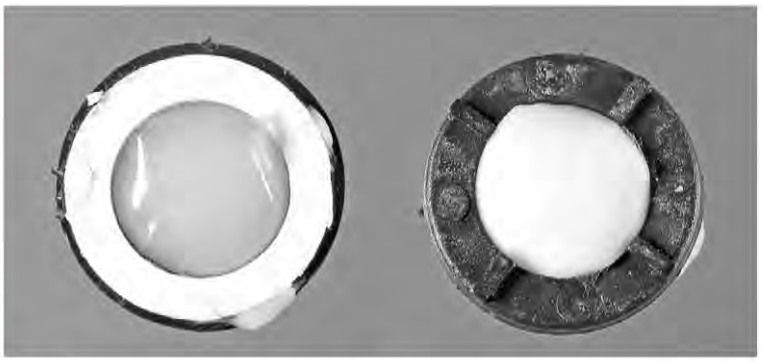

Fig. 2a

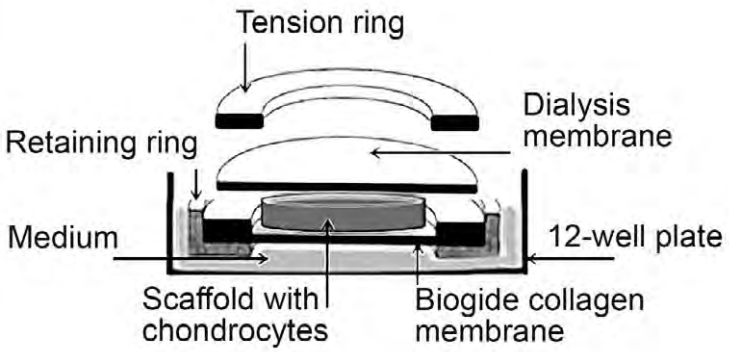

Fig. 2b

a) Photograph showing the 3D chondrocyte-Spongostan chambers (Minusheets; left, view from above; right, view from below), and b) – Diagram showing the experimental set-up.

mated in the chondrocyte Spongostan matrix and the concentration of the Spongostan powder was optimised to yield a matrix with sufficient mechanical stability. In order to characterise the differentiating/de-differentiating effects of the matrix, the expression of type-II collagen and other cartilage-specific markers was analysed[14] using the quantitative real-time polymerase-chain reaction (PCR), which allowed a precise quantification of the mRNA expression in a cartilage scaffold/matrix system for the first time.[4,15] In addition, we detected the respective proteins by immunohistology with glycosaminoglycans (GAG) being quantified in the culture mass using the dimethylene-blue (DMB) assay.[16]

Materials and Methods

Cartilage preparation. The knees of cows with a mean age of 24 months were obtained from the abattoir. They were skinned, disinfected and opened horizontally at the level of the ventral joint space under sterile conditions. Slices of cartilage of approximately 1.0 cm^2 to 1.5 cm^2 were removed from the subchondral bone of normal femoral condyles and collected in Dulbecco's modified Eagle's medium containing gentamicin (DMEM-G; Sigma, Steinheim, Germany 50 µg/ml). All the samples were inspected macroscopically and microscopically to ensure that they were free of any defects.

Cartilage digestion. The cartilage slices were initially incubated with serum-free pronase E (Merck, Darmstadt, Germany, 1 mg/ml) in DMEM-G for one hour at 37°C, with 5% CO_2, and with 95% humidity under constant stirring. After several washes in DMEM-G, they were incubated for 17 hours at 37°C in DMEM containing 5% fetal calf serum (FCS; Sigma-Aldrich, Taufkirchen, Germany) and collagenase P (0.1 mg/ml; Roche, Mannheim, Germany). Chondrocytes were then harvested by centrifugation at 1500 rpm for five minutes followed by removal of the supernatant and by washing with phosphate-buffered saline (PBS) containing gentamicin (50 µg/ml). The cells were counted and transferred to DMEM-G with 5% FCS for four hours at 37°C.

Chambers for 3D culture. The system consisted of a Minusheet chamber, a collagen membrane (Biogide; Geistlich, Baden-Baden, Germany), a dialysis membrane (Membra-cel dialysis tubing; molecular weight cut-off 3500 D; Serva Electrophoresis, Heidelberg, Germany), and approximately 25 mg to 30 mg (dry weight) of Spongostan.

Based on preliminary experiments, a mixture of Spongostan and DMEM (weight ratio of 1:6) was prepared in order to obtain a matrix with sufficiently high viscosity and mechanical stability. Chondrocytes ranging in concentration between 4×10^6 and 1×10^7 cells/chamber were then applied to the surface of the matrix which was mixed mechanically for 120 seconds in order to achieve a homogeneous distribution. Finally, the Spongostan-chondrocyte mass was shaped into a sphere of about 3 mm in diameter using a sharp spoon, centred on the rough side of the collagen membrane and covered with the autoclaved, round dialysis membrane. The culture chamber was closed by pressing the smaller white ring with the dialysis membrane (inner diameter 6.5 mm) into the larger black ring holding the collagen membrane (inner diameter 9 mm; Fig. 2). The chamber was then cultured in 12-well plates (3 ml medium/well) for seven, 14 or 28 days at 37°C in 5% CO_2 and with 95% humidity.

Table I. Primer, product sizes and gene bank accession numbers of real-time polymerase chain reaction-validated genes

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	T_A(°C)/ t_{ampl}* (sec)
GAPDH (NM-001034034)	CAT CAC TGC CAC CCA GAA GA	CCT GCT TCA CCA CCT TCT TG	60/20
Aggrecan (NM-173981.2)	ACT TCC GCT GGT CAG ATG GA	TCT CGT GCC AGA TCA TCA CC	60/20
Co1 IIa1 (NM-001001135.2)	CAT CTG GTT TGG AGA AAC CAT C	GCC CAG TTC AGG TCT CTT AG	60/20
Co1 Ia2 (NM-174520.2)	CTC AGA CCC AAG GAT TAT GAA G	GGA TCC TTG CAG AAT GAC AG	60/20

* T_A, annealing temperature; t_{ampl}, amplification time

Chondrocyte monolayer culture. Bovine chondrocytes (2×10^6) were cultured in monolayer in 75 cm^2 flasks with 25 ml of DMEM containing 5% FCS and 0.3 mg/ml of Spongostan. Culture was performed for seven, 14 or 28 days without prior expansion and the cells were characterised for mRNA expression of aggrecan, the alpha-2 chain of type-I collagen, and the alpha-1 chain of type-II collagen. In the case of the 28-day culture with a high density of cells, a change of the culture medium was performed after 14 days and the GAG concentrations were assessed in pooled supernatants. In all other cases, changes in the medium were deliberately avoided by using 750 ml culture bottles.

Histological examination. Cryostat sections were used for histological analysis. The culture chambers were cut in half, embedded in Tissue-Tek (Sakura Finetic Europe BV, Zoeterwande, Netherlands) and frozen in liquid nitrogen. Cryostat sections between 6 μm and 20 μm in thickness, were then prepared using a HM500 cryostat (Microm International, Waldorf, Germany). After drying in air for 24 hours, the sections were stained with haematoxylin and eosin, 4.6-diamidino-2-phenylindol (DAPI) or propidium iodide. They were analysed using an Axiovert 200M microscope and the respective software AxioVision 4.7 (both Carl Zeiss, Jena, Germany).

Immunohistological examination. Type-II collagen was detected using the monoclonal antibody clone II-4C11 (Calbiochem, San Diego, California). The sections were first treated with PBS containing 0.1% hyaluronidase in order to unmask collagen fibres and then incubated with the antibody (1:50 dilution in 30% Tris albumin) overnight at 4°C. After repeated washing in PBS, they were incubated with the fluorescence-labelled secondary antibody, either Alexa Fluor 488 anti-mouse IgG or Alexa Fluor 594 anti-mouse IgG (Invitrogen, Karlsruhe, Germany) for three hours at room temperature. Type-I pro-collagen was detected accordingly using the monoclonal anti-human type-I pro-collagen antibody clone 1913 (Fitzgerald, Concord, Massachusetts). In both cases, detection of the cell nuclei was performed by counterstaining with DAPI.

Aggrecan was detected using the monoclonal anti-human aggrecan antibody clone HAG7D4 (Acris Antibodies, Hiddenhausen, Germany). After unmasking of antigenic epitopes by incubation with 0.5 M NaOH for ten minutes and washing with PBS (five times), the sections were incubated overnight at 4°C with the primary antibody (1:20 dilution in 3% Tris albumin). After washing in PBS, the sections were incubated with the fluorescence-labelled secondary antibodies as above. Cell nuclei were counterstained using propidium iodide.

Isolation of RNA and cDNA synthesis. Chondrocytes (approximating 4×10^6 cells/chamber) which had been cultured in Spongostan for different periods of time were lysed, denatured by the addition of 1 ml of TRIzo (Invitrogen) and centrifuged for ten minutes at 12 000 *g* at 4°C. After dissolving the pellets in 20 volumes of nuclease-free water containing RNAase out (Invitrogen), RNA concentrations were measured using a Smart Spec 3000 spectrometer (Bio-Rad Laboratories GmbH, Munich, Germany) and the samples denatured at 65°C for five minutes.

The RNA was then transcribed to cDNA using reverse transcriptase and subsequently adjusted to a standard concentration of 1 μgCDNA/20 μl for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR was performed using a Multicolor Real-time Detection System (Bio-Rad Laboratories) after diluting the cDNA 1:10 in nuclease-free water (template 9.5 μl, Mastermix: SYBR Green (Bio-Rad Laboratories) 10 μl, upstream primer 0.3 μl, downstream primer 0.3 μl). The specific primers for the different genes and the respective PCR conditions are listed in Table I. Data were expressed as the threshold cycle at which the fluorescence intensity of the PCR product exceeded background levels for the first time, normalised to the respective gene standard and the threshold cycle of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Quantification of GAG. The GAG content of the cell fraction of the monolayers and the chondrocyte-Spongostan cultures was determined using the DMB (Serva, Heidelberg, Germany) assay after digestion with the cystein protease papain at a concentration of 125 μg/ml with 5 mM L-cystein buffer overnight at 60°C. As a reference standard, no commercially available GAG preparation was used, but instead the A1/D1 (A: associative, D: dissociative centrifugation) fraction of a cartilage proteoglycan preparation was utilised. The A1/D1 fraction contains fully sulphated GAG and therefore does not generate systematic errors because of potentially undersulphated standards, common to alkaline tissue extracts of GAG.[17]

Table II. mRNA expression in cells and supernatants of chondrocyte monolayers and Minusheet 3D cultures at 7, 14 and 28 days. The results are expressed as the mean and SEM

	7			14			28		
mRNA expression (arbitrary units)	**Monolayer S-**	**Monolayer S+**	**Minusheet**	**Monolayer S-**	**Monolayer S+**	**Minusheet**	**Monolayer S-**	**Monolayer S+**	**Minusheet**
Type-II collagen	0.22 (0.01)	0.08 (0.00)*	0.37 (0.09)	0.06 (0.00)†	0.06 (0.00)†	0.40 (0.10)‡	0.01 (0.00)†,§	0.002 (0.00)*,†,§	0.04 (0.02)†,‡
Type-I collagen	0.16 (0.01)	0.14 (0.01)*	0.01 (0.00)‡	0.13 (0.01)†	0.13 (0.01)	0.03 (0.01)‡	0.06 (0.00)†,§	0.02 (0.00)*,†,§	0.03 (0.01)
Aggrecan	0.23 (0.01)	0.14 (0.01)*	0.09 (0.02)	0.05 (0.00)†	0.06 (0.00)*†	0.10 (0.02)	0.03 (0.00)†,§	0.02 (0.00)*,†,§	0.03 (0.02)
Type-II: Type-I collagen ratio	1.35 (0.03)	0.56 (0.01)*	52.07 (22.77)‡	0.43 (0.00)†	0.50 (0.02)*	15.53 (1.65)‡	0.09 (0.01)†,§	0.11 (0.01)*,†,§	4.03 (2.66)†
Aggrecan: Type-I collagen ratio	1.39 (0.04)	0.95 (0.03)*	15.66 (6.98)	0.40 (0.02)†	0.45 (0.03)†	4.46 (0.86)†	0.48 (0.01)†,§	1.08 (0.01)*,†,§	3.34 (3.32)

* $p \leq 0.05$ for the comparison with the Monolayer S-

† $p \leq 0.05$ for the comparison with the preceding time point

‡ $p \leq 0.05$ for the comparison with the Monolayer S+

§ $p \leq 0.05$ for the comparison with the seven-day result

In the case of the monolayer supernatants, no digestion was performed. An influence of the papain buffer on the DMB assay was excluded by identical results for the GAG content in aliquots of supernatant in culture medium or aliquots transferred to papain buffer by dialysis. The culture mass (50 mg) of Minusheets was digested with 300 µl of the enzyme solution. The GAG concentration was determined by comparison with a standard curve as previously published.[16,18] The absorption was measured at 525 nm using a microplate reader (Bio-Rad Laboratories).

Statistical analysis. All measurements are presented as mean values and the SEM. Statistical analysis was performed using the software package SigmaStat 3.0 (SPSS Inc., Chicago, Illinois) and the non-parametric Mann-Whitney U test. Statistical significances were accepted and displayed for levels of $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$.

Results

Monolayer cultures. Chondrocytes cultured in monolayers for seven, 14, and 28 days with Spongostan-containing medium (S+; 2×10^6 cells/well) showed no reduction in vitality compared with cultures without Spongostan (S-) (based on comparable mRNA and protein expression in S- and S+ cultures, in particular on day 14; see below). In the S+ cultures after seven, 14 or 28 days, the mRNA expression for the alpha-1 chain of type-II collagen and aggrecan was only marginally altered in comparison with the S- monolayer cultures (2×10^6 cells/well, Table II (Fig. 3)).

By contrast, the mRNA expression for the alpha-2 chain of type-I collagen in S+ chondrocyte monolayer cultures was somewhat reduced in comparison with the S-monolayer cultures ($p \leq 0.05$ for day seven and 28, Table II, Fig. 3).

However, the resulting ratio of collagen type-II/type-I mRNA or aggrecan/collagen type-I mRNA was only marginally higher in S+ chondrocyte monolayer cultures than in S- monolayer cultures ($p \leq 0.05$ for collagen type-II/type-I ratio on day 14; $p \leq 0.05$ for aggrecan/collagen type-I ratio on day 28, Table II, Fig. 3). Therefore merely adding Spongostan to the chondrocyte culture medium does not appear to have a major effect on the differentiation of the cells *in vitro*.

Chondrocyte-Spongostan chambers. After 14 days of cultivation in chondrocyte-Spongostan chambers, the mRNA expression for type-II collagen, type-I collagen and aggrecan and, as a consequence, the ratios of type-II to type-I collagen or aggrecan to type-I collagen, did not change in comparison with the seven-day cultures (Table II, Fig. 3). By contrast, after 28 days of culture, type-II collagen mRNA and, subsequently, the collagen type-II to type-I ratio significantly decreased in comparison with the 14-day cultures ($p \leq 0.05$, Table II; Fig. 3).

Comparison of chondrocyte-Spongostan chambers with chondrocyte monolayers (2D S+). Collagen type-II mRNA showed a clear increase in Minusheet compared with that in S+ monolayer cultures (6.6-fold on day 14; 20-fold on day 28; both $p \leq 0.05$). By contrast, aggrecan mRNA was unchanged in Minusheets compared with S+ monolayer cultures (Table II, Fig. 3). Strikingly, there was a marked reduction of type-I collagen mRNA in the culture chambers compared with the monolayer cultures (14-fold on day 7; 4.3-fold on day 14; $p \leq 0.05$). However, the resulting collagen type-II to type-I (93-fold increase on day seven; $p < 0.01$; 31-fold increase on day 14; $p < 0.05$) and the aggrecan to collagen type-I ratios (16.5-fold increase on day seven ; $p < 0.01$; 9.9-fold increase on day 14; $p < 0.05$) were only significantly different in some cases (Table II; Fig. 3).

Histological and immunohistological examination. Aggrecan, as well as type-I and type-II collagen was detectable in S+ and S- monolayer cultures and Minusheets cultured for seven, 14 or 28 days. All proteins were observed in a predominantly cell-associated fashion, but were also distributed diffusely in the matrix (Fig. 4; data shown for 14 days).

Production of GAG. Whereas S+ and S-monolayer cultures showed high GAG production, with only sporadic differences between S+ and S-, Minusheets produced considerably lower amounts of GAG than monolayer cultures (S-/S+; $p \leq 0.001$ for all intervals). The chondrocyte-

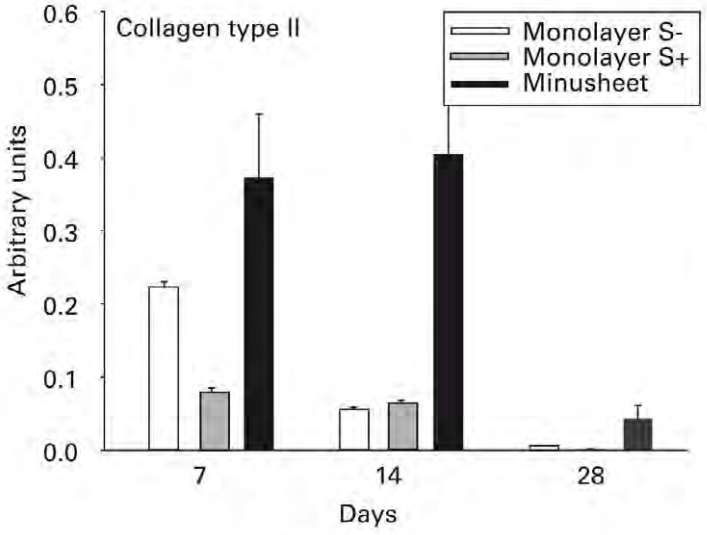

Fig. 3a

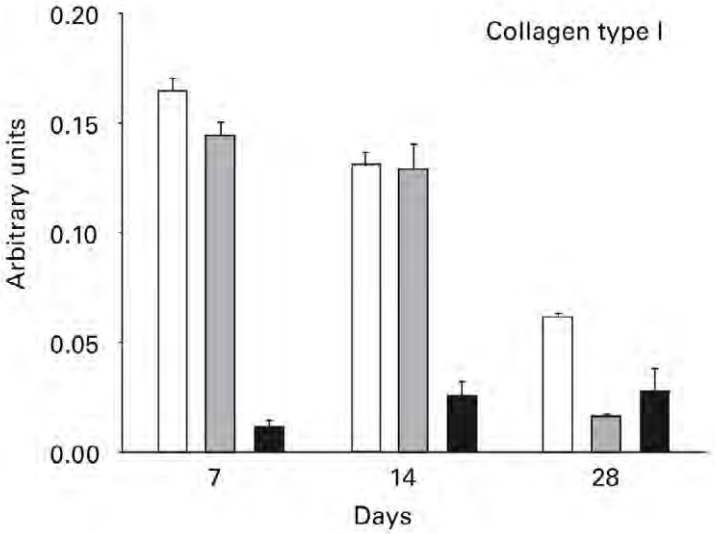

Fig. 3b

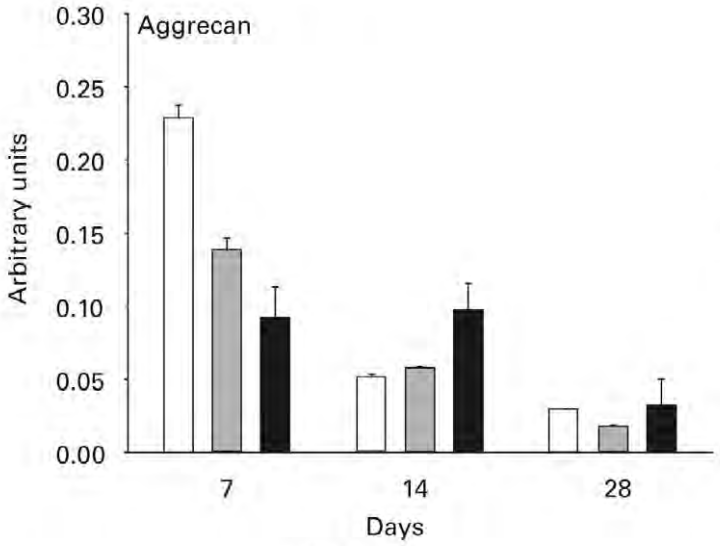

Fig. 3c

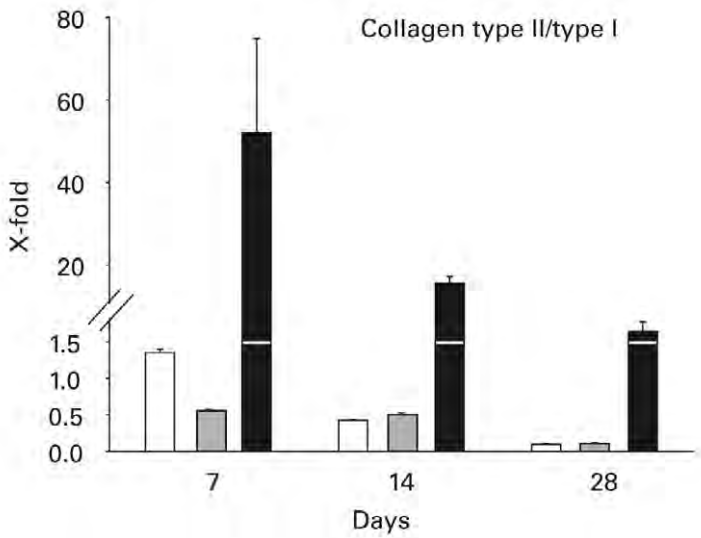

Fig. 3d

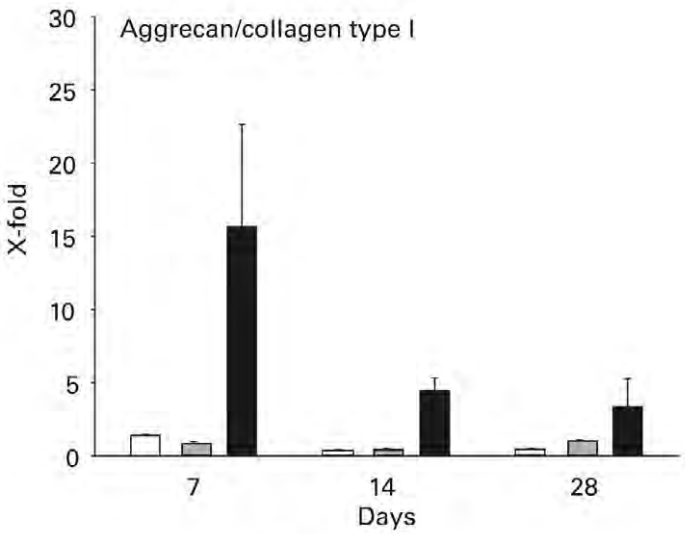

Fig. 3e

Bar charts showing polymerase chain reaction quantitative real-time for a) collagen type II, b) collagen type I, and c) aggrecan. The results were expressed as the threshold cycle, nomalised to the respective gene standard and the threshold cycle of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (mean and SEM for 3 to 8 cultures). Subsequently, the ratios of d) collagen type II to type I and e) aggrecan to collagen type I were calculated.

Spongostan chambers contained a GAG concentration of 0.29 ± 0.02 μg/10^6 cells after culture for seven days and a peak mean concentration of 0.83 ± 0.08 μg/ 10^6 cells after culture for 14 days. After 28 days, the GAG concentration was again decreased to a mean of 0.25 ± 0.02 μg/ 10^6 cells (Fig. 5, Table III).

Discussion

Our study describes for the first time the preparation and characterisation of a 3D highly shapeable cartilage replacement scaffold composed of the gelatine-based haemostyptic Spongostan, FCS-containing DMEM medium, and chondrocytes (4 × 10^6 cells/30 mg of Spongostan). The

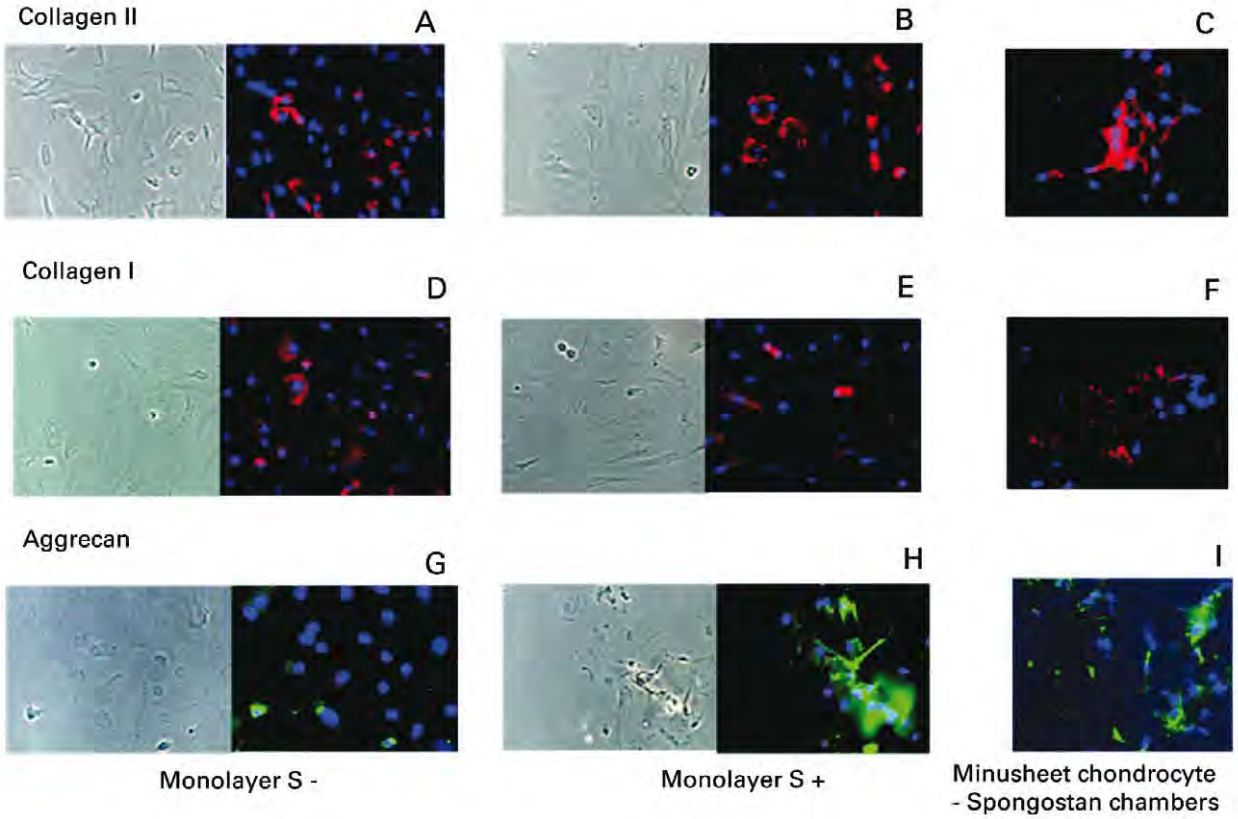

Fig. 4

Immunohistochemical detection of type-II and type-I collagen, as well as aggrecan in monolayer culture with (S+) or without Spongostan (S-) and chondrocyte-Spongostan chambers (in all cases cultured for 14 days). Collagen type II (A, B, C; red fluorescence, nuclear counterstaining with DAPI) or collagen type I (D, E, F; red fluorescence, nuclear counterstaining with DAPI), as well as aggrecan (G, H, I; green fluorescence, nuclear counterstaining with propidium iodide) were detectable in all cultures. All proteins were observed in a predominantly cell-associated fashion, but were also distributed diffusely in the matrix (× 200).

composite was cultured in Minusheets sealed with a collagen and a dialysis membrane, thereby creating a bioactive culture chamber. In this 3D culture chamber, the cartilage-specific differentiation markers type-II collagen and aggrecan were deposited in the pericellular matrix and very high ratios of type-II to type-I collagen and aggrecan to collagen type-I mRNA were observed. These ratios were greatly increased compared with those seen in time-matched monolayer cultures with or without the addition of Spongostan. By contrast, the expression of mRNA for collagen type I in S+ chondrocyte monolayer cultures was only marginally reduced in comparison with the S- monolayer cultures. Thus, the culture in a 3D Spongostan scaffold reduces dedifferentiation of the cells *in vitro* and shows its potential to stabilise the chondrocytic phenotype.[4,19,20] These results confirm and support those in other types of 3D cultures, such as pellet, micromass or aggregate models, whereas conventional monolayer culture regularly results in a loss of the differentiated phenotype.[21]

In our study, the mRNA levels of aggrecan and collagen type II in Minusheets remained stable until day 14. Also, there were limited changes in the ratios for collagen type II to type-I or aggrecan to collagen type I between seven and 14 days, and the level of GAG reached its maximum after 14 days in line with, although not completely comparable

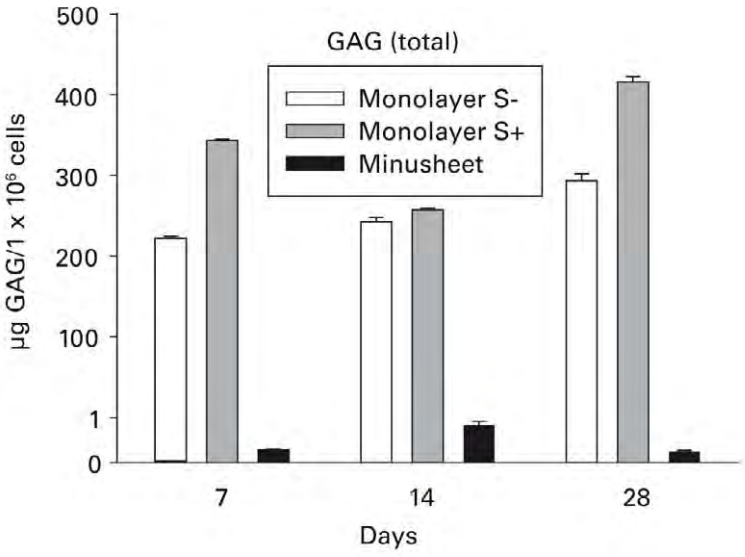

Fig. 5

Bar chart showing the determination of the GAG of monolayer culture with (S+) or without Spongostan (S-), as well as in Minusheet chondrocyte-Spongostan chambers. The results were expressed as GAG in µg/l × 10^6 cells (mean and SEM for 3 to 8 cultures).

with other findings.[22] Therefore, *in vitro* culture for 14 days in a 3D chamber may be the best point to obtain an optimised matrix before surgical implantation, an interval already used for the production of commercial cartilage

Table III. Concentration of glycosaminoglycan (GAG) in cells and supernatants of chondrocyte monolayers and in Minusheet 3D cultures at 7, 14 and 28 days. The results are expressed as the mean and SEM

	7			14			28		
GAG (µg/10^6 cells	Monolayer S-	Monolayer S+	Minusheet	Monolayer S-	Monolayer S+	Minusheet	Monolayer S-	Monolayer S+	Minusheet
Cells	14.25 (0.13)	15.01 (0.93)	0.29 (0.02)	15.58 (0.41)*	14.88 (0.32)	0.83 (0.08)*	17.43 (0.52)*†	13.06 (0.16)*,‡,§	0.25 (0.02)*
Supernatant	208.88 (1.10)	327.98 (0.55)§	-	226.97 (5.72)*	242.43 (2.19)*,§	-	276.00 (8.34)*,†	402.44 (6.42)*,†,§	-
Total	223.13 (1.23)	342.99 (1.49)	0.29 (0.02)§,¶	242.55 (6.14)	257.32 (2.51)	0.83 (0.08)§,¶	293.42 (8.86)	415.51 (6.58)	0.25 (0.02)§,¶

* $p \leq 0.05$ for the comparison with the preceding time point
† $p \leq 0.05$
‡ $p \leq 0.05$ for the comparison with the seven-day result
§ $p \leq 0.05$ for the comparison with the Monolayer S-
¶ $p \leq 0.01$ for the comparison with the Monolayer S+

replacement materials.[23] This is in good agreement with findings in the literature which show a maximum mRNA expression for aggrecan after 3D culture for 16 days.[24]

Using a sponge-based collagen type-I matrix, other investigators have recently obtained very promising results.[23] First the decrease in the collagen type-II to type-I and the aggrecan to collagen type-I mRNA ratios between seven and 14 days of culture was markedly smaller than in the respective monolayer culture, indicating the suitability of a 3D culture. In addition, the production of GAG, as assessed by the DMB assay and ^{35}S-sulphate incorporation, increased in 3D culture until day 14 (Fig. 5). These results further support the concept that 14 days may be optimal for the 3D culture of matrix-associated ACT samples.

It is of note that there was a considerably lower production of GAG in Minusheets 3D chambers than in monolayer cultures. However, this is in accordance with previous results from other 3D models showing comparably low GAG levels of approximately 1.5 µg/l × 10^6 cells.[25] Also, the levels of GAG in both the cell layer and supernatant of the monolayer culture in our study showed the same order of magnitude as those in a recent study.[26] Since to our knowledge the level of GAG production has never been directly compared, the reasons for this difference presently remain a matter of speculation. Possible mechanisms include cell density,[26] accessibility to soluble chemical signals[26] and/or a positive or negative feedback of the surrounding insoluble matrix.[25,27,28]

In both day-seven and day-14 3D chambers, the chondrocytes were evenly distributed and type-II collagen was detected in a homogeneous pericellular distribution. The observations are in line with previous findings for biodegradable polymer fleeces and demonstrate that Spongostan may be equally suitable as a replacement scaffold.[6,15] A major advantage of powder-based Spongostan in this context is its ability to be moulded into a plastic material, allowing tight form-filling even in the case of an irregularly-shaped cartilage defect.

The procedure of matrix-associated ACT requires a balance between the *in vitro* expansion of the chondrocytes often associated with significant dedifferentiation and the availability of sufficient numbers of chondrocytes for implantation. Since at least 1 × 10^6 cells/cm^2 of the surface area of the defect are recommended by the German Orthopaedic Society[29] (resulting in a volume-adjusted cell density of approximately 3 × 10^6/cm^3 in a defect of 0.3 cm depth),[25] the size of the biopsy samples from healthy cartilage may have to be increased in order to avoid *in vitro* expansion in monolayer cultures. However, in view of the results from this study and those of others, the aim of providing a homogeneously populated replacement scaffold of sufficient volume without prior cell expansion/dedifferentiation makes this approach highly attractive for clinical practice.[6]

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