Generation of a scaffold free cartilage-like implant from a small amount of starting material

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Abstract

Introduction: An autologous cellular based treatment of a traumatic cartilage injury requires a procedure whereby a biopsy of healthy cartilage is removed from the patient and the cells isolated and expanded by monolayer passage. This increases the cell number to required levels but also leads to a de-differentiation of the cells. We aim to produce a scaffold-free, de-novo implant from a biopsy of cartilage. Methods: Bovine chondrocytes were isolated from a small biopsy and expanded. The chondrocytic phenotype of the monolayer expanded cells was recovered during a period of culture in alginate and the effect of factors such as IGF1, TFGβ1 and dexamethasone was investigated. Results: During the alginate culture period a pre-treatment with IGF1 and dexamethasone was shown to have little effect. IGF1 however increased the glycosaminoglycan/DNA (GAG/DNA) content on day 14 to 84.95 ± 5ng/ng compared with 37.3 ± 1.8ng/ng in the controls ($P < 0.001$). 35S labeling demonstrated an increased GAG synthesis in the presence of IGF1 ($P < 0.001$). IGF1 also induced a increase of DNA content 1383 ± 314ng/bead compared to 512 ± 19ng/bead in the controls ($P < 0.001$). The cells were released from the alginate and cultured in a silicon mould for a further 14 days to obtain a three dimensional implant. Releasing the cells from the alginate and casting in a mould produced an implant of defined shape which contained no foreign material. After 31 days of culture the implants contained 152.4 ± 13.14ng/ng GAG/DNA and 42.93 ± 10.23ng/ng collagen II. Discussion: We believe alginate released chondrocytes provide a real alternative to artificial scaffolds.

Keywords: chondrocyte • cartilage repair • implant • autologous cells • tissue engineering

Introduction

The generation of an autologous *de novo* cartilage-like implant requires the sampling of a small biopsy and the isolation of the chondrocytes contained within. Due to the small number of isolated cells it is likely a monolayer step will be required to expand cell number. The monolayer passaging of chondrocytes is known to negatively influence their capability to re-constitute a cartilage like material *in vitro*, a process known as de-differentiation [1]. This process can be reversed by a period of 3-dimensional culture, such as agarose [1] or alginate [2–4] in order to regain the chondrocyte phenotype.

A source of debate is whether a three dimensional implant requires the use of a pre-formed artificial scaffold. A large number of groups are focusing on

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the production of a suitable scaffold for *de novo* cartilage synthesis with chondrocytes or stem cells [5–11] for review see Bonassar 2004 [12]. The implanted material should not induce any inflammatory response and it must be degradable over a suitable time frame *in vivo* with no toxic intermediate or end products. It should also not interfere with the typical chondrocyte phenotype. As we believe solving these issues will provide considerable difficulties, we have concentrated efforts into producing a scaffold free implant. A scaffold free implant must possess enough inherent mechanical stability to withstand the mechanical forces applied during rehabilitation after implantation. The method must also allow for the formation of various shapes and sizes without the need for a structural backbone normally provided by the artificial scaffold material. There are currently very few reports of scaffold free cartilage-like implants in the literature [13–17] and these are either uncontrolled in size and shape [17] or rely on a membrane insert of a fixed diameter for determination of shape [13–16]. The original method as described by Masuda [14] was performed using primary cells and as such is not suitable for clinical use due to the low numbers of cells normally obtained.

We describe a method whereby a small amount of starting material can successfully be used to produce a larger piece of cartilage-like tissue. While it is not of the same quality as normal cartilage, it is robust enough to be handled with forceps and be sutured or glued. It is easily cast into any shape with thicknesses up to 5 mm possible. As proof of principle the chondrocytes were expanded beyond the number of population doublings realistically required. This was in order to maximally stress the cells and should provide a "worst case" scenario. A crucial step is a period of growth in alginate culture after the monolayer expansion. This enables re-differentiation to occur and allows for deposition of abundant extra-cellular matrix. The cells are then easily recovered and can be further cultured to produce a coherent implant.

Methods

Intact bovine knee joints, from 10 month old calves were obtained from a local slaughterhouse. Cartilage tissue was dissected from the subchondral bone within a few hours after slaughter. Chondrocytes were liberated by sequential digestion with 0.4% pronase (Sigma, Buchs, Switzerland) for 1½ h and 0.025% collagenase-P (Böhringer Mannheim, Germany) for 16 h. Cells were isolated by filtration through a 40 μm-filter and repeated wash cycles of centrifugation-re-suspension.

Chondrocyte culture

The protocol for the production of cartilage-like implants includes three steps.

Step 1
Isolated bovine chondrocytes were plated at a density of 1000 cells/cm^2 and fed complete medium consisting of 1:1 Ham's F12/DMEM (Gibco, Basel, Switzerland), 50 μg/ml gentamycin (Sigma, Buchs, Switzerland), 5 ng/ml TGFβ1 (Research Diagnostics Inc, New Jersey, USA), 5 ng/ml bFGF (Research Diagnostics Inc, New Jersey, USA) and 10% foetal calf serum (FCS) (Vitronex, Vilshofen, Switzerland). Cells were harvested by trypsinisation and a portion were subjected to a second monolayer passage, again at a density of 1000 cells/cm^2, and fed complete medium as before. On reaching confluence the cells were harvested by trypsin/EDTA digest.

Step 2
Cells harvested from monolayer culture were encapsulated in alginate gel using a slightly modified version [18] of the method described originally by Guo *et al.* [19]. Cells were resuspended at a density of 4×10^6 per ml in sterile filtered low-viscosity alginate solution (1.2%, w/v, Keltone LV, Kelco, Chicago, USA), then slowly expressed through a 22 gauge needle in a dropwise fashion into a gently agitated 102 mM CaCl$_2$ solution. After instantaneous gelation, the polymerization was completed for a period of 10 min in the 102 mM CaCl$_2$ solution. Beads, containing approximately 40,000 cells, were washed 4 times in 10 volumes of 150 mM NaCl and once in 1:1 Ham's F12/DMEM medium. Finally, the beads were cultured in either control medium consisting of 1:1 Ham's F12/DMEM, 25 μg/ml ascorbic acid (Sigma, Buchs, Switzerland), 50 μg/ml gentamycin and 10% FCS or IGF1 medium consisting of control medium containing 50 ng/ml IGF1 (Research Diagnostics Inc, New Jersey, USA). Two groups were pre-treated for 7 days with medium consisting of 1:1 Ham's F12/DMEM, 25 μg/ml ascorbic

	d0 - d7	d7 - d28
Group 1:	Control Medium	Control Medium
Group 2:	Control Medium + 50 ng/ml IGF1	Control Medium + 50 ng/ml IGF1
Group 3:	Control Medium + 2.5 ng/ml TGFβ1 + 40 ng/ml DEXA	Control Medium
Group 4:	Control Medium + 2.5 ng/ml TGFβ1 + 40 ng/ml DEXA	Control Medium + 50 ng/ml IGF1

acid, 50 µg/ml gentamycin, 2.5 ng/ml TGFβ1, 40 ng/ml Dexamethasone (DEXA) (Sigma, Buchs, Switzerland) and 10% FCS. After 7 days the cells were transferred to either control medium or IGF1 medium. The cultures were maintained in 24-well plates at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every 2 days and samples taken for analysis every 7 days.

Step 3

After 14 days of culture the beads were dissolved by mild citrate treatment and the recovered cells washed once with mild citrate (150 mM NaCl / 55 mM sodium citrate pH 6.8) and twice with Hanks buffer (Gibco, Basel, Switzerland). The cell pellet was resuspended in 100% FCS, centrifuged at 100g for 10 min and the excess serum removed. The cell slurry was then cast either into a 1.5 cm^2 by 1 mm thick square rubbery silicon form or a circular rubbery silicon form 5mm in diameter and 1 mm thick (Fig. 4) cut from a sheet of silicon (Angst + Fister, Zurich , Switzerland), and then overlaid with dialysis tubing (MWCO 100 kDa). The exact number of cells placed into the mould will vary slightly as it is largely determined by the amount of matrix accumulated by the cells during culture in alginate bead. The tissue was grown in this mould for 7 days before being removed and placed in a 6 well plate, the base of which had been previously covered with 3% agarose gel to prevent any cells from attaching. The *de novo* tissue was fed every 2 days with the same medium used for bead culture.

Quantification of proteoglycan biosynthesis

After 14 days of culture in alginate beads, three beads were fed with fresh culture medium containing ^{35}S-sulfate (50 µCi/ml, Na$_2$SO$_4$ salt, ICN Biomedicals Inc,

Eschewege, Germany) to label for glycosaminoglycans [20]. After 16 hrs, the medium was harvested and analyzed. The beads were then digested with 400 µl papain (Sigma, Buchs, Switzerland) before being extracted by combining with 400 µl 8M GuHCl, 0.1M sodium actetate trihydrate, 0.02M EDTA, 0.2M 6-Aminocaproic acid. The extract was chromatographed on a Sephadex G-25 column (Pharmacia) in order to remove free isotope. The radioactivity incorporated in ^{35}S-proteoglycan was determined by liquid scintillation counting and is expressed as counts/hr/µg DNA.

Biochemical analyses

Cell number was determined by measuring total DNA using Hoechst 33258 fluorescent dye (Sigma, Buchs, Switzerland) and calf thymus DNA as a standard [21] (Sigma, Buchs, Switzerland). Accumulation of extracellular matrix macromolecules was determined by measuring contents of proteoglycan by a modified alcian blue precipitation assay [22]. Native Collagen II was quantified in implants using the Chondrex native type II collagen detection kit (MD Biosciences, Zürich, Switzerland). Samples were prepared as described in the kit protocol and quantified in duplicate with 1:100 and 1:1000 dilutions.

Cryosectioning and alcian blue staining of alginate beads

The alginate beads cultured using the optimal factor combination, were placed in an eppendorf tube containing Tissue Tek OCT cryo-compound (Digitana, Horgen, Switzerland) and left to incubate for 30 min at room temperature. The samples were then frozen using liquid nitrogen and 12 µm cryosections were cut. Sections were then incubated in 3% acetic acid in

0.1 M CaCl$_2$ for 3 min, followed by 30 min in alcian blue staining solution consisting of 1 g alcian blue 8GX (Fluka, Buchs, Switzerland), 3% acetic acid, 0.08 M HCl, 0.1 M CaCl$_2$. Sections were then rinsed 10 times in 0.1 M CaCl$_2$ with shaking, followed by 10 min incubation in 0.1 M CaCl$_2$. The slides were finally rinsed with 50 mM CaCl$_2$ and then covered with a water based mountant to which 5 mM CaCl$_2$ was added and coverslipped.

Histology

Samples were placed in 4% buffered formalin at 4°C overnight. After washing in dH$_2$O the samples were then dehydrated through a series of alcohols, followed by xylene and then paraffin embedded. Sections were cut 4 μm thick and re-hydrated prior to being stained with haemotoxylin and eosin (for a general overview) or Safranin O (for GAG content). Bovine articular cartilage was also stained as a positive control.

Immunohistochemistry

Paraffin embedded samples were sectioned to 6 μm and antigen retrieval was performed at 95°C for 30 min using 10 mM sodium citrate buffer pH 6.0. All subsequent steps were carried out at room temperature using the protocol of Milz [23]. After a hyaluronidase digestion (1.5 IU/ml, Sigma, Buchs, Switzerland) sections were blocked with rabbit serum. Labelling for collagen II was performed for 30 min at room temperature using 6.8 μg/ml CIICI mouse monoclonal antibody. The monoclonal antibody developed by Holmdahl and Rubin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA52242. After a 30 min incubation with biotinylated anti-mouse IgG (Horse anti-mouse, Vectastain elite ABC kit, Vector Laboratories, PK-6102) and a 30 min incubation with Vectastain elite ABC reagent, the sections were reacted for 4 min with DAB substrate containing nickel chloride (Vector Laboratories, SK-4100). Slides were then dehydrated in ascending grades of alcohol, cleared in xylene and mounted in DPX (Fluka, Buchs, Switzerland). Negative controls lacking primary antibody were included and no counterstain was performed.

Statistics

Samples were analyzed by two way ANOVA with Tukey pairwise analysis, P< 0.05 was considered significant. Results are from three experiments unless otherwise stated.

Results

Cartilage digest

The digest of the bovine cartilage tissue yielded 3.6 x 10^4 cells per mg ± 2% from an average of 112.8 mg ± 5.37 mg giving an average total yield of approximately 4.1 x 10^6 cells from each biopsy (n = 3). From this a portion (1.75 x 10^5 cells) was placed into monolayer culture and the rest discarded.

Monolayer culture

Passage 1
Cells took approximately 11 days to reach confluence. On recovery of the cells from passage 1 the cells had undergone and average of 6.3 ± 0.88 population doublings increasing cell number 111.15 ± 21.7 fold.

Again only a portion of these cells (7.75 x 10^5) were used for further monolayer passage.

Passage 2
Cells took approximately 8 days to reach confluence. On recovery of the cells from passage 1 the cells had undergone an average of 7.98 ± 0.21 population doublings increasing cell number 253.3 ± 35.9 fold.

The total number of population doublings undergone by the cells over the two monolayer passages was 14.75 which corresponds to a 2.75 x 10^4 fold increase in cell numbers.

Alginate culture

Passage two monolayer expanded cells were exposed to four combinations of factors during alginate culture (see table in methods section) in order to determine their effects during the re-differentiation of the monolayer expanded cells.
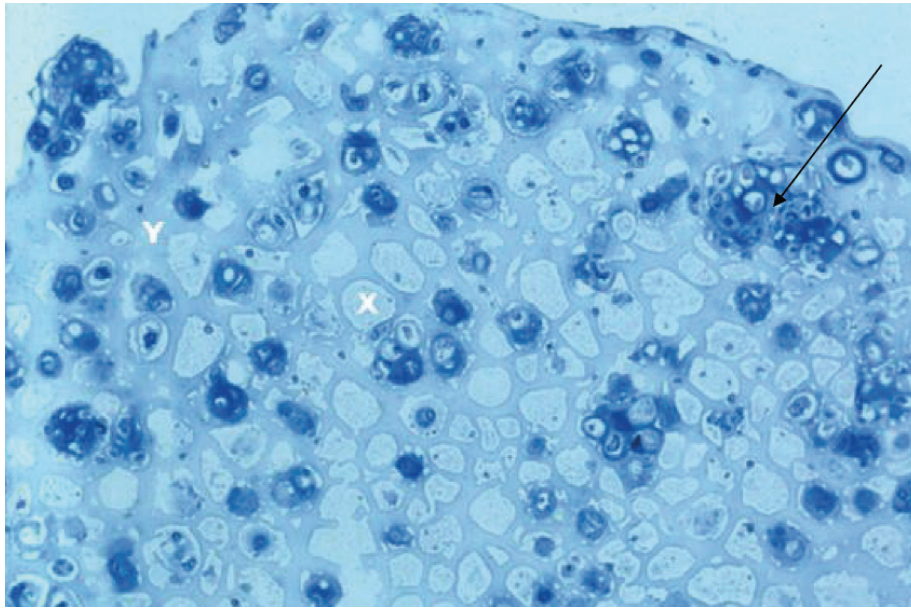
Fig. 1 Representative image of chondrocytes encapsulated in an alginate bead after culture in control medium containing IGF1, demonstrating the phenotype commonly seen in alginate culture. After staining for glycosaminoglycans with alcian blue, darkly stained cells can be seen interspersed with lighter stained further removed matrix (arrow) and unstained areas (**X**). There is a slight background staining of alginate (**Y**).

Effect of factors on DNA content

The effect of a pretreatment with TGFβ1 and dexamethasone is dependant on the presence or absence of IGF1 (Fig. 2). Pre-treatment with TGFβ1 + dexamethasone had very little effect on DNA content when samples were treated in conjunction with IGF1 (Gr. 4), only on day 7 can any difference be seen, with TGFβ1 + DEXA pre-treated samples (Gr. 4) having a reduced DNA content compared with IGF1 treated alone (Gr. 2). Samples not exposed to additional IGF1 showed an enhanced DNA content when pre-treated with TGFβ1 + dexamethasone (Gr. 3). This increase was most notable on day 21 when the TGFβ1 + DEXA pre-treatment (Gr. 3) led to an increase of 199% over control sample (Gr. 1) (1676 μg compared with 844 μg, P < 0.001, n=6). An increase can still be seen by day 28, albeit not so great (2772 μg compared with 2179 μg, P < 0.01, n=6). Addition of IGF1 plays a much greater role in increasing DNA content. Both samples treated with IGF1 (Gr. 2 and Gr. 4) have a greater than 3 fold increase in DNA over control medium (Gr. 1) by day 21 (4070 μg IGF1 alone, 4039 μg TGFβ1 + DEXA pre-treatment to IGF1 compared with 844 μg control).

Effect of factors on GAG/ DNA content in alginate bead

A pre-treatment with TGFβ1 + DEXA had little or no effect on the final GAG/ DNA content of any of the conditions tested (Fig. 2b). Samples later treated with IGF1 (Gr. 4) actually exhibit a reduced GAG/DNA during the first 7 days of TGFβ1 + DEXA pre-treatment compared with samples treated with IGF1 alone (Gr. 1). Over the following 14 days the accumulation in TGFβ1 + DEXA treated cells did exceed that of the IGF1 only treated cells. Constant treatment with IGF1 (Gr. 1) increases GAG/DNA content from 44.7 ± 3.9 ng/ng to 93.6 ± 0.7 ng/ng (209%) between day 7 and 21 (n=6). A seven day pre-treatment with TGFβ1 + DEXA (Gr. 4) increases GAG/DNA content from 30.7 ± 3.3 ng/ng to 92.6 ± 3.9 ng/ng (301%) over the same time period (P < 0.001, n=6). This recovers the lag initially displayed by the TGFβ1 + DEXA treated group.

The presence or absence of IGF1 is the critical factor in determining GAG/ DNA content. After 14 days of culture the addition of IGF1 (Gr. 2) increased GAG/DNA to 84.95 ± 5.0 ng/ng compared with 37.3 ± 1.8 ng/ng in the control (P < 0.001, n=6). The increase induced by IGF1 is still apparent at day 21 (93.6 ± 0.7 ng/ng *vs* 64.2 ± 7.7 ng/ng, P < 0.001, n=6) and day 28 (93.6 ± 0.7 ng/ng *vs* 64.2 ± 7.7 ng/ng, P < 0.001, n=6). By day 28 there are no apparent differences between the group treated with IGF1 constantly (Gr. 2) and that pre-treated with TGFβ1 + DEXA for 7 days followed by control medium containing IGF1 (Gr. 4).

Incorporation of radioactive S35

The S35 content was determined in beads radiolabeled on day 14 (Fig. 3). All combinations of fac-

Fig. 2a Monolayer expanded chondrocytes were encapsulated in alginate beads and cultured for 28 days. Gr. 1 was fed control medium, Gr. 2 control medium containing IGF1, Gr. 3 was pretreated for 7 days with control medium containing TGFβ1 and DEXA then transferred to control medium and Gr. 4 was pretreated for 7 days with control medium containing TGFβ1 and DEXA then transferred to control medium containing IGF1. Groups exposed to IGF1 had a greater increase in DNA per sample than those in control medium (P < 0.001). The TGFβ1 + DEXA pretreatment enhanced DNA per sample only in samples not exposed to IGF1. (* P < 0.001 over control culture)
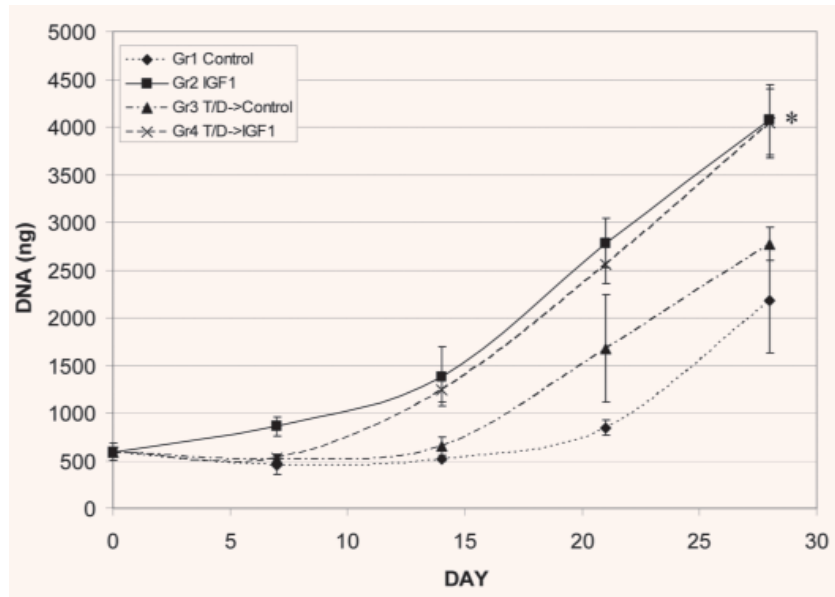
tors that included IGF1 increased the S^{35} incorporation. The greatest increase was produced in cells treated with TGFβ1 and DEXA for 7 days, followed by 7 days of control medium containing IGF1 (Gr. 4, 111156 compared with 43456 counts/hr/μg DNA, P < 0.001, n=6). Cells treated for 14 days with control medium with IGF1 alone (Gr. 2) demonstrated a 178% increase over control (77384 compared with 43456 counts/hr/μg DNA, P < 0.001, n=6). Cells pre-treated with TGFβ1 + DEXA for 7 days followed by 7 day culture with control medium containing IGF1 (Gr. 4) had a statistically significant greater incorporation than IGF1 treatment alone (Gr. 2) (111156 compared with 77384 counts/hr/μg DNA, P< 0.001 n=6). Pre-treatment with TGFβ1 + DEXA had no effect when cells were later maintained with control medium (44810 compared with 43456 counts/hr/μg DNA).

As the pretreatment with TGFβ1 and DEXA did not appear to offer any added benefit over the period of culture investigated, the standard treatment in bead was considered to be Control medium + 50 ng/ml IGF1.

Alcian blue staining of alginate beads

Beads cultured in control medium + 50 ng/ml IGF1 were stained for glycosaminoglycans with alcian blue. This staining demonstrated a typical chondrogenic phenotype with a ring of darkly staining pericellular matrix around the chondrocytes. Mild stain-

ing of the further removed matrix was also seen (arrowhead, Fig. 1.). A slight background staining of the alginate can still be seen (Y).

Collagen and glycosaminoglycan content within implants grown in control medium containing 50 ng/ml IGF1

After 14 days of bead culture in control medium + 50 ng/ml IGF1, isolated cells were cast into silicon moulds and further cultured. After 14 days of further culture the implants had 89.5 ± 4.1 ng/ng GAG/DNA (n=3) and 23.19 ± 6.57 ng/ng collagen II/ DNA (n=3). After 31 days of culture the implants had 152.4 ± 13.14 ng/ng GAG/DNA and 42.93 ± 10.23 ng/ng collagen II (n=3). The GAG/DNA values compare favorably with the 84.95 ± 5 ng/ng GAG/DNA seen after 14 days culture in bead, indicating that GAG accumulation continues within the implant after the cells are released from alginate.

Histology of implants cultured in control medium containing 50 ng/ml IGF1

Haematoxylin and eosin staining on 14 day of implant culture demonstrated that there were a number of nucleated cells interspersed within large areas of weakly staining matrix (Fig. 6a). Staining
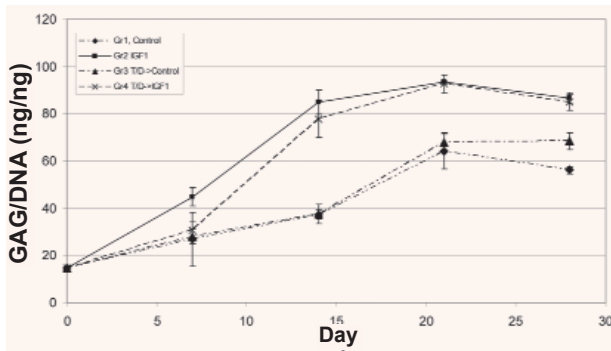
Fig. 2b Treating monolayer expanded chondrocytes with IGF1 increased GAG/ DNA content over control medium, most notably on day 14 (208–228% of control (P < 0.001)). This increase is not as great by day 28 but an increase is still seen (151–154% of control, P < 0.001)). TGFβ1 + DEXA pretreatment enhanced the rate of GAG/ DNA accumulation between day 7 and day 21 for samples treated with IGF1 compared with IGF1 samples not treated with TGFβ1 + DEXA (201% increase over day 7 content compared with 109% increase in untreated samples, P < 0.001) but by day 28 both contained comparable GAG/ DNA. The TGFβ1 + DEXA did not appear to have a noticeable effect on samples not exposed to IGF1.

intensity was similar to the transitional zone of cartilage (Fig. 6b, arrowhead).

Safranin O staining (specific for GAG) on day 14 of implant culture demonstrated that the implants produced were heterogeneous with respect to extracellular matrix content (Fig. 6c). Some areas did not stain for GAG (Fig. 6c - *), some areas stained poorly (Fig. 6c - x) while a large area of the

central part of the implant stained very strongly (Fig. 6c - y). At higher magnification areas staining strongly for GAG can be seen to contain rounded chondrocytes surrounded by a darkly stained cell associated matrix, interspersed with areas of further removed matrix which is less intensely stained (Fig. 6d). A thin layer of more flattened cells can be seen on the upper surface of the implant.

Immunostaining demonstrated the presence of collagen II. As with the safranin O staining for GAG's, the intensity of collagen II stain varied within the tissue.

Role of alginate step

In order to determine whether the alginate step was required to produce a scaffold free implant of defined shape, cells released from monolayer were cultured directly in the silicon mould in control medium containing IGF1 without pre-culture in alginate. The cells rapidly contracted forming a shrunken cell pellet which did not resemble the original form used (Fig. 8). The final implants shrunk to 15–20 % of the size of the original mould used.

Discussion

The main aim of this study was to produce a piece of *de novo* cartilage-like tissue which is larger in size than the biopsy taken as the start material. The
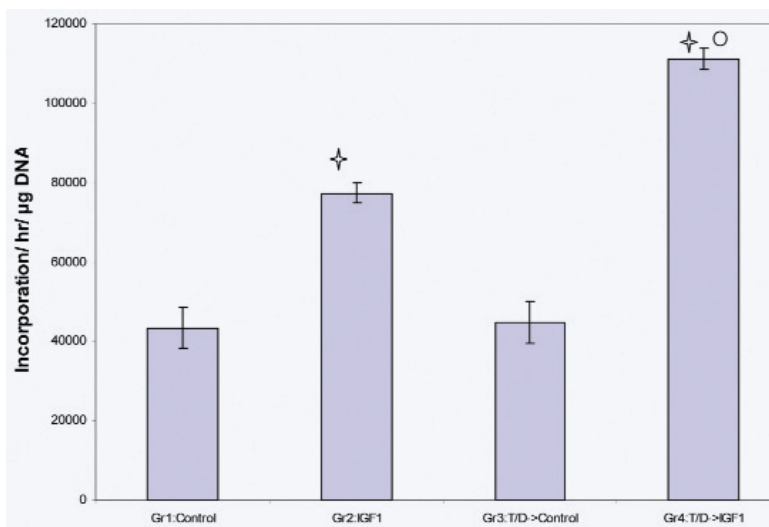

Fig. 3 S[35] incorporation was measured after 14 days of culture. Groups 3 and 4 were pretreated with TGFβ1 + DEXA in control medium for 7 days before being transferred into control medium with or without IGF1. TGFβ1 + DEXA pretreatment did not change incorporation in cell not exposed to IGF1 later. Continual treatment with IGF1 led to a higher incorporation than control medium (P< 0.001), but was less than TGFβ1 + DEXA pretreated cells later exposed to IGF1. TGFβ1 + DEXA pretreatment for 7 days led to a higher incorporation than control (P < 0.001) and IGF1 treatment alone (P < 0.001). (✛ = P < 0.001 compared to control, ○ =P < 0.001 compared with Gr2)
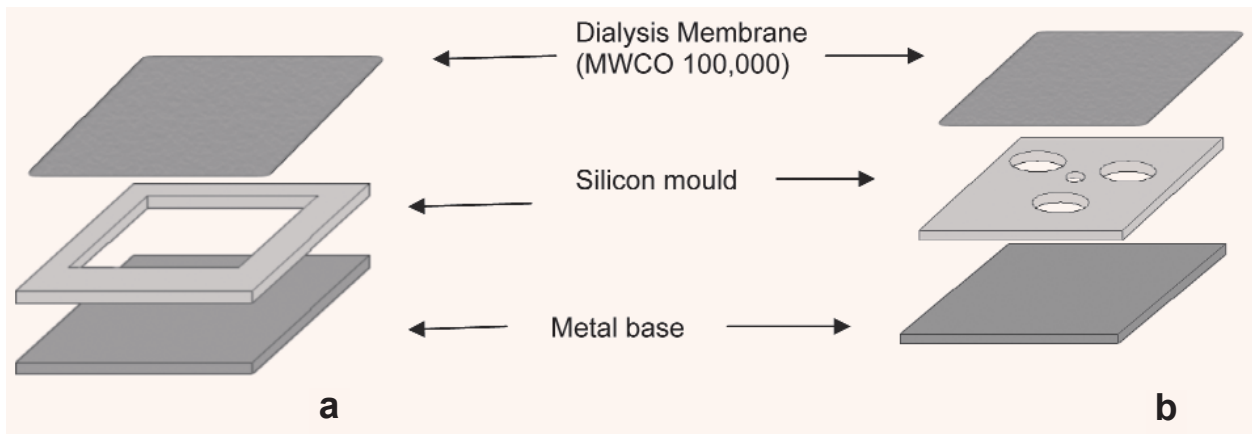
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Fig. 4 Overview of mould apparatus. The silicon part can be prepared in a number of shapes. Demonstrated here are a 2 cm x 2 cm square (a) or 3 x 5 mm diameter disks (b).

requirement of producing more *de novo* tissue than start material used implies that a monolayer step is a likely requirement. As previous studies have demonstrated that TGFβ1 and FGF2 in monolayer are highly beneficial [24], these factors were used during all monolayer steps. In order to maximally stress the cells, a portion was discarded at every stage. This was done to provide a "worst case" scenario. It is likely that for use in an actual therapy in human patients much fewer cell divisions will be required. The fact that a cartilage-like tissue can be

generated after so many population doublings indicates that a degree of safety is built into the system when the combination of factors described is used.

A total of 4.6×10^6 cells (estimated using the value 7.7 pg DNA per bovine chondrocyte [25]) were used to produce an implant of $2.25 \text{ cm}^2 \times 0.1$ cm (0.225 cm^3). This equates to 20.78×10^6 cells per g wet weight. This is greater than that in native human knee cartilage [26] where 4.6×10^6 chondrons per cm^3 were seen, but less than the cell density found in young bovine cartilage [27, 28].
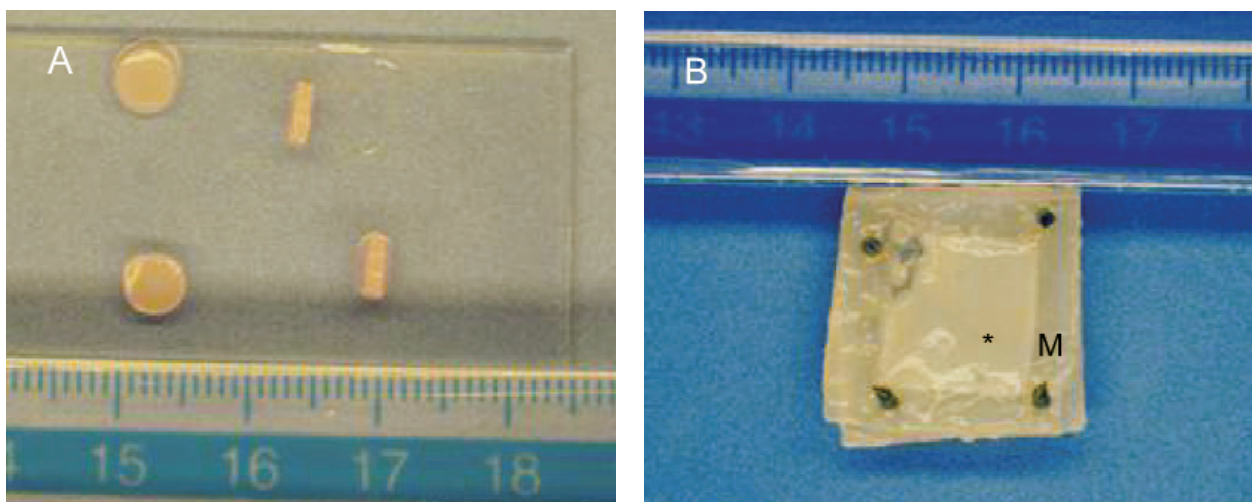

Fig. 5 Scaffold free cartilage-like material produced from passage 2 monolayer expanded cells (up to 14.75 population doublings). Cells were cultured for 14 days in alginate followed by 14 days (A) or 31 days (B) in the silicon mould. Implants (5 mm in diameter) are shown after release from the silicon mould (A). Larger implants can be produced by varying the mould (B). The 2.25 cm^2 implant (*) is still within the silicon mould (M).
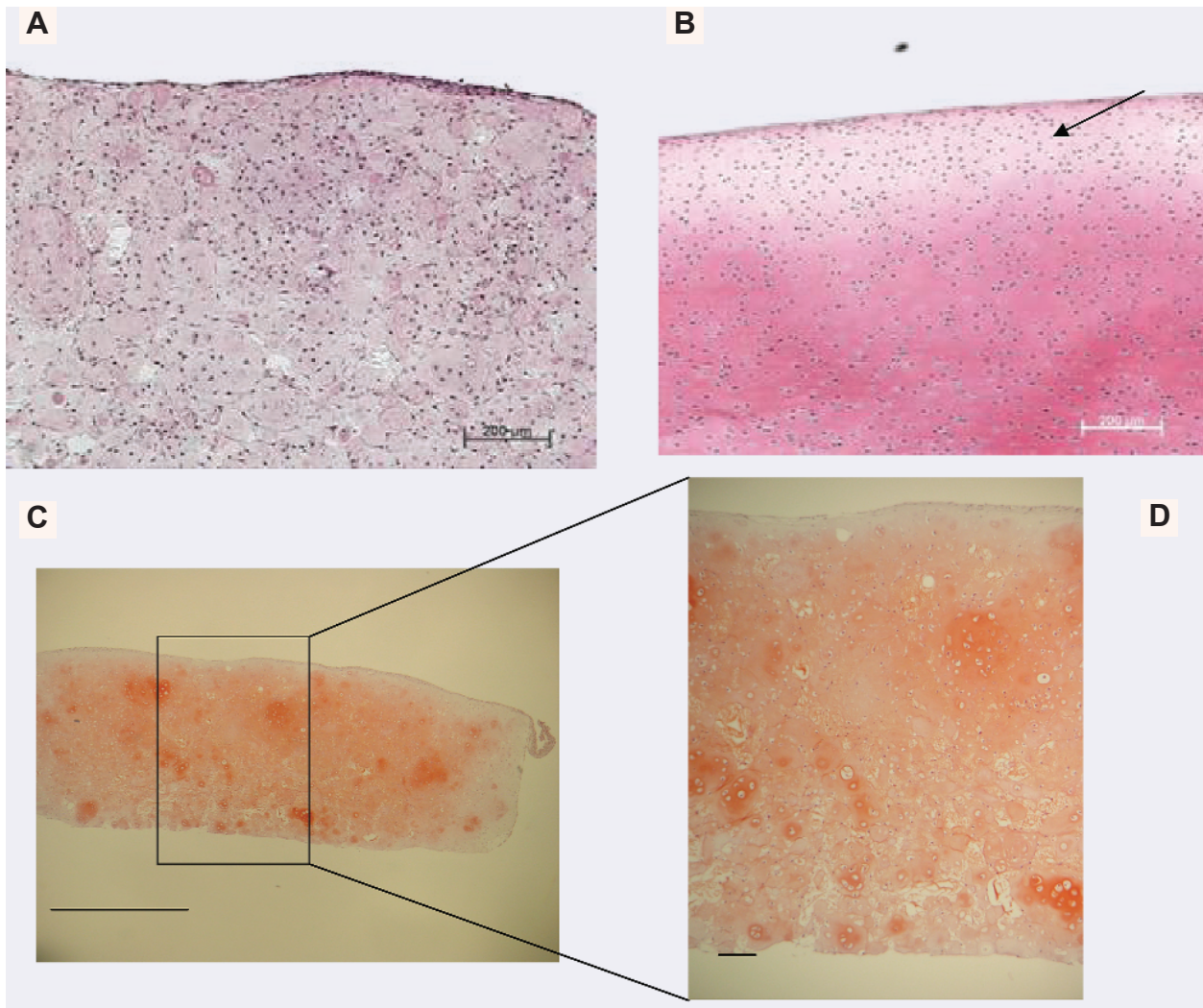
Fig. 6 Chondrocytes were monolayer passaged twice (14.28 population doublings) and then cultured in alginate beads for 14 days before being released from the alginate and cultured in a 5mm x 1 mm circular silicon mould for a further 14 days. Staining with haematoxylin and eosin demonstrated a number of nucleated cells separated with lightly staining matrix (**A**). The intensity of staining was similar to that of the transitional zone of bovine cartilage (**B**, arrowhead) and lighter than that of deep zone cartilage. The implant was also stained with Safranin O for GAG (**C**, **D**). Intensity of staining varies within the implant with a number of areas staining intensely (y), areas that stain less intensely (x) and areas that did not stain (*).

After the initial monolayer expansion phase the de-differentiated cells were embedded in alginate to stimulate the re-expression of matrix proteins associated with the chondrocytic phenotype [2, 3]. Omitting this step leads to the formation of a dense cell pellet which did not resemble the original dimensions of the implant within the silicon mould (Fig. 8). Although the resultant tissue was chondrogenic, the uncontrolled shrinkage made it impossible to design an implant of pre-determined dimen-

sions. This would suggest that the alginate culture step is crucial in the production of scaffold free *de-novo* cartilage of defined shape. It is likely that the accumulation of matrix is an important part of the alginate step and therefore we investigated the effects of factors during this stage.

The combination of TGFβ1 and dexamethasone is commonly used to induce mesenchymal stem cells (MSC's) to become chondrocytes [29–32]. As it has been shown that heavily expanded chondro-
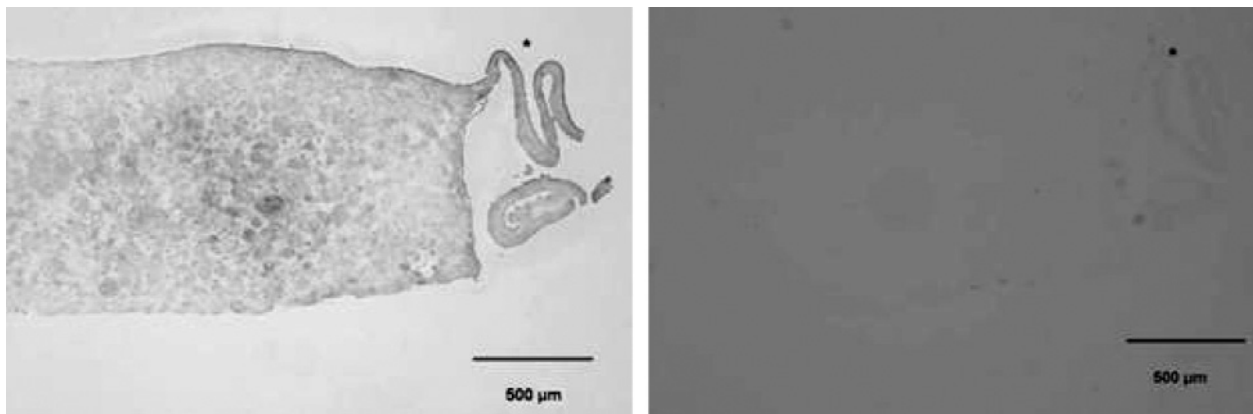
Fig. 7 Immunostaining demonstrating the presence of collagen II in implants cultured with control medium containing IGF1 both in bead and in implant culture (**A**). Negative control of the serial section (**B**) shows a light staining of the thin overlaying fibrous layer (*). As demonstrated with the Safranin O staining the intensity varies within the implant. The more central areas appear to stain with a greater intensity.

cytes have a phenotypic plasticity [33], we investigated whether the addition of TGFβ1 and dexamethasone during the early stages of alginate culture would enhance the re-differentiation of the expanded chondrocytes. Initial seven day incubation with TGFβ1 and dexamethasone led to an increased S^{35} incorporation when followed by seven days exposure to IGF1 (Gr. 4), compared to IGF1 treatment alone (Gr. 2) (111156 compared with 77384 counts/hr/µg DNA, P<0.001, n=6). IGF1 treatment alone (Gr. 2) enhanced S^{35} incorporation over control (77384 compared with 43456 counts/hr/µg DNA, P<0.001, n=6). The increased ^{35}S incorporation induced by IGF1 can be seen in the GAG accumulated within the cell matrix, as indicated by increased GAG/DNA (Fig. 2b). By day 28 there was little difference in the GAG/DNA content of groups pre-treated with TGFβ1 and DEXA over those that were not. Therefore the combination of these two factors did not appear to have any major effects on monolayer expanded bovine chondrocytes after 28 days of culture under the conditions described. The presence of unknown factors in serum may have mediated any response within the alginate culture.

On encapsulation in alginate, the cells adopt a three dimensional rounded phenotype normally associated with the chondrocyte. With time it has been shown that a return to an expression pattern associated with cartilage is also achieved [2–4, 34]. Following encapsulation, treatment with IGF1 (Gr.

2 and Gr. 4) further stabilized the cells and led to increased matrix synthesis when compared with culture not exposed to IGF1 (Gr. 1) (increased by 25–50% over controls depending on time in culture, P < 0.001, Fig. 2b). Alginate has the advantage that its mesh like structure helps retain matrix products within the bead. Up to 98% of newly synthesized ^{35}S-labelled GAG's are retained within the alginate bead, which compares favorably with agarose where only 88% are retained [18]. The porous nature of scaffolds, required to allow seeding with cells, has the added drawback that a large proportion of matrix molecules are then released into the medium. Studies have shown that up to 78% of newly synthesized proteoglycans are released into the medium [35].

Chondrocytes cultured in alginate produce matrix that is either cell associated (*i.e.* mechanically attached to the cell), which remains with the cells when they are released, or further removed matrix which is released into solution when the alginate is dissolved as it is not attached to the cell in a mechanically stable fashion [18]. Fig. 1 demonstrates the darkly staining cell associated matrix surrounded by the lighter staining further removed matrix (Y). This pattern of staining is also seen within the final implant (Fig. 6c and d). As the cells are pelleted after release, the proportion of matrix incorporated into the cell associated matrix is critical for this method. It has been demonstrated that IGF1 increases the proportion of matrix that is cell associated [36], there-
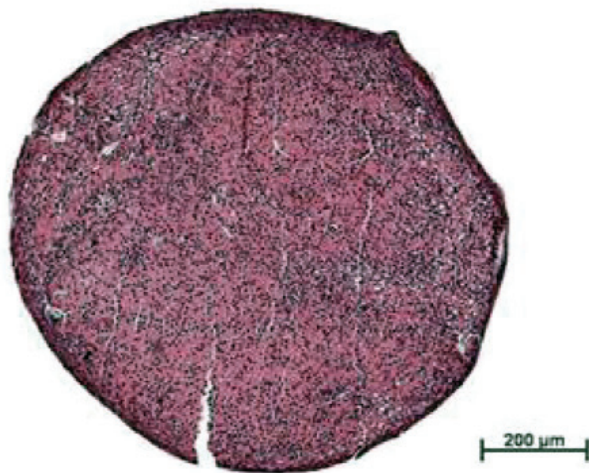
Fig. 8 Bovine chondrocytes were monolayer passaged twice and then cultured in a 5mm x 1 mm silicon mould for a further 14 days without an alginate step. The cells rapidly contracted leading to a shrinkage of the material within the mould. H&E staining of the resultant tissue shows a tightly packed cell pellet which is predominantly cellular material. The diameter of the pellet is approximately 800 µm which is much smaller than the 5 mm wide mould used.

fore providing added benefit to alginate released cells. Time spent in alginate not only helps to stabilize the cells by enhancing re-differentiation but the matrix laid down also acts as a spacer when the cells are finally brought together in a scaffold free environment. As the density of cells is greater than that found in normal cartilage [26] there is a potential for growth providing the cells remain viable.

Of all the described combinations of factors tested within the alginate bead culture, control medium containing 50 ng/ml IGF1 resulted in the greatest accumulation of GAG's. For this reason this media was used to further culture the implants. Safranin O staining for GAG's after 14 days of culture demonstrated areas of the implant that stained darkly for GAG while other areas were not as intensely stained (Fig. 6c). The same pattern was observed when immunostaining for collagen II (Fig. 7). This would suggest that a portion of cells re-differentiate and a portion does not, or the speed of re-differentiation varies for different cell populations. It is interesting to note that the central portion of the implant stained most heavily for GAG and collagen

II. Whether this is a result of cellular signaling, nutrition or oxygen gradient, or some other factor is as yet unclear.

Due to the varying nature of repair tissues produced by the different cartilage repair methodologies, long term animal studies are required to determine the robustness of the repair. Initial studies involving scaffold free tissue produced from monolayer expanded ovine chondrocytes has also been produced and tested within a sheep animal model. Initial results indicate that the repair tissue integrates well and produced a superior repair to periost flap alone (Personal communication Axel Jubel, Cologne).

The ability to recover cells grown in alginate is well documented [3, 14–16, 18, 36, 37] and yet the use of alginate culture in tissue engineering is uncommon. The implantation of a mix of cells plus alginate has been described [38–40] but this may lead to unwanted immunological and foreign body responses, at least in a minipig animal model (Personal communication Karola Messner and Ernst Hunziker). The use of alginate recovered chondrocytes (ARC) has been described using primary [14, 15] and monolayer expanded cells [16]. With the ARC method chondrocytes released from alginate are seeded onto Millipore inserts and further cultured until a solid consistency is achieved. We have taken this concept further by the use of factors to decrease the monolayer expansion time [24] and to increase the level of matrix produced during the bead stage (Fig. 2b). More importantly, we have also used custom built moulds that enable the shape of the resultant implant to be by design, enabling more complex shapes to be developed. A further advantage of scaffold free constructs is the lack of any complications caused by the structure or composition of the scaffold itself. One issue in the production of scaffold free implants that requires further work is the time required in culture to generate enough matrix. As this method needs substantial ECM there must be a period of culture between harvesting the monolayer expanded cells and implantation. The use of scaffolds does not require this step as cells can be seeded within the scaffold and implanted immediately, although protocols involving scaffolds often include a period of culture between monolayer expansion and implantation.

We believe therefore that alginate recovered cells provide a viable alternative to scaffolds in the production of three dimensional cartilage-like implants.

Acknowledgements

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