Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle

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Summary

Objective: Adult articular cartilage shows a limited intrinsic repair response to traumatic injury. To regenerate damaged cartilage, cell-assisted repair is thus viewed as a promising therapy, despite being limited by the lack of a suitable technique to deliver and retain chondrogenic cells at the defect site.

Design: We have developed a cytocompatible chitosan solution that is space-filling, gels within minutes, and adheres to cartilage and bone in situ. This unique combination of properties suggested significant potential for its use as an arthroscopically injectable vehicle for cell-assisted cartilage repair. The primary goal of this study was to assess the ability of this polymer system, when loaded with primary articular chondrocytes, to support cartilage formation in vitro and in vivo. The chitosan gel was cultured in vitro, with and without chondrocytes, as well as injected subcutaneously in nude mice to form subcutaneous dorsal implants. In vitro and in vivo constructs were collectively analyzed histologically, for chondrocyte mRNA and protein expression, for biochemical levels of glycosaminoglycan, collagen, and DNA, and for mechanical properties.

Results: Resulting tissue constructs revealed histochemical, biochemical and mechanical properties comparable to those observed in vitro for primary chondrocytes cultured in 2% agarose. Moreover, the gel was retained after injection into a surgically prepared, rabbit full-thickness chondral defect after 1 day in vivo, and in rabbit osteochondral defects, up to 1 week.

Conclusions: The in situ-gelling chitosan solution described here can support in vitro and in vivo accumulation of cartilage matrix by primary chondrocytes, while persisting in osteochondral defects at least 1 week in vivo.

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Key words: Tissue engineering, Cartilage, Chitosan, Hydrogel, Implants, Rabbit model.

Introduction

Developments in therapeutic strategies for cartilage repair have increasingly focused on the promising technology of cell-assisted repair (reviewed in 1,2), proposing to use autologous chondrocytes or other cell types to regenerate articular cartilage in situ. The method of cell implantation within the articular joint poses special challenges, including strong shear forces and mechanical loads, a fluidic environment, and a graft site composed of stiff, irregular, and often bleeding surfaces not easily amenable to surgical attachment of tissue flaps or insertion of biomaterials. Moreover, biomaterials to be used as cell-delivery vehicles must also meet stringent biocompatibility requirements in that the material should be non-toxic, non-immunogenic, and biodegrade without generating toxic by-products or debris that could subsequently injure the joint. The biomaterial must either integrate in a mechanically and physiologically harmonious manner with the repair tissue, or degrade without leaving gaps or fissures in the tissue. Thus, one function of a biomaterial acting as a cell carrier for cartilage repair is to serve as a temporary scaffolding that permits the accumulation of extracellular matrix by delivered cells, while leading to congruent attachment with the perimeter of the cartilage defect and the subchondral bone.

In Autologous Cell Implantation (ACI), a currently practiced cell-based therapy to repair cartilage defects, autologous chondrocytes are recovered from the patient but are considered too sparse for direct re-implantation 3. To overcome cell scarcity, chondrocytes are amplified in tissue culture prior to re-implantation 4,5. After one in vitro passage (two cell doublings), chondrocytes can still form some hyaline cartilage as a nude mouse implant 6–7. However,
chondrocytes are typically propagated in monolayer by three to four population doublings for human ACI therapy. After three to four doublings, chondrocytes can no longer produce cartilage matrix in the nude mouse implant model using canine6, pig7, or human chondrocytes 8. Therefore, in tissue-engineered constructs, the greatest biochemical accumulation of cartilage matrix has been typically demonstrated using primary chondrocytes.

A wide array of cytotolerant matrices or hydrogel carriers have been seeded with articular chondrocytes in order to regenerate cartilage matrix *in vitro*, including scaffolds made of proteins (collagen, fibrin), polysaccharides (agarose, algin, hyaluronic acid, chitosan), and polymers (polyethylene glycol, polylactic acid) (reviewed in9). A quantitative *in vitro* accumulation of cartilage matrix has been demonstrated biochemically for chondrocytes cultured in collagen10,11, agarose12, poly-glycolic acid13, algin14, fibrin glue15, polyethylene glycol dimethacrylate16, and self-assembling peptides17. Most of these matrices also support neocartilage matrix deposition *in vivo* when placed as implants into nude mice, and include poly-glycolic acid17, algin18,19, fibrin glue5, and polyethylene oxide20,22.

Since primary chondrocytes appear to retain function and phenotype in a multitude of cell carriers *in vitro* and *in vivo*, a major obstacle in cell-assisted cartilage repair therapies does not appear to be the generation of a chondrocyte-compatible matrix, but rather the delivery and retention of the cell-delivery vehicle within an articular defect. Although semi-solid or solid matrices may reside in specifically tailored animal defects, by press-fitting23,24,25, suturing or tacking26,27,28, or by using biologic or synthetic glues29,30, such pre-formed implants cannot be designed to adequately fit the precise shape and form of a naturally occurring defect, and typically lead to repair tissue with poor attachment and poor integration29,31. By another approach, cells have been delivered underneath a periosteal patch sutured over the defect24,25,6 with resulting damage to the joint defects in living animals. Three to four population doublings for human ACI therapy 3. Chondrocytes are typically propagated in monolayer by three to four population doublings for human ACI therapy. After three to four doublings, chondrocytes can no longer produce cartilage matrix in the nude mouse implant model using canine6, pig7, or human chondrocytes 8. Therefore, in tissue-engineered constructs, the greatest biochemical accumulation of cartilage matrix has been typically demonstrated using primary chondrocytes.

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**Institutional Animal Care and Use Committee (University**

**Materials and methods**

**INJECTABLE HYDROGEL-FORMING CHITOSAN SOLUTIONS**

Chitosan was obtained either from BioSyntech (Laval, QC, Canada), with a 90% DDA, free base form, average molecular weight 216 kDa by GPC before autoclave, or from Pronova (Oslo, Norway, Product No UP213 84% DDA, 16% HCl, 1% solution viscosity 143 mPa·s). BioSyntech chitosan was dissolved in 88 mM HCl (Sigma, Tissue Culture grade) at 1.8% (w/v) and autoclave sterilized. Protasan UP213 powder was sterilized by exposure to UV light for 2 h in a cell culture laminar flow hood, and then dissolved in sterile water at 1.7% (w/v) chitosan (Pronova UP213, batch No. 607-783-02, taking into account HCl content and loss on drying). These chitosan—HCl solutions were individually mixed with concentrated stock solutions of filter-sterilized disodium β-glycerophosphate (GP, Sigma, Tissue Culture grade, St. Louis, MO), and glucosamine (Sigma, Tissue Culture grade), to yield a liquid chitosan—GP—glucosamine solution with 1.5% (Pronova) or 1.6% (BioSyntech) chitosan, 0.75 mM disodium β-glycerophosphate, 18 mM glucosamine, final pH 6.8 (Pronova chitosan) or pH 6.7 (BioSyntech chitosan). Chitosan gels were formed by mixing 10% chitosan—GP—glucosamine solution with 2.5 mL of a cross-linking solution consisting of 2.5% (w/v) in Dulbecco’s Modified Eagle’s Medium (DMEM) filter-sterilized hydroxyethyl cellulose (Fluka, Ulm, Switzerland, medium viscosity), and incubating at 37°C.

**CHONDRocyte ISOLATION, ENCAPSULATION, HYDROGEL CULTURE INJECTION IN NUDE MICE**

Primary calf chondrocytes were isolated by enzymatic digestion of femoral-patellar groove cartilage of two, 2-week post-natal calf knees within 6 h of sacrifice, as described. A pellet containing 130 million freshly isolated primary chondrocytes (~0.75 ml packed volume) was resuspended with 2 ml filtered hydroxyethyl cellulose (25 mg/ml) and mixed with 10.5 ml of 4°C chitosan—GP—glucosamine to generate a solution of 10 million cells/ml which was poured into 100 mm culture dishes and permitted to gel for 20 min in a humidified, 37°C, 5% CO2 incubator for four 20-min washes with DMEM. Chondrocytes were also cast in 100 mm culture dishes at the same cell density in a 2%, low-melting point agarose gel (SeaPlaque, FMC Bioproducts, Rockland, MA) according to Buschmann et al. Control gels (chitosan and agarose) were cast without cells. A biopsy punch was used to core 6-mm-diameter and 1.0 mm (chitosan) to 1.5 mm (agarose) thick discs from the gel slabs. Discs were cultured in individual wells of 48-well plates on top of washed, sterile 1000 μm nylon mesh grids ( Spectrum, Laguna Hills, CA), in 0.5—1.0 ml of complete media [DMEM Sigma D5648; non-essential amino acids Sigma, M2025; 0.4 mM proline Sigma, P4655; an additional 2 mM glutamine Sigma, G5763; 100 U/ml PenStrep Sigma P0728; 10% Fetal Bovine Serum Sigma, and 50 μg/ml fresh ascorbic acid Sigma, 134-03-2], replaced daily. Gel constructs without cells were carried through identical culture conditions as constructs with cells, including daily fresh media changes. Culture dishes were shaken slowly on an orbital platform in humidified 5% CO2, at 37°C. Cell viability was determined using a calcein AM/ethidium-homodimer-1 fluorescent assay.

**NUDE MOUSE IMPLANTS**

All procedures and protocols were approved by an Institutional Animal Care and Use Committee (University
of Montreal, QC). A pellet containing 65 million or 130 million freshly isolated primary chondrocytes was resuspended with 1 ml filtered hydroxyethyl cellulose (25 mg/ml) and mixed with 5.0 ml of 4°C chitosan-—GP—glucosamine to generate a solution of 10 million cells/ml (3 week implants) or 20 million cells/ml (7 or 9 week implants) which was injected within 5 min of mixing. A 1 cc syringe and 26 g ½ inch needle was used to generate one 400 µl implant with (n = 2) or without cells (n = 2) in the subcutaneous dorsum of nude mice (25 g male CD1-Crl nu/nu, Charles River, St Constant, QC) and allowed to develop for 3 weeks. In other nude mice, four, 100 µl implants were injected in the four corners of the mouse dorsum either with (n = 5) or without cells (n = 5) and allowed to develop for 7 or 9 weeks. Individual implants were recovered, dissected from the capsule, and divided into portions that were either fixed, or frozen in liquid nitrogen, or cored with a 3 mm biopsy punch for multiple analyses (histology, biochemistry, RNAse protection, mechanical).

DETERMINATION OF COLLAGEN, GLYCOSAMINOGLYCAN, AND DNA CONTENT

Individual 40–60 mg cultured discs, or 5–25 mg implant samples, or fresh bovine articular cartilage explants were frozen in liquid nitrogen, and stored at −80°C. Samples were either extracted in guanidine salts [4 M guanidine HCl/50 mM Tris pH 7.2 (GuCl), followed by 4 M guanidine isothiocyanate/50 mM Tris pH 7.2 (GITC)], as previously described, or digested in 1 ml papain at pH 7.5 in a manner similar to Kim et al. Total collagen content was determined by hydroxyproline assay (conversion factor of 7.6 mg collagen/mg hydroxyproline). GuCl extracts and papain digests were submitted directly to the DMMB assay to determine glycosaminoglycan (GAG) content, and to the Hoechst 33258 DNA assay to estimate cell density (conversion factor of 7.7 pg DNA/cell).

RNA ANALYSIS BY RNASE PROTECTION ASSAY

4 M GuCl/50 mM Tris pH 7.2 and 4 M GITC/50 mM Tris pH 7.2 extracts were combined in equal proportions and 20 µl was submitted to direct mRNAse protection assay with a commercial kit (Ambion, Austin, TX) using bovine-specific RNAse protection probes for collagen type II, aggrecan, and glyceraldehyde phosphate dehydrogenase (GAPDH), as described previously. Bovine tendon and kidney guanidine extracts were used as controls.

PROTEIN ANALYSIS BY WESTERN BLOTTING

Combined GuCl/GITC extracts were precipitated with five volumes ethanol, and submitted to 7.5% acrylamide, SDS-PAGE followed by parallel Western blot analyses using monoclonal anti-vimentin V9 (Sigma, 1:5000 dilution), monoclonal anti-Proliferating Cell Nuclear Antigen (anti-PCNA) (Sigma, 1:2000), polyclonal anti-C-propeptide of type II collagen (CP2) (R160, gift of R. Poole, Shriners Hospital, Montreal, Canada, 1:10,000), monoclonal anti-link protein (polyclonal antisera R110 or R295, respectively, gifts of A. Robin Poole, Shriners’ Hospital, Montreal, QC, Canada). Aggrecan and link protein antibodies were diluted at 1:100 and 1:200, respectively, with 5% normal goat serum containing 0.2% bovine serum albumin in TBST buffer, and then incubated with secondary antibody (biotinylated goat anti-rabbit IgG; Caltag Laboratories, Burlingame, CA) diluted 1:200 with 0.2% bovine serum albumin in TBST for 45 min at room temperature. After additional washing with TBST, sections were further treated with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) for 45 min at room temperature. Color development was achieved with an 8-min treatment of Fast Red TR/Naphthol AS-MX phosphate (Sigma) containing 1 mM Levamisole as an inhibitor of endogenous alkaline phosphatase. After washing with distilled water, sections were counterstained with methyl green for 2 min, washed with distilled water and mounted under coverslips using Kaiser’s glycerol jelly. Negative controls consisted of the same treatment with the omission of the primary antibody in the first incubation.

BIOMECHANICAL PROPERTIES

Biomechanical properties of 3-mm- to 6-mm-diameter discs were assessed via stress-relaxation tests in uniaxial, unconfined compression using the Mach-1™ micromechanical testing system (BioSyntech, Laval, QC). Sample diameter and thickness were determined using a micrometer. For gels cultured in vitro, two successive compression ramps each with an amplitude corresponding to 10% of the sample thickness were applied at a strain rate of 1%/s. For all samples, equilibrium was estimated when the load changed by less than 0.05 g/min. Chitosan in vivo implants were submitted to a sequence of compression ramps of 2.5% of the sample thickness, at a strain rate of 0.25%/s. Stress relaxation curves from the second ramp (10–20% strain) of in vitro discs and the eighth ramp of the in vivo implants (17.5–20% strain) were fit to a fibril reinforced poroelastic model to obtain the compressive equilibrium modulus (Ee), tensile fibril modulus (Et) and hydraulic permeability (k).

RABBIT FEMOROPATELLAR ARTICULAR DEFECTS

New Zealand White rabbits (n = 6), 4–7 months old (Charles River), were anesthetized by intramuscular...
injection of ketamine/xylazine/buprenorphine, followed by isoflurane gas anesthesia. A medial parapatellar incision exposed the joint, followed by joint capsule incision, and patellar displacement. Exposed cartilage was irrigated with Phosphate Buffered Saline (PBS). All animals received bilateral arthrotomies to generate articular cartilage defects on the patellar (n = 1) or trochlear surfaces (n = 5). One animal received a full thickness (~0.3 mm deep) 4 × 3 mm chondral-only defect on the patellar surface of the left knee, and a 3 mm deep osteochondral patellar defect in the right knee and was allowed to heal for 1 day. Another animal received chondral-only defects on left and right trochlear surfaces with a microsurgical knife and was allowed to heal for 1 day. In other animals (n = 4), 3–5 mm deep bilateral osteochondral defects were created in the trochlea by removing the cartilage with a scalpel, then using a hand-held high-speed drill and a 3 mm diameter drill bit with constant 4 °C PBS irrigation to avoid thermal necrosis. Sterile liquid chitosan gel without cells was loaded into both patellar defects, or into one of the trochlear defects using a 1 cc syringe and 20 g 1 inch needle to assess gel retention and residency. In contrast with one injection in chondral-only defects, liquid chitosan implant was re-injected a second or third time into all four trochlear osteochondral defects before defect hemostasis was observed. In situ gelation and the arrest of bleeding occurred after a 5–10 min delay, after which the patella was replaced and the capsule, muscle, and skin were sequentially closed with 5-0 black braided silk (Harvard Apparatus, Holliston, MA). Bilateral osteochondral trochlear defects were allowed to heal for 1 day (n = 1) or 1 week (n = 3).

STATISTICAL ANALYSES

Quantitative biochemical (cell density, GAG concentration, collagen concentration) and biomechanical (E_m, E_i, k) outcomes were taken as dependent variables while scaffold type (chitosan vs agarose) and day of culture were independent variables (predictors). Multivariate analysis of variance (MANOVA) using the GLM routines of Statistica (Statsoft, Tulsa, OK) was used to assess the significance (P < 0.05) of the main effects (scaffold, day of culture) with and without their interaction on dependent outcomes. Contrast analysis for planned comparisons was then used to determine the significance (P < 0.05) of main effects on specific outcomes.

Results

IN VITRO CULTURES

During a 3-week culture period in vitro, primary calf chondrocytes produced comparable amounts of de novo cartilage matrix in the chitosan and agarose gels, as assessed by histological, molecular, and biochemical criteria. Chondrocytes remained >90% viable in the solid chitosan matrix immediately post-casting [Fig. 1(A)], and throughout the 21-day, in vitro culture period [Fig. 1(B)]. Cells cultured in chitosan or agarose accumulated pericellular sulfated GAG-containing matrix, as measured by toluidine blue staining [Fig. 1(D,G)], in addition to pericellular aggrecan (not shown) and pericellular link protein [Fig. 1(E)], aggrecan mRNA [Fig. 2(B)], and extracted link protein expression [Fig. 2(A)]. Total GAG concentration was lower (P < 0.05) in chitosan (11.3 μg/mg at day 20) than in agarose (15.6 μg/mg at day 20) while collagen concentrations were similar (Table I). No evidence of mineralization was seen by von Kossa staining (data not shown). Chitosan scaffold was still present after 21 days in culture [Fig. 1(D), open arrow]. The histological appearance of chitosan gel cultured from 1 to 14 days without cells was similar to that of chitosan gel with cells at day 1 [Fig. 1(C), open arrow], indicating that the gel did not biodegrade during 3 weeks in culture. Chondrocytes in chitosan cultures or in implants produced type II collagen, as reflected by C-propeptide II expression [Fig. 2(A)], and abundant type II mRNA expression [Fig. 2(B)]. Of note, calf chondrocytes cultured in chitosan expressed only one CP2 isoform, as did fresh adult articular cartilage explants, whereas calf chondrocytes in agarose and calf explants expressed two CP2 isoforms (Fig. 2).

Type I collagen mRNA expression was detected by Reverse Transcriptase-Polymerase Chain Reaction in both agarose and chitosan gels at 21 days (data not shown). Cells were observed to divide during the 3-week cultures as determined by quantification of absolute DNA content (Table I). Cell density in chitosan cultures doubled at 14 days and then tapered off to a level that was significantly higher (P < 0.05) relative to agarose cultures where cell density linearly increased by about half over 3 weeks. As the hydrogel disc wet mass did not decrease significantly during the culture (Table I), the apparent increase in DNA content was not caused by gel shrinkage, but may have been partly due to a greater proliferation of cells that had a fibroblast shape on the surface of the chitosan discs [Fig. 1(G, H)]. Chondrocytes cultured in chitosan or agarose expressed PCNA, a cell cycle-associated protein [Fig. 2(A)]. PCNA was detected in 2-week calf articular cartilage, but not in adult cartilage [Fig. 2(A)].

After 3 weeks in culture, an age-dependent increase in stiffness and decrease in hydraulic permeability (or pore size) was observed for both chitosan and agarose discs (Table I), which could be attributed to the accumulation of GAG and collagen in the hydrogel pore spaces. Statistical analysis showed significant (P < 0.05) time- and scaffold-dependent changes for all mechanical properties of in vitro samples. Although the final mechanical properties were superior for cells grown in agarose compared to chitosan (Table I), it must be noted that the initial 2% agarose hydrogel was much stiffer (~5×) and less permeable (~30×) than 1.3% chitosan gel (Table I). By comparison, the fold-increase in mechanical properties over time was always higher for chitosan, compared to agarose. For instance, during 15 days in culture, the equilibrium modulus of gels with chondrocytes increased by 9.2-fold for chitosan (days 4–19), and 4.5-fold for agarose (days 5–20, Table I).

NUDE MOUSE IMPLANTS

Chondrocytes were encapsulated in liquid chitosan/hydroxyethyl cellulose and injected subcutaneously in five different nude mice, where implants were allowed to develop for 3, 6, or 9 weeks in vivo (Fig. 3, Table I). Additional mice received chitosan implants without cells. Solely the implants with chondrocytes acquired a pearly white appearance of hyaline cartilage, inside of which chondrocytes were surrounded by a variable ring of pericellular GAG (Fig. 3). A fibrous capsule containing fibroblasts, neutrophils and blood vessels formed around all implants. Murine neutrophils, blood vessels, and capsular fibroblasts were seen to grow into the margins of implants with no cells [Fig. 3(G)], while implants with chondrocytes showed reduced cellular
Fig. 1. Histology of chondrocytes cultured in chitosan- (A, B, C, D, E, H) or agarose- (F, G, I) based hydrogels. Viability of chondrocytes freshly encapsulated (A) and cultured for 20 days (B) was assessed with fluorescent calcein AM (green live cells) and ethidium homodimer 1 (red nuclei, dead cells). Toluidine blue-stained, LR White semithin sections are from day 0, immediately post-casting (C, F), and from day 20 of culture (D, G) showing cell nuclei (arrows), GAG-rich extracellular matrix (arrowheads), and chitosan (open arrow). Immunohistochemistry with anti-link protein antibody shows accumulation of pericellular link protein, an abundant cartilage extracellular matrix protein (E). Fibroblastic cells grew in a variable layer on the surface of in vitro constructs (H, I). Scale bars = 50 μm.
infiltration limited to the periphery [Fig. 3(F)]. Interestingly, an ~100 μm wide zone immediately adjacent to the capsule was devoid of chondrocytes [Fig. 3(F)]. Chitosan scaffold was still visible in semithin plastic sections after 7–9 weeks [Fig. 3(C,E), open arrow]. Compared to day 19 in vitro chitosan cultured samples, in vivo implants with chondrocytes had slightly lower average GAG content, but higher equilibrium stiffness, comparable fibril modulus, and reduced permeability (Table I). Implants with no cells were friable and unsuitable for mechanical testing. As seen previously in several studies, mechanical properties of these in vitro and in vivo constructs remain significantly inferior to those of native tissue (P < 0.05, Table I).

**LIVE RABBIT ARTICULAR DEFECT RESIDENCY**

When polymerized within ex vivo cartilage defects in pig or rabbit joints, the chitosan gel adhered strongly to bone and cartilage after articulation (unpublished results). To test the manner in which the in situ-gelling chitosan implant would reside in live articular defects, the chitosan cell carrier solution without cells was loaded into an osteochondral defect, or into a shallow, but full-thickness chondral defect in living rabbit joints. The gel was delivered to a 3-mm-diameter, 3- to 5-mm-deep, bleeding defect in the femoral-patellar groove of four, 4-month male rabbits, and permitted to reside there for 1 day (n = 1), or 7–8 days (n = 3) in vivo. The contralateral knee received an identical defect with no implant. The gel was also delivered to an osteochondral patellar defect (n = 1) and allowed to heal for 1 day. At the time of surgery, excessive bleeding from the drill hole in several joints washed the liquid chitosan out of the defect, requiring a second or third injection before the gel was able to visibly solidify in the bleeding defect. The chitosan liquid appeared to be mildly hemostatic, in support of previous observations. No effort was made to immobilize the joint post-surgery.

At 1 day post-surgery, control defects contained a blood clot packed with red blood cells [Fig. 4(A,C)], while defects with chitosan gel at 1 day contained variably shaped implant with blood clot covering the surface and also mingling with the base of the implant [Fig. 4(B,D)]. At 1 week post-surgery, the control defect contained a red tissue [Fig. 4(E)] which consisted in highly vascularized granulation tissue [Fig. 4(G,I,K)]. At 1 week post-implantation, all three defects with a chitosan gel implant were filled with an off-white tissue [Fig. 4(F)] that contained variable amounts of chitosan gel [Fig. 4(H)] covered with a layer of fibrin.
Table I

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<th>Agarose NC</th>
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<td>GAG (µg/mg tissue; n = 8)</td>
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Data represented as mean ± SD for all samples n ≥ 3. Range of values for samples with n = 2: a1) 5.5–8.6; a2) 198–243; a3) 113–116; b1) 2.7–3.0; b2) 171–188; b3) 154–177; c1) 1.4–1.5; c2) 8486–9430; c3) 77–93; d1) 1.4–1.6; d2) 12,452–15,508; d3) 40–46; e1) 4.3–4.6. Symbols: – = not done; NC = no cells. The same samples were analyzed in Figs. 2 and 3; * from Ref.11, ** from Ref.36 except that collagen:hydroxyproline conversion factor = 7.6, instead of 8.3; † from Ref.11 (Table 2); ‡ from Ref.53 (Fig. 3). All biochemical values given per wet weight.

[Fig. 4(N)]. At 1 week, some cellular infiltration into the chitosan gel was observed [Fig. 4(L), black arrowhead]. A thick granular repair tissue containing cell debris, neutrophils, and fibroblasts surrounded the chitosan implant [Fig. 4(J,L,M)]. A loose vascular tissue was detected peripheral to the granulation tissue [Fig. 4(M)]. In contrast with the subcutaneous chitosan implants, a fibrous capsule was absent. Histology of osteochondral defects allowed to heal for up to 1 month (data not shown) suggested that the cross-linked chitosan gel implant inhibited new bone formation.

Chitosan gel was also injected into shallow, cartilage-only defects. In vivo retention of the chitosan matrix was assessed in a 4 × 3 mm², full-thickness chondral defect of the patella in a 7-month-old male rabbit [Fig. 5(A)]. The depth of such a defect in an adult rabbit is around 0.3 mm. Liquid gel was injected into the defect, allowed to solidify for 5–10 min [Fig. 5(B)], and the knee joint was sutured. The gel was permitted unrestrained motion and load-bearing for 24 h, after which the patella was dissected, and processed for histology. In toluidine blue-stained LR White sections, most of the polymer that filled the defect was retained. The gel was partly adhering to bone, and partly adhering to clotted blood present at the edge of the defect [Fig. 5(C,D)]. Some neutrophils were present on the surface of the chitosan gel (data not shown). Similar chitosan gel
residency in a trochlear chondral-only defect was observed after 1 day of unrestricted load-bearing (data not shown). These results demonstrated the retention of chitosan gel delivered to chondral defects during which time the experimental animal was allowed unrestricted motion and full load-bearing for 1 day.

Discussion

A recently developed, injectable and autogelling chitosan-based cell carrier was studied to determine whether primary chondrocytes can be effectively delivered using this carrier, and whether the carrier can adhere and reside in defects of living animals. From the studies presented here, it was determined that the chitosan self-gelling solution can 1) preserve chondrocyte viability and phenotype after injection and solidification, 2) serve as a scaffolding to help build tissue with mechanical properties, and 3) reside in articular defects in live animal joints for at least 1 week after implantation. These observations suggest the unique potential of this system for cell-based tissue repair.

Chitosan–glycerol phosphate solutions gel by an endothermal mechanism. It has subsequently been discovered that the hydroxethyl cellulose, originally used as a bulking agent, harbors a bifunctional cross-linker that also participates in the gelation mechanism without compromising cell viability (unpublished data, in preparation). Previous
work has shown that chondrocytes can grow on the surface of chitosan films and continue to produce cartilage-specific molecules. Collective results presented here further show that chondrocytes can be homogenously encapsulated at high density within an injectable chitosan hydrogel and develop mechanical properties over a relatively short in vitro culture period. Alginate liquid hydrogel has been previously used to deliver allogeneic chondrocytes to rabbit osteochondral defects, although chitosan’s known blood compatibility, mucoadhesivity, and wound-healing properties may prove beneficial compared to other carriers. To our knowledge, the chitosan–glycerol phosphate hydrogel described here is the only cell-compatible, injectable, in situ-gelling cell-delivery system shown to adhere to and reside within full-thickness, chondral articular defects after load-bearing.

Newborn bovine primary articular chondrocytes in vitro gave rise to comparable though not identical tissues when seeded and cultured in 2% agarose (Table I), in 1.3% chitosan gels (Table I), and in other scaffolding materials when grown under static conditions. Newborn primary chondrocytes appear to have an intrinsic synthetic capability to deposit matrix, with optimal accumulation in agarose (Table I), near 1 ng GAG per chondrocyte after a 2–6 week culture period. Our 3-week chitosan cultures yielded 0.5 ng GAG per chondrocyte at 2 weeks. The higher GAG/cell for agarose could be attributed to its smaller pore size, allowing greater retention of proteoglycan. In addition, a lower GAG/cell for chitosan could have been partly due to the varying presence of a layer of fibroblast-like cells growing on the matrix surface being about two cell layers thick for agarose, vs over five cell layers thick at the eroding chitosan gel surface. The type I collagen mRNA detected in both agarose and chitosan cell-laden constructs may have originated from these fibroblast-like cell layers. Such in vitro de-differentiation at the construct surface has been seen for other biodegradable scaffoldings such as fibrin and PGA. In vitro artifacts of surface proliferation, giving rise to cells that either produce little chondrocyte matrix, or that perhaps secrete catabolic proteases, could thus partially skew the global properties of in vitro constructs. It has been suggested that perfusion or agitation of the cultures could remove these catabolic factors.

Fig. 4. In vivo delivery, retention of chitosan gel, and inflammatory response in example 3 mm deep osteochondral defects in 4-month-old NZW rabbits. Osteochondral holes 3 mm diameter and 3 mm deep were drilled into the femoral-patellar groove (A–N) with one control defect allowed to bleed (A, C, E, G, I, K) and the other defect loaded with chitosan gel (B, D, F, H, J, L–N). After 1 day (A–D) or 8 days (E–N) of normal load-bearing, the joints were recovered and processed for histology. Control defects at 1 day were filled with a blood clot (A, C). Control defects at 8 days were filled with a red (E) vascular granulation tissue (G, I, K), as previously seen. Chitosan gel implant of variable shape (B, arrows) was retained in osteochondral defects at 1 day. Chitosan gel was covered with a blood clot at 1 day (D) and a layer of fibrin at 8 days (N). Defects with chitosan gel at 8 days were filled with an off-white tissue (F). Cell-mediated degradation of the chitosan implant (open arrow in H, J) was seen at the implant-granular tissue interface (L, M). Loose vascular tissue containing neutrophils (M, black arrowhead) was seen adjacent to the granulation tissue surrounding the chitosan. The Growth Plate (Gr Pl) is visible at the base of section (H).
The mechanical properties measured in chondrocyte-seeded chitosan gels could be almost wholly attributed to cell-produced matrix, given the initial weak chitosan gel stiffness, high porosity, and overt loss of mechanical strength by constructs without cells over time (Table I). Although chitosan—cell mechanical properties of the mouse implants appear weaker than those of previous studies\textsuperscript{19,51} it is important to note that these previous studies employed a 2.5 higher cell density, longer culture times (8–30 weeks) and also performed confined rather than unconfined compression, all of which could lead to higher apparent equilibrium moduli. Unconfined compression tests reveal both tensile properties ($E_f$) and compressive properties ($E_m$), while only compressive properties can be obtained from confined compression.

Differences were observed between the inflammatory response to the chitosan gel in subcutaneous implants and osteochondral defects, potentially due to differential vascular involvement. Although the chitosan gel adhered well to \textit{ex vivo} cartilage and bone, \textit{in vivo} subchondral defects bled in such a manner that direct and complete bonding of the gel with bone was prohibited. Moreover, uncontrolled mixing of liquid chitosan with subchondral blood resulted in implants with a variable shape. In contrast, subcutaneous chitosan implants, which induced no bleeding after injection, were enveloped by a fibrous capsule populated by a thin layer of neutrophils. The chitosan implant in osteochondral defects after 1 week was interfaced with a much thicker layer of granulation tissue containing neutrophils, compared to subcutaneous implants, with no fibrous capsule formation. Given that an 100 $\mu$m zone adjacent to the nude mouse capsule was noticeably devoid of chondrocytes, it is possible that the more prolific osteochondral granulation tissue could have a detrimental effect on transplant cell viability or phenotype. Collectively, these observations indicate that scaffold properties seen in subcutaneous implants or \textit{in vitro} culture will not necessarily predict scaffold integration, degradation or inflammatory responses in full-thickness articular cartilage or osteochondral defects. Since implantation of the cross-linked chitosan cell carrier alone appeared to inhibit bone regeneration in osteochondral defects, this indicates that the use of this chitosan gel to repair osteochondral defects could additionally require the delivery of bone-forming cells or factors.

Our data in living animal defects show that the chitosan gel can reside at least 1 day in a full-thickness chondral defect, and for at least 1 week in a mobile osteochondral defect. It is uncertain whether this time frame would be sufficient to initiate tissue repair by delivered cells. Cartilage repair therapies using this chitosan hydrogel will additionally require protection of the initially mechanically fragile implant from high shear forces. Using the chitosan gel described here in humans, with controlled post-operative mobility and an appropriate cell source or growth-factor/cell combination, delivered cells could gradually produce a viable and mechanically stable repair tissue at the defect site and

Fig. 5. \textit{In vivo} delivery and retention of chitosan gel in an $\sim$0.3 mm deep full-thickness chondral-only defect of an adult NZW rabbit patella (A). Liquid chitosan gel was injected into the $4 \times 3$ mm$^2$ full-thickness chondral defect (B). The patella was recovered after 24 h of normal load-bearing and processed for histology. Toluidine blue-stained sections show gel retention and adherence, even after load-bearing to subchondral bone (arrows in C, D), calcified cartilage (open arrows in C, D) and adjacent articular cartilage (arrowheads in C, D).
thereby contribute to cell-based arthroscopic treatments for lesions of the articular cartilage.

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References