Chitosan–glycerol phosphate/blood implants increase cell recruitment, transient vascularization and subchondral bone remodeling in drilled cartilage defects

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Objective: Marrow-stimulation techniques are used by surgeons to repair cartilage lesions although consistent regeneration of hyaline cartilage is rare. We have shown previously that autologous blood can be mixed with a polymer solution containing chitosan in a glycerol phosphate (GP) buffer (chitosan–GP), and that implantation of this polymer/blood composite onto marrow-stimulated chondral defects in rabbit and sheep leads to the synthesis of more chondral repair tissue with greater hyaline character compared to marrow-stimulation alone. In the current study, we examined the modulation of cell recruitment and repair tissue characteristics at early post-surgical time points (from day 1 to 56) in a rabbit model to elucidate potential mechanisms behind this improved repair outcome.

Design: Thirty-three skeletally mature New Zealand White rabbits underwent bilateral arthrotomies, with each trochlea receiving a cartilage defect (3.5 mm × 4.5 mm) bearing four microdrill holes (0.9 mm diameter, ~4 mm deep) into the subchondral bone. One defect per rabbit was treated with a chitosan–GP/blood implant, while the other defect was left as a microdrilled control. Repair tissues were stained by histochemistry, for collagen types I, II, and X by immunohistochemistry and analyzed using quantitative stereological tools.

Results: Histological analyses demonstrated that control defects followed a typical healing sequence observed previously in marrow-stimulation animal models while chitosan–GP/blood implants led to three significant modifications in the healing sequence at early stages: (1) increased inflammatory and marrow-derived stromal cell recruitment to the microdrill holes, (2) increased vascularization of the provisional repair tissue in the microdrill holes, and (3) increased intramembranous bone formation and subchondral bone remodeling (BR).

Conclusions: These results suggest that the greater levels of provisional tissue vascularization and BR activity are main factors supporting improved cartilage repair when chitosan–GP/blood implants are applied to marrow-stimulated cartilage lesions.

Key words: Chitosan, Cartilage, Cartilage repair, Bone repair, Biodegradation, Biocompatibility, Chemotaxis, Angiogenesis

Abbreviations: BR Bone remodeling, BV Blood vessels, DDA Degree of deacetylation, EDTA Ethylenediaminetetraacetic acid, GP Glycerol phosphate, Ig Immunoglobulin, IL Interleukin, IM Intramuscular, NWB New woven bone, NZW New Zealand White, PBS Phosphate buffered saline, PDGF Platelet-derived growth factor.
cartilage defect whereupon it solidifies, adheres and remains voluminous by impeding platelet-mediated clot retraction, in order to more effectively drive subchondral bone-mediated cartilage repair10,11. Chitosan is composed of glucosamine and N-acetylglucosamine monomers, is biocompatible, biodegradable, non-toxic, and has been shown to improve wound healing in connective tissues of several species12,13. Chitosan induces aggregation and activation of platelets14 and promotes blood coagulation15,16. Furthermore, chitosan is a polycation that can bind to biological surfaces that are rich in anionic glycosaminoglycans17, including cartilage11, and is therefore particularly promising for orthopedic applications. In dermal wound healing models, chitosan has been found to induce transient chemotaxis of inflammatory cells18-20 that is often accompanied by an increase in the synthesis of collagen in the granulation tissue20, by a process believed to be partly dependent on secretion of growth factors by macrophages21. Chitosan is chemoattractive for endothelial cells in vitro22 and induces angiogenesis in vivo23. Similarly as in dermal wound healing models, the stimulatory effects of chitosan on cell recruitment and angiogenesis could promote more effective cartilage repair in lesions that communicate with subchondral bone.

A well known property of chitosan is its solubility at acidic pH (<6) and insolubility at neutral pH, making its use in solution with living cells and tissues problematic. However, glycerol phosphate (GP) buffers can be used to bring chitosan solutions to a cytocompatible near-neutral pH, while maintaining chitosan solubility14,24. We recently demonstrated that mixing of this physiological chitosan–GP solution with whole blood generates a chitosan–GP/blood solution that coagulates in situ within 15 min, when applied to cartilage defects10,11. Furthermore, these coagulated chitosan–GP/blood clots are adhesive and contract much less than whole blood clots, thereby maintaining a voluminous scaffold10,11. Chitosan–GP/blood implants were applied to marrow-stimulated chondral defects in sheep and rabbit cartilage repair models, where they induced greater fill of chondral defects with repair tissue compared to marrow-stimulation alone11 and, in addition, produced a more cellular and hyaline repair cartilage well integrated with a porous subchondral bone structure10,11. This led us to hypothesize that chitosan–GP/blood clots stimulated specific early events in osteochondral healing that led to more hyaline repair.

In the current study, we have examined cell recruitment and repair tissue characteristics at early post-surgical time points in a rabbit repair model to further understand the influence of these implants on cartilage repair. We tested the hypotheses that chitosan–GP/blood implants would (1) initially reside in the defects and then be completely degraded without generating chronic inflammation, (2) increase bone marrow cell recruitment, (3) increase vascularization of the provisional repair tissue, and (4) modulate subchondral repair tissue development. For this, we used a previously developed rabbit model10, where bilateral trochar cartilage defects were perforated with microdrill holes into the subchondral bone, and then either treated with the chitosan–GP/blood implants or left to bleed from subchondral bone as the control. Implant residency and biodegradation, recruitment of host cells, vascularization of subchondral tissue and osteochondral healing events were assessed with histochemical stains, immunohistochemical collagen typing, and stereology tools at time points ranging from 1 to 56 days post-surgery.

Materials and methods

ANIMALS

Three groups of skeletally mature New Zealand White (NZW) rabbits (Charles River, St. Constant, QC, Canada) housed individually in cages were used for the study (Table I). The housing, care and study protocol were approved by the Animal Care Committee of the University of Montreal. The animals were randomized according to sex and weight for distribution to treatment group and to each time point. The treated knee (left or right) was also randomized.

PREPARATION OF CHITOSA–GP SOLUTIONS

Chitosans (BioSyntech Inc, Laval, Quebec, Canada) with degree of deacetylation (DDA) between 77% and 83% (Table II) were used to treat the three groups of rabbits. Sterile chitosan–GP solutions were prepared by first dissolving 150 or 170 mg (after drying) chitosan powder in 9 ml of 66 ± 5 mM HCl (W3202-2, VWR, Mississauga, Ontario, Canada) followed by autoclave sterilization, and addition of 1 ml filter-sterilized 1.35 M disodium β-glycerophosphate (C5H7Na2O6P2) (G-9891, Sigma, Oakville, Ontario, Canada) to obtain a final chitosan–GP solutions consisting of 1.5% or 1.7% (w/v) chitosan with 135 mM disodium β-glycerophosphate and 59 mM HCl. The chitosan concentration, pH and osmolality of the chitosan–GP solutions used to treat each animal group are indicated in Table II. Chitosan–GP solutions were distributed in 1.18 ml aliquots into sterile, de-pyrogenized 4 cc Wheaton glass vials containing six sterile surgical stainless steel beads (0.39 g each, #9736, Salem Specialty Ball Co, Canton, CT, USA), flash-frozen with liquid nitrogen and stored at −80°C until use. The steel beads aided in mixing with blood during manual shaking as described below.

SURGICAL PROCEDURES

Following intramuscular (IM) injection of an anesthetic cocktail (keta mine–xylazine–buprenorphine), the animals were placed under gas anesthesia with 3% isoflurane. Steroidal anesthesia. The animals were randomized according to sex and weight for distribution to treatment group and to each time point. The treated knee (left or right) was also randomized.

### Table I

**Number of animals evaluated in each group at each time point**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>1*</td>
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<tr>
<td>Age at surgery</td>
<td></td>
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<tr>
<td>8 Months</td>
<td>15 Months</td>
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<tr>
<td>Average weight at surgery</td>
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<tr>
<td>4.7 kg</td>
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<tr>
<td>Sacrifice at 7 days</td>
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<tr>
<td>Sacrifice at 14 days</td>
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</tr>
<tr>
<td>Total</td>
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</tr>
</tbody>
</table>

*Sixty minutes recovery from anesthesia.
†Four hours recovery from anesthesia.
‡Male and female rabbits were equally distributed among all groups at each time point studied.
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Table II

Characteristics of chitosans and chitosan–GP solutions used to make chitosan–GP/blood implants for each of the three groups of animals

<table>
<thead>
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<td>Chitosan batch</td>
<td>PCCH0002</td>
<td>PCCH00025</td>
<td>CH10064</td>
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<tr>
<td>DDA of chitosan (%)</td>
<td>78.5</td>
<td>77.1</td>
<td>82.6</td>
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<tr>
<td>Endotoxin units</td>
<td>&lt;3000</td>
<td>&lt;375</td>
<td>&lt;500</td>
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<tr>
<td>per mass of chitosan (EU/g)</td>
<td>1.7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Chitosan concentration (w/v) in chitosan–GP solution (%)</td>
<td>6.82</td>
<td>6.82</td>
<td>6.75</td>
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<tr>
<td>pH of chitosan–GP solution</td>
<td>520</td>
<td>464</td>
<td>444</td>
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</tbody>
</table>

Fig. 1. Trochlear cartilage defects were microdrilled and either left alone as untreated controls (A) or filled with chitosan–GP/blood implants for each of the three groups of animals. No treatment-specific effusions or other overt signs of infection were seen for the remaining rabbits.

PREPARATION AND APPLICATION OF CHITOSAN–GP/BLOOD MIXTURE

Immediately prior to mixing and application of the implants, autologous blood was aseptically withdrawn from the rabbit ear artery and 3.5 ml added to the vial containing 1.18 ml sterile chitosan–GP solution and steel beads. For mixing, the vial was closed and shaken vigorously by hand for 10 s. The chitosan–GP/blood mixtures consisted of autologous blood mixed with the chitosan–GP solutions in a 3:1 ratio to yield final concentrations of 0.4% (w/v) chitosan and 34 mM disodium phosphate. A 3 cc syringe equipped with an 18 gauge needle was used to withdraw approximately 3 ml of the chitosan–GP/blood solution and one hanging drop of this solution was applied to the drilled cartilage defect [Fig. 1(B)]. In 23 of 33 treated defects, the liquid implant flowed outside the defect and was in potential contact with other joint tissues. Coagulation was allowed for 5–8 min before closing the knee. All implants formed a solid clot covering the entire defect before closing the knee.

HISTOPROCESSING AND STAINING OF OSTEOCHONDRAL TISSUES

After sacrifice, the distal portions of the femurs were clipped off using a bone cutter and fixed in 4% (w/v) paraformaldehyde (P-6148, Sigma, Oakville, Ontario, Canada), 0.1 M sodium cacodylate (BP 325-50, Fisher Scientific, St-Laurent, Quebec, Canada), pH 7.3 at room temperature.
for 24 h and then placed at 4°C for a further 48 h. The femurs were decalcified in 10% (w/v) ethylenediaminetetraacetic acid (EDTA) (EDTA50, Sigma, Oakville, Ontario, Canada) in PBS with 0.1% (w/v) paraformaldehyde pH 7.2 for 9 weeks at 4°C. The condyles were trimmed off and the trochlea was prepared for cryosectioning as previously described.26 Cryosections (10–12 μm thickness) were taken (Microm, Model Cryo-Star HM 560 M) and collected on SuperFrost Plus slides (12-550-15, Fisher Scientific, St-Laurent, Quebec, Canada).

For Safranin O/Fast Green staining, the sections were sequentially immersed in 0.04% (w/v) Fast Green (F-7252, Sigma, Oakville, Ontario, Canada) and 0.1% (w/v) Safranin O (S-2255, Sigma, Oakville, Ontario, Canada) in water. For Gomori Trichrome staining, the sections were immersed in 0.6% (w/v) Chromotrope 2R (C-3143, Sigma, Oakville, Ontario, Canada) and 0.3% (w/v) Fast Green in 0.6% (w/v) phosphotungstic acid (P-4006, Sigma, Oakville, Ontario, Canada).

COLLAGEN TYPING OF OSTEOCHONDRAL TISSUES BY IMMUNOHISTOCHEMISTRY

Cryosections were subjected to antigen retrieval27 by boiling in 10 mM Tris pH 10 and enzymatic pre-treatment with 2.5% (w/v) hyaluronidase EC 3.2.1.35 (H-3506, Sigma, Oakville, Ontario, Canada) diluted to 22 μg/ml (C-2456, Sigma, Oakville, Ontario, Canada) in PBS with 0.1% Triton X-100 for 60 min at room temperature and incubated with the primary antibody of interest diluted with 10% goat serum in PBS with 0.1% Triton X-100. The primary antibodies were (1) monoclonal anti-collagen type I IgG1 clone COL-1 diluted to 37 μg/ml (C-2456, Sigma, Oakville, Ontario, Canada), (2) monoclonal anti-collagen type II IgG1 diluted 1:10 (II-II6B3, DSHB, Iowa, USA), and (3) monoclonal anti-collagen type X IgM clone COL-10 diluted to 575 μg/ml (C-7974, Sigma, Oakville, Ontario, Canada). Sections were rinsed and incubated with biotinylated goat anti-mouse IgG (Fab specific) (B-7151, Sigma, Oakville, Ontario, Canada) diluted to 22 μg/ml with 10% goat serum in PBS with 0.1% Triton X-100 for 60 min at room temperature. Histochemical detection was performed with the Vectastain ABC-Alkaline Phosphatase (AP) system and AP staining medium (SP15-100, Fisher Scientific, St-Laurent, Quebec, Canada). Exclusion of primary antibody or use of isotype-matched immunoglobulin (Ig) resulted in no staining. All sections were counterstained with Weigert Iron Hematoxylin (HT-10-7 and HT-10-9, Sigma, Oakville, Ontario, Canada), rapidly dehydrated in graded ethanol and mounted in Permount mounting medium (SP15-100, Fisher Scientific, St-Laurent, Quebec, Canada).

HISTOMORPHOMETRIC AND STEREOLOGICAL ANALYSES

Digital images of stained sections were acquired with a Zeiss Axioslab microscope equipped with either a Sony analogue camera, a digital Q Imaging MicroPublisher 3.3 RTV camera or a digital Hitachi HV-F22F camera. Software for histomorphometric and stereological analyses included Northern Eclipse (Mississauga, Ontario, Canada) and BIOQUANT OSTEO (Nashville, TN, USA).

The extent of defect debridement was quantified at 1 day post-surgery (n = 10 defects) by measuring the proportion of the defect covered with calcified cartilage on one side of the defects as a guide. Then, the total area (mm²) of tissue present above the projected tidemark was measured using Northern Eclipse software by thresholding on tissues filling the defect and then normalizing to the width of the defect (mm).

To quantify volume density (Vv = volume per tissue volume) of neutrophils and bone marrow-derived stromal cells in the microdrill holes, point (P) counting was performed on Safranin O/Fast Green stained sections, on 0.11 mm² microscopic fields taken at three systematic positions (0.5 mm, 1.25 mm and 2.5 mm below the projected tidemark) in each of the four microdrill holes, and applying Vv = ∑Pc type/∑Ptotal.28 For each knee, Vv was calculated in 12 fields. The grid size to the area (mm²) of tissue present above the projected tidemark was measured using Northern Eclipse software by thresholding on tissues filling the defect and then normalizing to the width of the defect (mm).

To quantify volume density (Vv) of subchondral cartilage in Safranin O/Fast Green stained sections, the area of subchondral cartilage (mm²) was obtained by thresholding on subchondral cartilage tissue (Safranin O positive) below the projected tidemark using Northern Eclipse software and then normalizing to the total area of subchondral tissue (mm²).

To quantify volume density (Vv) of subchondral bone in Gomori Trichrome stained sections, the area of subchondral bone (mm²) was obtained by thresholding on subchondral bone tissue (Safranin O positive) below the projected tidemark using Northern Eclipse software and then normalizing to the total area of subchondral tissue (mm²).

To quantify trabecular bone surface density (SV = surface area per tissue volume) in Gomori Trichrome stained sections, the perimeter (Per) of subchondral bone (mm) was obtained by thresholding on subchondral bone (excluding other subchondral tissues such as clot, fibrous tissue, cartilage and marrow) using BIOQUANT OSTEO software and then normalizing to the total area of subchondral tissue (mm²).

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cartilage and marrow) using BIOQUANT OSTEO software. The total area of subchondral tissue (mm²), known as the area of reference ($A_{ref}$), was also obtained using BIOQUANT OSTEO software. Sv was then calculated as $\sum \text{Per} \times A_{ref} \times \frac{4}{\pi}$. 

STATISTICAL ANALYSES

Statistical analyses were performed using the paired Student’s t-test (pairing treated and untreated for this bilateral model), analysis of variance (ANOVA) and multivariate ANOVA in the general linear model, after checking for normality of data, as well as using non-parametric tests such as the Mann–Whitney U Test and the Wilcoxon matched pairs test, as implemented in Statistica version 6.1 package (Statsoft Inc, Tulsa, OK, USA). Since normal probability plots suggested most data to respect normality and non-parametric tests were for the most part concordant with parametric tests, only results from parametric tests were presented.

Results

CHITOSAN–GP/BLOOD CLOTS WERE PRESENT FOR UP TO 1 MONTH POST-SURGERY

Chitosan–GP/blood implants were solidified in treated drilled cartilage defects [Fig. 1(B)], while the contralateral control defects were drilled and simply allowed to bleed [Fig. 1(A)]. At 1 day post-surgery, control and treated defects were equally debrided of calcified cartilage in the zone midway between the drill holes where 53% ± 31 vs
respectively, of the defect base was still covered with calcified cartilage. Acute repair events were studied in the sections traversing the microdrill holes where residual calcified cartilage was only present outside the holes. Controls from 1 to 14 days post-surgery contained a fibrin network and blood clot in the defects and microdrill holes [Fig. 2(A)]. At these same time points, chitosan–GP implants were detected in treated defects as dense material staining a dark color with Fast Green [Fig. 2(B)]. All but one treated defect (23 of 24) taken between 1 and 14 days post-surgery contained chitosan–GP implant. At 21 days post-surgery, the residual chitosan–GP implant could only be detected subchondrally in the microdrill holes of treated defects [Fig. 2(D)], and was not observed at all thereafter at 35 and 56 days. Five microdrill holes from treatment groups 2 and 3, from a total of 128 microdrill holes (32 treated defects with four holes each) contained fibrous capsules at 14, 21 and 35 days post-surgery, some of which enclosed chitosan particles (data not shown). The average thickness of chitosan–GP implants in the chondral region of treated defects was significantly greater than the average thickness of fibrin clots in control defects from 1 to 14 days (* $P < 0.01$ using treatment and day as predictors from day 1 to 14, as well as paired t-tests for days 1, 3 and 14). Chitosan–GP implants and regular blood clots were for the most part degraded by 21 days post-surgery in the chondral area, after which chondral repair tissue of increasing thickness was synthesized in treated and control defects. Data are expressed as mean ± SD with $n = 5$ (Day 1, 3, 7), $n = 8$ (Day 14) and $n = 3$ (Day 21, 35, 56). Horizontal arrowhead represents the average thickness of trochlear articular cartilage from unoperated age-matched rabbits.

Fig. 3. The average thickness of chitosan–GP implants in treated defects was significantly greater than the average thickness of regular clots in control defects from 1 to 14 days post-surgery (* $P < 0.01$ using treatment and day as predictors from day 1 to 14, as well as paired t-tests for days 1, 3 and 14). Chitosan–GP implants and regular blood clots were for the most part degraded by 21 days post-surgery in the chondral area, after which chondral repair tissue of increasing thickness was synthesized in treated and control defects. Data are expressed as mean ± SD with $n = 5$ (Day 1, 3, 7), $n = 8$ (Day 14) and $n = 3$ (Day 21, 35, 56). Horizontal arrowhead represents the average thickness of trochlear articular cartilage from unoperated age-matched rabbits.

Fig. 4. Safranin O/Fast Green staining showing increased recruitment of neutrophils (N) to the chitosan–GP implants of treated defects (B) vs to the regular blood clots of control defects (A) at 1 day post-surgery. Some neutrophils in treated defects co-localized around and within implant chitosan particles (I) (B). Chitosan–GP/blood implants increased subchondral recruitment of neutrophils (N) to the microdrill holes of treated defects (D) vs control defects (C) at 7 days. Bone marrow-derived stromal cells (S) were also observed migrating into the microdrill holes starting at day 7 (C, D). By 35 days post-surgery, repair tissues of continually increasing thickness were detected in the chondral region of treated and control defects.
Repair tissues were better integrated to the subchondral bone in treated defects compared to control defects at 35 days [Fig. 2(F vs E)].

**CHITOSAN—GP/BLOOD IMPLANTS INCREASED CELL RECRUITMENT**

At 1 day post-surgery, neutrophils (N) were observed in the chondral region of all treated defects, and some neutrophils were co-localized with chitosan particles and aggregates [Fig. 4(B)]. Fewer neutrophils were recruited to the fibrin clots of control defects [Fig. 4(A)]. Neutrophils were seldom observed in the subchondral repair tissue of control defects after 7 days [Fig. 4(C)] while an abundance of neutrophils was seen between 3 and 21 days in treated defects [Fig. 4(D)]. Chitosan—GP/blood implants increased volume density (Vv) of neutrophils in the microdrill holes of treated defects until 21 days post-surgery ($P<0.001$ at day 3, $P<0.01$ at day 7 and $P<0.05$ at day 14 for paired t-tests) [Fig. 5(A)]. Synovial fluid leukocyte differential profiles also showed a significant increase in the proportion of neutrophils in treated knees compared to control knees from 3 to 21 days post-surgery, which returned to normal at 35–56 days (data not shown).

Bone marrow-derived stromal cells were observed migrating into the microdrill holes of control and treated defects starting at 7 days [Fig. 4(C, D)]. Volume density (Vv) of bone marrow-derived stromal cells was significantly higher in microdrill holes of control defects at 7 days post-surgery ($P<0.05$ using the paired t-test), but was higher in treated defects from 14 days post-surgery and thereafter ($P<0.05$ using the paired t-test on day 21; also $P=0.072$ from ANOVA of days 14–56 using Day as a co-variate) [Fig. 5(B)]. Numerical integration of total numbers of stromal cells recruited to sampled sites up to day 56 indicated a net increase of 35% in stromal cells recruited to treated defects vs control.

**CHITOSAN—GP/BLOOD IMPLANTS INCREASED SUBCHONDRAL VASCULARIZATION**

New BV filled with erythrocytes were first observed in the bottom of the microdrill holes of treated and control defects at 7 days post-surgery. By day 14, greater numbers of BV were observed in the provisional repair tissue of microdrill holes below treated defects [Fig. 6(B, D)] compared to controls [Fig. 6(A, C)]. Length density (Lv) of BV was higher in microdrill holes of treated defects from 14 to 35 days post-surgery ($P<0.05$ for ANOVA using all days taking into account Day as a co-variate, while $P<0.05$ for the paired t-test at 14 days post-surgery) (Fig. 7).

**CHITOSAN—GP/BLOOD IMPLANTS DELAYED AND PARTLY INHIBITED SUBCHONDRAL CARTILAGE FORMATION WHILE PROMOTING SUBCHONDRAL BONE REMODELING (BR)**

Subchondral cartilage formation initially occurred in the microdrill holes of six of eight control defects at 14 days [Fig. 8(A)]. At 21 days post-surgery, cartilage formation could be observed throughout the microdrill holes of control defects [Fig. 2(C)]. Treated defects were almost devoid of subchondral cartilage at 14 days [Fig. 8(B)] and only contained small islands of subchondral cartilage at 21 days [Fig. 2(D)]. Subchondral cartilage volume density (Vv) was therefore higher in control defects at 14 and 21 days post-surgery ($P<0.05$ by the paired t-test at 14 days post-surgery) [Fig. 9(A)].

Subchondral cartilage formation was followed by chondrocyte hypertrophy, as revealed by type X collagen immunostaining (data not shown), endochondral ossification and terminal differentiation to bone. By 35 days post-surgery, subchondral cartilage was observed in treated and control defects in equal quantities [Fig. 9(A)], near the top of the microdrill holes [Figs. 2(E, F), 9(A)].

New woven bone (NWB) formation was initiated in control and treated defects at 7 days, and was initially restricted to the bottom of microdrill holes. Starting at 14 days post-surgery, chitosan—GP/blood implants showed much more extensive remodeling of cancellous bone in areas surrounding the microdrill holes [Figs. 2(D), 8(D)].

Treatment was a significant predictor of increased trabecular bone surface density (Sv) below defects from 14 to 56 days post-surgery ($P<0.05$ from ANOVA with Day as a co-variate between 14 and 56 days) [Fig. 9(B)]. There was no difference in total bone volume density (Vv) between control and treated defects at any time point (data not shown).
Discussion

The objective of this study was to examine modifications of early events in osteochondral repair following application of chitosan–GP/blood implants in cartilage defects that contain access channels to subchondral bone, in skeletally mature rabbits. Our previous work has established the ability of these implants to increase the volume and hyaline quality of cartilage repair tissue, but key events controlling this improved repair were not previously investigated in detail. Our results here revealed that chitosan–GP/blood implants reside for approximately 3 weeks post-surgery in this rabbit model and that degradation is in part mediated by host-derived neutrophils. During this period, chitosan–GP/blood implants increased subchondral cell recruitment and vascularization in the microdrill holes. Increased BR and new bone formation by an intramembranous pathway were also observed, rather than endochondral ossification requiring a cartilaginous intermediate. These subchondral events preceded cartilage repair tissue synthesis and could explain the previously described significant correlation between porous bone underlying the synthesis of a hyaline repair tissue in the chondral compartment.

Microdrilled control defects in this study healed through a similar sequence to that previously reported in rabbit for drill or microfracture holes communicating with the cancellous bone. Healing in the microdrill holes of control defects in our model closely resembled healing of 3-mm diameter osteochondral defects observed previously, starting with blood clot formation, inflammatory and marrow-derived stromal cell migration, synthesis of a vascularized granulation tissue, intramembranous bone formation in the deeper regions of the microdrill holes, as well as subchondral cartilage formation followed by endochondral ossification in the more superficial regions of the microdrill holes. In microdrilled cartilage defects of skeletally mature rabbits, these events led to the synthesis of bone in the bony compartment and of principally fibrocartilaginous repair tissue emerging from the microdrill holes into the cartilage compartment.
Chitosan–GP implant residency was confirmed by histology in treated defects until 21 days post-surgery (Fig. 2). In general, more tissue was macroscopically apparent in the treated defects compared to controls, especially around the drill holes, which appeared to anchor the implants. Chitosan–GP clots adhered to defect surfaces at 1 day post-surgery (Fig. 2), extending the previous finding of adhesion of chitosan–GP implants to the defect surfaces at 2 h post-surgery in sheep microfracture defects\(^1\). We have previously shown in vitro that chitosan–GP can physically stabilize the blood clot by generating clots that retract less than whole blood clots\(^1\). Consistent with this observation was our finding here of a greater average thickness of chitosan–GP implants compared to regular clots in control defects until 14 days (Fig. 3). Interestingly, most of the chitosan–GP implants in treated defects and the regular clots in control defects were degraded by 21 days, and tissue regrowth into the chondral zone occurred thereafter (Figs. 2, 3). It appears then that early stages of bone marrow-derived cartilage repair primarily depend on processes occurring in the subchondral bone and that subsequent tissue repair in the chondral zone occurs afterwards by an appositional mechanism, growing up from the subchondral bone.

Chitosan–GP/blood implants increased recruitment of neutrophils to the microdrill holes of treated defects (Figs. 4, 5) and to the synovial fluid of treated knees, possibly due to the chemotactic potential of chitosan demonstrated previously in vitro\(^3\) and in vivo\(^2\). Neutrophils appeared to migrate mainly from the bone marrow in conjunction with bone marrow-derived stromal cells, but may have also migrated from the vasculature in the granulation repair tissue and from the synovial fluid. Numerous neutrophils were observed co-localizing with chitosan particles and aggregates, suggesting that these cells are mainly responsible for the phagocytosis and degradation of the implants. Some micro-drill holes from treated defects contained fibrous tissues surrounding chitosan particles that resolved over time. Fibrous capsules like these have been shown to form around slowly degradable biomaterial implants\(^4\), suggesting that ideal residency for the chitosan implant may be similar to the residency of the normal clot, as we observed here. Neutrophils are believed to have short life spans, but their survival can be lengthened by certain cytokines\(^5\). Chitosan scaffolds implanted in soft-tissue of mice elicited a sustained neutrophil presence up to 4 weeks post-implantation\(^6\). In our model, neutrophil infiltration from the marrow and synovial fluid ceased by 35 days post-surgery in treated defects, coinciding with the complete degradation of the implant.

Chitosan–GP/blood implants increased subchondral vascularization of granulation tissues in treated defects (Figs. 6, 7) consistent with the ability of chitosan to promote endothelial cell migration in vitro\(^7\) and angiogenesis in vivo\(^8\). Additionally, chitosan has been shown to synergistically increase the proliferative effect of platelet-derived growth factor (PDGF) on vascular smooth muscle cells\(^9\). In our model, increased vascularization occurred simultaneously with a period of inflammatory cell recruitment, suggesting that these inflammatory cells may secrete factors that promote angiogenesis. Alternately, increased recruitment of marrow-derived stromal cells may affect angiogenesis. Chitosan has been shown to stimulate secretion of the angiogenic chemokine interleukin (IL)-8 by fibroblasts in vitro\(^10\) and may

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stimulate local joint fibroblasts to secrete angiogenic factors as well.

Chitosan—GP/blood implants favored intramembranous bone formation in the microdrill holes (Figs. 8, 9) rather than endochondral ossification, perhaps through increased vascularization of the granulation tissue. For example, an increase in blood supply during distraction osteogenesis has been associated with intramembranous bone formation\(^1\). Angiogenesis in the microdrill holes appeared to be directly related to delayed subchondral cartilage formation. Microdrill holes of control defects had few BV at 14 and 21 days post-surgery and extensive subchondral cartilage formation. In comparison, microdrill holes of treated defects had, on average, twice as many BV, continued angiogenesis and marrow-derived stromal cell recruitment at the same time points. Delayed initiation and displacement of chondrocyte and marrow-derived stromal cell recruitment at the same time points. Delayed initiation and displacement of chondrocyte and marrow-derived stromal cell recruitment at the same time points. Delayed initiation and displacement of chondrocyte and marrow-derived stromal cell recruitment at the same time points. Delayed initiation and displacement of chondrocyte and marrow-derived stromal cell recruitment at the same time points. Delayed initiation and displacement of chondrocyte and marrow-derived stromal cell recruitment at the same time points.

Although some BR was observed under our control defects, as has been observed under cartilage defects treated with microfracture\(^1\), chitosan—GP/blood implants increased subchondral BR compared to controls (Figs. 2, 8, 9). Other cartilage repair procedures have been reported to induce subchondral bone reactions, leading to a net loss of bone\(^1\), invasion of the bony compartment with cartilaginous and fibrous tissues\(^1\) and distortion of the trabecular structure\(^1\). In contrast, we found focal areas of bone resorption below the implants were quickly replaced with a vascularized granulation repair tissue and NWB. New bone trabeculae were finer and more numerous (Fig. 8), leading to an increase in trabecular surface density under treated defects (Fig. 9) without any apparent increase in bone volume density. The latter is in contrast to increased subchondral bone porosity found at 8 weeks in a more superficial region (250 μm below the tidemark)\(^1\) compared to our more deeply sampled regions here reaching down to 4 mm below the tidemark, reinforcing the importance of accounting for depth when analyzing subchondral bone responses. Of note, an increased number of multinucleated cells, possibly osteoclasts, were observed within bone marrow cavities of defects treated with chitosan—GP/blood implants in our rabbit model at 56 days (unpublished observations), suggesting that remodeling is still on-going at this early end-point. Taken together, these observations suggest that chitosan—GP/blood implants induce angiogenesis and BR which leads to a porous subchondral bone structure with well vascularized marrow cavities. The presence of this type of porous and vascularized subchondral bone may be essential for effective cartilage repair in methods that rely on bone marrow-mediated cartilage regeneration\(^1\).

Solutions of chitosan in GP can be mixed with freshly drawn autologous blood and applied to debrided cartilage lesions containing conduits to subchondral marrow spaces to facilitate cartilage repair. Previous studies showed that chitosan—GP/blood implants solidify and adhere to cartilage defects, inhibit clot retraction and lead to more chondral repair tissue with greater hyaline quality. The current study added to this knowledge that chitosan—GP/blood implants increase cell recruitment, transient vascularization and remodeling in the subchondral bone region and suggests that these three events are in part responsible for increased quantity and quality of repair tissue in the chondral zone. These results highlight the importance of examining acute and intermediate events in cartilage repair, the predominant role of subchondral bone in effecting cartilage repair, and the potential benefits of adopting a cartilage repair strategy that modulates acute and intermediate events in the subchondral bone in order to improve final cartilage repair outcome.

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