CHONDROCYTE TRANSPLANTATION USING A COLLAGEN BILAYER MATRIX FOR CARTILAGE REPAIR

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We have developed a novel, two-layered, collagen matrix seeded with chondrocytes for repair of articular cartilage. It consists of a dense collagen layer which is in contact with bone and a porous matrix to support the seeded chondrocytes. The matrices were implanted in rabbit femoral trochleas for up to 24 weeks. The control groups received either a matrix without cells or no implant.

The best histological repair was seen with cell-seeded implants. The permeability and glycosaminoglycan content of both implant groups were nearly normal, but were significantly less in tissue from empty defects. The type-II collagen content of the seeded implants was normal. For unseeded implants it was 74.3% of the normal and for empty defects only 20%. The current treatments for articular injury often result in a fibrous repair which deteriorates with time. This bilayer implant allowed sustained hyaline-like repair of articular defects during the entire six-month period of observation.

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The clinical need for repair of lesions of the articular cartilage is increasing, but there is no satisfactory method of surgical repair. Abrasion arthroplasty, excision and drilling, cartilage debridement, and arthroscopic shaving have all been used.[1-4] These procedures generally evoke repair responses in which the damaged site is filled with fibrocartilage.[5,6] This may provide temporary pain relief, but the tissue is often mechanically inadequate, resulting in arthritis.[7,8]

Transplantation of osteochondral grafts,[9] periosteum,[10] and more recently cultured chondrocytes[11] and mesenchymal stem cells[12] to induce repair have not produced long-lasting hyaline cartilage. Tissue-engineering concepts have been applied to a variety of biomaterials to give chondrocyte-seeded implants for repair. Among the materials used have been demineralised bone,[13] polylactic acid,[14] polyglycolic acid,[15] hydroxyapatite/Dacron composites,[16] fibrin,[17] collagen gels,[18-20] and collagen fibres.[21,22] Some allowed formation of a repair tissue that resembled normal cartilage but the repair was often accompanied by substantial fibrocartilage formation.

We report the use of a novel, biodegradable, biocompatible, two-layered collagen material, seeded with chondrocytes to repair full-thickness lesions of articular cartilage. The layer in contact with the subchondral bone is a dense collagen which prevents fibroblast ingrowth from below while allowing the influx of endogenous tissue factors that promote cell growth.[23] The second layer is a porous matrix optimised to support chondrocyte growth and metabolism.[21]

We performed studies in vitro to determine whether the cells in the porous component of the implant retained the chondrocyte phenotype,[24,25] and to evaluate the ability of the dense component of the implant to prevent fibrous ingrowth. Matrices were implanted into surgically-created cartilage defects in rabbit femora for periods of up to six months. We then performed biochemical, mechanical and histomorphological analyses on the resulting repair tissue.

MATERIALS AND METHODS

Chondrocytes were isolated by primary culture. Cartilage from the knees of New Zealand White rabbits was digested by clostridial collagenase, deoxyribonuclease, testicular

hyaluronidase and penicillin-streptomycin (all from Sigma, St Louis, Missouri) at 37°C for 18 hours.[25] The released chondrocytes were incubated in RPMI-1640 with 10% fetal bovine serum. They were transferred at confluence to the bovine type-I collagen matrix (Integra LifeSciences, Plainsboro, New Jersey) and grown for 10 to 14 days before implantation or testing in vitro. Matrices 4 mm in diameter were seeded with 850 000 cells.

In vitro studies. Immunohistochemical and histological evaluation of the cultured chondrocyte-matrix complexes was undertaken at 3, 7, 10 and 14 days after cell seeding. The histological sections of the matrices were stained with Safranin-O/Fast Green to determine glycosaminoglycan (GAG) production and with S-100 antibody (DAKO Immunoglobulin, Glostrup, Denmark) to detect expression of the chondrocyte phenotype.[25,26]

The mean GAG content of matrices, repair tissue, and normal cartilage was determined using a method modified from De Palma et al.[27] Interfering proteins were removed with a 1% sodium dodecylsulphate (SDS) solution and digested with Proteinase-K (Bio-Rad, Richmond, California). The proteins were precipitated and discarded. Solubilised GAGs were extracted with 2:1 chloroform/methanol, precipitated with cold ethanol/potassium acetate and quantified using Alcian Blue dye and the optical density determined at 620 nm. This assay does not differentiate between GAG types but is a sensitive assay to determine quantitative differences of GAGs in repair compared with normal tissue.

The surfaces of the porous and dense components of the matrices were seeded with fibroblasts, cultured for 3, 6 and 10 days, and sectioned to determine the penetration of fibroblasts into each component. In addition, a modified Boyden chamber assay (ADAPS Inc, Dedham, Massachusetts) was performed to quantify the passage of fibroblasts through the dense layer. Cells were placed in the upper compartment of the chamber, with fibroblast growth factor (100 ng/ml) in the lower compartment to act as a chemoattractant. A barrier material was placed between the upper and lower compartments. Nitrocellulose filters were used as a positive control (Millipore, Bedford, Massachusetts). They were placed between the compartments, and plated with neutrophils or fibroblasts in the upper compartment. Neutrophils migrate at a known rate through these filters. The third set of chambers used the dense component of the implant matrix as the barrier material and was plated with fibroblasts. The number of cells in the lower compartment was counted after four hours.

In vivo studies. We used 51 skeletally mature male New Zealand White rabbits. An osteochondral defect 3 mm in diameter was drilled in the femoral trochlea into the subchondral bone (Fig. 1). The dense layer of the matrix was packed into the defect followed by the cell-seeded layer. The patella was reduced and the wound closed. A control group of knees received the bilayer matrix with no cells or no implant. The animals were allowed unlimited movement.

At 6 (n = 26), 12 (n = 34) and 24 weeks (n = 42) postoperatively, the animals were killed by an overdose of pentobarbitol.

Histological analysis. Histological specimens were scored blindly by a grading system modified from O'Driscoll, Comisso and Fitzsimmons.[28] The criteria included the percentage of hyaline cartilage, surface regularity, thickness, cellularity, cluster formation, bonding to native cartilage, and degeneration of adjacent cartilage. The maximum score was 24 points.

Mechanical testing. Specimens of repair tissue with a short length of subchondral bone attached were cored from the defect and tested in the load frame of a custom-built device.[29] A step load sufficient to cause a stress of 0.1 MPa was applied and deformation of the cartilage layer was measured. The equilibrium modulus and the permeability of the specimens were determined by non-linear regression analysis using the equation of Mow et al.[30]

Analysis of collagen type. Type-II collagen in both repair and normal samples was measured using SDS-PAGE, as modified from the method of Barr et al.[31] The samples were treated with pepsin and 1 g/ml of cyanogen bromide in 70% formic acid for four hours at 30°C. The resulting peptides were separated by SDS-PAGE on a continuous gradient gel, stained with Coomassie Blue, and destained with methanol/acetic acid/water. Gels were digitised using image analysis and the major peaks integrated. A calibration curve for type-II collagen content, normalised to total protein content, was made for each gel. The results were expressed as a percentage of the normal values.

Statistical analysis. We performed Student's *t*-test to determine the statistical significance of the data.

RESULTS

In vitro studies. The matrix sections exposed to Safranin-O showed a diffuse pink stain in the pericellular matrix,

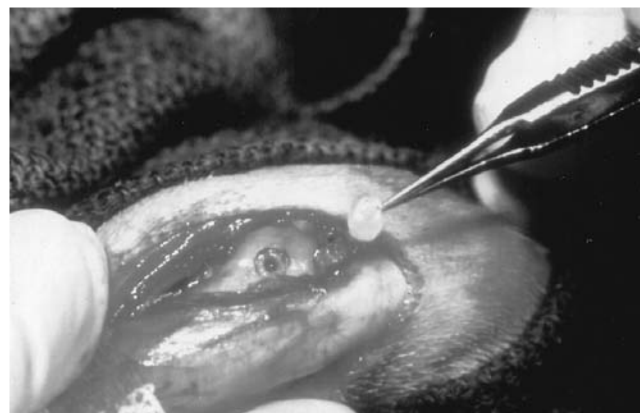

Fig. 1

A cell-seeded collagen implant, ready to be placed in a surgically-created defect. The porous layer is visible with the dense layer below.

indicative of GAG secretion by the cells. The mean GAG content of the implants was 0.015 ± 0.002 μg/μl. Immunostaining indicated that cells grown within the matrix for 14 days were S-100 positive and were thus phenotypic chondrocytes.

Fibroblasts seeded onto the dense collagen layer of the matrix were not able to penetrate this material. In the Boyden chamber study, neutrophils and fibroblasts migrated through the nitrocellulose control membrane at the same rate. There was a mean of 1×10^6 cells in the lower chamber after four hours. The dense collagen matrix successfully prevented fibroblast penetration, with only 4×10^4 cells (4% of the control value) passing through it.

Table I. Histological characterisation of articular cartilage repair sites according to the grading system of O'Driscoll et al[28] (see text)

	Weeks		
Type	**6 (n = 8)**	**12 (n = 8)**	**24 (n = 7)**
Cell-seeded	17.11 ± 1.27	18.00 ± 2.83	18.57 ± 2.37
Unseeded	9.43 ± 4.76	10.00 ± 3.46	10.20 ± 3.77
Empty defect	--	11.50 ± 2.12	6.00 ± 0.01

In vivo studies

Histological analysis. At six weeks cell-seeded implants produced a significantly better repair ($p < 0.01$) than unseeded implants (Table I). The percentage of hyaline cartilage and reconstitution of the subchondral plate in cell-seeded specimens were at least twice as good with cell-seeding compared with the control group. At 12 weeks cell-seeded grafts again gave much better surface regularity, percentage of hyaline cartilage, congruence in thickness with the host cartilage, and columnar organisation of cells (Fig. 2a). The repair with unseeded grafts appeared immature, with a random arrangement of chondrocytes. The cartilage layer extended well below the level of adjacent normal tissue into an area that should have been occupied by bone (Fig. 2b). Empty defects had very irregular surfaces with poor bonding and many chondrocyte clusters (Fig. 2c).

At 24 weeks there was a clearly superior repair with cell-seeded implants (Fig. 3a; $p < 0.01$). Bonding to host tissues was excellent and the surfaces remained smooth. The tidemark and columnar chondrocytes were evident. Repair with unseeded implants was well integrated into the native tissue

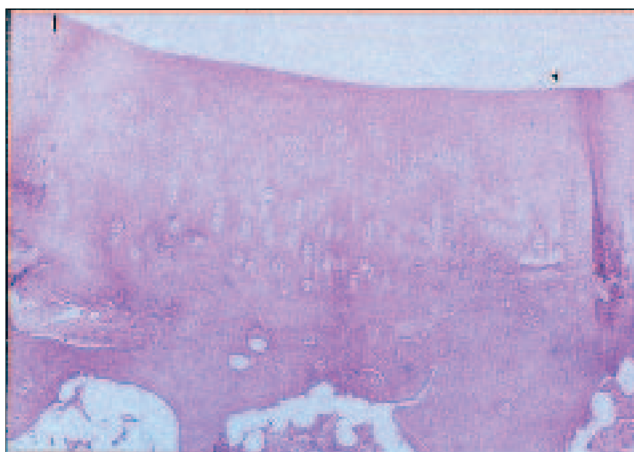

Fig. 2a

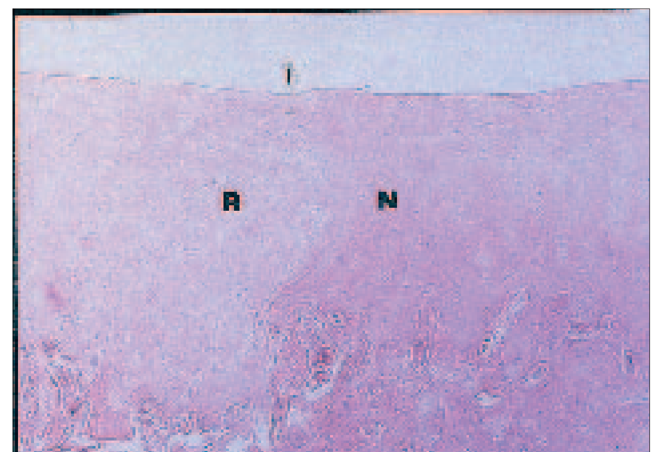

Fig. 2b

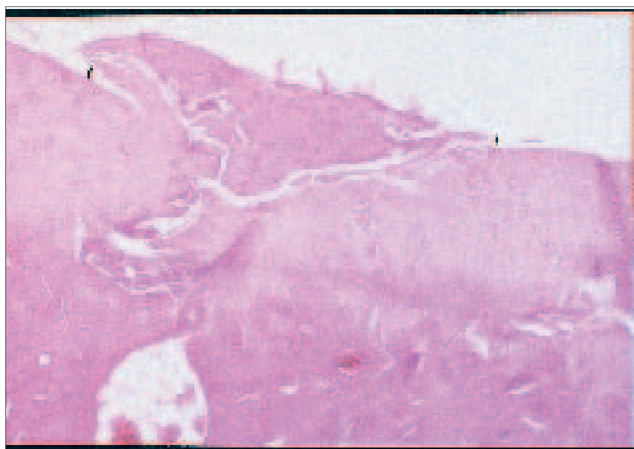

Fig. 2c

Tissue recovered from a 12-week specimen. Figure 2a – A cell-seeded graft. Columnar organisation of chondrocytes and a smooth surface are evident. The arrows indicate the margin of the defect. Figure 2b – An unseeded graft. Repair cartilage (R) extends well below the level of the adjacent tissue into an area which should be occupied by bone. The arrow indicates the interface between host and repair tissue (N, native tissue). Figure 2c – An empty defect. There are surface clefts, discontinuity of deeper tissue, and many cell clusters. The arrows indicate the margin of the defect (haematoxylin and eosin ×56).

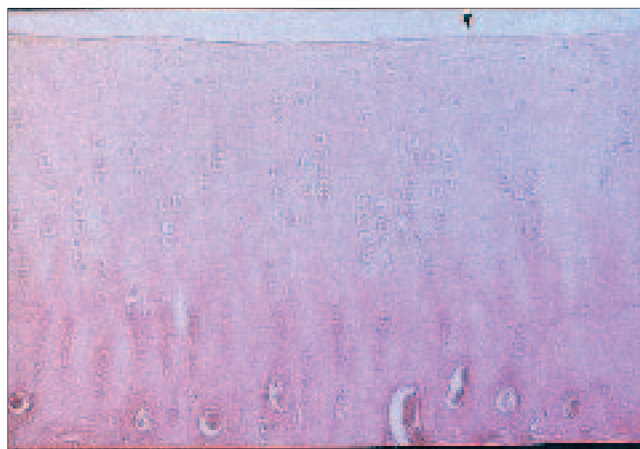

Fig. 3a

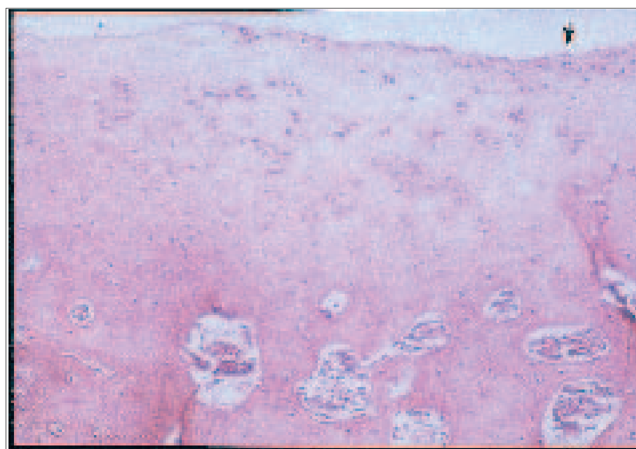

Fig. 3b

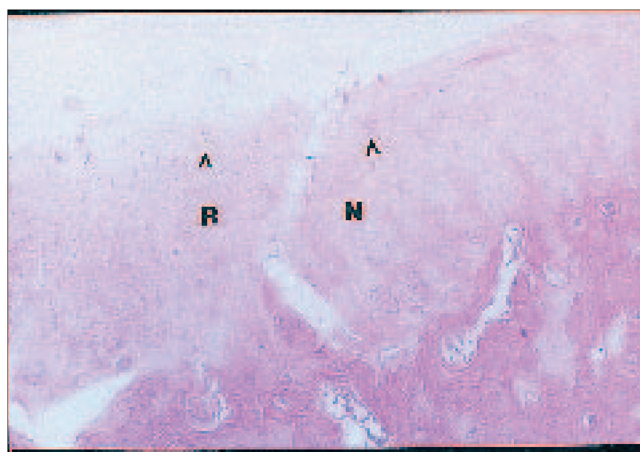

Fig. 3c

Tissue recovered from a 24-week specimen. The arrow indicates the interface between host and repair tissue, with repair tissue to the left of the arrow. Figure 3a – A cell-seeded graft. The repair resembles normal hyaline cartilage, with reconstitution of the tidemark and subchondral bone. Figure 3b – An unseeded graft. The tissue is hypercellular and immature with few cells surrounded by well-defined lacunae. Figure 3c – An empty defect. The surface is fibrillated and chondrocyte clusters are present in native (N) and repair (R) tissue (arrowheads). The repair has failed to bond to native tissue (haematoxylin and eosin ×90).

and appeared to have improved survival over time, neither of which was true of empty defects. Unseeded implants had little hyaline-containing cartilage (Fig. 3b). The score for empty defects declined significantly from 12 weeks ($p < 0.05$) and was lower than the score for unseeded implants at 24 weeks (Table I).

Mechanical testing. Repair tissue is less stiff and more permeable than normal tissue.[30] We measured aggregate modulus (stiffness) and permeability at 12 and 24 weeks. At 12 weeks unseeded specimens had a greater permeability (7.30 ± 6.24 M^4/Ns) than seeded (3.57 ± 1.86 M^4/Ns); both were higher than normal tissue values (1.58 ± 1.10 M^4/Ns). Differences between all groups at 12 weeks were not significant ($p = 0.05$). The same was true at 24 weeks.

At 24 weeks, the permeability of both seeded (1.06 ± 0.37 M^4/Ns) and unseeded (1.93 ± 1.51 M^4/Ns) specimens did not differ significantly from that of normal tissue (1.58 ± 0.95 M^4/Ns). Specimens from empty defects, however, were significantly more permeable than any other group (5.75 ± 0.10 M^4/Ns).

Collagen analysis. The amounts of type-I and type-II collagen were expressed as percentages of normal values and the ratio of type I to type II found in each specimen was also calculated (Table II). Ratios of less than 0.1 probably represent background amounts of type-I collagen.

At six weeks, type-II collagen in seeded and unseeded specimens approached normal values; empty defects contained only 56% of the normal value. The ratio for specimens from empty defects was significantly higher than for other specimens, indicating a greater proportion of type-I collagen in the empty defects.

The percentage of type-II collagen in seeded specimens

Table II. Collagen analysis of articular cartilage repair sites at 6 and 24 weeks

	Type-II content (%)		Type I: type II ratio	
Type	**6 weeks**	**24 weeks**	**6 weeks**	**24 weeks**
Cell-seeded implant	*92.1*	*103.0*	0.1 ± 0.1	0.1 ± 0.0
Unseeded implant	*87.1*	*74.3*	0.2 ± 0.3	0.8 ± 0.4
Empty defect	*56.8*	*20.0*	0.8 ± 0.3	2.4 ± 0.0*
Normal tissue	--	--	0.1 ± 0.1	0.1 ± 0.1

* $p < 0.05$

Table III. Glycosaminoglycan (GAG) content of articular cartilage repair sites

	GAG content (% wet weight of specimen)		GAG content ratio defect/normal tissue	
Type	**6 weeks**	**24 weeks**	**6 weeks**	**24 weeks**
Cell-seeded implant	*3.84 ± 1.40*	*5.44 ± 0.46*	1.02 ± 0.66	1.03 ± 0.13
Unseeded implant	*3.31 ± 1.80*	*4.10 ± 2.35*	0.71 ± 0.35	0.94 ± 0.27
Empty defect	*1.07 ± 0.15**	*1.32 ± 0.40**	0.27 ± 0.05	0.37 ± 0.10
Normal tissue	*4.33 ± 1.22*	*4.44 ± 1.20*	--	--

* $p < 0.05$

at 24 weeks was almost normal; for unseeded specimens, it was 74.3% of normal and in those from empty defects it was 20% of normal. For both empty defects and unseeded specimens the ratio of type-I to type-II collagen was significantly higher than normal, indicating overproduction of type-I collagen.

Glycosaminoglycan content. GAG content was expressed as a percentage of the wet weight of the specimen (Table III). The ratio of the GAG content of repair site tissue to that of normal tissue from the same animal was also calculated. At 6 and 24 weeks there was no statistically significant difference between seeded, unseeded, and normal specimens. Both seeded and unseeded specimens, however, had significantly greater amounts of GAG than those from empty defects. The ratio of the GAG content of repair site tissue to that of normal tissue showed a similar pattern: 1.03 for seeded implant sites, 0.94 for unseeded implant sites, and 0.37 for empty defects.

DISCUSSION

Untreated full-thickness cartilage lesions may undergo partial self-repair, but such repair tissue is typically fibrocartilage which is usually mechanically inadequate and temporary.[8] The structural, biochemical, and mechanical differences between the fibrocartilage and the normal articular surface will cause this neocartilage to erode. In addition, invasion of cells from subchondral bone and from the synovium which produces fibrous tissue may compromise attempts at joint resurfacing that employ chondrocyte transplantation. Our in vitro studies confirm the utility of a barrier between the repair site and the subchondral plate. The dense component of the collagen implant, which also confers shape-retaining and favourable handling properties on the implant, acts as a barrier to fibroblast entry into the transplant from the subchondral bone, while remaining permeable to the humoral factors that may promote healing.

Cell-transplantation techniques may require a period of monolayer culture to expand the number of cells available for transplantation. Chondrocytes in monolayer dedifferentiate and stop secreting GAG and type-II collagen.[24] Cells transplanted in the absence of a three-dimensional cell carrier are thus dedifferentiated cells, such as those used in the human study by Brittberg et al.[11] We have shown that cells cultured in our matrix are indeed phenotypic chondrocytes, which release GAG into the matrix. The use of a carrier may also facilitate retention of transplanted cells at the implantation site.

A number of methods have been developed to determine the proportion of collagen types in various tissues, particularly type-I, type-II and type-III collagens.[28,31,32] These generally involve isolation of the collagen fraction of the extracellular matrix, followed by chromatographic separation of cyanogen-bromide-digested peptides (CB-peptides). They are highly specific and each collagen type isolated to date has its own unique CB-peptide pattern. The work of O'Driscoll et al[28] and Barr et al[31] has shown the high degree of accuracy which can be achieved in determining the ratio of type-I to type-II collagen in articular cartilage. In our study, the histological observations were strongly supported by the analysis of collagen production. Specimens from cell-seeded implants contained nearly-normal amounts of type-II collagen; empty and unseeded defects had at least 25% less than the normal amount of type-II collagen and much greater than normal amounts of type-I collagen. Regenerated tissue formed by unseeded implants, while not as good as that seen with seeded implants, did not show the same sharp deterioration with time as did repair tissue in empty defects.

Freed et al[33] suggested that controlled release of growth factors from implanted scaffolds may improve the quality of repair when such implants are cell-free and subsequently populated by host cells. This has been verified by recent preliminary studies in which collagen fibre matrices containing bone morphogenetic protein but no cells were implanted in vivo.[34,35] The results show early formation of hyaline-appearing cartilage, using implants containing no chondrocytes.

While our six-month results are very promising, examination of the repair at one year and later is necessary to determine the long-term survival of both seeded and unseeded implants and their potential for resurfacing larger defects of articular cartilage.

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