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TISSUE ENGINEERING OF ARTICULAR CARTILAGE: PERICHONDRIAL CELLS IN OSTEOCHONDRAL REPAIR

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Abstract

Efforts to expand treatment options for articular cartilage repair have increasingly focused on the implantation of cell polymer constructs. Primary cells cultured from perichondrium, a chondrogenic tissue, were found to survive *in vitro* within a biodegradable porous polylactic acid matrix. These perichondrocyte-polylactic acid composite grafts were then implanted within osteochondral defects drilled into 3.7 x 5 mm of the left medial femoral condyles of 82 adult New Zealand white rabbits. The repair tissue was evaluated grossly, histologically, histomorphologically, biochemically, and biomechanical-ly at 6 weeks, 12 weeks, 6 months, and 1 year after implantation. On gross evaluation, cartilaginous material appeared to fill the defect in 70 experimental knees, for an overall repair frequency of 85%. None of the specimens were completely normal at 1 year. Only specimens with subchondral bone formation displayed a definable hyaline cartilage appearing surface with chondrocytes surrounded by dense matrix. Subchondral bone reformation was inconsistent, reaching 50% at 1 year. Biochemically, the repair tissue matured during a 1-year period into a hyaline Type II collagen dominant tissue, whereas glycosaminoglycan content remained low at all periods. The measured compressive properties of the repair tissue at 1 year were not significantly different from those of the contralateral knee that was not surgically treated. The treatment of osteochondral defects in the rabbit knee with allogenic perichondrium cell polylactic acid composite grafts yielded a high percentage of grossly successful repairs that showed inconsistent subchondral bone reformation.

Key Words: Tissue engineering, articular cartilage, osteochondral repair, perichondrial cells.

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Introduction

Articular cartilage is composed principally of matrix maintained by a small number of cells dispersed in low density throughout the matrix. The major structural element is type II collagen which entraps proteoglycan, a highly charged molecule that has a high affinity for water. The cells receive their nutrition from synovial fluid that filters in and out of the matrix. Cartilage has no nerve supply (Buckwalter *et al*., 1987a).

When the matrix is disrupted, the classical inflammatory response of vascular invasion and cell deposition does not occur because there is no blood supply (unless the disruption extends into the marrow). Typically, the chondrocytes are too few and are too tightly confined in the matrix to mount a reasonable repair response to any disruption. Lesions of articular cartilage tend to be progressive if present in a biomechanically adverse location, i.e., the weight-bearing surface of the joint (Buckwalter *et al*., 1987b).

Traumatic and degenerative lesions of articular cartilage are a frequent cause of physical disability (Kelsey *et al*., 1978). It is estimated that 22 million people in the United States suffer from some form of arthritis, of which 9.2% are severely disabled by the disease. There are 13 million people who have moderate to severe osteoarthritis, and 5 million people with rheumatoid arthritis. It is estimated that 3.9% of all people over the age of 55 are candidates for total hip replacements, and 0.2% of people between the ages of 35 and 55 are candidates for this procedure. Approximately 90,000-100,000 total hip procedures are performed in the United States every year, as well as approximately 100,000 total knee procedures (Praemer *et al*., 1992).

The wide recognition of the unsuitability of total joint replacements for young, active adults (Chandler *et al*., 1981; Dorr *et al*., 1983) provides the stimulus to search for alternatives in the field of biological resurfacing of joints. Biological resurfacing arthroplasty is used only rarely at the present time, although Brittberg *et al*. (1994) has indicated success with cultured autogenous cartilage cells transplanted into the knee joint of humans.

Current treatment options for clinically significant

full thickness defects include subchondral drilling, osteochondral allograft replacement, and total joint replacement. Because of young age and high activity level, total joint replacement is not an option for many people handicapped by traumatic osteochondral injuries. Even for those who are candidates for joint replacement, problems with reactions to metal and polyethylene wear particles lead to implant loosening and contribute to a variable rate of therapeutic failure (Chandler *et al*., 1981; Dorr *et al*., 1983). Because of the scarcity of cadaver tissue, the risk of infectious disease transmission, and the potential for an immune response limit the usefulness and acceptability of osteochondral allografting (Amiel *et al*., 1989; Beaver *et al*., 1992; Convery *et al*., 1991, 1994; Garrett, 1986). The results of subchondral drilling, like-wise, are unpredictable and unreliable (Beiser and Kanat, 1990; Kim *et al*., 1991). In essence, there are currently no widely applicable and long lasting methods for resurfacing damaged articular cartilage. Thus, development of techniques to stimulate or augment biological healing methods are needed in the quest to restore mobility to damaged articular surfaces.

Tissue engineering: A potential for biological solution?

The search for a biological solution to joint resurfacing has, in recent years, led to a marriage between the life sciences and polymer chemistry (Cima *et al*., 1991; Itay *et al*., 1987; Sah, 1995a; Vacanti *et al*., 1990, 1994). Identification of factors, such as, the presence of a fibrin scaffold, the need for sufficient chondrogenic repair cells, and the as yet insufficiently characterized interplay of growth factors in affecting articular cartilage repair has led to interest in fabricating tissue engineered cartilage healing units of varied composition (Amiel *et al*., 1985; Chu *et al*., 1997; Coutts *et al*., 1992; Grande *et al*., 1989). In general terms, a cartilage healing unit consists of a biocompatible or biodegradable carrier, a chondrogenic cell source, and potentially one or more combinations of growth factors and adhesion molecules. While much earlier work involved the use of biological carriers such as demineralized bone matrix, collagen and fibrin gels, and periosteal and perichondrial tissue grafts (Amiel *et al*., 1988; Billings *et al*., 1990; Coutts *et al*., 1992; von Schroeder *et al*., 1991; Wakitani *et al*., 1989, 1994), there is increasing emphasis on the use of biodegradable polymers, particularly, the alpha hydroxy esters, as carriers for chondrogenic cells, growth factors, and adhesion proteins.

With the use of perichondrium as the chondrogenic cell source and D,D-L,L-polylactic acid as the biodegradable carrier, perichondrocyte polylactic acid (PLA) composite grafts were assessed in preliminary studies (Chu *et al*., 1995a,b). These studies showed that high density perichondrium cell suspensions could be seeded within porous D,D-L,L-PLA (Chu, 1995a). These perichondrium cells remained viable and continued to proliferate within the scaffold through 4 days after cell seeding (Chu, 1995b). On the basis of these results, a long-term study was conducted to evaluate and assess the *in vivo* repair response during a 1 year period after implantation of allogenic perichondrium cell PLA composite grafts into 3.7 x 5 mm osteochondral defects drilled into the medial femoral condyles of 82 adult New Zealand White rabbits. This review describes the tissue engineering of articular cartilage using perichondrial cells in an osteochondral repair. The logistics from the *in vitro* implant preparation to the *in vivo* studies are presented in the communication.

Materials and Methods

In vitro implant preparation

Primary culture: Minced perichondrium obtained from the costal cartilage of 15 adult New Zealand white rabbits was plated on 100 mm diameter tissue culture grade plates (Corning Science Products, Corning, NY), incubated at 37°C in minimum essential media (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum, and allowed to form primary explant cultures. The cultures were grown to near confluence in approximately 3 weeks.

Collagen gene expression: Ten micrograms of RNA extracted using the guanidine thiocyanate-phenol extraction procedure (Chomczynski and Sacchi, 1987) from confluent cultures of perichondrial cells were subjected to agarose gel electrophoresis under denaturing conditions. Following subsequent transfer onto nitrocellulose membranes, the immobilized RNA was probed for the presence of types I and II procollagen messenger RNAs (mRNAs). The probes consisted of ^{32}P-labelled cDNA fragments (specific activity of 1-1.5 x 10^9 counts per minute per microgram of DNA) specific for pro α1(I) collagen mRNA (pCAL1U) and pro α1(II) collagen mRNA (pCAR3) (Sandberg and Vuorio, 1987). The labeled cDNA-mRNA hybrids were visualized by autoradiography. The specificity of the cDNA for their respective target mRNAs was established by probing RNA extracted from primary chondrocytes [pro α1(II) collagen mRNA] and from patellar tendon cells [pro α1(I) collagen mRNA].

PLA core preparation: Cubes measuring 2 x 1 x 1.5 cm that were synthesized from D,D-L,L-PLA (commercially marketed as a dressing material to be used after dental extractions in large animals; ADD Cube, OSMED, Duluth, MN) were prepared into 3.7 mm diameter x 10 mm cylindrical cores using a custom coring device. Five cores were obtained from each cube and placed in a sterile 20 ml scintillation bottle (Fisher Scientific, Pittsburgh, PA).

For electron microscopy, sample cores were prefixed in 4% formaldehyde, fixed in 4% glutaraldehyde, and then washed with 2% OsO_4 in aqueous solution on ice. Cores were then air dried with a desiccant and coated with gold

before observation in a Hitachi S-405A scanning electron microscope (SEM) at magnifications ranging from 30X to 1000X (Hitachi Ltd., Tokyo, Japan).

Cell seeding: Ten to twelve plates of nearly confluent perichondrocyte cultures were released with 0.5% trypsin (Gibco) and counted on a hemocytometer. The initial viability was determined by trypan blue exclusion. The resulting cell suspension was concentrated in fresh media containing 10% fetal bovine serum to between 13 to 16 million viable cells per 1.5 ml of media. The cell suspension was added to 5 sterile, dry PLA cores in a 20 ml scintillation bottle (Fisher Scientific) and rotated at 100 revolutions per minute for 2 hours at room temperature.

Kinetics of attachment: Six to twelve plates of near confluent perichondrocyte cultures were trypsinized and counted on a hemocytometer. The initial viability was determined through trypan blue exclusion. The resulting cell suspension was concentrated in fresh media containing 10% fetal bovine serum (FBS) to between 7-10 million viable cells per 1.5 ml of media. The void volume of the PLA cores was estimated by injecting media into 5 sample cores using a tuberculin syringe with a 25 gauge needle (Becton-Dickinson, Franklin Lakes, NJ) and noting the amount of media absorbed by each core. The cell suspension was then added to 5 sterile dry PLA cores in a 20 ml scintillation bottle (Fisher Scientific) and rotated at 100 revolutions per minute. The number of cells remaining in solution were counted at 5, 20, 50 and 110 minutes using a hemocytometer. This number was then used to calculate the amount of cells absorbed by the PLA cores.

Viability and survivability studies: ***In situ* double stain/confocal microscopy**: Live and dead cells were simultaneously viewed *in situ* using 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein, acetoxy-methylester or BCECF-AM (Molecular Probes, Eugene, OR) and propidium iodide followed by examination through a confocal microscope on Days 1 and 4. BCECF-AM, a fluorescein derivative that is metabolized to a fluorescent product by non-specific esterases present in viable cells, was used to stain live cells *in situ*. Propidium iodide, a nucleic acid stain, which is excluded by intact cell membranes, enabled the nuclei of dead cells to be seen. Cores were prepared into 1 mm slices and studied using a confocal microscopy (Biorad Laboritories, Richmond, CA; and Meridian Instruments, Inc., Okemos, MI).

Viability and survivability studies: **Thymidine uptake**: Cell seeded cores were labeled for 18 hours at either 4 or 37°C with ^{3}H-thymidine (2 μCi/ml) in media supplemented with 10% FBS on Days 2-5.

***In vivo* studies**

Surgical model: Eighty-two New Zealand white rabbits, 9 to 12 months old and weighing 3.7 to 4.4 kg, were used as experimental animals. Fifteen additional rabbits of the same ages and weights served as non-surviving donors of rib perichondrium that was harvested aseptically 1 month before each scheduled surgery. Allogenic tissue was used to minimize surgical interventions on experimental animals. Experimental animals were given an intramuscular injection of ketamine (100 mg/kg) and xylazine (8 mg/kg).

A medial parapatellar incision was made on the left knee and carried through the medial capsule. The patella was dislocated laterally until the medial femoral condyle was exposed. Surgical drill bits measuring 1, 2.5, and 3.7 mm were used sequentially to create a 3.7 mm diameter defect that was centered on the condyle and penetrated to a depth of 5 mm. This hole was drilled as far posteriorly on the condyle as possible with the knee in maximum flexion. This area is intermittently weight-bearing in the rabbit and contacts the tibial surface when the knee is in 70° flexion. In virtually all animals, the hole extended across the width of the condyle. The perichondrocyte PLA composite core was then divided into halves transaxially and one-half of a core (3.7 mm diameter x 5 mm) was implanted in each experimental knee. The core was press fit into the defect and trimmed with a scalpel to match the height of the adjacent cartilage. The joint capsule and skin were closed and the animals were returned to unlimited cage activity (1 x 0.75 m cages). Before surgery, the animals had been assigned randomly to 1 of 4 sacrifice dates (6 weeks, 12 weeks, 6 months, or 1 year after implantation) and to 1 of 3 post-sacrifice analysis groups (histology, biochemistry, or biomechanics). The gross morphology of all 82 specimens was assessed at the time of sacrifice.

Gross morphology: The biologic acceptability of the repair was determined during gross examination in accordance with the criteria set forth in previous studies (Amiel *et al*., 1985, 1988; Billings *et al*., 1990; Coutts *et al*., 1992). Biologically acceptable repairs were defined as smooth, firm repair tissue that filled the defect. The specimens were photographed and prepared for additional analysis (histologic, biochemical, or biomechanical) according to their preassigned designations. The biologically unacceptable specimens were excluded from additional study.

Histologic study: The right and left medial femoral condyles from the experimental animals assigned to histologic study were fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride for 1 week. The specimens were then decalcified in disodium ethylenediamine tetraacetate, embedded in paraffin, and cut along the midsagittal plane. Sections closest to the midsagittal plane were stained with hematoxylin and eosin for the study of morphologic detail and with safranin O and fast green to assess glycosaminoglycan distribution.

Histomorphometric analysis: Histologic sections were analyzed using an image analysis system and custom-

ized software according to previously described methods (Yoshioka *et al*., 1996). The specific parameters measured included repair height, root mean square roughness, percent repair, and repair attachment. The repair height is the mean of the repair cartilage thickness, from the ossification front to the top of the repair, at 5 different points along the defect site of the histologic section. Root mean square roughness is a quantitative measure of the deviation of the repair site surface profile from an idealized surface (Hacker *et al*., 1997). Percent repair measures the amount of repair tissue that fills an idealized cross sectional profile of the cartilage defect area. The measurement of repair attachment quantifies the amount of integration the repair makes with the surrounding normal cartilage and subchondral bone. Attachment is expressed as a percentage of the length of the subchondral and peripheral boundaries (Hacker *et al*., 1997). One section from the midsagittal plane was analyzed by 3 different investigators.

Biochemical analysis: After the animals were sacrificed, the repair tissue was dissected from the defect with a scalpel, taking care not to include any grossly evident bone. The relative amounts of Types I and II collagen comprising the repair tissue were determined by gel filtration high performance liquid chromatography of tissue aliquots after cyanogen bromide (CNBr) digestion according to previously published methods (Amiel *et al*., 1989). Marker CNBr peptides characteristic of collagen Types I and II were established from preparations of purified Type I collagen (patellar tendon) and Type II collagen (hyaline cartilage). Relative amounts of the 2 collagen types were determined by integrating the absorbance peaks at 220 nm of the respective marker peptides; α1(I) - CB7 and α1(I) - CB8 for Type I collagen, and α1(II) - CB10 for Type II collagen. Total glycosaminoglycan (GAG) was calculated by measuring hexosamine concentration.

Biomechanical analysis: The linear biphasic theory has been used to model the behavior of cartilage during static and dynamic compression at small strain (_ 25%) (Mow *et al*., 1980, 1984, 1991). The mechanical behavior of cartilage under confined compression conditions is described by the equilibrium confined compression modulus, whereas the interaction of the solid and the fluid is described by the hydraulic permeability. Osteochondral cores, 4 mm in diameter, were isolated from the repair tissue and contralateral control knees using a custom coring device. The cartilage thickness was measured by reflected light microscopy. The samples were placed in a phosphate buffered saline bath within a confined compression apparatus with the articular surface against a stainless steel filter and the subchondral bone supported on a rigid flat disc. The mechanical properties of the specimen were then tested. A load of 0.2 N was applied through the disc, and the sample was allowed to equilibrate for 1000 seconds. An additional load of 0.2 N was applied, and the displacement was measured during the next 1000 seconds. The data were fit to the biphasic theory (Mow *et al*., 1980) to determine modulus and permeability.

Statistical analysis: Results from the histomorphometric studies were evaluated using unpaired t-test. Results from collagen typing were expressed as relative percentages of Types I and II collagen with published properties of normal perichondrium provided for reference (Amiel *et al*., 1988). Results from glycosaminoglycan content and biomechanical evaluations were compared with that of the contralateral knee that was not operated on and analyzed statistically using the paired t-test setting $\alpha = 0.05$.

Results

In vitro studies

PLA cores: Randomly sized interspaces combined with a variegated surface architecture were observed by SEM (Fig. 1a). Sample PLA cores absorbed 100 µl of medium, exhibiting a porosity of 93%.

Viability studies: ***In situ* double stain/confocal microscopy**: After double staining of cell seeded cores, numerous round, BCECF-AM stained, green fluorescing cells were seen within the PLA interspaces on Day 1 in both surface and central regions of the cell seeded cores. By Day 4, many cells had flattened and appeared to outline the pores of the PLA matrix (Fig. 1b). Occasional propidium iodide staining nuclei were seen in all specimens. Ninety images were collected at 1.25 µm intervals using a 20x objective. Angle of separation between left and right image is 2.5°.

Viability studies: **Thymidine uptake**: Thymidine uptake increased from 1000 counts per minute (cpm) to 4500 cpm between Days 2 through 5 in the cell seeded cores incubated at 37°C. Cell seeded control cores incubated at 4°C and subjected to the same thymidine labeling process exhibited only baseline levels of radioactivity (Fig. 2). Perichondrocyte-PLA composites sectioned into 1 mm pieces and placed in fresh medium explanted into first passage cultures which grew to near confluence in 2 weeks.

Kinetics of attachment: Sample PLA cores were found to absorb 100 µl of media. For PLA cores seeded with perichondrocytes, virtually complete absorption of up to 2 million cells per 100 µl of PLA void volume occurred by 110 minutes (Fig. 3). Cell absorption was rapid, with the steepest uptake into the cores (80%) within the first 5 minutes. Within 50 minutes, 94% of cell absorption was seen, then a plateau curve was obtained for the remaining of the kinetic attachment.

Primary culture collagen gene expression: Prima-

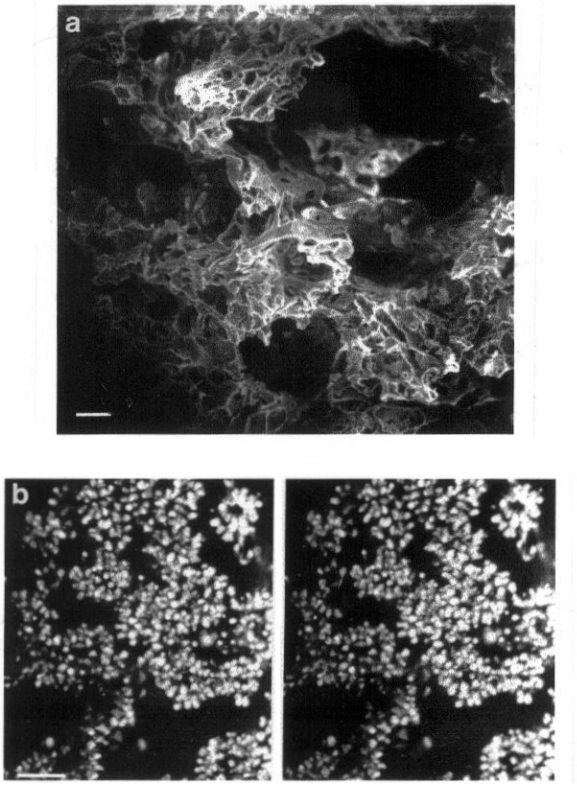

Figure 1. Porous polylactic acid cores before and after cell seeding. (**a**) This electron micrograph of a porous polylactic acid core before cell seeding demonstrates the large surface area available for cell attachment. Note the randomly sized pores. Bar = 318.7 μm. (**b**) Stereo images of perichondrocytes grown on polylactic acid (PLA) for 4 days after cell seeding. Live cells were stained with BCECF-AM as described under **Materials and Methods**. Ninety images were collected at 1.25 μm intervals using a 20x objective. Angle of separation between left and right image is 2.5°. Scale base = 100 μm. Reproduced from Chu *et al*. (1995b) with permission.

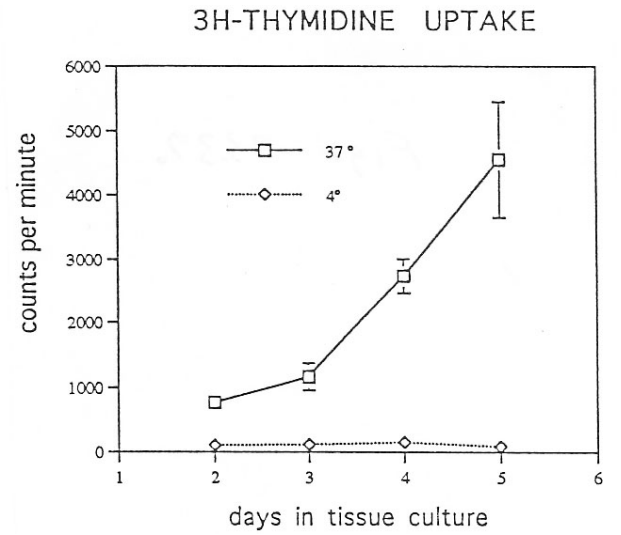

Figure 2. Thymidine uptake increased from 1000 to 4500 cpm between Days 2 and 5 in the cell-seeded cores incubated at 37°C. Cell-seeded control cores incubated at 4°C and subjected to the same thymidine labeling process exhibited only baseline levels of radioactivity. Reproduced from Chu *et al*. (1995b) with permission.

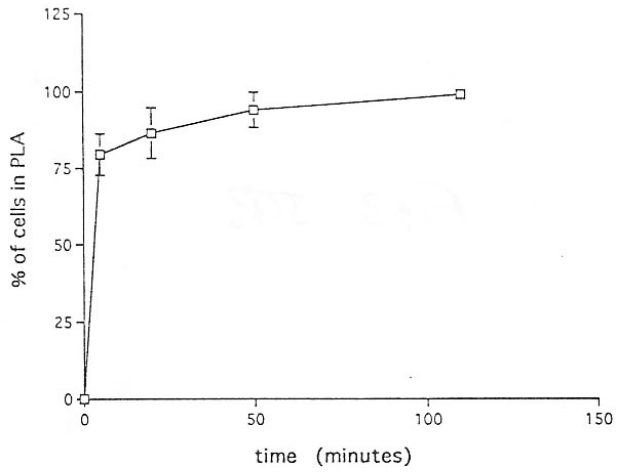

Figure 3. Absorption kinetics of perichondrocytes into cores prepared from porous PLA. Reproduced from Chu *et al*. (1995a) with permission.

ry perichondrial explant cultures grew to approximately 85% confluence within 21 days. Results of northern blot analysis revealed that these cultures expressed primarily pro α1(I) collagen mRNA with a much smaller signal for pro α1(II) collagen mRNA. The cDNA probes proved specific for their intended target mRNA, as no cross-hybridization was observed in primary tendon or primary chondrocyte cultures (Fig. 4).

***In vivo* studies**

Gross morphology: Biologically acceptable repairs were shown by 94% of the 6-week specimens. The repair site appeared completely filled with a white, firm cartilaginous tissue that was flatter (less convex curvature) than the surrounding cartilage. The percentages of biologically acceptable repairs were: 75% at 12 weeks, 86% at 6 months, and 90% at 1 year. The overall repair frequency was 85% (70 of 82 specimens had biologically acceptable repairs; the 12 biologically unacceptable specimens were excluded from additional analysis). The white repair tissue seen at 6 weeks

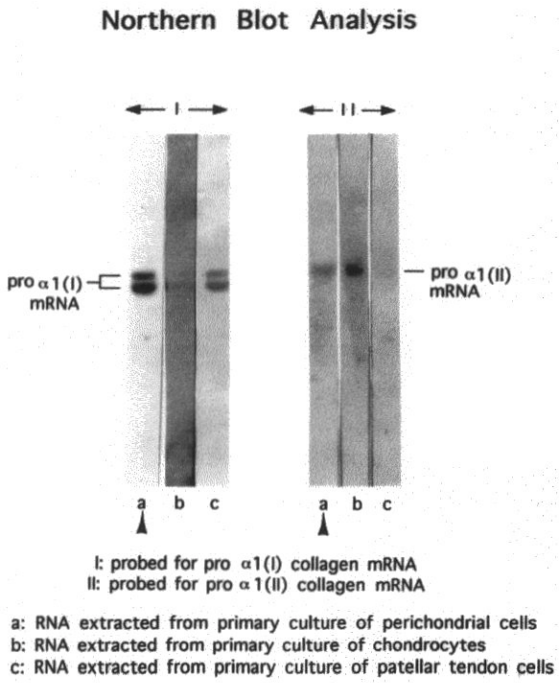

Figure 4. Northern blot analysis of the procollagen mRNA extracted from confluent primary perichondrocyte explant cultures (arrowhead) reveals a stronger signal for pro α1(I) collagen mRNA than pro α1(II) collagen mRNA. Reproduced from Chu *et al.* (1995a) with permission.

(Fig. 5b) matured into a firm, smooth neocartilage that appeared similar in color to the surrounding cartilage by 1 year after implantation (Fig. 5d). Little difference was observed when we compared the gross appearance of the 1 year repair (Fig. 5d) to the contralateral normal cartilage (Figs. 5a and 5c).

Histologic analysis: At 6 and 12 weeks after implantation, safranin O stained sections revealed chondrocyte-like cells surrounded by glycosaminoglycan containing matrix in the upper regions of the repair tissue. The cartilaginous tissue appeared to terminate in a region of the defect that corresponded to the area of subchondral bone in a normal condyle.

Safranin O staining of a 6-week repair reveals a concentration of glycosaminoglycan in the superficial regions of the repair tissue. The cartilage region of the repair consists of the portion of the repair extending from the metachromatic matrix-containing region to the articular surface. The neocartilage appears thicker than the adjacent cartilage and is slightly depressed (Fig. 6a). Hematoxylin and eosin-stained section also details nests of chondrocyte-like cells surrounded by safranin O staining metachromatic matrix (Fig. 6b).

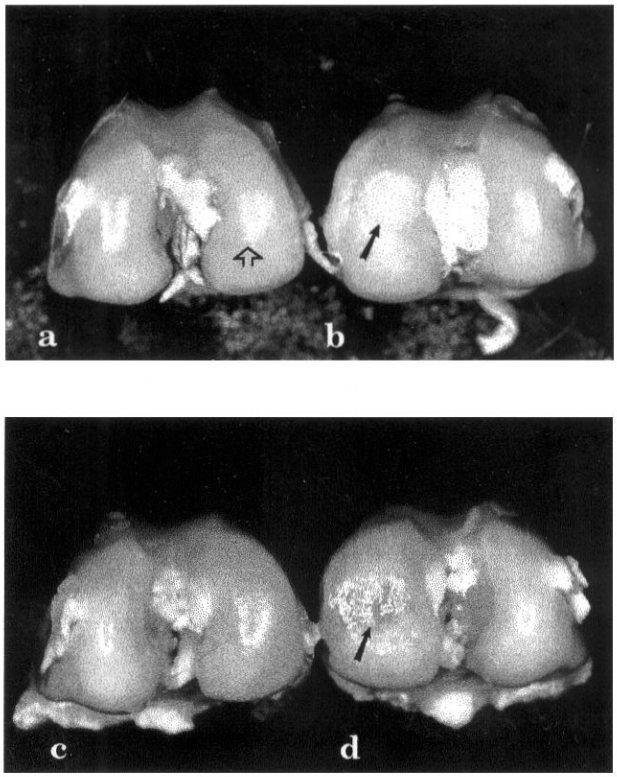

Figure 5. Cartilage regeneration. (**a**) 6 week unoperated right medial femoral condyle (open arrow) serves as a normal model. (**b**) 6 week post implantation (arrow) with smooth white neocartilage filling the defect. Reproduced from Chu *et al.* (1995a) with permission. (**c**) 1 year unoperated femoral condyle (left untreated knee). (**d**) 1 year after implantation the repair tissue (arrow) was similar in color and texture to the surrounding cartilage. Reproduced from Chu *et al.* (1997) with permission.

The histologic appearances of the 3 month, 6 month, and 1 year specimens were variable. Specimens with subchondral bone reformation had smoother surface profiles, near normal cartilage thickness, and chondrocyte-like cells within a dense matrix (Figs. 6c and 6e). In contrast, the poor specimens had a convoluted surface profile, irregular repair tissue appearance, poor restoration of the subchondral bone, and lacked osseous repair tissue centrally (Fig. 6d). At no time period was there any inflammatory response to the PLA scaffold, nor did there appear to be any adverse reaction by the surrounding or replacing tissues to the presence of the PLA. The PLA was absorbed by the 6-month time period.

Histomorphometric analysis: Analysis showed that the thickness of the repair of the articular surface gradually decreased with time. At 6 weeks, the repair height was 0.86 ± 0.21 mm; at 12 weeks, 0.89 ± 0.42 mm; at 26 weeks, 0.32 ±

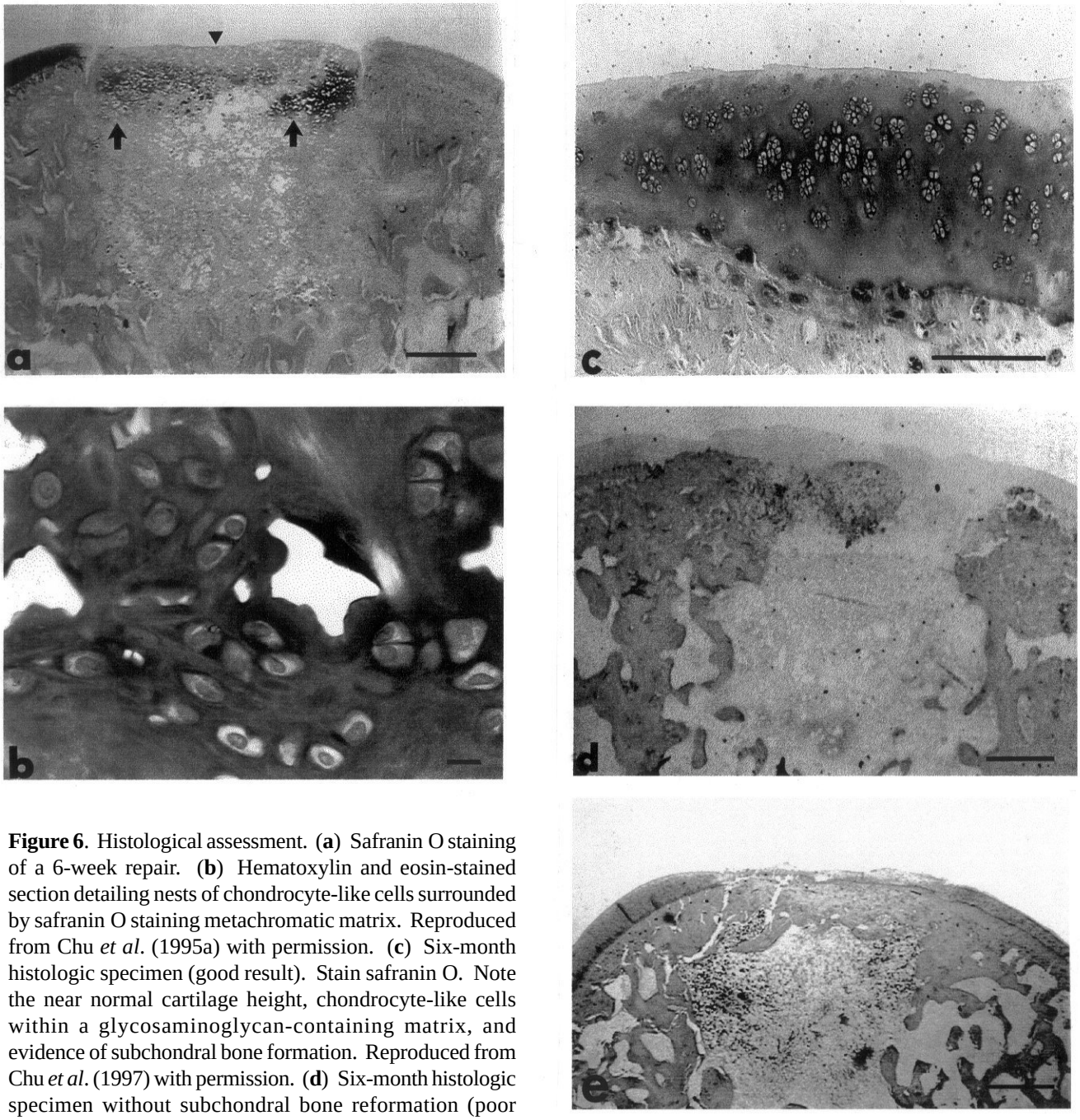

Figure 6. Histological assessment. (**a**) Safranin O staining of a 6-week repair. (**b**) Hematoxylin and eosin-stained section detailing nests of chondrocyte-like cells surrounded by safranin O staining metachromatic matrix. Reproduced from Chu *et al*. (1995a) with permission. (**c**) Six-month histologic specimen (good result). Stain safranin O. Note the near normal cartilage height, chondrocyte-like cells within a glycosaminoglycan-containing matrix, and evidence of subchondral bone formation. Reproduced from Chu *et al*. (1997) with permission. (**d**) Six-month histologic specimen without subchondral bone reformation (poor result). Stain safranin O. Note the convoluted surface profile, poor glycosaminoglycan staining at the surface of the repair tissue, and absence of a reconstituted tidemark. Reproduced from Chu *et al*. (1997) with permission. (**e**) Twelve-month histologic specimen (good result) staining hematoxylin and eosin. The peripheral and subchondral attachment measurements were 100% and 98%, respectively, with the adjacent tissue. Bars = 1000 μm (a and d), 500 μm (c and e), and 10 μm (b).

0.10 mm; and at 52 weeks, 0.44 ± 0.19 mm. The unoperated control height of normal cartilage was found to be 0.29 ± 0.06 mm. The percent repair gradually decreased from 12 weeks post-surgically to 52 weeks. The percent repair at 6 weeks was 59 ± 44; at 12 weeks, 95 ± 86; at 26 weeks, 65 ± 69; and at 52 weeks, 29 ± 17 (Fig. 7a). Subchondral bone formation was inconsistent, reaching 50% at 1 year (Fig. 7a).

The roughness of the osteochondral repair increased with time: 0.26 ± 0.16 mm at 6 weeks, compared with 0.35 ±

0.19 mm at 52 weeks. Unoperated joints had a roughness of 0.06 ± 0.05 mm (Fig. 7b). The percent of peripheral attachment and subchondral attachment for all time periods are described in Table 1. Note that at 1 year post implantation 91.2 ± 22.4% and 56.8 ± 47.6% described these respective percents ± standard deviation.

Biochemical findings: Analysis of the repair tissue formed after 6 weeks of implantation revealed that the relative proportion of type I and type II collagen was 81% and 19%, respectively. This ratio was reversed after 1 year of implantation with 82% Type II collagen and 18% Type I collagen. The articular cartilage of unoperated knees contained greater than 95% Type II collagen. Priority was given to collagen type analysis. In several animals, most notably at 12 weeks, there was insufficient tissue remaining after collagen typing to determine glycosaminoglycan content. For the specimens measured, the matrix glycosaminoglycan content was significantly less than that of the unoperated knees at all time periods. Although the repair tissue hexosamine content increased from 11.5 ± 1.3 mg hexosamine per gm of dry tissue at 6 weeks to 22.3 ± 3.1 mg hexosamine per gm dry tissue at 1 year, this represented only 55% of the 40.6 ± 5.1 mg hexosamine per gram of dry tissue found in the unoperated knees.

Biomechanical findings: The apparent hydraulic permeability of the repair tissue was very high at 6 weeks ($27.2 \pm 16.2 \times 10^{-15}$ m^2/Pascal·second compared with $3.6 \pm 1.7 \times 10^{-15}$ m^2/Pascal·second for the unoperated knees $p = 0.03$) but normalized at 12, 26 and 52 weeks ($4.0 \pm 2.1 \times 10^{-15}$ m^2/Pascal·second, $3.9 \pm 2.2 \times 10^{-15}$ m^2/Pascal·second, and $3.6 \pm 2.3 \times 10^{-15}$ m^2/Pascal·second, respectively). At all time periods, the apparent confined compression modulus of the repair tissue was similar to that of the unoperated knees (0.28 ± 0.11 MPa). In previous studies of macroscopically normal control cartilage cores with a diameter of 1.8 mm (Sah *et al*., 1995b, 1997) which provided a relatively flat articular surface that conformed well to the compression platen (compared to the 4 mm diameter samples tested here), the permeability ($0.64 \pm 0.35 \times 10^{-15}$ m^2/Pascal·second) was lower, and the modulus (0.61 ± 0.21 MPa) was higher than the values for both the experimental and contralateral control samples tested here.

Discussion

These results indicate that cells derived from perichondrial explant cultures are capable of attaching to and surviving within a porous D,D-L,L- PLA scaffold, which, in turn, becomes a tissue-engineered device for delivering reparative cells into an osteochondral defect. The novel application of a fluorescent viability stain protocol to cell-polymer constructs demonstrated that the perichondrocytes seeded within the polymer carrier were alive. After a 6-week implantation of this composite graft, early repair tissue formed in a significantly higher percentage of rabbits (96%, $p = 0.002$) than in previous studies using perichondrial grafts (Amiel *et al*., 1985, 1988; Coutts *et al*., 1992). This uniform filling of an osteochondral defect with a grossly cartilaginous-appearing tissue in nearly all experimental animals receiving perichondrocyte-PLA composite grafts represents an improved level of consistency in articular cartilage repair.

Histological and biochemical evaluation also revealed the presence of chondrocyte-like cells surrounded by matrix-containing cartilage defining components. The cellular alignment mimicked that of the scaffold, giving the neocartilage a porous appearance. The influence of scaffold architecture on the resulting tissue is readily seen in both the cell seeded PLA prior to implantation and in the repair tissue. This finding suggests that a modification in scaffold architecture to approximate that of articular cartilage may result in a neocartilage which is morphologically more similar to the native tissue (such as, having vertically oriented fibers for the cartilage component of the implant). The reproduction of organ level functions in structurally sophisticated tissues, such as, cartilage and liver may depend on the development of appropriate polymer architectures (Cima *et al*., 1991).

The porous architecture of the PLA surgical dressing also provides a large surface area for cell attachment. Nearly complete uptake of up to two million cells per 100 µl of scaffold volume within 2 hours suggests that porous D,D-L,L-PLA is a suitable carrier for high density cell suspensions. This suitability likely stems from both architectural and physicochemical considerations.

Unlike homopolymers of L(+)polylactic acid, which are crystalline in nature, D,D-L,L-PLA is amorphous (Kulkarni *et al*., 1971; Therin *et al*., 1992). While the methyl groups in PLA represent hydrophobic domains for both amorphous and crystalline PLA, the crystalline forms are more hydrophobic and more resistant to hydrolysis (Kulkarni *et al*., 1971; Pistner *et al*., 1993; Therin *et al*., 1992). Unlike cell seeding studies involving foams made of poly(L)lactic acid (Elgendy *et al*., 1993; Freed *et al*., 1993), prewetting was not necessary for this D,D-L,L-PLA dressing. It was, therefore, possible to seed the prepared cores dry. Initial cell entry into the scaffold appeared facilitated by the resulting capillary action. Confocal microscopy demonstrated penetration of cells into the interior of the scaffold using this method.

Following implantation, gross and histological analysis demonstrated the formation of a hyaline neocartilage in the cartilage regions of the repair. At the earliest time period of 6 weeks, the gross appearance of 96% of the defects were characterized as completely filled with a

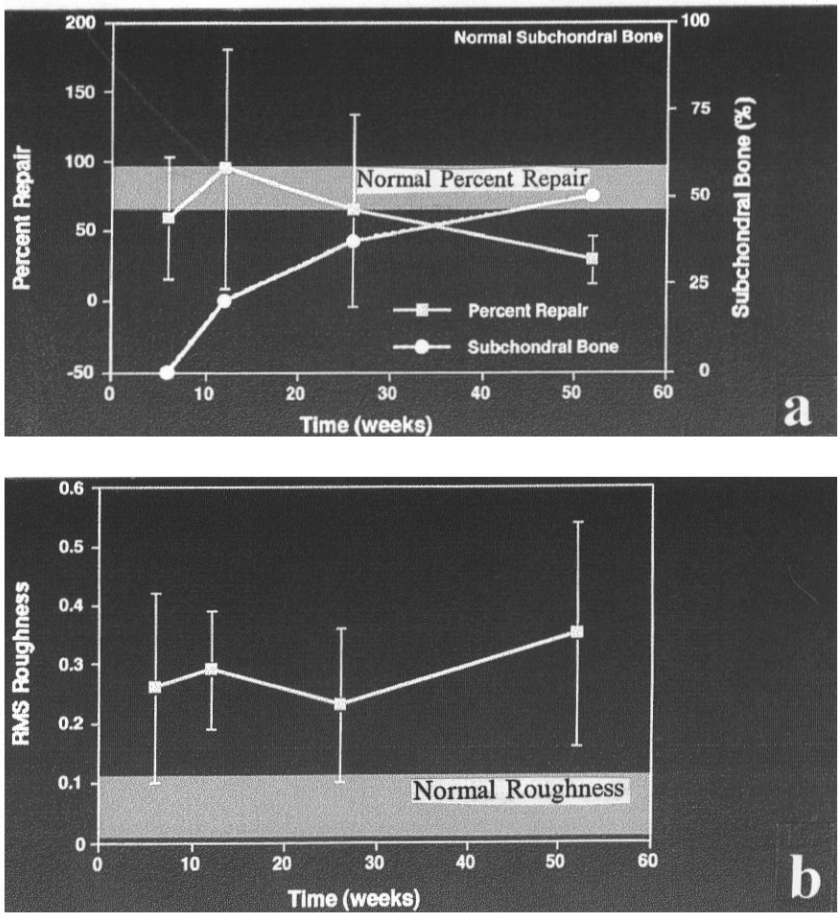

Figure 7. Histomorphometric results for allogeneic cells with PLA. The data at each time periods represent a mean average of 5 animals ± standard deviation. (**a**) Percent repair (squares) and the percent of subchondral bone formation (circles) at different time periods. The white band in the middle represents normal percent repair. (**b**) Root mean square (RMS) roughness in mm of the articular repair from 6 weeks to 52 weeks; note the increased roughness with time of post-surgical repair. The white band at the bottom represents normal roughness.

cartilaginous tissue. At one year, 90% of the specimens continued to demonstrate successful repairs by gross inspection. Biochemically, the immature repair tissue contained predominantly type I collagen. The proportion of Type II collagen increased with time, suggesting that the synovial environment stimulated repaired cells to differentiate along a chondrocyte lineage to form hyaline cartilage.

Taken in isolation, these results seemed to herald a breakthrough in articular cartilage research. In human studies, thorough multidisciplinary analysis of repair tissue characteristics beyond gross inspection through arthroscopy or arthrotomy cannot realistically be performed (Beiser and Kanat, 1990; Brittberg *et al*., 1994; Kim *et al*., 1991). In contrast, histological, histomorphometric, biochemical, and biomechanical analysis of the neocartilage in this long-term animal study presents a more complete picture than gross assessment alone.

Histologic results at the 12 week, 6 month and 1 year periods were variable, with none of the specimens appearing completely normal at 1 year. Although, some specimens exhibited near normal histologic structure, the absence of subchondral bone restoration in several other specimens was associated with reduced metachromatic staining and increased surface fibrillation. These findings suggest that healthy subchondral bone is important to the ultimate survival of articular cartilage. Efforts to enhance restoration of the subchondral bone may play an important role in future efforts to repair full thickness articular cartilage lesions.

The inconsistent restoration of the subchondral bone raises concerns about the immunologic acceptance by the host of an allogenic perichondrocyte PLA graft. A host immune response to the allogenic cells may have adversely affected the repair process in the poorer specimens. This supposition is supported by several previous studies. Kawabe and Yoshinao (1991) transplanted allogenic chondrocytes into defects in the rabbit knee and assessed the host immune response to these grafts. They demonstrated that host cell mediated cytotoxicity resulted in degeneration of the implanted cartilage (Kawabe and Yoshinao, 1991). Noguchi *et al*. (1994) compared the transplantation of isogeneic and allogenic chondrocytes. The allogenic group had an increased inflammatory cell infiltrate and a delay in subchondral bone formation.

Degradation of the PLA matrix has been shown in some studies to potentially adversely affect healing of bone voids (Lovell *et al*., 1989; Miyamoto *et al*., 1992). PLA is available in several different forms, each with different rates of degradation (Miyamoto *et al*., 1992). The porous D,D-L,L-PLA used in this study appeared to degrade over a 12-week period, correlating with an observed decrease in the gross repair frequency from 96% to 75%. While improvement of the gross repair frequencies to 86% and 90% at 6 months and 1 year, respectively, after surgery were observed, carrying the experiment to these later time points provided crucial information concerning the underlying quality of the repair tissue. The progressive decrease in the area of the idealized repair occupied by hyaline neocartilage from 95% at 12 weeks, to 65% at 6 months, and to only 29% at 1 year, coupled with the persistently low glycosaminoglycan content, suggest that the neocartilage degraded over time.

Assessment of hydraulic permeability and compressive modulus by confined compression testing of 4 mm diameter samples showed no significant difference between the repair tissue and normal cartilage after the 12 week time frame. These parameters appear less sensitive than the histologic and biochemical data which describe a more gradual and less complete process of repair tissue maturation. It is known that the compressive properties of bovine and human cartilage vary greatly with depth from the articular surface (Schinagl *et al*., 1996; Schneiderman *et al*., 1986). Thus, the assessment of repair tissue mechanical properties by traditional methods assuming homogeneous material properties may not clearly portray a repair process. In addition, and perhaps most important, mechanical testing could also have been complicated by the small radius of curvature of the rabbit femoral condyle and the altered tissue geometry inherent to a repair process (Sah *et al*., 1995b). The apparent modulus and permeability with the 4 mm diameter test specimens were markedly different than that for the more ideal (flat) 1.8 mm diameter specimens (Sah *et al*., 1997).

Potential explanations for the apparent degradation of repair tissue quality obtained in this long-term multidisciplinary study include adverse effects from the relative size and depth of the defects to that of the rabbit condyle, immunogenicity from allograft cells, adverse effect of polymer degradation, and cartilage degradation associated with inconsistent subchondral bone reformation.

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Discussion with Reviewers

J.A. Buckwalter: There is no control group. The absence of simultaneous control group of untreated osteochondral defects makes it difficult to know, using the authors' measures of outcomes, whether the graft actually represented an improvement over the natural repair process and whether or not grafts inhibited reformation of subchondral bone. Previous reports of the osteochondral repair process in experimental defects in rabbit knee suggest that a vast majority of these do reform a subchondral bone matrix. Thus, it would be of considerable interest and importance to have a simultaneous control group for the perichondral polylactic acid grafts.

G.M. Lee: Were there any control studies done where the deep hole was left unfilled or filled with a polylactic acid core without added cells? If not, how does one control for repair due to infiltration of mesenchymal cells?

Authors: As stated in our **Introduction**, the interest in fabricating tissue engineered cartilage units of varied composition was led by the fact that there are currently no widely applicable and long-lasting methods for resurfacing damaged articular cartilage. Amiel *et al*. (1985) published that a defect in the femoral condyle of the rabbit knee (0.4 cm in diameter; 0.8 cm in depth), where the bone core was denuded of cartilage, did not fill at 6 and 12 weeks post surgery unless a perichondrial graft was used. Also, Shapiro *et al*. (1993) reported on the full thickness defects of articular cartilage in the rabbit knee, and stated that degenerative changes were not evident until approximately 12 weeks after surgery. The progressive decrease in the area of the idealized repair occupied by neocartilage was demonstrated from 95% at 12 weeks, to 65% at 6 months, and to only 29% at 1 year. Also, our group (von Schroeder *et al*., 1991) has shown that after 12 weeks, a defect in the rabbit condyle of 3.7 mm diameter by 5 mm depth filled with PLA alone gave the following results: Histologically, small amounts of PLA remained under the neocartilage with the majority being replaced by bone. Biochemically, at 12 weeks post surgery, there was more type II collagen in the grafted knee (83%) than in the PLA alone (65%). At one year, most of the neocartilage of the PLA alone contained mainly type I collagen.

C.T. Laurencin: The authors biomechanically evaluated the healing graft using creep testing and biphasic analysis. Why was this method of testing chosen to evaluate the polymer/cartilage samples over static compression testing? Would the authors expect different results had static compression testing been used instead?

Authors: The data from the creep test is extrapolated by the curve fitting procedure to determine both the equilibrium modulus and the hydraulic permeability. Thus, the equilibrium modulus would likely be similar, whether dynamic or static mechanical data are used.

C.T. Laurencin: The authors proposed that the inconsistency seen in subchondral bone formation may be due to an immunologic response to the allogenic cells or the release of acidic by-products due to polymer degradation. It appears that these factors would elicit an all or none response and would not result in variability between samples. Do the authors have any other theories as to why bone formation was variable? Could it be linked to placement of the graft on the articulating surface? If the grafts were not placed in the exact same site, those grafts in areas of increased loads would show an increase in bone formation. Please comment.

Authors: The release of acidic byproducts or an immune response to allogeneic cells would not necessarily be an all-or-none type of response. There could be a variable amount of acid by-product from an implant, and there could be a variable response by the host to these factors. Of course, there may be other factors at play, such as, the age

of the animal or the repair response elicited by the drilling and PLA implantation. There could be variation in the nutrition to the repair site, and it could be affected by the presence of synovial fluid reaching the subchondral plate. There could be a variation in the mechanical factors acting on the implant, depending on activity of the animal on the location of the implant, however, the size of the implant fills the width of the rabbit femoral condyle, therefore, there is little variability in the centering of the holes. There was some variability in the location of the holes vis a vis the point in the flexion arc on the condyles. The knees were flexed maximally and the holes placed as far posteriorly as possible, since the rabbits rest with their knees in flexion, and it was felt desirable to get as close to the resting contact area as possible. Different rabbits exhibited different degrees of knee flexibility, so the point in the flexion arc probably did exist. In reality, however, we do not know exactly why the subchondral bone did not reform.

G.M. Lee: How does the depth and diameter of the hole drilled for the described implant experiments compare with the holes drilled through the subchondral bone in order to stimulate repair in human joints? Please respond in terms relative to the different sized joints, i.e., in terms of percent penetration of the subchondral bone and marrow?
Authors: There is really very little similarity between the defect created for these experiments and the multiple drill holes used in the treatment of human lesions. The single large hole used in the rabbits is designed to create a containment region for the implant, securing it to the area of desired repair. It does create access to the marrow and its source of pluripotential cells that can facilitate a repair process. But this feature is desired only to the extent that it would facilitate the repair of the bone. The cartilage repair should be effected by the perichondrial cells in the PLA plug.

Multiple drill holes through the subchondral plate are used to try to repair defects in human articular cartilage. The purpose of these holes is to allow cells to migrate from the marrow into a clot that forms from the bleeding induced by these drill holes. The depth of the holes are the same in this instance, as they are for the repair model that was utilized in the described experiments. The holes in the human treatment are proportionately much smaller in diameter than the single large hole and, in fact, the multiple holes in aggregate represent a much smaller area relative to the size of the condyle than the rabbit model.

G.M. Lee: What was the rationale for using a model with an osteochondral defect instead of a chondral defect?
Authors: The rationale for using an osteochondral model in the rabbit is related to the issue of implant fixation. Our initial studies on cartilage repair utilized a chondral defect with perichondrium sutured over the defect. Excellent cartilage growth could be obtained, but attachment of the repair tissue to the underlying bone proved problematic. We have subsequently focused considerable attention on the issue of implant fixation and repair cartilage attachment, which has resulted in the development of the osteochondral model. The hole in the bone serves the primary purpose of holding the implant while the cells grow the repair tissue. In the rabbit, the articular cartilage is only 300 μm thick, which presents a serious obstacle to the use of a chondral defect. There is not enough cartilage thickness available to hold an implant in place by press-fit, and we have not found any glues capable of adequately securing an implant. Suturing to this thin cartilage is extremely difficult so, although it would be desirable at some time to evaluate cartilage repair in a chondral model, it will be necessary to go to a larger animal with thicker cartilage to do this.

E.B. Hunziker: Could this material also be used for inducing repair of shallow full-thickness defects or partial thickness defects?
Authors: Please refer to the above answer to the question posed by Dr. Lee. The technology being developed for this engineered cartilage repair tissue could be modified to deal with a shallow lesion. But as noted above, there will need to be a method for obtaining fixation of the implant to the defect: either press-fit to the surrounding cartilage, glue, or suture method would have to be employed to keep the implant in place. There is clearly a need for this type of repair, and it is anticipated that future studies in large animals will be performed once the osteochondral repair has been developed.

E.B. Hunziker: Do the authors have any indication that polylactate degradation products may have an inhibitory effect on the formation of subchondral bone tissue?
Authors: It could be that the lactic acid released by the breakdown of the polylactic acid could inhibit this process of bone repair. However, the histology has never shown absence of tissue in the holes, only failure of the subchondral bone plate to reestablish itself in the proper location.

E.B. Hunziker: What is the role of the bone marrow derived blood imbibing the cell-matrix-composite at the time of surgery? Is it needed as a stimulatory component for successful repair?
Authors: In our experimental model, the bone marrow derived blood cells have no role proposed. The bone marrow derived blood cells could have a stimulatory component, but this has not been investigated in our presented data.

Reviewer V: One of the major difficulties of tissue-engi-

neered repair of articular cartilage seems to be in achieving complete integration at the interface of the neocartilage and the original tissue. How well was this integration achieved overall, and how important is this issue in the long-term success repair of cartilage defects using therapies such as the one presented here?

Authors: Table 1 shows the percent peripheral attachment and subchondral attachment of the repaired tissue from 6 weeks to 1 year. Integrative repair of articular cartilage may indeed be important in the long term integrity of the joint. While no degenerative changes were observed at 6 and 12 months in a rabbit model, evidence of general cartilage degeneration in rabbit knees subjected to partial laceration at a 2 year periods has been published (Ghadially *et al*., 1977).

Reviewer V: There is the suggestion that this type of therapy may potentially be used to treat osteoarthritis or other forms of progressive joint degeneration. What other considerations do the authors foresee before such a method can be applied to treat the loss of cartilage from osteo-arthritis?

Authors: The initial application of this type of engineered tissue repair will be for the treatment of isolated, relatively small defects of articular cartilage. Large deficits of articular cartilage will be more challenging, mainly because, it will be difficult to deal with the mechanical loading of the implant through a large arc of motion. In particular, the elderly will probably always have their end stage arthritic joints treated by total joint replacement, since that procedure is so effective and will usually survive the patient's life expectancy. For the young person with osteoarthritis, a biological repair would be ideal, and for this individual, it is hoped that the methodologies being worked on (as described in this paper) may evolve into a technique to treat large areas of arthritic joint surfaces.

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