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Combining Chondrocytes and Smooth Muscle Cells to Engineer Hybrid Soft Tissue Constructs

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ABSTRACT

Engineering new tissues using cell transplantation may provide a valuable tool for reconstructive surgery applications. Chondrocyte transplantation in particular has been successfully used to engineer new tissue masses due to the low metabolic requirements of these cells. However, the engineered cartilaginous tissue is too rigid for many soft tissue applications. We propose that hybrid tissue engineered from chondrocytes and smooth muscle cells could reflect mechanical properties intermediate between these two cell types. In this study, rat aortic smooth muscle cells and pig auricular chondrocytes were co-cultured on polyglycolic acid fiber-based matrices to address this hypothesis. Mixed cell suspensions were seeded by agitating the polymer matrices and a cell suspension with an orbital shaker. After seeding, cell–polymer constructs were cultured in stirred bioreactors for 8 weeks. The cell density and extracellular matrix (collagen, elastin, and glycosaminoglycan) content of the engineered tissues were determined biochemically. After 8 weeks in culture, the hybrid tissue had a high cell density (5.8×10^8 cells/cm^3), and elastin (519 μg/g wet tissue sample), collagen (272 μg/g wet tissue sample), and glycosaminoglycan (GAG; 10 μg/g wet tissue sample) content. Mechanical testing indicated the compressive modulus of the hybrid tissues after 8 weeks to be 40.8 ± 4.1 kPa and the equilibrium compressive modulus to be 8.4 ± 0.8 kPa. Thus, these hybrid tissues exhibited intermediate stiffness; they were less stiff than native cartilage but stiffer than native smooth muscle tissue. This tissue engineering approach may be useful to engineer tissues for a variety of reconstructive surgery applications.

INTRODUCTION

SYNTHETIC MATERIALS and tissue grafts have been used conventionally in plastic and reconstructive surgery to replace damaged or defective tissues. Synthetic prosthesis (*e.g.*, metals, ceramics, and plastics) can be fabricated reproducibly in large quantities with well-defined properties, and lead to aesthetic improvement. However, these materials do not faithfully mimic the properties of native tissues, and the long-term implantation of synthetic materials can give rise to infection and/or rejection from the body.[1] Alternatively, tissue transplantation (*e.g.,* cartilage) has often been used to replace damaged and defective tissues. For example, autogeneous cartilage used as a free graft for cosmetic improvement has produced excellent results.

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In plastic surgery, craniofacial and aesthetic surgeons have strived to use this technique for reconstructing osseocartilaginous craniofacial skeleton using cartilage and bone grafts.[2] However, the scarcity of usable tissue, donor site morbidity, and scarring limit the application of tissue transplants.

A new approach to generate tissues for reconstructive and plastic surgery is to engineer functional, natural tissues using three-dimensional scaffolds and cultured cells.[3] Expanding cells in culture may allow a virtually unlimited supply of tissue to be created from an initially small tissue biopsy. The scaffolds for tissue engineering can be fabricated from a variety of naturally derived materials (*e.g.,* type I collagen, alginate, hydroxyapatite) or synthetic biodegradable polymers [*e.g.,* polyglycolic acid (PGA), polylactic acid, and poly(lactic-co-glycolic) acid].[4] Cells seeded onto the scaffolds are either allowed to develop into a new tissue *in vitro* or transplanted into a patient to create a functional tissue that is structurally integrated with the body.[5] Tissue engineering principles are being applied to repair, replace, maintain, or enhance the function of a large number of different tissues and organs.[3]

Methods to engineer cartilage using biodegradable polymers and cultured cells have been extensively investigated because many aspects of chondrocytes make them ideal for tissue engineering.[2,6–11] These cells have a very low metabolic activity, and thus survive exceptionally well following transplantation with minimal nutrient availability. Chondrocytes are well adapted to conditions where the oxygen tension may be as low as 1% (compared with 24% in normal atmosphere). Indeed, pieces of cartilage several millimeters in thickness can be engineered by transplanting chondrocytes on biodegradable polymer matrices.[11,12] This contrasts with most cell types, which are susceptible to hypoxia and thus cannot be used to form tissues more than several hundred micrometers thick unless a vascular supply is provided.[13,14] An additional advantage of chondrocytes is that these cells can be readily multiplied in culture. However, while the high strength and stiffness of the tissues engineered with chondrocytes make them ideal for cartilage replacement, these properties make the tissues unsuitable for soft tissue replacement in many plastic and reconstructive surgery applications.

To engineer suitable tissues that can be used in the reconstruction of soft tissues, we propose to engineer tissues from a combination of chondrocytes and other cell types. These tissues may allow us to readily engineer large tissues (due to low metabolic activity of chondrocytes) that have mechanical properties intermediate to cartilage and soft tissues. In the present study, we determined whether the addition of smooth muscle cells would decrease the rigidity of tissues engineered using chondrocytes *in vitro*. In addition, chondrocytes derived from auricular cartilage were utilized, because auricular cartilage is typically less rigid than articular cartilage.

MATERIALS AND METHODS

Cell isolation

Porcine auricular chondrocytes (passage = 0 and 1) donated by Reprogenesis (Cambridge, MA) were used in all experiments. The chondrocytes were maintained in DMEM/F-12 (GIBCO, Gaithersburg, MD) containing 10% (vol/vol) fetal bovine serum (FBS; GIBCO) and 100 U/mL penicillin-streptomycin (GIBCO). Cells were cultured in tissue culture flasks (Corning Inc., Corning, NY) in a humidified 5% CO_2 incubator with growth medium changed once a week (twice a week when approaching confluence). Cells of passages 1–4 were used for all experiments. Smooth muscle cells (SMCs) were isolated from 300–350 g adult male Lewis Rats using an enzymatic dissociation and cultured as previously described.[15] SMCs were maintained in Medium 199 (GIBCO) containing 10% (vol/vol) FBS (GIBCO) and 100 U/ml penicillin-streptomycin (GIBCO) with the growth medium changed every other day. Cells of passages 12–18 were used for all experiments.

Polymer scaffolds

PGA fiber-based matrices (2-mm thick) with a bulk density of 50 mg/mL and porosity of approximately 97% were purchased from Albany International (Taunton, MA) and stored in a dessicator before use. The PGA matrices were cut (5 × 5-mm squares for biochemical assay and simple compression test specimens and 12.6-mm diameter disks for viscoelastic testing specimens) and sterilized under UV light for a 10-h pe-

riod on each side. They were prewet in a petri dish containing 70% ethanol and rinsed (4×) in phosphate-buffered saline (PBS; GIBCO). The scaffolds were then transferred into 50-ml tubes with 25 ml of the medium containing 45% (vol/vol) DMEM/F12, 45% (vol/vol) Medium 199, 10% (vol/vol) FBS, and 100 U/mL penicillin-streptomycin. The closed tubes were placed in an incubator for 24 h prior to seeding to allow the adsorption of proteins contained in the serum onto the PGA fibers for cell attachment.

Cell seeding and culture

Matrices were seeded with equal numbers of chondrocytes and SMCs (total cell concentrations in the seeding solution were 5.4×10^7 cells/mL for the biochemical assay and simple compression test specimens and 4.6×10^7 cells/mL for the viscoelastic testing specimens) using an agitated cell seeding method.[16]

To seed cells, the polymer matrices were placed into either 50-mL tubes with 0.3 mL of a cell suspension or into wells of a 24-well tissue culture plate (Falcon, Franklin Lakes, NJ) with 0.4 mL of a cell suspension. The polymers were then agitated with the cell suspension at 100 rpm for 20 h in a humidified 5% CO_2 incubator using an orbital shaker (Belco Glass Inc., Vineland, NJ). Ten hours after agitation had begun, a volume of media equal to that of the original cell suspension volume was added to each matrix. Following the agitation, the scaffolds were transferred to a 150 × 15-mm petri dish (Falcon) for 3 days with 50 mL of media changed every day. Following the cell seeding, cell–polymer constructs were maintained in 250-mL spinner flasks (Belco Glass, Inc.) stirred at 26 rpm in a humidified 5% CO_2 incubator for 8 weeks with medium containing 0.5 μg/mL ascorbic acid changed every other day. During the culture period, samples collected for analytical assays were washed with PBS prior to analysis.

Analytical assays

For histological analysis, samples were fixed in 10% (vol/vol) buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E), Verhoeff's, or Safranin-O.

The number of cells in the matrices was determined by measuring the amount of DNA in enzyme-digested samples with Hoechst 33258 dye and a fluorometer (Hoefer Dyna Quant 200, Pharmacia Biotech, Uppsala, Sweden) using a previously described technique.[17] In brief, the samples were washed with PBS, lyophilized for 24 h, and digested in proteinase K digestion solution (0.5 mg/mL proteinase K and 0.1 mg/mL SDS in a buffer solution of 50 mM Tris-HCl, 0.1 EDTA, 0.2 M NaCl, pH 7.4) for 12 h at 60°C with occasional gentle shaking. For measurement, 20–50 μL of sample was aliquoted into a cuvette and mixed with 2 mL of dye solution. A solution containing calf thymus DNA (Pharmacia Biotech) was used for calibration. The presence of the polymer scaffold does not interfere with the quantification of DNA in this assay. Assaying samples in which known amounts of DNA were added to blank polymer scaffolds leads to the expected DNA concentration (data not shown).

Commercially available assay kits (Fastin elastin, collagen, and glycosoaminoglycan (GAG) assay kits, Accurate Chemical & Scientific Corp., Westbury, NY) were used for the measurement of elastin, collagen, and GAGs within the samples. The elastin assay was designed for the measurement of insoluble elastin. The elastin–polymer constructs were solubilized by hot oxalic acid treatment for three 1-h intervals at 95°C. The samples were then precipitated overnight in a 5°C refrigerator with Fastin precipitating reagent to allow precipitation of the elastin. Following precipitation, the samples were centrifuged and the pellets were mixed with Fastin dye reagent. The elastin–dye complex was then collected by centrifugation. The dye bound to the elastin pellet was solubilized with the destain reagent, and the absorbance of the samples was measured at a wavelength of 513 nm using a UV spectrophotometer. A calibration standard containing α-elastin was used to obtain the standard curve for this experiment.

The GAG assay was designed for the measurement of soluble GAG within a tissue sample. GAG was extracted from the polymer constructs using a solvent system of 4 M guanidine-HCl, 0.5 M sodium acetate, pH 6, with 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM *N*-ethylmaleimide (NEM).[18,19] The constructs were added to the chilled solution with stirring on an orbital shaker for a 12- to 20-h period at 4°C. After the extraction, the samples were centrifuged to remove insoluble residue and the supernatant was collected and mixed with blyscan dye reagent (composed of 1,9-dimethylmethylene blue in an organic buffer) for 30 min. The glycosaminoglycan-dye complex was collected by

centrifugation. The dye bound to the pellet was solubilized by adding a dissociation reagent and mixing until the bound dye was released into solution. The absorbance of the samples was measured at a wavelength of 656 nm using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen content (salt, acid, and pepsin soluble fractions) of the tissues was also quantified.[20] The salt-soluble collagen was extracted from the tissue samples by incubating tissues at 5°C with a series of NaCl buffer solutions containing protease inhibitors (EDTA 20 mM, PMSF, NEM). The salt buffers were collected for later analysis. The acid-soluble collagen was subsequently removed from the remaining tissue by adding 0.5 M acetic acid to the samples and incubating at room temperature. The acid solution containing dissolved collagen was collected for later analysis, and the pepsin-soluble collagen was subsequently extracted from the remaining tissue. A solution containing pepsin (1 mg/10 mg tissue sample; Sigma, St. Louis, MO) was added to the tissues and incubated overnight. The salt-soluble, acid-soluble, and pepsin-soluble samples were collected and combined for total soluble collagen content. This sample was prepared for assay by mixing with Sircol dye reagent for 30 min on an orbital shaker. The samples were then centrifuged to collect the collagen–dye complex. The dye bound to the collagen pellet was solubilized with an alkali reagent, and the absorbance of the samples was measured at a wavelength of 540 nm using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment. ECM contents were normalized to the wet mass of the new tissues for all assays.

Mechanical testing

Following measurement of 8-week polymer-construct thickness and cross-sectional area with calipers, simple compression tests were performed using a MTS Bionix 100 (Model 810, MTS Systems Corp., Eden Prairie, MN) with a 10N load cell and a cross-head speed of 1 mm/min. Stress versus strain curves were then generated using the force and displacement data. The compressive moduli were obtained by measuring the slope of the first 5% of the stress-strain curve where the stress was non-zero ($n = 4$).

Viscoelasticity tests were also performed using a similar method employed for materials such as cartilage[21] and hydrogels.[22] The thickness and cross-sectional area of specimens to be tested were measured. Using a custom testing fixture, the specimens were submerged in medium at 37°C and placed at the bottom of an aluminum well 6.3 mm diameter by 3 mm deep. The specimens communicated with the medium through a 3.2-mm-thick porous stainless steel filter with an average pore size of 20 microns (SSI Technologies Inc., Janesville, WI). The compression tests were unconfined in the radial direction because specimen diameters were less than the well diameter. A confined compression test would be ideal, however, this test could not be used because the samples did not have an identical size and shape, and the tissues could not be cut due to their spatially inhomogeneous nature. Specimens were compressed at a rate of 5 mm/min in step strains of ~4%. After each step strain, the strain was held constant for 10 min or until an equilibrium stress state was reached. Equilibrium stress-strain curves were obtained and longitudinal moduli were calculated using the secant slope of the first 5% of the interpolated equilibrium stress-strain curves where the stress was non-zero ($n = 5$).

Statistical analysis for assays

All data were obtained from a minimum of triplicate samples. Data were expressed as the mean and standard deviation (SD). Statistical analysis was carried out using the unpaired Student's *t*-test (Instat, Graphpad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

A combination of equal numbers of chondrocytes and SMCs were seeded onto polymer matrices. The cells adhered to the polymer fibers as individual cells and cell clusters, and proliferated on the matrices. Solid new tissues resulted over the course of 8 weeks in spinner flask culture. Tissue samples were fixed, sectioned, and stained to histologically examine the engineered tissues. Observation of sections stained with H&E after 8 weeks in culture indicated the proliferation of cells throughout the polymer matrices and the

accumulation of an extracellular matrix in the engineered hybrid tissues (Fig. 1A). The engineered tissues had a higher density of cells on the outer layer of the constructs, and a lower density of cells in the center.

The major ECM molecules in cartilage and smooth muscle include elastin, collagen, and GAGs. Verhoeff's staining of the histologic sections indicated that the hybrid tissues contained substantial elastin at 8 weeks (Fig. 1B). More elastin was present in the outer layer of the hybrid tissue than in the inner portion, suggesting that SMCs are preferentially distributed to the outer layer rather than the center of these engineered tissues. Safranin-O staining was done to verify the presence of a cartilaginous tissue, and histological sections exhibited positive staining for GAG. The hybrid tissue exhibited a more prominent staining in the interior, again consistent with a preferential distribution of chondrocytes in the inner layer of the tissues (data not shown).

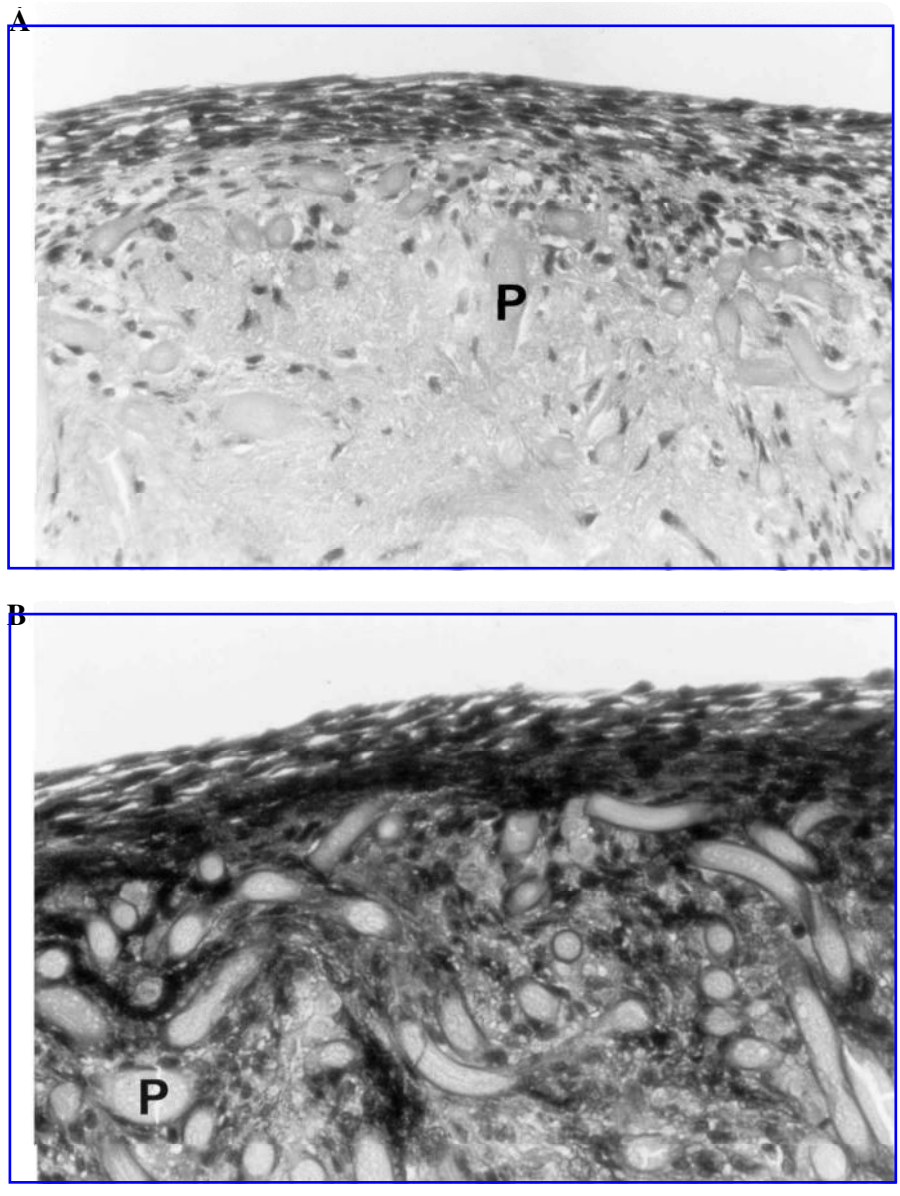

FIG. 1. Representative photomicrographs of H&E stained histologic sections (**A**) and Verhoeff-stained sections of tissues engineered from a combination of SMCs and chondrocytes after 8 weeks of culture (**B**). Polymer fibers (P) are visible. (Original magnification: 40× for both photomicrographs.)

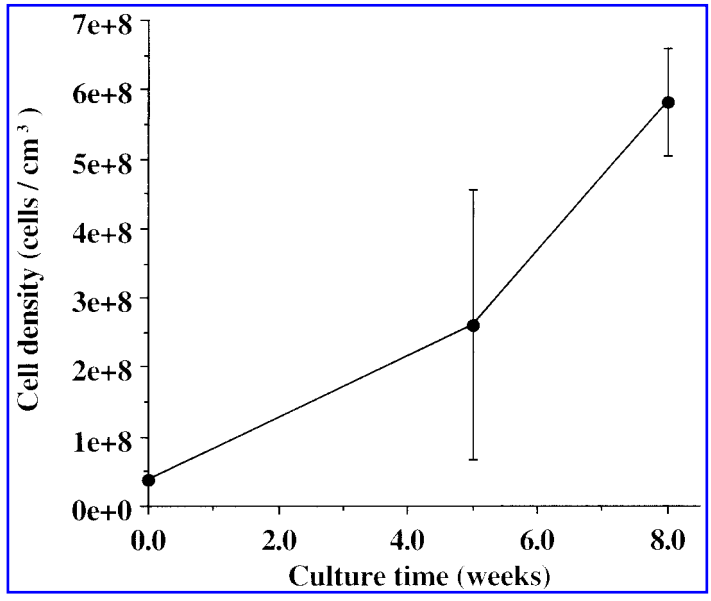

FIG. 2. The cell density of hybrid tissues cultured for 8 weeks. Data points represent the average ± standard deviation (SD) of triplicate samples.

The cell density and ECM composition of the engineered tissues were also quantified over time. The cell density of the engineered tissues was determined by measuring the DNA content in the tissue constructs. The cell density increased dramatically over time and after 8 weeks in culture the engineered tissues had a cell density of 5.8×10^8 cells/cm^3 (Fig. 2). The cell density had increased 123% from 5 to 8 weeks in culture. The extracellular matrix components also increased significantly during the culture of the hybrid tissues. The elastin, collagen, and GAG content of the hybrid tissue also increased over time to 8-week values of 519, 272, and 10 μg/g wet tissue sample, respectively (Fig. 3). The increases from 5 to 8 weeks were significant ($p < 0.003$ in all cases).

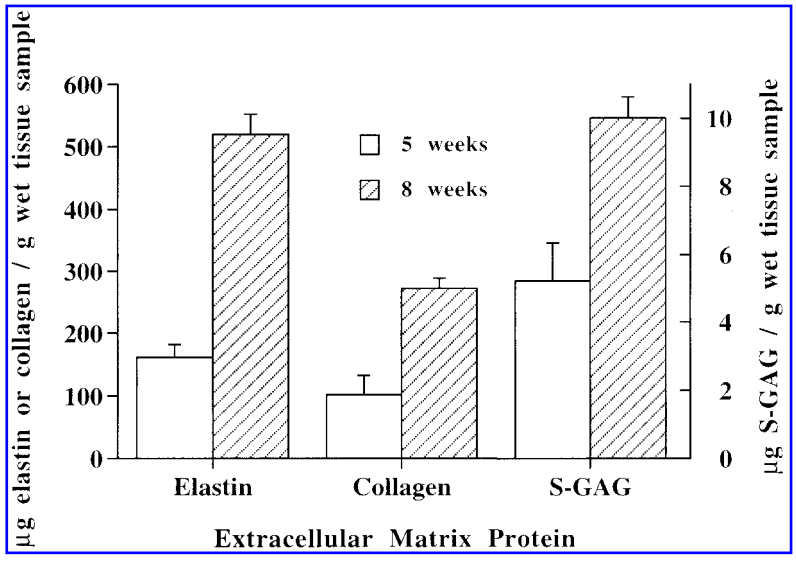

FIG. 3. Content of elastin, collagen, and sulfated glycosaminoglycan (S-GAG) per gram wet tissue sample after 5 and 8 weeks of culture for the hybrid tissues. Data points represent the average ± standard deviation (SD) of triplicate samples.

The elastic modulus of the engineered tissues was next measured using two different techniques. One group of specimens was subjected to simple uniaxial compression tests at a constant strain rate. Stress-strain curves (Fig. 4A) were constructed from load-displacement data and the average compressive modulus of the hybrid tissues was found to be 40.8 ± 4.1 kPa. Because moduli determined with this method are strain rate dependent, a more complex viscoelastic technique was then employed for another group of specimens. Small step strains were applied to the specimens and held constant until an equilibrium stress state was achieved. The specimens demonstrated significant stress relaxation after each step strain (Fig. 4B). The steady-state data were then used to construct equilibrium stress-strain curves (Fig. 4C) from which the average equilibrium compressive modulus was calculated (8.4 ± 0.8 kPa). This represents approximately a five-fold difference in modulus, depending on whether perfectly elastic behavior or viscoelastic characteristics of the tissues were assumed to perform the mechanical test.

DISCUSSION

Hybrid tissues composed of SMCs and chondrocytes were engineered using biodegradable polymer matrices. These hybrid tissues exhibited extensive matrix deposition that increased dramatically over time. Im-

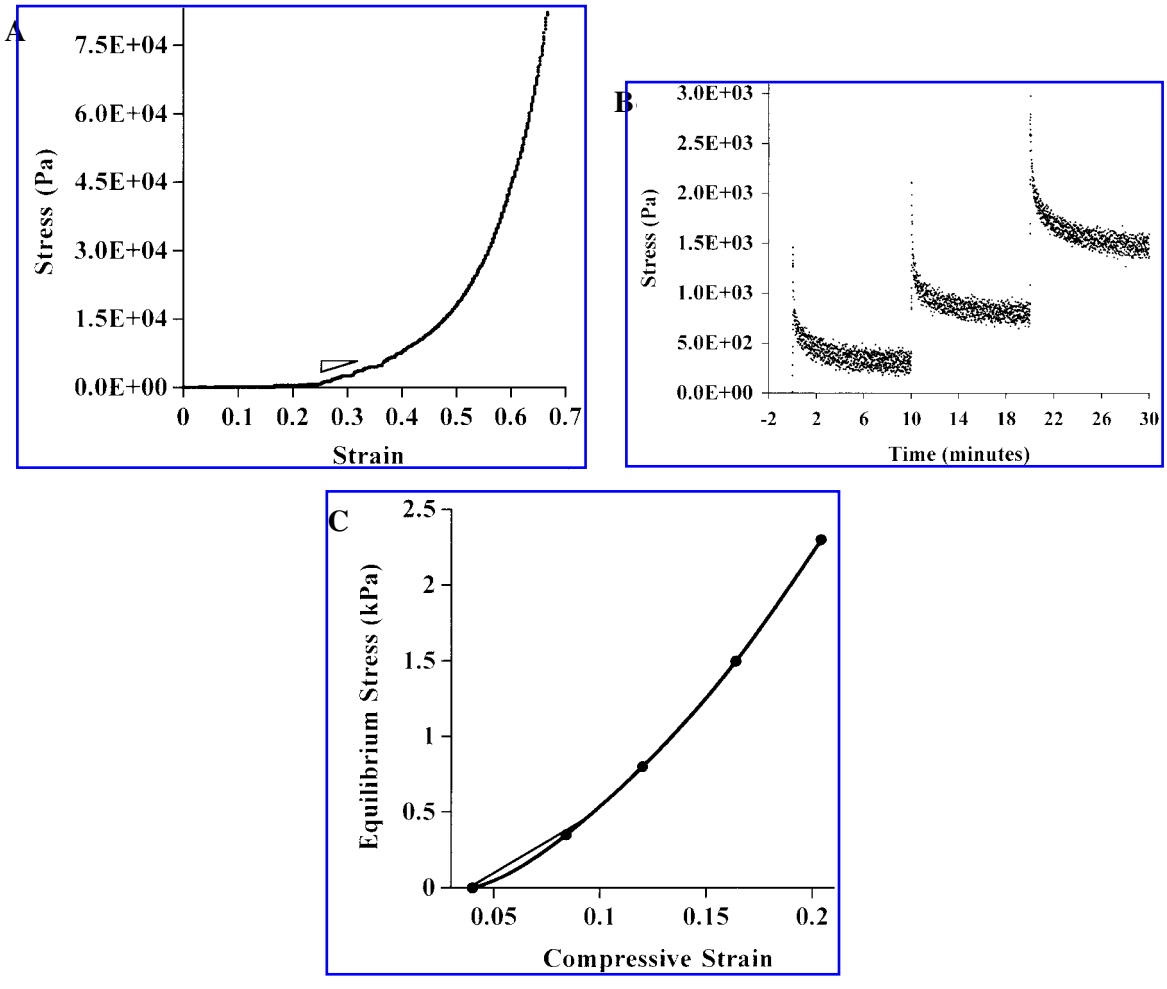

FIG. 4. A typical stress-strain curve obtained from a simple uniaxial compression test (**A**), stress relaxation curves obtained from a step strain viscoelastic test (**B**), and an interpolated equilibrium stress-strain curve constructed from a step strain viscoelastic test (**C**). The slope of the triangle hypotenuse in (**A**) and the secant line segment in (**C**) indicate the regions where the moduli were calculated.

portantly, the mechanical properties of the hybrid tissues showed they were less stiff than native or engineered cartilage.[21,23]

The cells seeded on the matrices proliferated over time, and deposited significant amounts of extracellular matrix. The density of cells in the hybrid tissues increased to a final value of 5.8×10^8 cells/cm^3 after 8 weeks. This value is much larger than the range of values found for bovine articular cartilage ($3.0–10.0 \times 10^7$ cells/cm^3)[24] and very similar to that of native smooth muscle tissue.[25] It is not surprising that the new tissues demonstrated significant elastin and collagen content, as SMCs typically produce large amounts of elastin on these polymer matrices,[16,26] and the chondrocytes, obtained from elastic cartilage which is found in the external ear and external auditory canal, are also known to produce elastin[27] and collagen[28] *in vivo*. The hybrid tissues also exhibited significantly increasing GAG content over time that is likely attributable primarily to the chondrocytes.

The hybrid tissue constructs were mechanically tested using two approaches, and these results demonstrate the importance of choosing an appropriate testing methodology when analyzing engineered tissues. The constant strain rate compression test led to a high modulus, likely because the modulus measured in this manner contains transient information pertaining to both the viscous and elastic properties of the tissues lumped together. These types of measurements can only be repeated or compared to other values in the literature if identical strain rates are used. In contrast, compression-stress relaxation measurements using step strains can be used to obtain equilibrium compressive moduli that are not as test condition dependent. The equilibrium compressive modulus was much smaller because the specimens were allowed to relax to a steady-state stress following each step strain. In this manner, the viscous response was dissipated and only the elastic properties of the tissues were measured. The hybrid tissues exhibited equilibrium compressive moduli that were one order of magnitude less than tissue-engineered cartilage formed from articular chondrocytes (55 kPa)[23] and two orders of magnitude less than native articular cartilage (760 kPa).[21] The lower compressional mechanical properties of the hybrid tissues compared to purely cartilaginous tissues may be a result of lower GAG content and higher elastin content. Also, in contrast to the preceding referenced studies, auricular chodrocytes from elastic cartilage were used in this study, and this may lead to a less-stiff cartilaginous tissue. Future experiments in which the cells are labeled before the seeding step and the proportion of the two cell types are varied in co-culture would yield valuable information about the final cell composition of the constructs and each cell type's relative contribution to the resultant hybrid tissues' extracellular matrix components and mechanical properties.

In summary, hybrid tissues can be grown *in vitro* using SMCs, chondrocytes, and synthetic, biodegradable polymers. The mechanics of engineered tissues can be readily modified by combining various cell types, and this approach may ultimately be useful for reconstruction of a variety of soft tissues.

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