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Bioreactor Cultivation Conditions Modulate the Composition and Mechanical Properties of Tissue-Engineered Cartilage

G. Vunjak-Novakovic, I. Martin, B. Obradovic, *S. Treppo, *A. J. Grodzinsky, R. Langer, and L. E. Freed

*Division of Health Sciences and Technology and *Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.*

Summary: Cartilaginous constructs have been grown *in vitro* with use of isolated cells, biodegradable polymer scaffolds, and bioreactors. In the present work, the relationships between the composition and mechanical properties of engineered cartilage constructs were studied by culturing bovine calf articular chondrocytes on fibrous polyglycolic acid scaffolds (5 mm in diameter, 2-mm thick, and 97% porous) in three different environments: static flasks, mixed flasks, and rotating vessels. After 6 weeks of cultivation, the composition, morphology, and mechanical function of the constructs in radially confined static and dynamic compression all depended on the conditions of *in vitro* cultivation. Static culture yielded small and fragile constructs, while turbulent flow in mixed flasks yielded constructs with fibrous outer capsules; both environments resulted in constructs with poor mechanical properties. The constructs that were cultured freely suspended in a dynamic laminar flow field in rotating vessels were the largest, contained continuous cartilage-like extracellular matrices with the highest fractions of glycosaminoglycan and collagen, and had the best mechanical properties. The equilibrium modulus, hydraulic permeability, dynamic stiffness, and streaming potential correlated with the wet-weight fractions of glycosaminoglycan, collagen, and water. These findings suggest that the hydrodynamic conditions in tissue-culture bioreactors can modulate the composition, morphology, mechanical properties, and electromechanical function of engineered cartilage.

Articular cartilage derives its form and mechanical function from its matrix, which consists of tissue fluid and a framework of structural macromolecules (collagens, proteoglycans, and noncollagenous proteins and glycoproteins). During the development, maintenance, and remodeling of cartilage, chondrocytes synthesize appropriate types and amounts of macromolecules and assemble them into a highly organized matrix (4). Adult articular cartilage has a limited capacity to repair damage resulting from injury or disease, and there have been many different approaches to restore tissue composition, structure, and function, including the development of engineered cartilage for potential implantation (5,27). Cartilaginous constructs have been grown *in vitro* with use of isolated chondrocytes, biodegradable polymer scaffolds, and bioreactors and implanted *in vivo* to form subcutaneous cartilage or to promote joint repair (16). Fibrous polyglycolic acid scaffolds permitted chondrocytes to maintain their differentiated phenotype and provided a three-dimensional framework for tissue regeneration (14), and bioreactors provided control over the conditions of cell seeding and tissue cultivation and affected construct structures and compositions (19,31).

We hypothesized that conditions of flow and mixing in tissue-culture bioreactors could be utilized to modulate the mechanical function of engineered cartilage. In particular, enhanced rates of mass transfer and hydrodynamic effects associated with dynamic laminar flow patterns in rotating vessels were expected to stimulate chondrocytes to regenerate a functional extracellular matrix. Although chondrocytes cultured on polyglycolic acid scaffolds in bioreactors have been shown to regenerate a cartilaginous extracellular matrix containing glycosaminoglycan and type-II collagen (18), mechanical and electromechanical behaviors of newly synthesized matrix have not yet been well characterized. Functional assessment would both permit evaluation of whether constructs grown *in vitro* might withstand physiological loading and enable further optimization of the cultivation of cartilage-like tissue substitutes.

The objectives of the present study were (a) to assess the effects of cultivation conditions on construct

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Address correspondence and reprint requests to G. Vunjak-Novakovic at Massachusetts Institute of Technology E25-342, 45 Carleton Street, Cambridge, MA 02139, U.S.A. E-mail: gordana@mit.edu

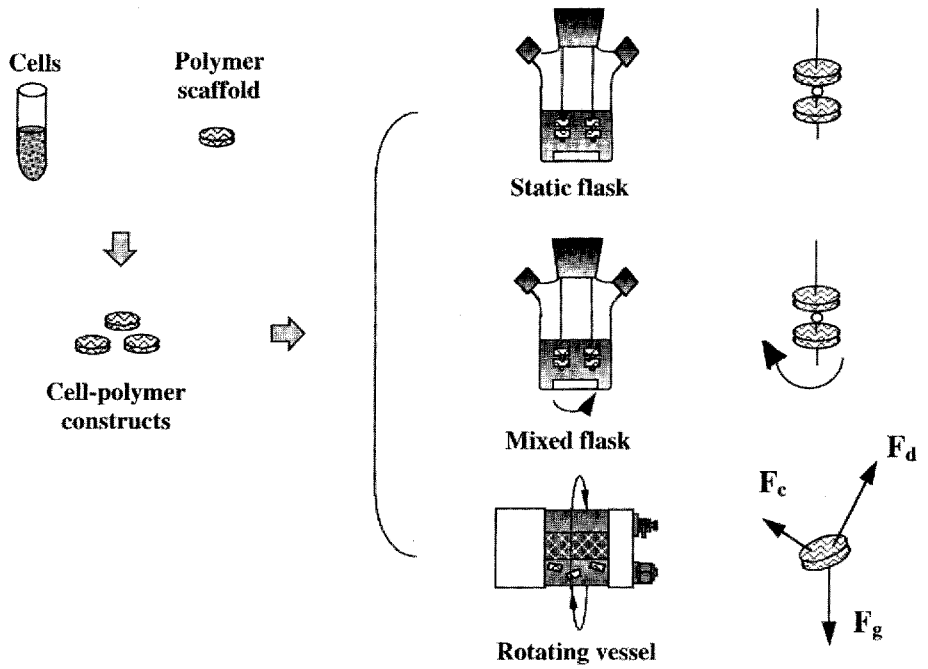

FIG. 1. Experimental setup. Cells isolated from bovine articular cartilage were seeded on 5-mm-diameter, 2-mm-thick, polyglycolic acid scaffolds (5×10^6 cells/scaffold) in well mixed flasks. The resulting cell-polymer constructs were cultured for 6 weeks in static flasks, mixed flasks, or rotating vessels. F_c, F_d, and F_g refer to centrifugal, drag, and net-gravity forces, respectively. Hydrodynamic conditions in each vessel are summarized in Table 1.

composition, morphology, and mechanical function and (b) to identify some of the relationships between the composition and mechanical properties of engineered cartilage. Bovine calf articular chondrocytes were cultured for 6 weeks on polyglycolic acid scaffolds under three sets of hydrodynamic conditions: static, turbulent, and laminar. The constructs were analyzed structurally to determine the amounts and distributions of tissue components and functionally (in static and dynamic confined compression) to determine the equilibrium modulus, dynamic stiffness, hydraulic permeability, and streaming potential. Mechanical properties of 6-week constructs were correlated to wet-weight fractions of glycosaminoglycan, collagen (hydroxyproline), and water and compared with properties of freshly explanted bovine calf articular cartilage.

MATERIALS AND METHODS

Tissue Harvest and Cell Isolation

Full-thickness articular cartilage was harvested from the femoropatellar grooves of 2-3-week-old calves (six knee joints from different animals) within 8 hours of slaughter. Cells were isolated with use of type-II collagenase (Worthington, Freehold, NJ, U.S.A.) and resuspended in Dulbecco's modified Eagle medium containing 4.5 g/L glucose, 584 mg/L glutamine, 10% fetal bovine serum, 50 U/cm^3 penicillin, 50 μg/cm^3 streptomycin, 10 m*M* HEPES, 0.1 m*M* nonessential amino acids, 0.4 m*M* proline, and 50 μg/cm^3 ascorbic acid (16). Control samples of natural cartilage (discs 5 mm in diameter and 2-mm thick) were harvested from the middle sections of full-thickness plugs with use of a dermal punch (Miltex Instruments, Lake Success, NY, U.S.A.), a custom-made vacuum holder, and a razor blade; rinsed in phosphate buffered saline supplemented with 100 U/cm^3 penicillin and 100 μg/cm^3 streptomycin; and analyzed immediately after explant.

Scaffold Seeding

Scaffolds were produced as previously described (14) at Albany International (Mansfield, MA, U.S.A.) by the extrusion of polyglycolic acid (weight-average molecular weight of 69 kDa) into 13-μm-diameter fibers and the processing of these into fibrous disks 5 mm in diameter and 2-mm thick (void volume: 97%; bulk density: 62 mg/cm^3). In brief, the fibers were (a) formed by polymer extrusion, stretching, and relaxation at elevated temperatures, (b) crimped and cut, (c) carded into a lofty web, (d) needled to form a nonwoven mesh, and (e) punched into discs (14).

The scaffolds were seeded with freshly isolated chondrocytes in well mixed flasks (Bellco, Vineland, NJ, U.S.A.) as previously described (31,32). In brief, the scaffolds were pre-wet in culture medium and threaded onto 8-cm-long, 22-gauge needles. Flasks containing four needles with three scaffolds apiece and 120 cm^3 of culture medium were mixed at 50 rpm with use of a nonsuspended magnetic stir bar (4-cm long and 0.8 cm in diameter) in a humidified, 37°C, 10% CO_2 incubator. Each flask was inoculated with 60×10^6 chondrocytes, corresponding to 5×10^6 cells per scaffold. Over 3 days, the cells attached throughout the scaffold volume, with an essentially 100% yield as previously demonstrated (32).

Construct Cultivation

After 3 days of cell seeding, the cell-polymer constructs were cultured for 6 weeks at 37°C in 10% CO_2 in three groups: static flasks, well mixed flasks, or rotating vessels (Fig. 1). The study was done with use of nine vessels (three per group), each containing 12 constructs in 110-120 cm^3 of culture medium. In the static and mixed flasks, the constructs were fixed in place and exposed to either static or turbulently mixed culture medium; gas was exchanged through loosened side arm caps. In the rotating vessels (Rotary Cell Culture System; Synthecon, Houston, TX, U.S.A.), the constructs were freely suspended in the annular space between an outer 5.75-cm-diameter polycarbonate cylinder and an inner 2-cm-

TABLE 1. *Hydrodynamic conditions in static and mixed flasks and rotating vessels*

Parameter	Static flask	Mixed flask	Rotating vessel
Vessel diameter (cm)	6.5	6.5	Outer/inner = 5.75/2
Vessel volume (cm^3)	120	120	110
Construct	Fixed in place	Fixed in place	Dynamically suspended
Stirring/rotation rate (s^{-1})[a]	0	0.83	0.25 (3 days)-0.67 (6 weeks)
Flow conditions	Static fluid	Turbulent[b]	Laminar[c]
Mechanisms of mixing/mass transfer	Diffusion	Convection[d]	Convection[e]
Fluid shear at construct surfaces	None	Steady, turbulent	Dynamic, laminar

[a] The fluid was magnetically stirred in the mixed flasks and underwent solid-body rotation in the rotating vessels.

[b] The smallest turbulent eddies had an estimated diameter of 250 μm and a velocity of 0.4 cm/sec, calculated according to Cherry and Papoutsakis (7) as shown in Vunjak-Novakovic et al. (31).

[c] The constructs were freely suspended and settling in a tumble-slide regimen in a rotational field, as predicted with use of nondimensional drag and inertia calculated with construct dimensions and settling velocity according to Clift et al. (8) as shown in Freed and Vunjak-Novakovic (15).

[d] Convection due to turbulence.

[e] Convection due to fluctuations in fluid velocity and vortex shedding around settling constructs.

diameter hollow cylinder covered with a 175-μm-thick silicone membrane; gas (10% CO_2 in air) was pumped through the inner cylinder at 0.7-1.2 L/min and exchanged by diffusion through the silicone membrane. The rotation speed of the vessel was adjusted throughout the period of cultivation to balance the forces acting on the growing constructs (i.e., gravity, buoyancy, and centrifugal and drag forces) to maintain each settling construct at a relatively steady position within the vessel (15,18). In all groups, the culture medium was replaced at a rate of 50% every 2-3 days, i.e., approximately 3 cm^3 per construct per day. The constructs were sampled at both 3 days and 6 weeks for structural and functional assessment.

Structural Analyses

Histological samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (8-μm thick). The sections (eight sections from two constructs per group) were stained with hematoxylin and eosin for cells and with safranin-O/fast green for glycosaminoglycan. Samples for biochemical analyses (six constructs per group) were frozen, lyophilized, and digested for 15 hours at 56°C with 1 mg/cm^3 proteinase-K solution in buffer (50 m*M* Tris, 1 m*M* EDTA, 1 m*M* iodoacetamide, and 10 $\mu g/cm^3$ pepstatin A) with use of 1 cm^3 of enzyme solution per 4-10 mg dry weight of the sample (21). The number of chondrocytes per construct was assessed from the DNA content measured with use of Hoechst 33258 dye, a spectrofluorometer (QM-1; Photon Technology International, South Brunswick, NJ, U.S.A.), and the following conversion factors: 7.7 pg DNA per chondrocyte (22) and 10^{-10} g dry weight per chondrocyte (14). Glycosaminoglycan content was determined spectrophotometrically (Perkin Elmer, Norwalk, CT, U.S.A.) with use of dimethylmethylene blue dye (10). Total collagen content was determined from the measured hydroxyproline content after acid hydrolysis (6 N HCl at 115°C for 18 hours) and reaction with p-dimethylaminobenzaldehyde and chloramine-T (36) with use of a hydroxyproline-to-collagen ratio of 1 to 10 (21). The amount of undegraded polyglycolic acid was calculated from the initial scaffold weight (2.8 mg) according to previously determined kinetics of polymer degradation *in vitro* (14).

Biomechanical and Electromechanical Evaluation

Disks 3 mm in diameter and 2-mm thick were harvested from the central regions of the explanted cartilage plugs or engineered constructs (n = 3-4 samples per group) and equilibrated for 10-15 minutes at room temperature in 0.15 *M* phosphate buffered saline (pH 7.4) supplemented with 100 U/cm^3 penicillin and 100 $\mu g/cm^3$ streptomycin; each disk was mounted in an electrically insulating cylindrical confining chamber as previously described (11). The chamber was mounted in a servo-controlled Dynastat mechanical spectrometer (Imass, Hingham, MA, U.S.A.), and the specimen was compressed between a porous polyethylene platen and an Ag/AgCl electrode, with an identical electrode placed in the surrounding bath. The disks were compressed at sequential increments of 10% strain to a maximum of 40% strain, which is in the range where equilibrium stress varied linearly with applied strain for both the engineered constructs and cartilage explants. In particular, the equilibrium modulus calculated for strains 10 to 40% was not significantly different from that calculated for strains 10 to 20%. After stress relaxation, the equilibrium stress was measured and plotted against the applied strain; the equilibrium modulus was determined from the slope of the best linear regression fit.

At a static offset strain of 30%, sinusoidal strains of 0.5% amplitude were applied at frequencies in the range 0.025-1 Hz. In all tests, the porous platen remained in contact with the sample. The amplitude of the applied strain was small enough to elicit a linear mechanical response, and the total harmonic distortion in the measured load was less than 10%. Dynamic stiffness was calculated as the ratio of the measured oscillatory load normalized by the flat circular area of the disc and the amplitude of the applied displacement. The amplitude of the oscillatory streaming potential was simultaneously measured and normalized by the amplitude of the applied strain. The equilibrium modulus and dynamic stiffness were used in conjunction with the method of Frank and Grodzinsky (12) to calculate the effective hydraulic permeability of the sample.

Statistical Analysis

Structural and functional parameters of engineered constructs and cartilage explants were expressed as the average ± SD of values measured for three to six individual specimens. Differences among experimental groups were assessed by fully factorial analysis of variance in conjunction with Tukey's studentized range test. The dependence of functional properties on construct composition was assessed by multiple linear regression. All statistical analyses were performed with use of Student Systat 1.0 (Systat, Evanston, IL, U.S.A.).

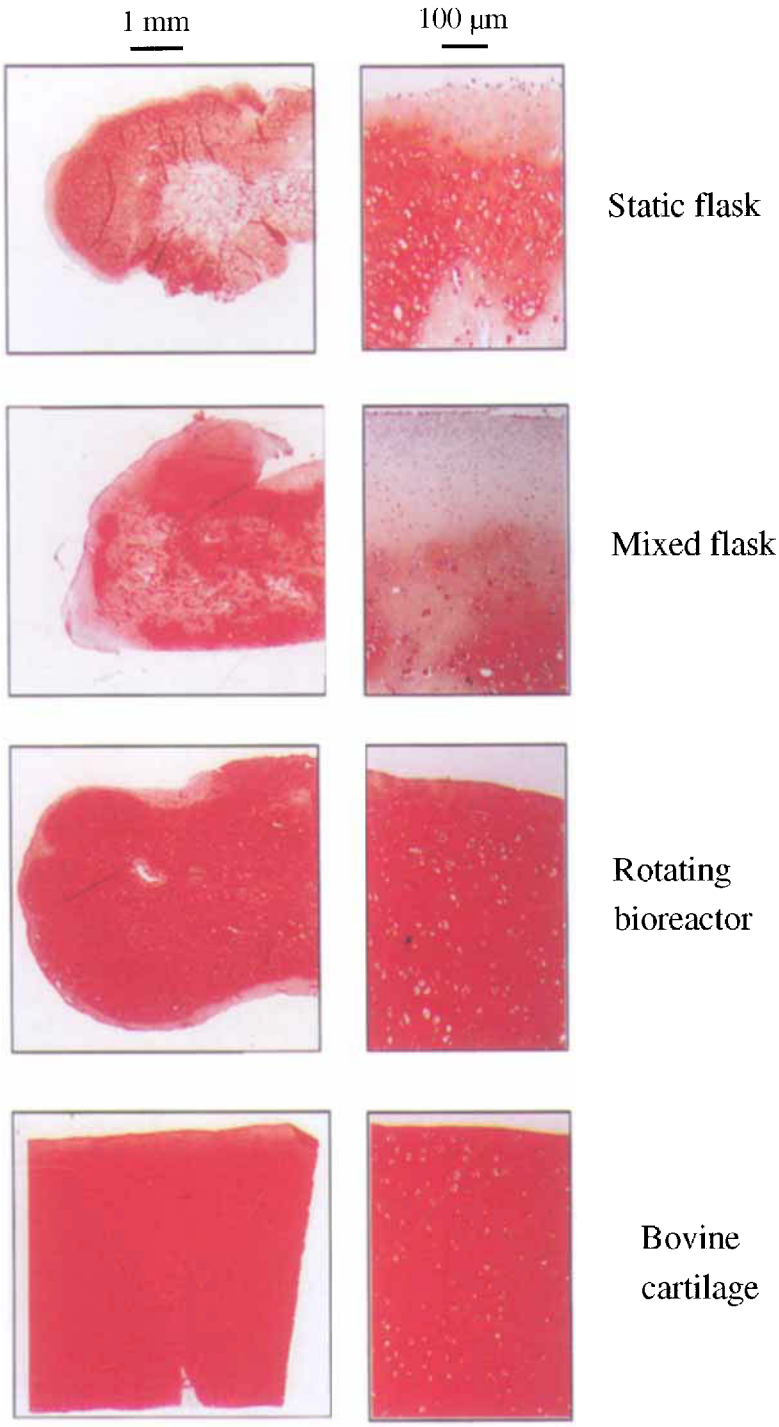

FIG. 2. Tissue morphology. Histological cross sections of 6-week constructs from static and mixed flasks, rotating vessels, and freshly explanted bovine calf articular cartilage (stain: safranin O/fast green).

RESULTS

Flow Conditions

The conditions of flow and mixing during cultivation in static flasks, mixed flasks, and rotating vessels are summarized in Table 1. In the static flasks, the constructs were fixed in place and cultured with diffusionally limited mass transfer of nutrients and gases and without hydrodynamic shear at tissue surfaces. In the mixed flasks, the constructs were fixed in place and exposed to a turbulent flow of medium (31), which enhanced the mass transfer of nutrients and gases but also caused shear at the construct surfaces. In the rotating vessels, the constructs were dynamically suspended in a laminar rotational flow field with their flat circular areas aligned perpendicular to the direction of motion (15). The constructs initially settled with small-amplitude, low-frequency oscillations about their diameters; over the cultivation period, the constructs

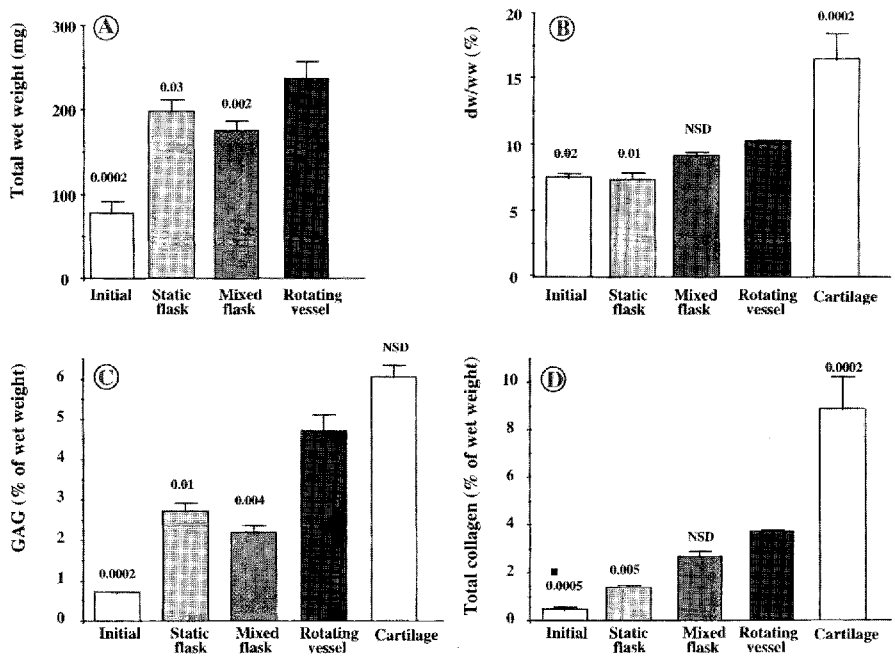

FIG. 3. Tissue growth and composition. **A:** Total wet weight (mg). **B:** Dry to wet-weight fraction (dw/ww, %). **C:** Glycosaminoglycan (GAG, % wet weight). **D:** Total collagen (% wet weight). Data represent average ± SD (n = 6) for the initial 3-day cell-polymer constructs, 6-week tissue constructs from static and mixed flasks and rotating vessels, and freshly explanted bovine calf articular cartilage. Numbers above bars are the p values of pairwise comparison probabilities between the rotating vessels and other experimental groups. NSD = no significant difference, $p > 0.05$.

began to settle with a larger amplitude pitching motion and tumble around their circular surfaces.

Construct Morphology and Composition

Construct morphology was affected by cultivation conditions as shown in Fig. 2. Statically grown constructs were thin, fragile, and irregular in shape; glycosaminoglycan accumulated mainly within an approximately 1.0-mm-thick outer layer. Constructs grown in mixed flasks contained more glycosaminoglycan in the inner tissue phase than did those grown statically. Turbulent mixing also induced the formation of an outer capsule, 100-400-μm thick, after 6 weeks of cultivation, that contained multiple layers of elongated cells and little glycosaminoglycan. Constructs grown in rotating vessels were thick and contained a continuous cartilaginous extracellular matrix over their entire cross sections, as assessed by visual inspection.

After 6 weeks of cultivation, the constructs from the rotating vessels had significantly higher wet weights than did those from either the static or mixed flasks or the initial 3-day constructs (Fig. 3A) and all constructs contained significantly more water than did the cartilage explants (Fig. 3B). Cell numbers increased from the initial five million cells seeded onto each scaffold to 9.66 ± 0.06, 16.6 ± 3.3, and 14.4 ± 2.0 million cells per construct in the static and mixed flasks and the rotating vessels, respectively. Glycosaminoglycan fractions in the 6-week constructs from the rotating vessels were lower, but not significantly, than those in the cartilage explants and were approximately 2-fold higher than those in constructs from either the static or mixed flasks (Fig. 3C). The fraction of total collagen in the 6-week constructs from the rotating vessels was comparable with that of the collagen in the mixed flasks, significantly higher than that in the static flasks, and significantly lower than that in the cartilage (Fig. 3D). The amount of polyglycolic acid decreased by approximately 60% (14), from 2.4 mg at 3 days (approximately 4% wet weight) to approximately 1 mg at 6 weeks (<0.6% wet weight). Wet-weight fractions of glycosaminoglycan and collagen in the 6-week constructs cultured in the rotating vessels were, respectively, 78 and 43% of those in the freshly explanted cartilage. Biochemically determined tissue components (cells, glycosaminoglycan, and collagen) composed 4.63 ± 0.13, 5.88 ± 0.33, and 9.04 ± 0.53% of the wet weight of the 6-week constructs from the static and mixed flasks and the rotating vessels, respectively, compared with 15.9 ± 2.45% of the wet weight of the freshly explanted bovine articular cartilage.

Construct Function

Mechanical and electromechanical characteristics of the constructs assessed in static and dynamic confined compression generally improved with increasing cultivation time and depended on the hydrodynamic conditions of cultivation. Initial 3-day constructs were too fragile to allow the measurement of mechanical properties (data not shown). After 6 weeks of cul-

TABLE 2. *Structure-function relationships for engineered cartilage constructs*

Function	Correlation coefficients (%, wet weight)							
			C_{GAG}		C_{coll}		C_{water}	
	r^2	p	SE	p	SE	p	SE	p
Equilibrium modulus, H_A (MPa)[a]	0.988	<0.00001	0.0053	<0.0001	0.0061	<0.1	0.00015	<0.001
Dynamic stiffness, H_D (MPa)[b]	0.956	<0.00005	0.18	<0.01	0.2	NSD	0.0052	<0.05
Hydraulic permeability, k (m^4/N-s)[c] $\times 10^{15}$	0.845	<0.005	5.9	NSD	6.79	<0.005	0.17	<0.005
Streaming potential, V (mV/%)[d]	0.958	<0.00005	0.0047	<0.005	0.0054	NSD	0.00014	<0.05

C_{GAG} = fraction of glycosaminoglycan in the construct, C_{coll} = fraction of collagen in the construct, C_{water} = fraction of water in the construct, SE = standard error, and NSD = no significant difference ($p > 0.05$).

[a] $H_A = 0.044\ C_{GAG} + 0.012 + C_{coll} - 0.00089\ C_{water}$.

[b] $H_D = 0.63\ C_{GAG} + 0.31\ C_{coll} - 0.013\ C_{water}$.

[c] $k = 3.4\ C_{GAG} - 18\ C_{coll} + 0.70\ C_{water}$.

[d] $V = 0.021\ C_{GAG} + 0.059\ C_{coll} - 0.00044\ C_{water}$.

tivation, the equilibrium modulus was comparable for the constructs grown in static and mixed flasks and about 4-fold higher for those grown in rotating vessels (Fig. 4A). In all of the groups, the 6-week constructs remained mechanically inferior to the cartilage explants. Hydraulic permeability was lower in the constructs that had higher equilibrium moduli (Fig. 4B). The permeability of the statically grown constructs was significantly higher than that of the constructs from rotating vessels and that of the cartilage and was higher, but not significantly, than that of the constructs from mixed flasks. The permeabilities of the constructs grown in mixed flasks and rotating vessels were not significantly different from each other or from that of the natural cartilage. Dynamic stiffness and streaming potential increased monotonically with frequency (Fig. 4C and D) as previously described for natural cartilage (11,13). At all frequencies, the dynamic stiffness was significantly higher for the constructs cultured in rotating vessels compared with those cultured in static or mixed flasks (Fig. 4C). The streaming potential was higher, but not significantly, for the constructs from rotating vessels compared with those from static and mixed flasks. The dynamic stiffness and streaming potential of the constructs from all of the groups were significantly

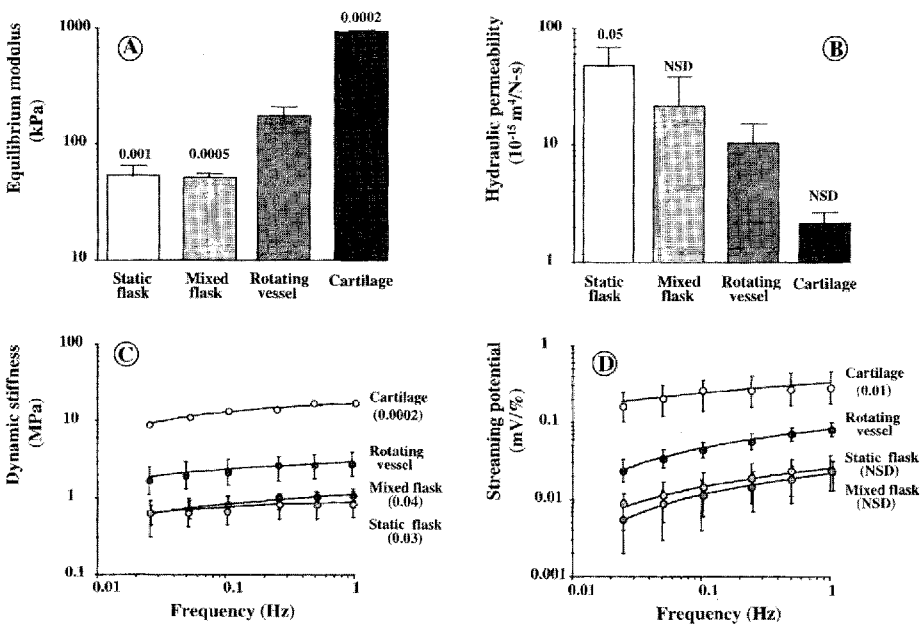

FIG. 4. Tissue biomechanics in static and dynamic confined compression. **A:** Equilibrium confined-compression modulus determined from the slope of the stress-strain curve between 10 and 40% strain. **B:** Hydraulic permeability at 30% strain, calculated with use of the equilibrium modulus and dynamic stiffness according to Frank and Grodzinsky (12). **C:** Dynamic stiffness calculated from the ratio of dynamic stress and the applied strain at 30% strain as a function of frequency. **D:** Streaming potential normalized by the amplitude of the applied strain at 30% strain as a function of frequency. Data represent average ± SD (n = 3-4) for 6-week constructs from static and mixed flasks, rotating vessels, and freshly explanted bovine articular cartilage. Numbers above bars and lines are the p values of pairwise comparison probabilities between the rotating vessels and other experimental groups. NSD = no significant difference, $p > 0.05$.

lower than those of natural cartilage (Fig. 4C and D).

Relationships between Construct Compositions and Mechanical Properties

Multiple regression analysis of data obtained for the 6-week constructs from all experimental groups detected that the equilibrium modulus (MPa), dynamic stiffness (MPa), hydraulic permeability (m^4/N-s) $\times$ 10^{15}, and streaming potential (mV/%) correlated with the % of wet-weight fractions of glycosaminoglycan, collagen, and water (Table 2).

DISCUSSION

Tissue engineering offers the possibility of creating functional cartilaginous equivalents for joint repair. Approaches have included transplanting collagen gels containing chondrocytes (33) or undifferentiated mesenchymal cells (34) or chondrocytes combined with periosteal grafts (3,20). Cartilage-like tissue can also be regenerated *in vitro* with use of chondrocytes or mesenchymal cells cultured on biomaterials (6,15-19,25,31). The latter approach has the potential advantage of transplanting a preformed functional tissue. However, cells tend to retain their differentiated phenotype *in vitro* only if cultured under conditions that resemble their natural *in vivo* environment (35). The mechanical and biochemical signals that affect *in vivo* tissue development, maintenance, and remodeling are likely to play similar roles during the *in vitro* cultivation of engineered tissue (19).

We previously showed that *in vitro* chondrogenesis progressed from the periphery of seeded scaffolds both outward and inward by the deposition of glycosaminoglycan and type-II collagen (18). After 6 weeks of cultivation in rotating vessels, the constructs were continuously cartilaginous over their entire cross sections (18). With further increase in cultivation time, the constructs continued to develop such that after 7 months of culture, their wet-weight fractions of glycosaminoglycan and their equilibrium moduli could not be distinguished from those measured for freshly explanted calf cartilage (17). In addition, the cultivation period of 6 weeks was previously found to be long enough to show the effects of cultivation parameters on tissue morphology and composition (19,31). In the present study, the hydrodynamic conditions during *in vitro* cultivation of tissue-engineered cartilage were varied with use of (a) static flasks (constructs fixed in place, static medium), (b) mixed flasks (constructs fixed in place, unidirectional turbulent flow), and (c) rotating vessels (constructs dynamically suspended in laminar flow) (Fig. 1 and Table 1). We report the effects of hydrodynamic factors on the development of functional cartilaginous matrix over 6 weeks of cultivation and the relationships between construct mechanical properties and compositions.

Fluid flow and mixing markedly affected the histomorphology and composition of engineered cartilage. In the static flasks, low diffusional rates of mass transport resulted in small constructs with low collagen and high water contents and glycosaminoglycan accumulation only at the periphery. In the mixed flasks, magnetic stirring generated turbulence at an intensity below that previously reported to cause cell damage (7,31), which induced the formation of fibrous capsules at the construct surfaces (Fig. 2) and increased the fractions of the tissue components, in particular that of collagen, as compared with constructs grown statically (Fig. 3). In the rotating vessels, fluid mixing was generated by the discoid constructs settling in the tumble-slide regimen as observed visually and predicted on the basis of the estimated values of inertia and drag (8,15) (Table 1). The associated fluctuations in fluid velocity (8,19) appeared to permit the chondrocytes to maintain their differentiated phenotype and form large cartilaginous constructs (Fig. 2) with glycosaminoglycan fractions not significantly different from those measured for natural cartilage explants (Fig. 3).

The effects of mixing on the composition of engineered cartilage can be compared with previously reported effects of hydrodynamic forces on cultured chondrocytes and cartilage explants. Steady hydrodynamic shear enhanced glycosaminoglycan synthesis in chondrocyte monolayers (30), whereas intermittent motion of medium in roller bottles stimulated chondrocytes to form cartilaginous nodules (23). Mixing during cell seeding and tissue cultivation resulted in increased fractions of tissue components in three-dimensional cartilaginous constructs (31). In particular, the constructs grown in rotating vessels had a continuous cartilaginous matrix with glycosaminoglycan fractions ranging from approximately 5% wet weight after 6 weeks of cultivation (18) (Figs. 2 and 3) to approximately 8.8% wet weight after 7 months of cultivation (17). Constructs cultured in rotating vessels were uniformly cartilaginous throughout their entire cross sections and had a very high, stable fraction of type-II collagen (92-99% of the total collagen of the construct) (18). It is possible that dynamic changes in hydrodynamic forces acting on freely settling constructs in rotating flow resemble some aspects of the dynamic mechanical loading, which enhanced glycosaminoglycan synthesis in cartilage explants (28), in contrast to static loading or the absence of loading, which caused cartilage matrix degradation (5). Fluid flow and mixing during *in vitro* cultivation generate forces (15,19,26,30,31) that are different in nature (mechanical compared with hydrodynamic) and several orders of magnitude lower than those resulting from physiological compression and shear in articular joints (1,5). Cultivation of constructs in the presence

of directly applied dynamic compression (28) or shear would allow a more direct comparison of tissue responses to acting forces *in vivo* and *in vitro*.

The effects of cultivation conditions and time on the equilibrium modulus, dynamic stiffness, streaming potential, and hydraulic permeability correlated with changes in the construct compositions (Table 2). Whereas 3-day constructs contained mostly cells and were too fragile to be mechanically tested, 6-week constructs could support load, presumably due to the accumulation of proteoglycans and collagen and their assembly into functional cartilaginous matrix. The constructs grown in rotating vessels, which had the highest wet-weight fractions of tissue components, also had the highest equilibrium modulus, dynamic stiffness, and streaming potential and the lowest hydraulic permeability (Figs. 3 and 4). Compared with those of chondrocytes cultured statically for 5 weeks in agarose gels (6), the glycosaminoglycan fractions of the chondrocyte/polyglycolic acid constructs cultured for 6 weeks in rotating vessels were approximately 3-fold higher and the equilibrium modulus and dynamic stiffness were approximately 2-fold higher. Compared with cartilage explants, the 6-week constructs from all groups were structurally and functionally inferior. In particular, the equilibrium modulus, dynamic stiffness, and streaming potential measured for the constructs from the rotating vessels were 20-25% as high and the hydraulic permeability was five times higher than the corresponding values measured for freshly explanted calf cartilage (Fig. 4). However, these same 6-week constructs contained about 78% as much glycosaminoglycan and 43% as much collagen as compared with fresh cartilage explants on a wet-weight basis, implying that either the accumulation of glycosaminoglycan and collagen preceded their assembly into a functional extracellular matrix or that extracellular matrix assembly in the constructs was different from that in the natural cartilage. This apparent lack of functional organization of the extracellular matrix in engineered constructs may be caused either by the use of very young cartilage (2-4-week-old bovine calf) or by the absence of specific factors or gene products in the *in vitro* culture environment that are normally present *in vivo*.

The mechanical properties of the constructs reflect the amounts and quality of matrix macromolecules and their ability to assemble into a functional extracellular matrix, as well as collagen-proteoglycan and fluid-matrix interactions. With additional cultivation, the constructs more closely approximated cartilage with respect to composition and function: the constructs cultured for 7 months in rotating vessels contained markedly more glycosaminoglycan and had better mechanical properties than those cultured for 3 months (17) or 6 weeks (Figs. 3 and 4). The cell-polymer-bioreactor system for tissue engineering can thus provide a basis for studying the structural and functional properties of the cartilaginous matrix during its development because tissue concentrations of glycosaminoglycan and collagen can be modulated by the time and conditions of tissue cultivation.

Correlations between matrix composition and mechanical function observed in the present study for engineered cartilage (Table 2) were qualitatively similar to those previously reported for natural cartilage immediately after explant or after *in vitro* cultivation (2,29), as well as to those for chondrocytes cultured in agarose gels (6). In particular, previously reported relationships include the following: (a) an increase in equilibrium modulus with increasing fractions of glycosaminoglycan and collagen in bovine cartilage explants (29) and with decreasing water content in human cartilage (2); (b) an increase in hydraulic permeability with increasing water content in bovine cartilage explants and no change in hydraulic permeability with changing fractions of glycosaminoglycan and collagen (29); and (c) an increase in streaming potential with increasing glycosaminoglycan fraction and decreasing collagen fraction in bovine cartilage explants (29) and increasing glycosaminoglycan fraction in matrix synthesized by chondrocytes in agarose gel cultures (6). The present study demonstrated similar relationships between the compositions (concentrations of glycosaminoglycan, collagen, and water) and physical properties (equilibrium modulus, dynamic stiffness, hydraulic permeability, and streaming potential) of the extracellular matrix synthesized by chondrocytes on polymer scaffolds (Table 2).

Possible mechanisms that may underlie the detected relationships between construct compositions and mechanical properties include the effects of (a) electrostatic forces, associated with proteoglycans, on equilibrium modulus (9,29) and (b) fixed-charge density (which has been correlated to glycosaminoglycan concentration in natural cartilage) on streaming potential (13,24,29). The observed effects of water fraction on physical properties of engineered cartilage may be explained by the decreased concentration of solid matrix, especially proteoglycan, available to resist the applied compressive load (2).

In summary, this study demonstrated that the morphology, composition, and mechanical function of engineered cartilage can be modulated by flow conditions during *in vitro* cultivation. The observed relationships between the composition and mechanical properties of tissue-engineered cartilage appeared consistent with those previously reported for young bovine and adult human cartilage.

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