Effects of Mixing on the Composition and Morphology of Tissue-Engineered Cartilage

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Cartilage constructs were grown using isolated chondrocytes and biodegradable polymer scaffolds made of fibrous polyglycolic acid in the form of 1-cm-dia × 5-mm-thick discs. The scaffolds were seeded in a mixed cell suspension and cultured for up to 8 weeks under static or mixed tissue culture conditions in petri dishes and spinner flasks. Turbulent mixing significantly improved the biochemical compositions and altered morphologies of the cartilage constructs, which were the thickest ones cultured to date in vitro. Constructs from mixed cultures were more regular in shape and contained up to 70% more cells, 60% more sulfated glycosaminoglycan, and 125% more total collagen when compared to constructs from static cultures. Mixing also induced the formation of an outer capsule with multiple layers of elongated cells and collagen fibrils around the inner tissue phase, while statically grown constructs consisted of round cells embedded in cartilaginous matrix. Mixing during cell seeding and tissue culture is thus an important parameter for the cultivation of tissue-engineered cartilage in a range of sizes, shapes and compositions for a variety of clinical applications (e.g., fibrous cartilage for reconstructive surgery or articular cartilage for joint resurfacing).

Introduction

Studies of tissue-engineered cartilage have been motivated by the clinical need for improved treatment options that could benefit an estimated one million patients per year (Langer and Vacanti, 1993). Current therapies include transplantation, which is limited by the availability of donor tissue, and artificial prostheses, which are often complicated by adhesive breakdown at the host-device interface. Cartilage tissue constructs with defined sizes and shapes have been grown *in vitro* using isolated chondrocytes and fibrous PGA scaffolds (Freed and Vunjak-Novakovic, 1995a). The scaffolds were shown to maintain the construct size and shape, degrade in parallel with the accumulation of tissue components, and permit chondrocytes to express their differentiated phenotype (Freed et al., 1994c). Constructs grown *in vitro* resembled normal cartilage both biochemically and histologically (Freed et al., 1994a), and continued to remodel following *in vivo* implantation, subcutaneously in nude mice (Freed et al., 1993a; Vacanti et al., 1991) and intra-articularly in rabbits (Freed et al., 1994b).

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Identification and control of key parameters that determine *in vitro* chondrogenesis are essential for cartilage tissue engineering, since the required size, shape, biochemical composition, and morphology of cartilage implants may vary from one case to another. Ideally, for a given clinical application (i.e., repair of fibro- or articular cartilage) the culture conditions could be optimized with respect to cell substrate (e.g., polymer scaffold), medium composition and renewal rate, and bioreactor fluid dynamics. The specific requirements of cartilage tissue cultivation include (1) a 3-D scaffold that allows spatially uniform cell attachment in conjunction with the maintenance of cell phenotype; (2) adequate mass-transfer rates of nutrients, oxygen, and carbon dioxide; and (3) a hydrodynamic environment that is permissive for chondrogenesis, or even designed to promote selected cell functions (Freed and Vunjak-Novakovic, 1995a).

Tissue morphogenesis is known to depend on exogenous forces (Thompson, 1977). In a quiescent environment, vascular endothelial cells tend to adopt a spherical shape that is associated with differentiated cell function, while environmental stress stimulates cell elongation and growth (Levesque et al., 1989). In cartilage, exogenous forces regulate cellular secretion of extracellular matrix (ECM) *in vivo*, the 3-D orga-

nization of which determines the biomechanical tissue properties (Mow et al., 1991). *In vitro* morphogenesis of engineered tissues is expected to depend on exogenous factors (e.g., flow and mixing) in at least two ways: (1) by direct hydrodynamic effects on cell shape and function, and (2) by flow-induced changes in mass-transfer rates of nutrients and metabolites. Mixing and turbulence are likely to be needed to cultivate clinically sized implants (e.g., 2–5-mm-thick cartilage), in order to achieve the required mass-transfer rates. Bioreactor design considerations must thus include the interaction between the growing cartilage tissue and its fluid-dynamic environment.

However, there has been little quantitative work on the effects of hydrodynamic forces on cells that is relevant for bioreactor cultivations of 3-D engineered tissues. Previous bioreactor studies have focused on the kinetics of cell growth under turbulent conditions, in suspension, or on microcarrier beads (Augenstein et al., 1971; Cherry and Papoutsakis, 1988, 1989, 1990; Croughan et al., 1987; McQueen et al., 1987; Merchuk, 1991; Papoutsakis, 1991). In contrast, the effects of flow on cellular morphology and biosynthetic rates have been studied for confluent cell monolayers (e.g., endothelial and kidney cells) in well-defined, laminar flow fields (Dewey et al., 1981; Dewey, 1984; Frangos et al., 1985; Franke et al., 1984; Krueger et al., 1971; Levesque and Nerem, 1985, 1989; Levesque et al., 1989, 1990; Stathopoulous and Hellums, 1984).

In the only previous study of the effects of turbulence on cell monolayers, Davies et al. (1986) observed that the nature of flow (laminar or turbulent) rather than the magnitude of the shear stress determined the turnover of vascular endothelial cells. In bioreactor cultures of chondrocytes on microcarrier beads, mixing promoted cell attachment and proliferation (Freed et al., 1993a). Orbital mixing in petri dishes improved cell seeding on polymer scaffolds and the composition of the resulting cartilage constructs (Freed et al., 1994a). More recently, hydrodynamic forces acting on freely suspended cartilage constructs in a well-defined rotational flow field resulted in construct morphologies different from those observed in control studies in spinner flasks (Freed and Vunjak-Novakovic, 1995b).

In this study, the effects of mixing in the tissue culture environment on the composition and morphology of 3-D cartilage constructs were studied using turbulent spinner flasks, orbitally mixed petri dishes, and static cultures. Our hypothesis was that fluid-dynamic conditions can modulate cell function in the engineered cartilage by affecting cell shape and mass-transfer rates of chemical species.

Materials

Knee joints from bovine calves were obtained from a local abattoir within 4 hours of slaughter. Type II collagenase was from Worthington (Freehold, NJ). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), *N*-2-HydroxyEthylPiperazine *N'*-2-EthaneSulfonicAcid (HEPES), and phosphate buffered saline (PBS) were from GIBCO (Grand Island, NY). Type III papain, penicillin, streptomycin, nonessential amino acids, proline, ascorbic acid, bovine chondroitin sulfate, and calf thymus DNA standard were from Sigma (St. Louis, MO). Hoechst 33258 dye was from Polysciences (Warrington, PA). Dimenthylmethylene blue dye was from Aldrich (Milwaukee, WI). Chloramine-T was from Mallinckrodt (Fair Lawn, NJ); *p*-dimethylaminobenzaldehyde (*p*-DAB) was from Fischer (Paris, KY). Agarose (high T_m) was from BioRad (Richmond, CA). Spinner flasks (100 cm^3 volume with a nonsuspended 4-cm-long stir bar), orbital shakers (type BTB), and magnetic stirrers (Multi Stir 4) were from Bellco (Vineland, NJ). Six-well tissue culture dishes were from Costar (Cambridge, MA). Needles [4-in. (102-mm)-long, 22-gauge] were from Metropolitan Hospital Supply (Cambridge, MA). Silicone stoppers (#10 1/2) and silicon tubing (#13) were from Cole Parmer (Niles, IL).

Methods

Chondrocytes

Full-thickness articular cartilage was harvested aseptically from the femoropatellar grooves of 2–3-week-old bovine calves. Chondrocytes were isolated by digestion with collagenase as previously described (Freed et al., 1993a), and resuspended in culture medium (DMEM), containing 4.5 g/L glucose, 584 mg/L glutamine, 10% FBS, 50 U/cm^3 penicillin, 50 μg/cm^3 streptomycin, 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM proline, and 50 μg/mL ascorbic acid.

Polymer scaffolds

Polyglycolic acid (PGA) mesh was produced at Albany International (Mansfield, MA) by extruding PGA into 12-μm-diameter fibers and processing these into fibrous scaffolds, 1 cm in diameter $\times$ 0.5 cm thick, with a void volume of 97% and a bulk density of 44 mg/cm^3. The scaffolds were sterilized with ethylene oxide and packaged under vacuum in a dry box (Freed et al., 1994c).

Petri dish studies

In petri dish studies, all constructs were seeded under mixed conditions and then cultured in either static or mixed petri dishes as follows (Figure 1). PGA scaffolds were prewetted in medium for 24 hours at 37°C and seeded with isolated chondrocytes in 35-mm dishes coated with a thin film of 1% agarose (Freed et al., 1994a). Dishes containing one PGA scaffold, 6 cm^3 of medium, and 7×10^6 cells per well were placed on a 50 rpm orbital shaker and incubated for 3 days at 37°C/5% CO_2 to allow for cell attachment to the PGA fibers. After 3 days, cell-PGA constructs were transferred to new 35-mm dishes (not agarose coated) and cultured for up to 8 weeks at 37°C/5% CO_2, either statically (experimental group 1) or on a 50 rpm orbital shaker (experimental group 2). Medium was replaced every 2–3 days (the medium renewal rate was 3 cm^3 per construct per day).

Spinner flask studies

In spinner flask studies, all constructs were seeded under mixed conditions and then cultured in either static or mixed flasks as follows (Figure 1). Spinner flasks were autoclave-sterilized for 45 min at 121°C. PGA scaffolds ($n = 8$ per flask) were prewetted in culture medium for 24 h at 37°C, threaded onto 4-inch-long, 22-gauge needles, and positioned using 0.3-

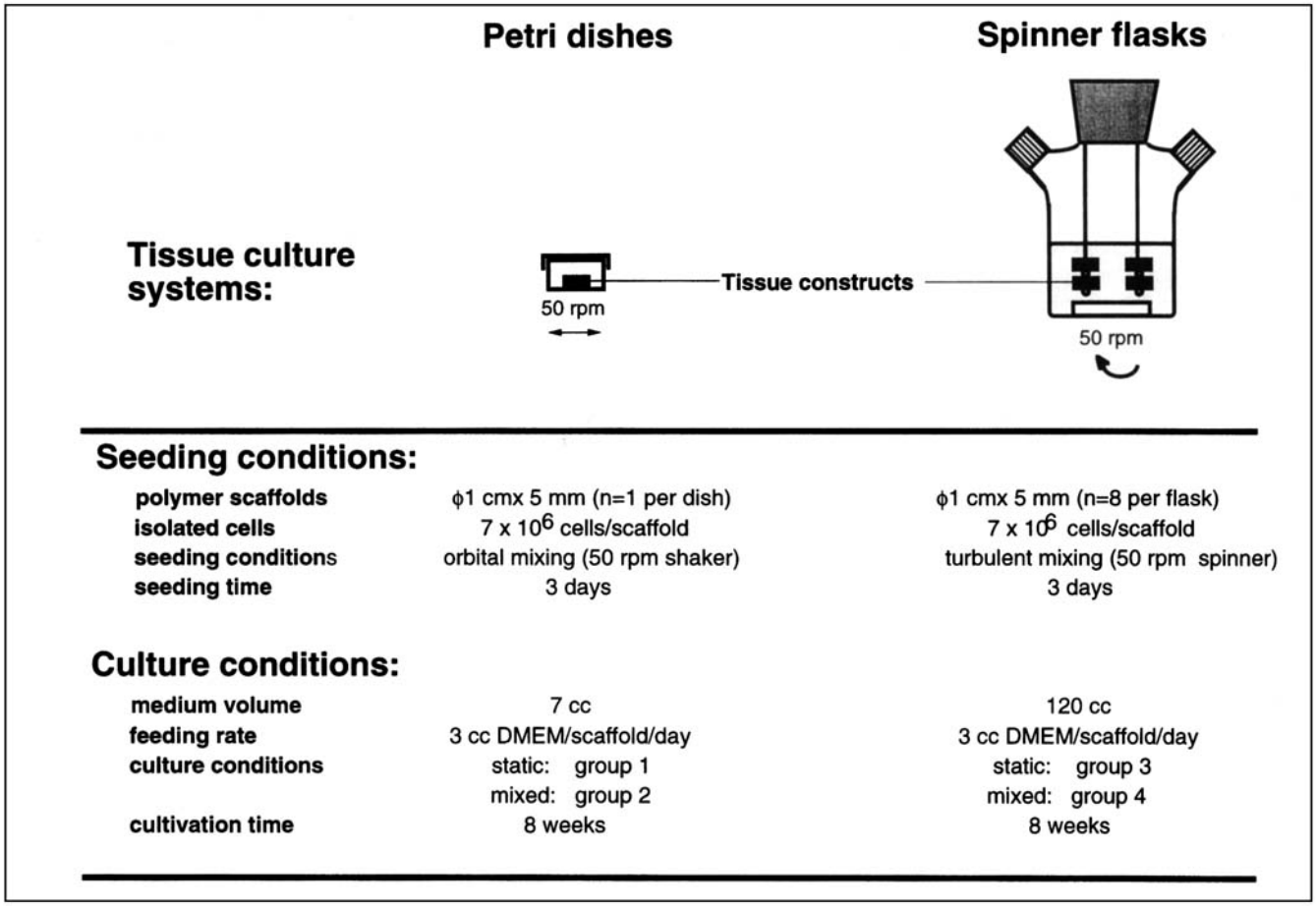


Figure 1. Experimental conditions.

Isolated primary chondrocytes were seeded onto fibrous, biodegradable polyglycolic acid (PGA) scaffolds under mixed conditions using petri dishes (orbital shaker, 50 rpm) and spinner flasks (magnetic stirrer, 50 rpm). In both systems, the resulting cell-polymer constructs were cultured *in vitro* under static and mixed culture conditions for up to 8 weeks.

cm-long segments of silicone tubing. Four needles with two scaffolds apiece were fixed to a 5-cm-diameter silicone stopper placed in the mouth of the flask (Figure 1). The flasks were filled with 120 cm^3 of culture medium and placed in a humidified 37°C/5% CO_2 incubator with the side-arm caps loosened such that the medium could equilibrate with the 5% CO_2 atmosphere. The flasks were mixed using a magnetic stir bar at 50 rpm and inoculated at time zero with freshly isolated chondrocytes (5.6×10^7 cells per flask corresponding to 7×10^6 cells per PGA scaffold). After 3 days, the constructs were cultured either statically (experimental group 3) or using a 50 rpm stirrer (experimental group 4). Medium was replaced at a rate of 50% every 2–3 days for up to 8 weeks (the medium renewal rate was 3 cm^3 per construct per day).

Assays

Histological samples were embedded in paraffin, cross-sectioned (8 μm thick) through the center of the construct, and stained with hematoxylin and eosin (H&E) for cells and safranin-O for glycosaminoglycan (GAG) (Lillie and Fullmer, 1976). Construct dimensions were measured on sectioned samples using an inverted microscope (Nikon Diaphot) fitted with an eyepiece reticle, a video camera (Hitachi), a frame grabber (Scion), and commercial image processing software (NIH Image 1.49 for MacIntosh). Construct diameter and height were assessed assuming a cylindrical construct shape. Capsule thickness was calculated from the cross-sectional areas of the construct and inner tissue phase that were determined by image analysis. The values of six independent measurements were averaged to obtain construct diameter and height and capsule thickness.

Samples for biochemical analyses (6 constructs per data point or 10 plugs of parent cartilage) were frozen, lyophilized, and digested with 2 cm^3 papain (Freed et al., 1993b). The number of chondrocytes per construct was assessed by measuring the amount of DNA using Hoechst 33258 dye and a spectrofluorometer (Aminco, Urbana, IL) (Kim et al., 1988). The mass of cells (mg dw) was calculated using the DNA content of a cell (7.7 pg/cell; Kim et al., 1988) and the mass of a single cell (10^{-10} g dw; Freed et al., 1994c). The sulfated GAG content was determined after reaction with dimethylmethylene blue dye using bovine chondroitin sulfate as a standard and spectrophotometer at 525 nm (Perkin Elmer, Oak Bridge, IL) (Farndale et al., 1986). The total collagen content was determined from the hydroxyproline content after acid hydrolysis (6N HCl at 115°C for 18 h) and reaction with *p*-DAB and chloramine-T, using a spectrophotometer at

Table 1. Hydrodynamic Parameters in Mixed Spinner Flasks

Flask diameter	$D = 6.5$ cm
Stirring bar diameter	$d_i = 4.0$ cm
Medium volume	$V = 120$ cm^3
Medium density*	$\rho = 1.03$ g/cm^3
Medium viscosity**	$\mu = 0.01$ g/cm·s
Mixing rate	$n = 50$ rpm $= 0.83$ s^{-1}
Impeller Reynolds no.	$Re_i = d_i^2 \cdot n \cdot \rho / \mu = 1373$
Integrated shear factor**	ISF$= 2\pi n d_i/(D - D_i) = 8.37$ (s^{-1})
Impeller tip speed	$T_s = \pi n d_i = 10.5$ cm/s
Max. time-averaged shear rate†	$Y_m = 0.4\ T_s = 4.19$ (s^{-1})
Dimensionless power no.‡	$N_p = 0.5$ for $Re > 1000$
Turbulent power dissipation/unit mass of fluid‡	$\epsilon = (N_p \cdot n^3 \cdot d_i^5)/V = 2.47$ cm^2/s^3
Size of the smallest turbulent eddies‡	$\eta = (\mu^3/\rho^3\epsilon)^{1/4} = 247\ \mu$m
Velocity of the smallest turbulent eddies‡	$v = (\mu\ \epsilon/\rho)^{1/4} = 0.395$ cm/s

*Freed and Vunjak-Novakovic (1995b).
**Croughan et al. (1987).
†According to Croughan et al. (1987) for undisturbed mixed flow in spinner flasks without tissue constructs.
‡Cherry and Papoutsakis (1988).

550 nm as previously described (Woessner, 1961). The amount of collagen was calculated using the hydroxyproline-to-collagen conversion factor of 0.143 (Woessner, 1961). The mass of undergraded PGA in tissue constructs was calculated based on the previously measured scaffold degradation kinetics (Freed et al., 1994c).

Results

Hydrodynamic conditions

Effects of mixing on tissue-engineered cartilage were studied for cell–polymer constructs grown for 8 weeks in mixed spinner flasks, mixed petri dishes, and static cultures (Figure 1). In both tissues culture systems, polymer scaffolds were seeded with cells under mixed conditions. In petri dishes, mixing during cell seeding was achieved by orbital motion of the shaker (50 rpm, 4-cm amplitude), which maintained the cells in a nonuniform, off-bottom suspension without causing motion of the scaffold. In spinner flasks, mixing during cell seeding was achieved by magnetic stirring (50 rpm, 4-cm diameter stir bar), which maintained a uniform cell suspension around scaffolds that were fixed in place. The resulting cell–polymer constructs were cultivated either statically in petri dishes (group 1) and spinner flasks (group 3), or under the same mixing conditions as during cell seeding in dishes (group 2) and spinner flasks (group 4).

Hydrodynamic conditions during cell seeding and tissue culture in mixed spinner flasks are summarized in Table 1. The impeller Reynolds number, Re_i, of 1373 corresponded to conditions of turbulent mixing around the scaffolds during cell seeding and the resulting tissue constructs during cultivation. The integrated shear factor (ISF), previously used as a measure of the average shear intensity in the radial fluid flow between the stirring bar and the vessel wall (Croughan et al., 1987), was 8.37 s^{-1}. The maximum time-averaged shear rate for mixed flow in spinner flasks without tissue constructs, Y_m, calculated according to Croughan et al. (1987), was 4.2 s^{-1}.

The Kolmogorov scale of the turbulence spectrum was estimated assuming that the presence of tissue constructs does not significantly affect the dissipation of kinetic energy in the viscous subrange. Approximation of the actual fluid flow in spinner flasks by isotropic turbulence as suggested by Cherry and Papoutsakis (1988) resulted in the volume-averaged turbulent power dissipation per unit mass of fluid, ϵ, of 2.47 cm^2/s^3. Using the preceding assumptions, the estimated size and velocity of smallest turbulent eddies throughout the fluid volume were $\eta = 250\ \mu$m and $v = 0.395$ cm/s, respectively (Table 1).

Construct size, weight, and histological appearance

The diameter of 8 week constructs remained close to that of the original scaffold (i.e., 10 mm) in all experimental groups (Table 2). Constructs grown in mixed dishes and spinner flasks better maintained the shape and dimensions of the original polymer scaffold when compared to statically grown constructs (Figure 2). Construct dry weights (mg dw) increased from the initial scaffold dry weight of 15 mg to 22–46 mg, where higher values corresponded to spinner flask cultures and mixed culture conditions (Figure 3a). The construct dry-to-wet-weight ratio (mg dw/mg ww) was also higher for constructs grown in spinner flasks and under mixed culture conditions, but generally lower than that for natural cartilage (Table 3).

Constructs were significantly thicker in mixed than in static cultures, and in static or mixed spinner flasks than in the corresponding petri dishes (Figure 3b). After 8 weeks of *in vitro* tissue culture, only constructs grown in mixed spinner

Table 2. Dimensions, Structures, and Compositions of 8-week Cartilage Tissue Constructs

	Petri Dishes			Spinner Flasks		
	Static	Mixed	p* 1 vs. 2	Static	Mixed	p* 3 vs. 4
Experimental Group	1 ($n = 6$)	2 ($n = 6$)		3 ($n = 6$)	4 ($n = 6$)	
Construct diameter (mm)	9.95 ± 0.11	9.69 ± 0.34	NSD	11.3 ± 0.36	9.45 ± 0.01	NSD
Thickness: construct (mm)	2.72 ± 0.02	3.57 ± 0.11	< 0.001	3.66 ± 0.40	4.76 ± 0.01	< 0.001
capsule (μm)	N/A	306 ± 54		N/A	334 ± 140	
Cell number ($\times 10^{-7}$/construct)	1.15 ± 0.02	1.61 ± 0.04	< 0.001	2.1 ± 0.30	3.55 ± 0.27	< 0.001
GAG (mg dw/construct)	2.96 ± 0.21	2.69 ± 0.68	NSD	4.46 ± 0.43	7.08 ± 0.35	< 0.001
Collagen (mg dw/construct)	3.85 ± 0.33	6.08 ± 0.39	< 0.001	6.77 ± 0.43	15.3 ± 2.8	< 0.001

NSD = not significantly different; N/A = not applicable; * = according to a two-sided Student T-test.

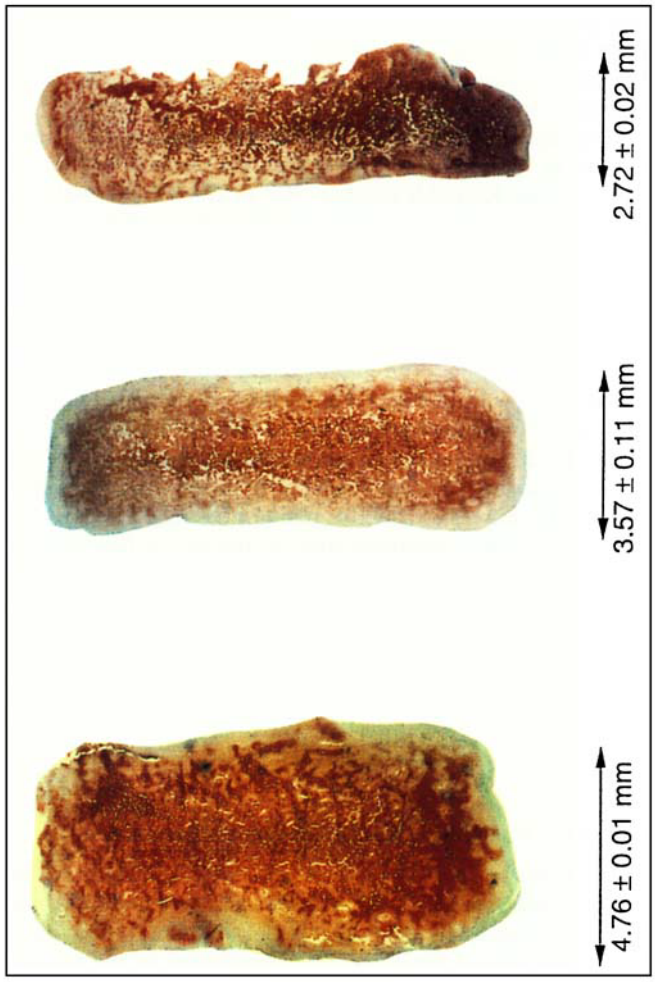


Figure 2. Effects of mixing on the shape and size of 8-week constructs.

Full cross-sections are shown for constructs cultivated in (a) static petri dishes, (b) orbitally mixed petri dishes, and (c) turbulent spinner flasks. Stain: safranin-O; original magnification × 10.

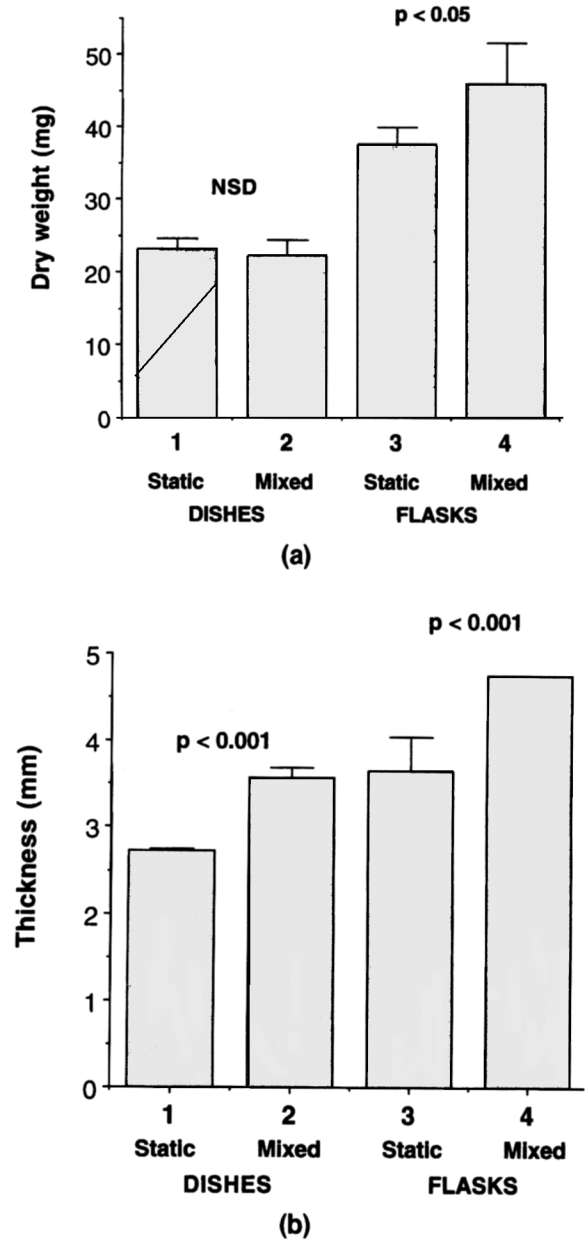


Figure 3. Effects of mixing on construct physical properties.

(a) Dry weight and (b) thickness. Data are the average ± SD ($n = 6$) for 8-week constructs.

flasks maintained the approximate thickness of the original polymer scaffold (5 mm), while all other constructs were significantly thinner (Table 2). The reported thicknesses, assessed by image processing of histological cross sections of the constructs, were up to 30% lower than the thicknesses of unprocessed constructs.

Mixing also affected the appearance and morphology of 8-week tissue constructs. Macroscopically, statically grown constructs had rough surfaces and appeared soft and friable, while constructs from mixed cultures had smooth surfaces and appeared stiff and elastic. Histologically, statically grown constructs consisted of round cells embedded in a nonhomogeneous, patchy cartilaginous matrix that stained red with safranin-O and was denser at the upper construct surface, which was exposed to the culture medium (Figure 2a). In contrast, two histologically distinct regions were observed in constructs grown in mixed cultures: a fibrous capsule around an inner cartilaginous tissue phase (Figures 4b and 4c).

In 8-week constructs from mixed dishes and flasks, the capsules were 306–334 μm thick (Table 2) and accounted for 26–30% of the construct volume. The capsule consisted of multiple layers of elongated cells that were embedded in collagen but did not stain for GAG (Figures 4a and 4b). The cellularity of the capsule was higher than that of the inner tissue phase (Figures 4b and 4c). In contrast, in statically grown constructs the cells at the surfaces remained spherical and were surrounded by both GAG and collagen (Figures 4a and 4b), and the cellularity at the surface was comparable to that of the inner tissue phase (Figure 4b vs. 4c). In both systems, the overall histological appearance of the inner tissue phase was similar for constructs grown under static and mixed conditions, except for stronger safranin-O staining of the cartilaginous matrix in mixed cultures (Figure 4c).

Table 3. Effects of Mixing on the Biochemical Composition of 8-week Cartilage Constructs

	Petri Dishes			Spinner Flasks			Parent Cartilage
	Static	Mixed	p^* 1 vs. 2	Static	Mixed	p^* 3 vs. 4	
Experimental Group	1 ($n=6$)	2 ($n=6$)		3 ($n=6$)	4 ($n=6$)		($n=10$)
Construct dry weight (dw, mg)	23.2±1.4	22.3±2.1	NSD	37.7±2.4	46.0±5.7	<0.05	7.9±1.8
Dry/wet weight ratio (dw/ww, %)	6.10±0.32	7.12±0.20	<0.001	6.09±0.31	9.27±0.32	<0.001	23.6±4.5
Cells (% dw)	4.96±0.23	7.24±0.75	<0.001	5.55±0.57	7.03±0.60	<0.02	4.5±1.4
GAG (% dw)	12.80±1.0	13.42±1.2	NSD	10.6±0.51	15.6±1.9	<0.002	37.7±4.8
Collagen (% dw)	16.62±0.75	26.7±1.1	<0.001	18.0±0.70	31.5±1.2	<0.001	45.4±6.6
PGA (% dw)	17.48±1.0	19.2±1.8	NSD	11.2±0.72	9.29±1.1	<0.002	0
Cells + GAG + Collagen (% dw)	36.38±1.7	47.43±1.7	<0.001	35.4±2.4	53.9±1.7	<0.001	87.6±4.0
GAG/cell (mg/mg dw)	2.58±0.18	1.87±0.30	<0.01	2.15±0.30	2.11±0.30	NSD	7.61±1.7
Collagen/cell (mg/mg dw)	3.36±0.25	3.72±0.23	NSD	3.25±0.27	4.63±0.50	<0.002	9.42±2.4
Collagen/GAG (mg/mg dw)	1.30±0.08	2.00±0.22	<0.001	1.52±0.14	2.26±0.35	<0.01	1.27±0.31

NSD = not significantly different; * = according to a two-sided Student T-test.

Biochemical composition of the constructs

At 3 days, cell-polymer constructs seeded in mixed dishes (groups 1 and 2) or mixed spinner flasks (groups 3 and 4) contained $(3.85 \pm 0.13) \times 10^6$ or $(9.63 \pm 0.52) \times 10^6$ cells/construct, respectively. The 3-day cell numbers corresponded to 55% and 138% of the number of cells added at time zero to the dishes and spinner flasks, respectively (7×10^6) and reflected the combined effects of mixing on cell attachment and the initial cell proliferation in the two systems. The initial distribution of the attached cells was more uniform in spinner flasks than in dishes, as assessed from histological cross-sections of 3-day constructs.

In both tissue culture systems, mixing increased the total amounts of tissue components when compared to the respective static cultures. The total amounts of cells, GAG, and collagen (mg dw per construct) were all significantly higher in 8-week constructs grown in spinner flasks than in the corresponding petri dish cultures (Table 2). Differences in the total amounts of GAG and collagen between constructs in mixed and static cultures increased with increasing cultivation time (Figures 5a and 5b).

The % dw amounts of cells, GAG, and collagen were also significantly higher for 8-week constructs grown in mixed cultures than in the corresponding static cultures, and in flasks than in dishes (Table 3). The differences in % dw amounts of GAG and collagen between mixed and static cultures also increased with increasing cultivation time (Figures 6a and 6b). When compared to parent calf cartilage, all constructs contained more cells, less GAG, and less collagen (Table 3). In particular, the constructs grown in mixed flasks, which most closely resembled parent cartilage, contained about 150% as many cells, 40% as much GAG, and 70% as much collagen on a % of dw basis as the parent cartilage (Table 3).

After 8 weeks of cultivation, the amount of GAG per unit of dry cell mass, GAG/cell, was similar in all experimental groups and significantly lower than that in parent cartilage (GAG/cell = 2–2.5 or 7.6, respectively). The amounts of collagen per unit dry cell mass, Coll/cell, were similar in static cultures and mixed dishes and significantly higher in mixed flasks (3.2–3.7 or 4.6 mg dw collagen/mg dw cell mass, respectively), but still much lower than that in the parent cartilage (Coll/cell = 9.4). The collagen to GAG ratio (mg dw/mg dw) in static cultures was close to that for the parent cartilage, and higher than normal in both mixed culture systems (Table 3). The total and % dw amounts of tissue components (cells, GAG, collagen) in 8-week constructs were significantly higher in mixed than in static cultures, and in flasks than in dishes (Figures 7a and 7b). The total amount of identified tissue components in constructs grown for 8 weeks in turbulent spinner flasks was about 60% that of the parent calf cartilage (54.1 and 87.6%, respectively, Table 3).

Discussion

The long-term goals of our studies are twofold: (a) to improve our understanding of *in vitro* chondrogenesis, and (b) to cultivate cell–polymer cartilage implants with a range of defined sizes, shapes, and compositions for a variety of clinical applications. The proposed system for cartilage tissue engineering is based on the use of isolated chondrocytes and 3-D fibrous PGA scaffolds in conjunction with tissue culture bioreactors. In our previous studies, mixing promoted cell seeding and tissue growth in petri dishes (Freed et al., 1994a) and bioreactors (Freed and Vunjak-Novakovic, 1995a). The present study was aimed at assessing the effects of mixing on the biochemical composition and morphology of cartilage constructs, in order to identify fluid-dynamic conditions that can stimulate chondrogenesis.

Mixing during cell seeding suspended the isolated cells and provided relative velocity between the cells and the scaffolds. The higher cellularities and more uniform cell distributions in 3-day constructs seeded in flasks when compared to dishes could be attributed to differences in flow patterns between the two systems (i.e., turbulent mixing vs. orbital fluid motion). The probable mechanism by which cells populate the scaffold interior is convective motion of suspended cells into the highly porous (97%) structure followed by inertial impacts between the cells and the fibers and cell attachment. Higher initial (3-day) celluarities of constructs seeded in flasks than dishes resulted in higher final (8-week) amounts of GAG and collagen (groups 3 and 4 vs. 1 and 2, respectively, Table 2). This finding can be attributed to cooperative cell–cell and cell–matrix interactions (Gurdon, 1988; Tacchetti et al., 1992; Watt and Dudhia, 1988).

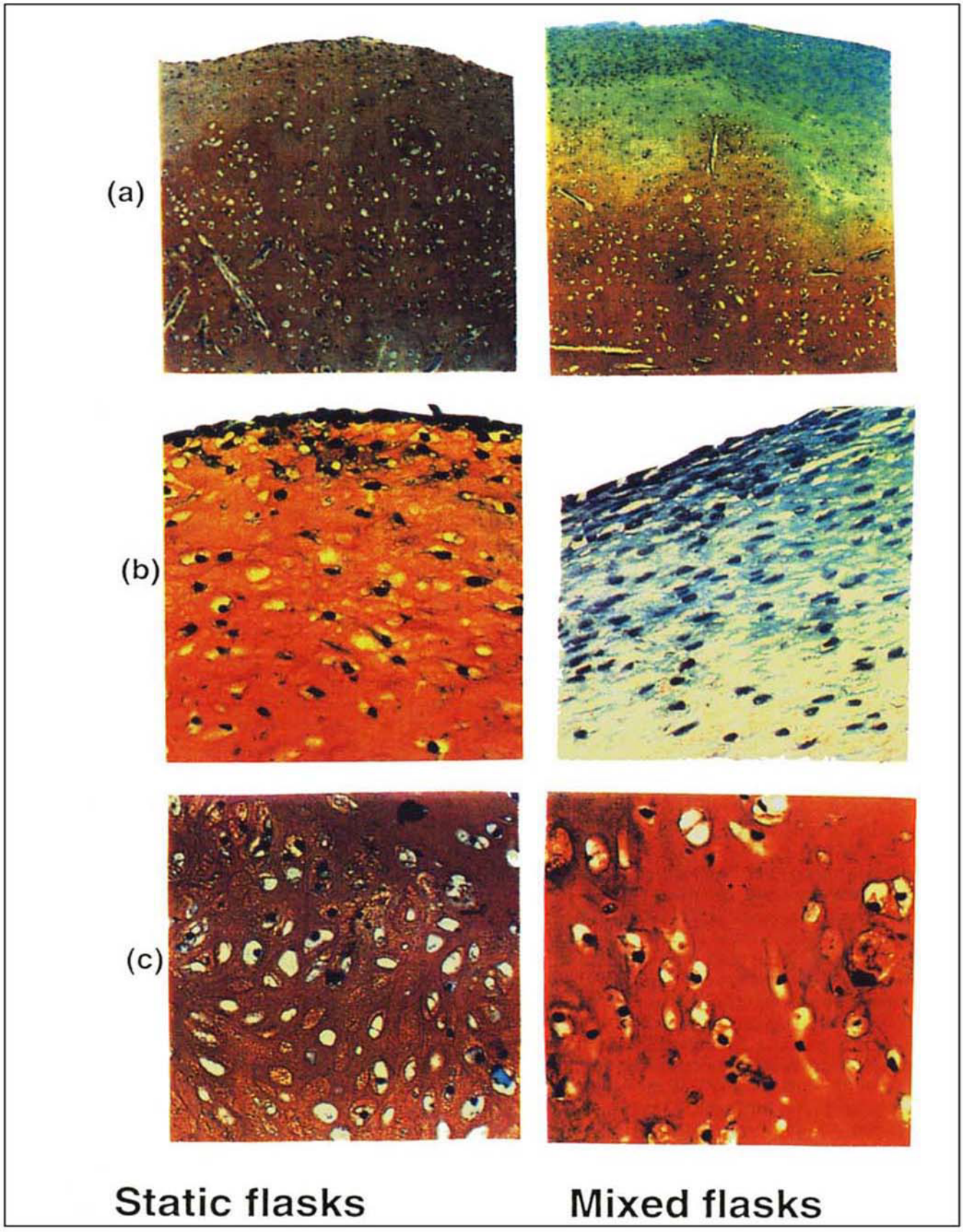
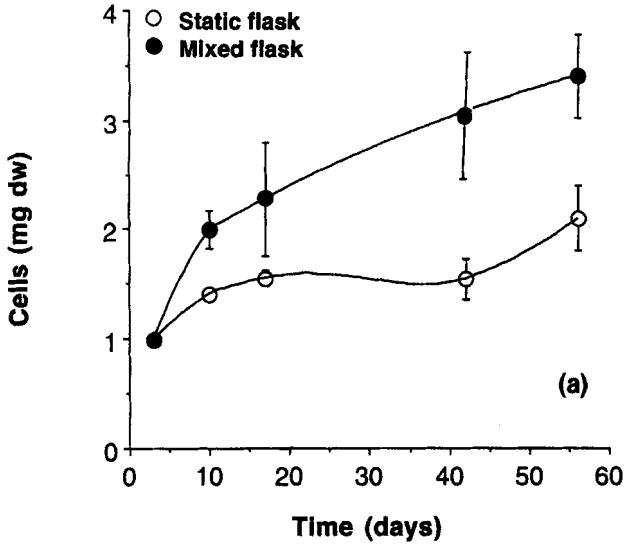
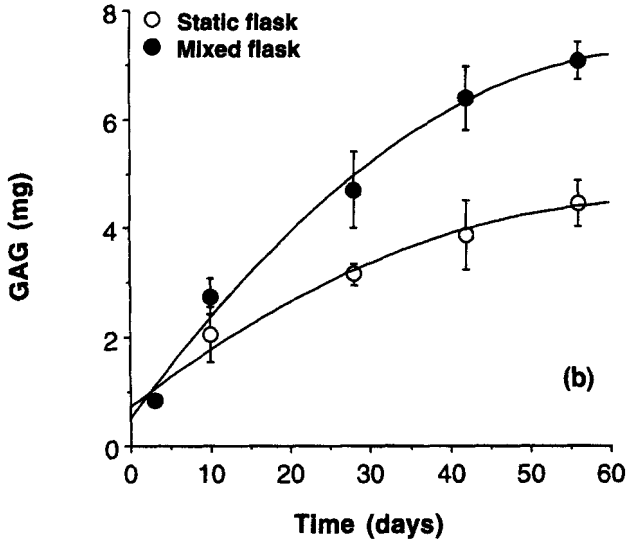


Figure 4. Effects of mixing on the morphology of 8-week tissue constructs.

(a) Uniform cartilaginous tissue or fibrous capsule at the construct surface (original magnification: × 100). (b) round or elongated cells at the construct surface (original magnification: × 400), and (c) inner tissue phases (original magnification: × 400) for static or mixed spinner flask cultures, respectively. Stain: safranin-O.





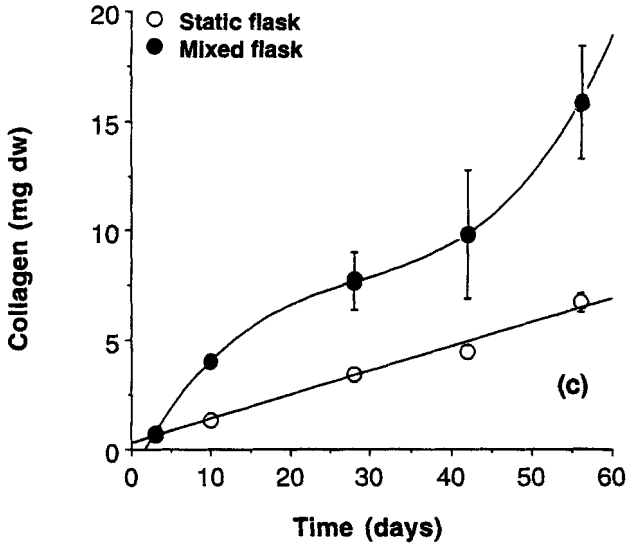
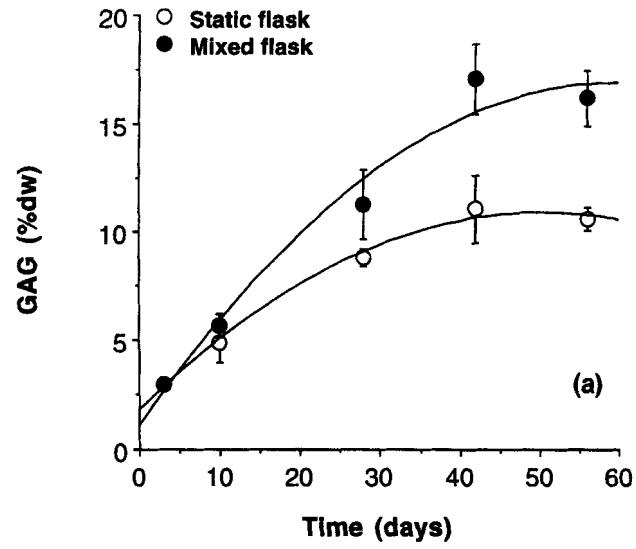


Figure 5. Amounts of (a) cells, (b) GAG, and (c) collagen as a function of cultivation time.

Data represent the average (mg dw) ± SD ($n = 6$) for constructs grown in static and mixed spinner flasks.



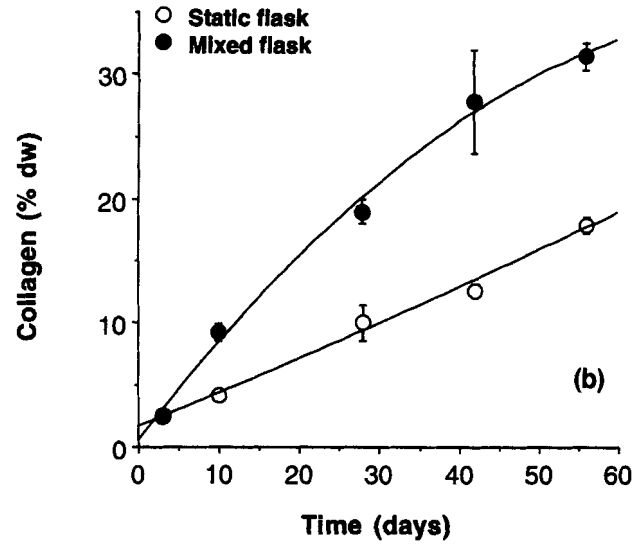


Figure 6. Effects of mixing on the fractions of (a) GAG and (b) collagen.

Data represent the average (% dw) ± SD ($n = 6$) for constructs grown in static or mixed spinner flasks.

Mixing during tissue culture significantly improved the biochemical compositions (Figures 6 and 7) and affected the histological appearances of the cartilage constructs (Figure 4), which were the thickest ones cultured to date *in vitro* (1 cm in diameter × 4.8 mm thick). Constructs grown in mixed cultures were thicker (Figure 3b), more regular in shape (Figure 2), and contained up to 70% more cells, 60% more GAG, and 125% more total collagen than the respective statically grown constructs (Table 3).

The observed effects of mixing on construct composition were consistent with the relative intensity and mass-transfer rate associated with turbulent mixing (i.e., flasks) as compared to orbital fluid motion (i.e., dishes). In dishes, orbital mixing increased the amounts of cells and collagen but not GAG in 8-week constructs (groups 1 and 2, Tables 2 and 3), while in flasks, turbulent mixing increased the amounts of all tissue components (groups 3 and 4, Tables 2 and 3, Figures

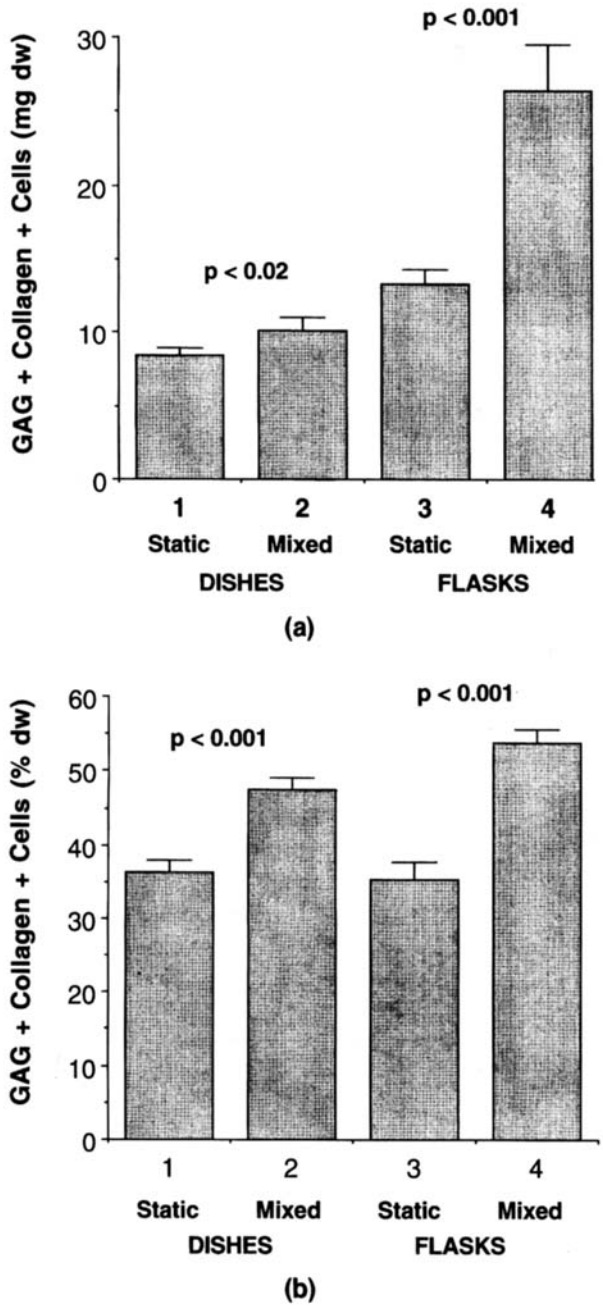


Figure 7. Composition of 8 week constructs: (a) cells + GAG + collagen (mg dw) and (b) cells + GAG + Collagen (% dw).

Data represent the average ± SD ($n = 6$) for constructs grown in dishes and spinner flasks under static and mixed culture conditions.

7a and 7b). In order to assess the effects of mixing on the mass transfer of chemical species into constructs, mass-transfer rates of small and large molecules (e.g., glucose and dextran, respectively) are being quantified in our current studies for a range of construct morphologies.

The rates of GAG and collagen synthesis and retention are in part determined by mass-transfer rates at the construct surface, which in turn depend on mixing intensity and by the diffusional construct permeability. Constructs from mixed flasks contained 60% more GAG than constructs from static flasks at the same amount of GAG/cell (Table 3). Possible explanations for the relatively high GAG and collagen contents of constructs grown in turbulent spinner flasks are (a) higher GAG synthesis rates due to more efficient mass transfer of small noncharged molecules (e.g., glucose, oxygen), and (b) decreased GAG loss into the culture medium since the outer capsule could act as a selective diffusional barrier for large charged molecules (e.g., chondroitin sulfate).

Natural articular cartilage contains two subpopulations of chondrocytes: (1) flat surface cells that surround themselves with collagen fibrils and little GAG, and (2) round deep-zone cells that surround themselves with both GAG and collagen (Zanetti et al., 1985; Aydelotte and Kuettner, 1988). Previous studies showed that these subpopulations, when cultured under identical conditions, continued to express the different phenotypes just cited (Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Haeuselmann et al., 1994). The present study shows that hydrodynamic conditions affected the shape and metabolic activities of mixed populations of chondrocytes as follows. In static cultures, round differentiated cells were seen both at the construct surface and in the inner tissue phase, while in mixed cultures cells at construct surface flattened, proliferated to high concentrations, and produced collagen but very little GAG (Figure 4b).

These findings are consistent with previous studies of environmental effects on cell shape and function as follows. Chondrocytes were previously shown to express their differentiated phenotype under conditions that maintain their spherical shape (Solursh, 1989). *In vitro*, chondrocyte shape was determined by its interactions with extracellular matrix (Daniels and Solursh, 1991; Watt, 1986) and the cell attachment substrate (Glowacki et al., 1983). Adherent cells exposed to external forces tend to flatten and activate stress-protection mechanisms (e.g., form cytoskeletal stress fibers) in order to remain firmly attached to substrate (Franke et al., 1984). Cell flattening and adherence to a substrate (e.g., PGA fibers) can increase cell stiffness and apparent viscosity, presumably due to the structural rearrangement of the cytoskeleton (Wang and Ingber, 1994). Flat chondrocytes proliferated faster than spherical chondrocytes (Ingber et al., 1994) and secreted type I collagen (Daniels and Solursh, 1991; Zanetti and Solursh, 1989), which can be organized into the form of an outer capsule (Harris et al., 1981).

Moreover, certain cells, including chondrocytes, respond to the characteristics of hydrodynamic forces in addition to their magnitudes. Unsteady flow environments (e.g., pulsatile laminar flow, turbulent flow) produced greater changes in cell shape and function than steady-state laminar flow at comparable average levels of hydrodynamic stress (Davies et al., 1986; Dewey, 1984; Frangos et al., 1985; Levesque et al., 1989). For example, proteoglycan synthesis in cultured chondrocytes and articular cartilage explants, which is known to be inhibited by static stress, was stimulated by dynamic stresses of presumably physiologic magnitude (Parkkinnen et al., 1993; Sah et al., 1989). Constructs grown in rotational fluid flow had thinner capsules than those grown in turbulent spinner flasks (Freed and Vunjak-Novakovic, 1995b).

The average intensity of turbulence in mixed spinner flasks was below the level that was previously shown to cause cell damage or detachment, according to several criteria. The

stirring speed of 50 rpm was below the range of 150–300 rpm at which cell damage was observed in microcarrier cultures (Cherry and Papoutsakis, 1990). The integrated shear factor (ISF) of 8.4 and maximum shear rate (Y_m) of 4.2 were 2- to 4-fold lower than the values that damaged anchored epithelial or endothelial cells in laminar flow (Stathopoulos and Hellums, 1984; Dewey, 1984), or various mammalian cells on microcarriers in turbulent flow (Croughan et al., 1987; Croughan and Wang, 1989; Merchuk, 1991; Papoutsakis, 1991). In the present study, the magnitudes of hydrodynamic forces associated with mixing appeared to be low enough not to cause cell death, but sufficient to stimulate chondrocytes at the construct surface to flatten, proliferate, and form a fibrous capsule.

It is reasonable to postulate that the observed hydrodynamic effects on cell shape and function in cartilage constructs are caused by the time and space fluctuations in fluid velocity and pressure at the construct surface that are associated with viscous dissipation of turbulent eddies. The basic structure of turbulence involves a spectrum of eddy sizes, where larger eddies pass their kinetic energy to smaller ones (Hinze, 1972). Even the viscous sublayer was shown to contain turbulent structures in the form of laminar flowing, randomly renewed fluid elements (Banerjee, 1992; Musschenga et al., 1992).

The Kolmogorov scale turbulent eddies release their kinetic energy by viscous dissipation and thus can affect cell shape and function (Papoutsakis, 1991). In the present study, the smallest eddies (250 μm) were large when compared to the cell size (10–15 μm dia.), but much smaller than the construct size (5–10 mm). Due to the lack of cell mobility, attached chondrocytes were exposed to pressure and velocity fluctuations associated with the eddies, which presumably caused the observed effects of turbulent mixing (e.g., cell flattening, proliferation, and formation of an outer capsule). Previous studies of endothelial cells also showed that turbulence stimulated cell proliferation (Davies et al., 1986) and affected cell–cell interactions (Satcher et al., 1992).

In summary, mixing and turbulence in the tissue culture environment improved the biochemical composition and altered the morphology of cartilage constructs. Further studies of the 3-D cell-polymer model system are expected to offer more insight into the nature of the responses of chondrocytes to hydrodynamic forces under conditions that are typical of tissue engineering bioreactors. The effects of increased mass transfer in mixed cultures on construct composition and morphology have to be distinguished from those of hydrodynamic forces on cell shape and function in order to eventually provide a basis for the custom-engineering of cartilage implants.

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