Wavy-Walled Bioreactor Supports Increased Cell Proliferation and Matrix Deposition in Engineered Cartilage Constructs

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ABSTRACT

Hydrodynamic forces in bioreactors can decisively influence extracellular matrix deposition in engineered cartilage constructs. In the present study, the reduced fluid shear, high-axial mixing environment provided by a wavy-walled bioreactor was exploited in the cultivation of cartilage constructs using polyglycolic acid scaffolds seeded with bovine articular chondrocytes. Increased growth as defined by weight, cell proliferation and extracellular matrix deposition was observed in cartilage constructs from wavy-walled bioreactors in comparison with those from spinner flasks cultured under the same conditions. The wet weight composition of 4-week constructs from the wavy-walled bioreactor was similar to that of spinner flask constructs, but the former were 60% heavier due to equally higher incorporation of extracellular matrix and 30% higher cell population. It is most likely that increased construct matrix incorporation was a result of increased mitotic activity of chondrocytes cultured in the environment of the wavy-walled bioreactor. A layer of elongated cells embedded in type I collagen formed at the periphery of wavy-walled bioreactor and spinner flask constructs, possibly as a response to local shear forces. On the basis of the robustness and reproducibility of the extracellular matrix composition of cartilage constructs, the wavy-walled bioreactor demonstrated promise as an experimental cartilage tissue-engineering vessel. Increased construct growth in the wavy-walled bioreactor may lead to enhanced mechanical properties and expedited *in vitro* cultivation.

INTRODUCTION

ARTICULAR CARTILAGE DAMAGE, mostly a result of arthritis or sport injuries, presents a persistent challenge to orthopedic medicine. Current therapies include joint resurfacing, biological autografts, and total joint replacement, all of which present limited long-term viability and success rates.[1] The problem is aggravated by the inability of articular cartilage to undergo self-repair. Engineered cartilage constructs that mimic the function of native articular cartilage have the potential to provide complete and permanent relief to a diseased joint, but they must first meet specific criteria related to biochemical composition, mechanical properties, immunological compatibility, and integration capability.

Engineered cartilage constructs are traditionally generated by a cell–scaffold–bioreactor approach. Cartilage cells are seeded onto porous and biodegradable polymer scaffolds, and cultivated in the controlled environment of bioreactors.[2] After a few weeks in culture, the cells proliferate and synthesize extracellular matrix, yielding tissue constructs that resemble native cartilage. The limitations of engineered cartilage constructs, compared with native tissue, often include (1) insufficient extracellular matrix incorporation, particularly collagen,[3–5] (2) inferior mechanical properties,[4,6,7] and (3) lack of integration with surrounding tissue.[8] Robust matrix production, mechanical strength, and the ability to integrate with native tissue are all necessary elements for the successful use of cartilage constructs as therapeutic implants. Poor me-

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chanical properties, in particular, impair construct functionality and often result from insufficient extracellular matrix deposition.[7,9] Prolonging tissue cultivation increases extracellular matrix deposition, thus improving construct biochemical and mechanical properties.[10,11] However, an extensive culture period introduces economic and therapeutic disadvantages. There exists a need for strategies to increase the production and incorporation of extracellular matrix in engineered cartilage constructs within a culture period of reasonable duration.

The bioreactor fluid environment greatly influences the biochemical and mechanical properties of engineered cartilage constructs, by ensuring efficient mass transfer conditions, uniform cell seeding onto three-dimensional scaffolds, and maintenance of chondrocyte phenotype, and also by providing physical stimuli.[12–17] Bioreactors used for the cultivation of cartilage constructs include conventional spinner flasks,[5,18] rotating-wall vessels,[19] concentric cylinder bioreactors,[20] and perfusion bioreactors.[13,21–23] Within these bioreactors, distinct fluid flow patterns around developing cartilage constructs promote changes in cell morphology, and in the exchange of nutrients and metabolites between culture medium and constructs.[24] These changes result in constructs with different biochemical and mechanical properties across bioreactors. Although numerous reports on the effect of hydrodynamic forces on cartilage constructs exist,[12,20,24–26] results are often contradictory and it remains difficult to accurately predict the impact of a specific hydrodynamic environment on final construct properties.

The spinner flask, widely used for cartilage tissue engineering, has been characterized extensively in terms of its fluid environment,[27] and the properties of the cartilage constructs it produces.[3,12,14] Spinner flasks yield cartilage constructs containing up to 60% more sulfated glycosaminoglycans and 125% more collagen than static cultures, suggesting that the high shear stress fluid regimen in this bioreactor[26] stimulates chondrocyte proliferation and extracellular matrix deposition. However, tissue growth in spinner flasks is nonuniform, and a fibrous capsule develops on the construct surface as a result of local high shear forces.[14] Nonetheless, the turbulent mixing environment in spinner flasks transports the cells into the scaffolds by convection.[14] The efficiency of chondrocyte seeding onto polyglycolic acid scaffolds in spinner flasks has been reported to reach 100% within 1 day,[28] and thus cell seeding is often performed in spinner flasks and construct cultivation is carried out in a different vessel, thereby introducing additional handling steps and complexity to the process. A low-shear bioreactor with improved or comparable cell-seeding efficiency and more uniform tissue growth than the spinner flask would successfully confine construct development to a single vessel. One possible approach is a bioreactor design that exploits the benefits of turbulent mixing at moderate shear stress levels.

The wavy-walled bioreactor (WWB) was designed to enhance mixing while minimizing fluid shear. The grooves of the WWB, which mimic baffles, introduce complex fluid patterns, create secondary motions in the radial direction, increase axial velocities,[29] and promote fluid recirculation regions.[30] We previously demonstrated that the fluid environment in the WWB enhanced chondrocyte aggregation in suspension. The kinetics of cell aggregation was 45% faster and the density of large cell aggregates was 2- to 4-fold higher in the WWB in comparison with the spinner flask (SF).[30] Chondrocyte aggregation leads to the maintenance of the desired phenotype, and the secretion of extracellular matrix molecules,[31,32] and promotes faster attachment to polymer scaffolds in comparison with nonaggregated single chondrocytes.[28] We hypothesize that the WWB provides a more amenable fluid environment to chondrocytes and developing cartilage constructs, resulting in efficient cell seeding and increased extracellular matrix deposition in comparison with SFs.

In the present study, the WWB was used for the first time for the cultivation of cartilage constructs generated by seeding isolated chondrocytes onto degradable polyglycolic acid scaffolds. Construct development in the WWB was compared with that in the conventional smooth-walled SF.

MATERIALS AND METHODS

Materials

Knee joints from 2- to 6-week-old calves were obtained within 6 h of slaughter from Research 87 (Marlborough, MA). All enzymes were purchased from Worthington Biochemical (Lakewood, NY). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), and fetal bovine serum (FBS) were from Invitrogen (Grand Island, NY). Sigmacote, penicillin–streptomycin, ethylenediaminetetraacetic acid (EDTA), nonessential amino acids (NEAAs), L-proline, ascorbic acid, amphotericin B, trypan blue solution, dimethylmethylene blue dye, and chloramine-T were purchased from Sigma (St. Louis, MO). A PicoGreen double-stranded DNA (dsDNA) quantitation kit was purchased from Molecular Probes/Invitrogen (Eugene, OR). Nitex mesh (150 μm) was purchased from Sefar Printing Solutions (Lumberton, NJ). Spinner flask bioreactors (capacity, 125 cm^3; diameter, 6.5 cm; stir bar, 0.8 cm in diameter and 3.8 cm long) and magnetic stirrers (Multi Stir 4) were from Bellco (Vineland, NJ). Wavy-walled bioreactors (capacity, 125 cm^3; average diameter, 6.7 cm; stir bar, 0.8 cm in diameter and 3.8 cm long) were made to design by Finkenbeiner Glass Blowers (Waltham, MA). Needles (4
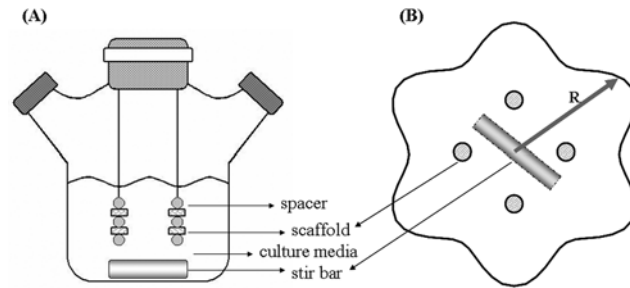
FIG. 1. (**A**) Front view and (**B**) cross-section of the experimental setup of the wavy-walled bioreactor. Four needles penetrate the stopper at the mouth of the flask; two scaffolds threaded onto each needle are spaced by 0.5-cm sections of silicone tubing. The radius of the wavy-walled bioreactor, R, is a function of the radial angle, ϕ, as follows: $R(\phi) = R_{ave} + A \sin (N\phi)$, where $N = 6$ waves, $A = 0.45$ cm is the amplitude of the grooves and R_{ave} is the average radius, 3.35 cm.

in. long, 22 gauge), stir bars, and Ehrlich's reagent were purchased from Fisher Scientific (Paris, KY). Silicone stoppers (no. $10^1/_2$) and silicone tubing (no. 13) were from Cole-Parmer Instrument (Vernon Hills, IL). Polyglycolic acid (PGA) scaffolds were supplied as disks (5 mm in diameter and 2 mm thick) composed of a mesh of 13-μm diameter fibers, 97% void volume, and a bulk density of 73 mg/cm^3 by Albany International Research (Mansfield, MA). Type II collagen and procollagen type I monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody was from BD Biosciences Pharmingen (San Diego, CA).

Methods

Cartilage extraction and chondrocyte isolation. Full-thickness articular cartilage sections were explanted from the femoropatellar groove of calf knee joints within 6 h of slaughter. Explants were diced into 1- to 2-mm^3 pieces and digested in collagenase II solution (DMEM with 13% FBS and 4–5 U of enzyme per cm^3) at 10 cm^3/g tissue. Tubes filled with digestion material were horizontally fixed to an orbital shaker placed inside a 37°C, 5% CO$_2$ incubator and mixed for 16 h at 50 rpm. The digestate was filtered through a 150-μm Nitex mesh to retain undigested tissue, and centrifuged at $200 \times g$ for 10 min. The cell pellet was washed three times with 0.02% EDTA–5% $1\times$ penicillin–streptomycin in PBS. Isolated chondrocytes were resuspended in chondrocyte culture medium: DMEM (with glucose at 4.5 g/L and glutamine at 584 g/L), 9% FBS, penicillin (50 U/cm^3), streptomycin (50 μg/cm^3), amphotericin B (2.5 μg/cm^3), 10 mM HEPES, 0.1 mM NEAAs, 0.4 mM proline, and ascorbic acid (50 μg/cm^3).

Bioreactor seeding. The interiors of assembled WWBs and SFs were treated with Sigmacote to prevent cell adherence, and steam sterilized for 25 min at 121°C. PGA scaffolds were sterilized by washing once in 70% ethanol for 10 min and three times in DMEM for 5 min each. Two scaffolds were threaded onto each 4-in.-long needle, and fixed with silicone tubing spacers. Four needles were fixed to the rubber stoppers in the mouth of each bioreactor, for a total of eight scaffolds per bioreactor (Fig. 1). Bioreactors were filled with 120 cm^3 of culture medium and allowed to stabilize by overnight mixing at 50 rpm inside a humidified, 37°C, 5% CO$_2$ incubator with the sidearm caps loose to allow gas exchange.

After equilibration, freshly isolated chondrocytes in suspension were added to the bioreactors via the sidearm caps at 5 million chondrocytes per scaffold.[24,28] This cell density is above that found in native cartilage; high seeding densities promote rapid *in vitro* chondrocyte proliferation and growth of tissue-engineered cartilage.[20] Medium volume was kept at 120 cm^3. Cell seeding proceeded undisturbed for 72 h inside the incubator under the previously described conditions.

Construct cultivation and sampling. Seventy-two hours after cell seeding, an aliquot of medium was extracted from each bioreactor and examined to assess cell concentration and viability via trypan blue exclusion. The total medium volume in each bioreactor was then replaced with fresh culture medium, marking the start of the tissue culture phase. Approximately 50% of the medium volume (9 cm^3/scaffold) was replaced every 3 days thereafter to maintain nutrient supply and to remove waste products. Construct samples (four constructs per bioreactor) were aseptically removed from the bioreactors at 3 and 28 days of culture.

Biochemical analyses. Constructs intended for biochemical analyses were transferred to tared 1-cm^3 tubes, weighed (wet weight), and frozen. Frozen constructs were lyophilized for 72 h, weighed (dry weight), and digested for 16 h at 60°C with 1 cm^3 of a 125-μg/cm^3 solution of papain enzyme (10 mM EDTA, 100 mM phosphate, 10 mM cysteine) per construct.
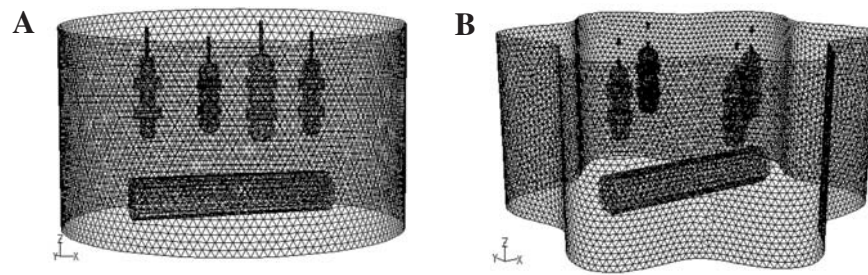
FIG. 2. GAMBIT software was used to generate these meshed geometries, simulating the bioreactor setup used in cartilage construct cultivation experiments. (**A**) Spinner flask; (**B**) Wavy-walled bioreactor.

The number of chondrocytes per construct was assessed by DNA content of papain digests, using a PicoGreen dsDNA kit. The DNA:chondrocyte ratio was 7.7 pg of DNA per cell[33] and to assess the contribution of cellular material to construct weight, the weight of one chondrocyte was assumed to be 100 pg.[34] The glycosaminoglycan content of constructs was determined by dimethylmethylene blue dye-binding assay,[35] using chondroitin sulfate as a standard. The total collagen content of constructs was determined from their hydroxyproline concentration, using a standard ratio of hydroxyproline to collagen of 1:10.[36] Hydroxyproline concentration was determined spectrophotometrically after alkaline hydrolysis and reaction with chloramine-T and Ehrlich's reagent.[37]

Histological analyses. Constructs were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned in 5-μm-thick slices. The sections were fixed on glass slides and stained with hematoxylin and eosin for cells, with safranin O for glycosaminoglycans, and with Masson's trichrome for collagen. Sections for immunohistochemistry were treated with 0.5% pepsin for 10 min at 37°C followed by blocking with 1% BSA in PBS. The sections were then exposed to a 1:1 dilution of primary antibody in mouse host (type II collagen or procollagen type I) in 1% BSA for 1 h. After wash, sections were treated for 30 min in the dark with FITC-conjugated goat anti-mouse IgG secondary antibody at 1:400 dilution in 1% BSA. Slides were mounted with a toluene-based solution and stored in the dark. Epifluorescence images were acquired with an inverted TE2000U microscope (Nikon, Melville, NY) at a magnification of ×10.

Statistical analyses. Results represent three separate experiments with the WWB and the SF operated in parallel. In each experiment, bioreactors were seeded with chondrocytes explanted from the same knee joint. Four construct samples were taken from each bioreactor at each time point; two were used for biochemical analyses and the other two for histological assessment. Biochemical assays were performed in triplicate. For each data set, values represent means ± SEM. Statistical significance was calculated by Student t test and reported as p values ($p < 0.05$).

Computational fluid dynamics modeling. Bioreactor geometries mimicking the cartilage construct cultivation conditions were created with GAMBIT mesh generation software (Fluent, Lebanon, NH) for the SF (Fig. 2A) and the WWB (Fig. 2B). Fluid flow inside the WWB and the SF was modeled with the realizable k–ε turbulence model[27] in FLUENT software (Fluent) to generate velocity flow fields. The average shear stresses applied by the fluid flow on constructs were calculated using the built-in wall shear stress function as surface integrals on the surface of the constructs.

RESULTS

Table 1 details the composition of 3- and 28-day constructs from the WWB and the SF. Cell-seeding efficiency and 3-day construct properties were similar in the WWB and the SF, with more than 90% of the 5 million cells initially seeded per scaffold attached within 72 h. After 4 weeks of culture, however, enhanced chondrocyte proliferation and matrix deposition were observed in constructs from the WWB when compared with constructs from the SF.

Macroscopic properties

Three-day constructs from either bioreactor were wet, and retained the spongy, flat appearance of the original scaffolds. After 28 days of culture, constructs were larger than the original scaffolds; they were solid, rounded, and shiny. From day 3 to 28 of culture, the wet weight of cartilage constructs increased from 72 ± 5 to 151 ± 10 mg in the WWB, and from 68 ± 5 to 93 ± 3 mg in the SF (Fig. 3). At 4 weeks, constructs from the WWB weighed approximately 60% more than those from the SF when wet, and 50% more after drying (dry data not shown). The water content of WWB constructs stayed approximately constant at 91% through 28 days of culture,

TABLE 1.　DETAILED COMPOSITION OF CARTILAGE CONSTRUCTS AND NATIVE ARTICULAR CARTILAGE[a]

	Wavy-walled bioreactor		Spinner flask		Native cartilage[b]
	3 days	28 days	3 days	28 days	
Construct wet weight (mg)[c]	72 ± 5	151 ± 10	68 ± 5	93 ± 3	90.1 ± 13.3
Water (mg)[c]	66.2 ± 7.0	136.7 ± 10.2	63.8 ± 5.9	83.8 ± 3.1	—
Water (%ww)[c]	90.0 ± 0.4	90.5 ± 0.1	93.8 ± 1.2	89.7 ± 0.2	82.8 ± 0.9
Cells per construct ($\times 10^{-6}$)[c]	4.8 ± 0.2	23.9 ± 2.1	5.1 ± 0.3	17.9 ± 0.4	—
Cells (%ww)[c]	0.7	1.6 ± 0.2	0.7 ± 0.1	1.9 ± 0.1	0.7 ± 0.1
GAG (mg/construct)[c]	2.7 ± 0.2	5.3 ± 0.5	2.9 ± 0.1	3.5 ± 0.1	—
GAG (%ww)	3.8 ± 0.5	3.5 ± 0.6	4.3 ± 0.5	3.8 ± 0.2	7.1 ± 0.6
Collagen (mg/construct)[d,e]	1.1 ± 0.3	5.7 ± 0.9	0.5 ± 0.2	3.6 ± 0.5	—
Collagen (%ww)	1.5 ± 0.5	3.8 ± 0.8	0.7 ± 0.4	3.9 ± 0.2	10.7 ± 0.9
Cells + collagen + GAG (mg)[d]	4.3 ± 0.5	13.4 ± 1.6	3.9 ± 0.3	8.9 ± 0.6	—
PGA (%ww)[b]	4	0.9	4	0.9	0
Mass balance (%ww)	101.0 ± 1.4	100.3 ± 1.7	103.5 ± 2.2	100.2 ± 0.7	101.3 ± 2.5
mg GAG/cell ($\times 10^{7}$)	5.6 ± 0.7	2.2 ± 0.4	5.6 ± 0.6	1.9 ± 0.1	7.6 ± 0.7[f]
mg collagen/cell ($\times 10^{7}$)[e]	2.4 ± 0.6	2.4 ± 0.6	0.9 ± 0.5	2.0 ± 0.3	9.4 ± 2.4[f]
Collagen/GAG (mg/mg)	0.4 ± 0.3	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.2	1.3 ± 0.3[f]

[a]Data for WWB and SF constructs represent means ± SEM of six independent measurements.
[b]Native cartilage composition from Freed *et al.*[43]
[c]$p < 0.01$, significant difference between 4-week WWB and SF constructs.
[d]$p < 0.05$, significant difference between 4-week WWB and SF constructs.
[e]$p < 0.05$, significant difference between 3-day constructs from the WWB and the SF.
[f]From Vunjak-Novakovic *et al.*[14]

whereas constructs from the SF decreased in water content from 94% at 3 days to 90% at 28 days of culture (Table 1).

Cell proliferation

Initial chondrocyte proliferation in the WWB and the SF was similarly slow, so that after 3 days of cultivation, the cell number per construct was essentially the same as
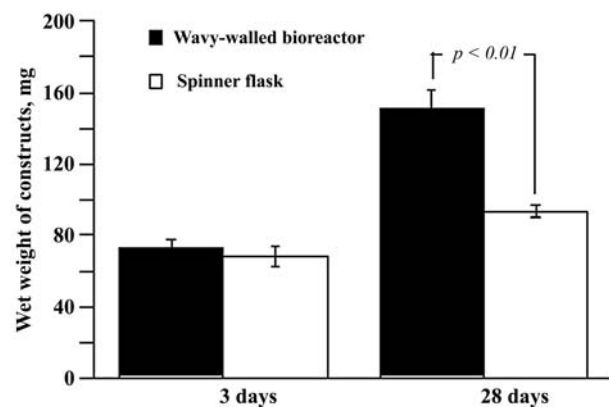

FIG. 3.　Weight of constructs from the wavy-walled bioreactor and the spinner flask. Constructs were aseptically removed from the bioreactors and immediately weighed for wet weight determination.
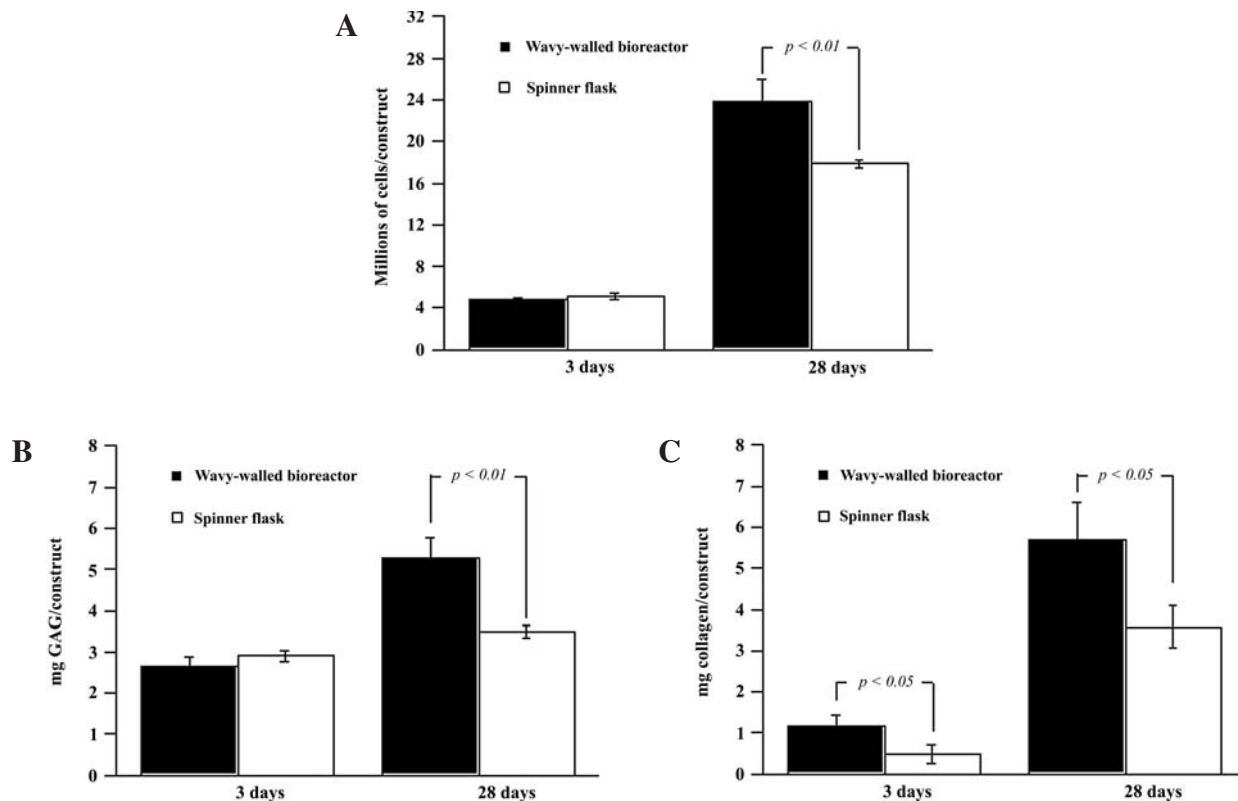
at the time of cell seeding (Fig. 4A). Over the long term, however, important differences in chondrocyte proliferation were observed between bioreactors, with significantly more cells in WWB constructs than in SF constructs. At 4 weeks of cultivation, wavy-walled constructs contained 23.9 ± 2.1 million cells per construct (Table 1), indicating that at least two cell population doublings occurred in the period after day 3 and before day 28. In comparison, SF constructs contained 17.9 ± 0.4 million cells per construct at 4 weeks (Table 1), indicating that fewer than two population doublings took place in the same period.

Matrix incorporation

The glycosaminoglycan (GAG) content of constructs from the WWB increased from 2.7 ± 0.2 mg of GAG per construct at 3 days of cultivation to 5.3 ± 0.5 mg of GAG per construct at 28 days (Fig. 4B). Whereas the GAG content of 3-day SF constructs was similar to that in the WWB, 4-week SF constructs had approximately 33% less GAG than did WWB constructs. The weight fraction of WWB constructs attributed to GAG was similar to that of SF constructs at both 3 and 28 days (Table 1).

Figure 4C shows that total collagen content in WWB constructs was significantly greater than in SF constructs at both 3 and 28 days of cultivation. Three-day constructs contained 1.1 ± 0.3 mg of collagen per construct for the WWB and 0.5 ± 0.2 mg of collagen per construct for the

FIG. 4. Cell proliferation and biochemical composition of cartilage constructs. (**A**) Construct cell numbers obtained by DNA content assessment for constructs seeded with 5 million chondrocytes per scaffold at time 0 days, (**B**) glycosaminoglycan incorporation per construct, and (**C**) total collagen incorporation per construct.

SF. In 4-week constructs, total collagen content increased to 5.7 ± 0.9 mg/construct in the WWB and to 3.6 ± 0.5 mg/construct in the SF (Table 1). On average, the collagen content of 4-week constructs in the WWB was 1.6-fold higher than in the SF. As in the case of GAG incorporation, collagen wet weight fractions in WWB constructs were not significantly different from those in SF constructs at 3 or 28 days. Although the total collagen content of constructs was increased in the WWB, it is not clear which proportion of it was cartilage-specific collagen (type II). Total collagen presented here reflects all types of collagen present in the constructs.

Chondrocyte biosynthesis

As reported in Table 1, when milligrams of GAG per construct results were normalized against cells per construct, no significant differences were observed between the WWB and SF at either 3 or 28 days, and a significant decrease in milligrams of GAG per chondrocyte from days 3 to 28 of cultivation was observed in both WWB and SF constructs. Construct GAG normalized as milligrams per cell at both 3 and 28 days corresponded to a fraction of the amount reported for native cartilage: 74% at 3 days and 29% at 28 days.[14] In terms of collagen, normalized collagen (mg):cell ratios were signifi-

cantly higher in 3-day constructs from the WWB than in those from the SF, suggesting that chondrocytes in the WWB secreted more collagen at the beginning of the culture period. This collagen (mg):cell ratio was unchanged after 28 days of cultivation in WWB constructs, whereas in SF constructs it doubled between days 3 and 28. At 4 weeks, milligrams of collagen per cell was similar in constructs from both bioreactors, and it amounted to only about 25% of the ratio reported for native cartilage explants.[14]

Histological analyses

Figure 5a–c shows that in 3-day constructs from the WWB, most chondrocytes attached to the borders of the scaffolds and fewer cells populated the interior of constructs. Little collagen or GAG was present in these constructs. Similar observations were made for 3-day constructs from the SF (not shown). After 28 days of culture, chondrocytes in both bioreactors had proliferated and populated the center of scaffolds, and they had secreted extensive amounts of extracellular matrix (Fig. 5d–m). Twenty-eight day constructs from the WWB (Fig. 5d–f) appeared to have a higher cell density and stained more intensely for collagen and GAG than did constructs from the SF (Fig. 5i–k).
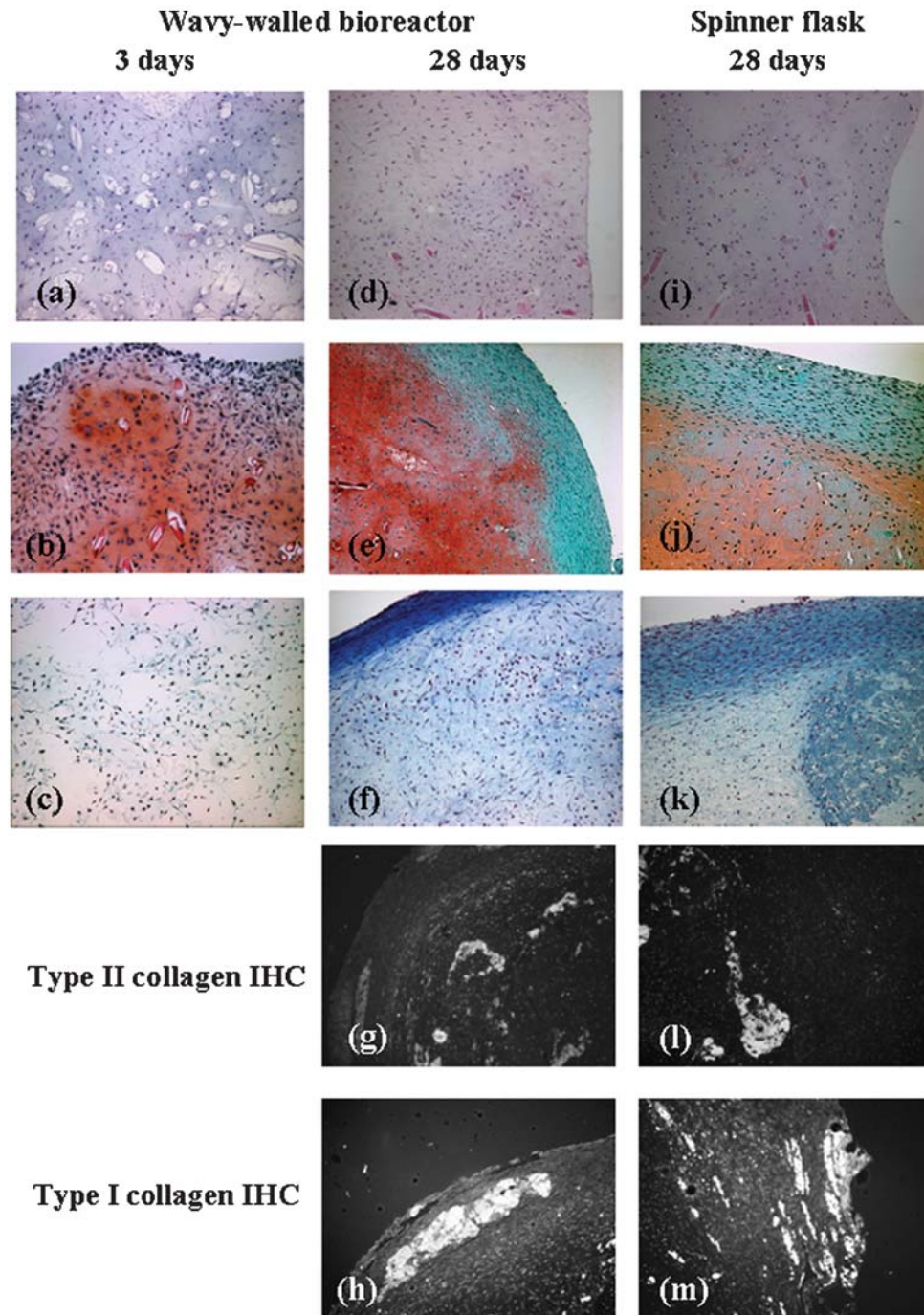
FIG. 5. Histology and immunohistochemistry (IHC) images of 3-day and 4-week cartilage constructs seeded with 5 million chondrocytes per scaffold and cultured in either wavy-walled bioreactors or spinner flasks. Histology sections were stained with standard hematoxylin and eosin to visualize cells (**a**, **d**, and **i**), safranin O and fast green for GAG (**b**, **e**, and **j**), and Masson's trichrome for collagen (**c**, **f**, and **k**). Remaining PGA fibers are visible as tubular structures. Histology images were acquired at an original magnification of ×20. Immunohistochemistry sections from 4-week cartilage constructs cultured in the wavy-walled bioreactor (**g** and **h**) and spinner flasks (**l** and **m**) were treated with type II collagen (**g** and **l**) or type I collagen (**h** and **m**) monoclonal antibodies. Images were acquired with mercury bulb epifluorescence at an original magnification of ×10.

As expected, early in culture, WWB constructs were composed mostly of cells and polymer fibers (Fig. 5a–c). Partial degradation of the scaffold occurred over time nonuniformly, so that 4-week constructs contained few polymer fibers at the periphery (Fig. 5e and f), and exhibited a higher density of PGA fibers toward the center (Fig. 5d). Scaffold degradation and accumulation of extracellular matrix accounted for the decrease in the scaf-

A

Path Lines Colored by Axial Velocity (cm/s)

Feb 08, 2005

FLUENT 6.1 (3d, segregated, rke, unsteady)

B

Path Lines Colored by Axial Velocity (cm/s)

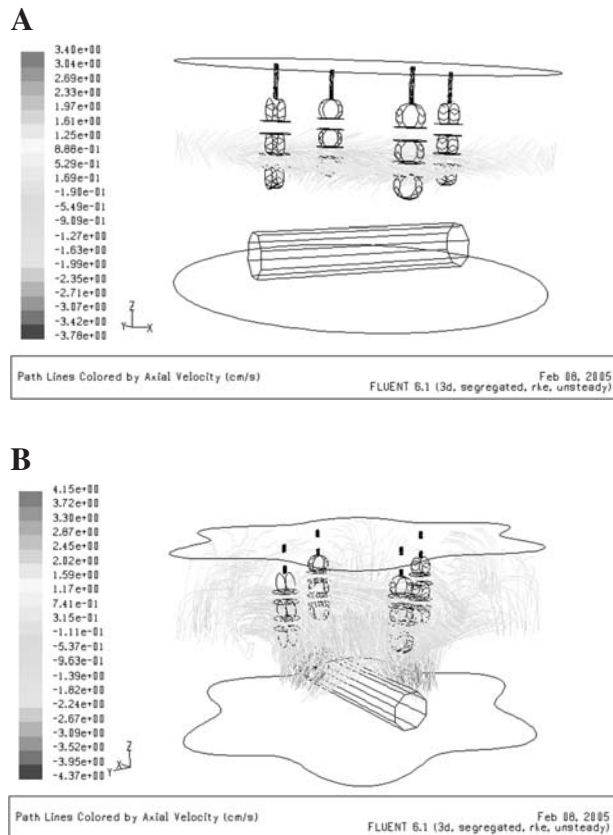Feb 08, 2005

FLUENT 6.1 (3d, segregated, rke, unsteady)

FIG. 6. FLUENT software was used to generate fluid path lines for a fluid particle released at the horizontal plane where bottom constructs were located in the (**A**) spinner flask and (**B**) wavy-walled bioreactors. The fluid path lines are colored by axial velocity magnitude.

fold contribution to overall construct weight: after 3 days of culture the scaffold accounted for approximately 4% of the construct wet weight, and was estimated to account for only 0.9% of the construct wet weight after 4 weeks.[34]

Twenty-eight day constructs from both the WWB and the SF were surrounded by a layer with a high density of cells elongated in the direction of fluid motion (Fig. 5e, f, j, and k). Such a layer has been reported for spinner flask constructs and is referred to as a fibrous outer capsule.[14] GAG deposition was not observed within the fibrous outer capsule of constructs from either bioreactor (Fig. 5e and j); instead, there was strong collagen incorporation in this area (Fig. 5f and k). Strong GAG deposition was evident in the interior of all constructs, with the exception of some areas in SF constructs, as seen in Fig. 5j. Fluorescence immunohistochemistry using type II collagen (Fig. 5g and l) and procollagen type I (Fig. 5h and m) monoclonal antibodies revealed that type I collagen was present in the fibrous capsule, and type II collagen was confined to the inner areas of both WWB and SF 4-week constructs.

Fluid dynamics

Fluid flow velocities computed by computational fluid dynamics (CFD) modeling revealed that, in agreement with Natarjan *et al.*,[29] mixing was mostly tangential in the SF (Fig. 6A), whereas the WWB geometry enhanced axial mixing as depicted by increased axial velocity magnitudes and particle path lines in Fig. 6B. The magnitude of the average shear stress applied on constructs exposed to the fluid environment in the SF was simulated as twice that in the WWB (0.049 versus 0.024 N/m^2), confirming our earlier hypothesis of reduced fluid shear in the WWB. Although these computational results serve as a guideline, the need for their experimental validation using velocimetry techniques remains.

DISCUSSION

The long-term goal of our studies is to further understand the influence of the hydrodynamic environment on the development of engineered cartilage in order to predict optimum bioreactor design and operating conditions that may lead to clinically relevant cartilage constructs. In support of this aim, in this study the WWB was used for the first time for cartilage construct cultivation.

Although the WWB and the SF are similar in operation and appearance, specific advantages of the WWB have been demonstrated. Enhanced mixing and the possibility to reduce turbulence by operating at lower rotation rates in the WWB were previously demonstrated.[29] In chondrocyte suspension cultures, kinetics of chondrocyte aggregation was up to 45% faster and the density of large chondrocyte aggregates was up to 4-fold higher in the WWB.[30] Computational fluid dynamics (CFD) simulations revealed that the WWB provided a significantly lower shear environment for cartilage construct cultivation than did the SF. As expected from these findings, the WWB provided efficient attachment of chondrocytes to scaffolds, and these chondrocytes proliferated and remained metabolically active, synthesizing extracellular matrix over a period of 4 weeks (Figs. 4 and 5). In comparison with the SF, the WWB supported increased cell proliferation and deposition of glycosaminoglycans and collagen, and produced cartilage constructs with reproducible characteristics in independent experiments. The increases in compositional properties of 4-week WWB constructs over SF constructs range consistently from 1.3- to 1.6-fold (Table 1), suggesting that the hydrodynamic environment in the WWB, rather than the promotion of differential increases in distinct matrix components, supports increased growth of whole cartilage constructs. The particular aspects of the hydrodynamic environment in the WWB that distinguish it from the SF, and may account for the increased cartilage construct

growth observed in this study, include (1) a significant reduction in the fluid shear applied on constructs, (2) fluid recirculation, and (3) increased axial mixing.

Plausible explanations for the increased extracellular matrix deposition observed in WWB constructs include (1) increased mitotic potential of chondrocytes in the WWB, with no significant differences in the amount of matrix secreted per chondrocyte in the WWB when compared with the SF, and/or (2) differential influences of the WWB and SF hydrodynamic environments on the synthetic activity of the chondrocytes. Understanding how the synthesis of extracellular matrix by chondrocytes is affected by the signals provided by bioreactors, and how in turn this affects matrix composition, is an area of interest in cartilage tissue engineering. The synthetic function of chondrocytes has been stimulated using signals provided by bioreactors, and has been observed to decrease with matrix accumulation.[38] In this study, a decrease in the GAG (mg) per cell from day 3 to 28 of cultivation in both WWB and SF constructs was accompanied by a significant increase in cell population, matrix accumulation, and appearance of elongated chondrocytes in the periphery of constructs. These elongated chondrocytes did not appear to incorporate any GAG, providing a possible explanation for the decreased GAG (mg) per cell, and instead produced detectable amounts of collagen type I. The apparent drop in GAG synthetic activity in chondrocytes between days 3 and 28 may be due to a combination of factors including accumulation of matrix, changes in cell morphology, and possibly GAG release into the fluid environment (not quantified in these experiments). As for collagen content, it was higher in 3-day constructs from the WWB than in those from the SF, suggesting that the same number of chondrocytes secreted more collagen at the beginning of the cultivation period in the WWB than in the SF. This supports explanation (2), at least in the case of early collagen synthesis. On the basis of milligrams of matrix per cell results and analysis of the wet weight fractions of constructs attributed to cells, GAG, and collagen (Table 1), we suggest that in WWB constructs, higher GAG incorporation was due to higher construct cellularity rather than to increased chondrocyte biosynthesis, whereas increased collagen incorporation was most likely due to a combination of bioreactor environment effects during early cultivation, and increased chondrocyte proliferation late in culture. That the matrix (mg) per cell was below those of native tissue suggests that, during *in vitro* cartilage development, chondrocytes undergo mechanisms of synthetic regulation different from those for *in vivo* chondrocytes.

In this study, chondrocytes initially attached preferentially to the surface of the scaffolds in both the WWB and the SF, such that in 3-day constructs, the density of cells at the surface of scaffolds was higher than within the interior. These findings are consistent with previous studies reporting a surface zone with cell concentrations 60–70%[28] and 2.7-fold[39] higher than those in the bulk volume of the constructs. Over 28 days, robust cell density was achieved throughout the thickness of the scaffold. The fibrous capsule with elongated cells, no visible GAG deposition, and distinctly intense collagen staining observed in 4-week constructs from both the WWB and the SF were also consistent with previous reports.[14] Chondrocytes at the surface of constructs respond to local shear stresses introduced by the stir bar by producing the fibrous capsule and synthesizing collagen I as a form of protection.[40,41] The intense collagen staining of the fibrous capsule was attributed to type I collagen by immunohistochemistry, because type I collagen was consistently found within the capsule, and type II collagen was limited to the interior of constructs (Fig. 5g, l, h, and m). A mechanism other than the stir bar to achieve uniform agitation, or a geometry that allows for shielding the constructs from high local shear stresses, could represent feasible solutions to this problem. The fibrous capsule in WWB constructs (Fig. 5e and f) appeared thinner and was limited to the periphery of constructs, whereas in SF constructs the capsule occasionally extended into the interior (Fig. 5j and k). The WWB may present a more amenable hydrodynamic environment for minimizing the thickness of the fibrous outer capsule, because of the effect of its grooves in diminishing fluid shear applied on constructs.

Comparisons among experimental cartilage tissue-engineering bioreactors must be made cautiously because of the wide range of scaffolds, cells, and cultivation periods used in different studies. In a review, Martin *et al.*[42] compared several well-characterized bioreactors for cartilage tissue engineering in terms of cell seeding, mass transfer, tissue growth, and fluid environment. Turbulent mixing in spinner flasks reduces mass transfer limitations, hence improving construct composition, but it also promotes nonuniform tissue growth and release of soluble GAG into the culture medium. Fluid flow in direct perfusion bioreactors enhances mass transfer, uniformity of cell and extracellular matrix distribution, and GAG deposition; however, cells align in the direction of flow and construct permeability decreases as extracellular matrix is deposited.[26] Low-shear rotating wall vessels (RWVs) have high mass transfer rates[42] and yield constructs with improved compositional properties relative to those obtained in spinner flasks[43]; however, irregular tissue growth occurs as a result of only one construct surface being exposed to fluid flow.[44] The concentric cylinder bioreactor accommodates a large number of constructs[20]; however, one face of the constructs is not exposed to fluid flow. The WWB is a nonuniform shear vessel, with turbulent, high-axial yet reduced shear mixing, and fluid flow over all of the construct surfaces. The following

comparisons are based on the dry weight composition (%dw) of cartilage constructs: 4-week construct composition has been reported as 25%dw GAG and 15%dw type II collagen for direct perfusion bioreactors,[23] 40%dw GAG and 27%dw type II collagen (after 40 days) in RWVs,[43] 13%dw GAG and 22%dw total collagen in concentric cylinder bioreactors,[20] 37%dw of each GAG and total collagen in SFs, and 37%dw GAG and 39%dw total collagen in WWBs. Collagen content reported here for SF and WWB constructs includes both type I and type II collagen. Overall, construct compositional properties obtained in the WWB were within the range of those obtained in other cartilage tissue-engineering bioreactors, supporting the use of the WWB as an experimental vessel for the cultivation of cartilaginous constructs.

We have demonstrated that the WWB can be used successfully to cultivate cartilage constructs that resemble native tissue and exhibit improved matrix deposition relative to constructs cultivated in the conventional SF. Our results suggest that the reduced shear, increased axial mixing environment imposed by the grooves of the WWB directly affected cell proliferation and increased matrix deposition. Under identical conditions, the hydrodynamic environment of the WWB produced more robust cartilage constructs with uniformly increased weight, cell proliferation, and GAG and collagen deposition than did the SF. The achievement of faster construct growth suggests that mechanical functionality could be reached more rapidly in the WWB. Finally, the WWB provided a distinct fluid environment that could be characterized and used as a tool to investigate the relationship between hydrodynamic forces and extracellular matrix deposition, and to improve engineered cartilage properties through directed bioreactor design. Further studies are needed to understand the kinetics of construct growth in the WWB and to develop potential strategies that fully exploit an environment that supports faster construct growth.

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