IGF-I and Mechanical Environment Interact to Modulate Engineered Cartilage Development

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Bovine calf articular chondrocytes were seeded onto biodegradable polyglycolic acid scaffolds and cultured for four weeks using in vitro systems providing different mechanical environments (static and mixed Petri dishes, static and mixed flasks, and rotating vessels) and different biochemical environments (medium with and without supplemental insulin-like growth factor I, IGF-I). Under all conditions, the resulting engineered tissue histologically resembled cartilage and contained its major constituents: glycosaminoglycans, collagen, and cells. The mechanical environment and supplemental IGF-I (a) independently modulated tissue morphology, growth, biochemical composition, and mechanical properties (equilibrium modulus) of engineered cartilage as previously reported; (b) interacted additively or in some cases nonadditively producing results not suggested by the independent responses, and (c) in combination produced tissue superior to that obtained by modifying these factors individually.© 2001 Academic Press

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Tissue engineering of articular cartilage is motivated by serious limitations of current articular cartilage repair techniques and could potentially benefit an estimated one million patients per year (1). Seeding chondrocytes on three-dimensional biodegradable polymeric supports and culturing the resulting cell-polymer constructs in vitro yields cartilaginous tissue that contains the primary extracellular matrix (ECM) components of cartilage (i.e., proteoglycans and collagen type II) and functions mechanically (2–5). The clinical utility of engineered cartilage will likely depend on the ability to generate an adequate tissue mass of engineered cartilage in an acceptable time and the extent to which the tissue resembles native cartilage. As the biochemical (6–10) and biomechanical (11–13) environments are both key regulators of the development and function of cartilage in vivo, researchers have manipulated either the mechanical environment (5, 14–21) or the biochemical (22–29) environments individually in attempts to improve cartilage engineered in vitro. Whereas many of these manipulations have improved specific characteristics of the engineered cartilage (e.g., increased the total mass of tissue generated from a given number of cells or the tissue fraction of GAG or collagen), improvements in one characteristic are often associated with no change or a worsening in other characteristics. We hypothesize that in addition to acting individually, the mechanical and biochemical environments interact to modulate the structural and functional properties of cartilage engineered in vitro. To investigate the effects of simultaneously manipulating the mechanical and biochemical environments, engineered cartilage was cultured in five different experimental model systems previously used for in vitro culture of engineered cartilage (i.e., static and mixed Petri dishes (24, 28), static and mixed flasks (14, 21, 30), and rotating vessels (4, 5, 15, 31) with and without supplemental IGF-I. IGF-I was chosen as a model growth factor as it is generally regarded as the dominant growth-stimulating agent for cartilage in vivo (6–8) and can modulate the development of engineered cartilage in vitro (28).

MATERIALS

Recombinant human IGF-I was from R&D Systems (Minneapolis, MN). Knee joints from 2- to 4-week-old bovine calves were obtained.
from a local abattoir within 8 h of slaughter. Polyglycolic acid (PGA) scaffolds were from Albany International (Mansfield, MA). Spinner flasks and magnetic stirrers were from Bellco (Vineland, NJ). Needles (4 in. long, 22 gauge) were from Metropolitan Hospital Supply (Cambridge, MA). Silicone tubing (#13) was from Cole Palmer (Niles, IL). Rotating vessels (Slow Turning Lateral Vessels, RCCV-110) were from Synthecon (Houston, TX). Type II collagenase was from Worthington (Freehold, NJ). High glucose Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid (Hepes), and calcium- and magnesium-free phosphate buffered saline (PBS) were from Gibco (Grand Island, NY). Proteinase K, penicillin, streptomycin, nonessential amino acids (NEAA), proline, ascorbic acid, bovine chondroitin sulfate, calf thymus DNA, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Hoechst 33258 dye was from Polysciences (Warrington, PA). Dimethylmethylene blue dye was from Aldrich (Milwaukee, WI). Chloramine-T was from Mallinckrodt (Fair Lawn, NJ); p-dimethylaminobenzaldehyde was from Fisher (Paris, KY).

METHODS

Isolation of chondrocytes. Primary chondrocytes were isolated from full-thickness bovine calf articular cartilage by digestion with type II collagenase and resuspended in culture medium (DMEM containing 10% FBS, 10 mM Hepes, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 μg/ml ascorbic acid, 50 U/ml penicillin, and 50 μg/ml streptomycin, as previously described (2)). Chondrocytes used in each individual experiment were isolated from 2 to 3 different knee joints.

Preparation of cell-polymer constructs. The PGA scaffold was previously described in detail (32). In brief, PGA was extruded into 13 μm diameter fibers, processed to form a 97% porous nonwoven mesh with a bulk density of 62 mg/cm³, die punched into discs 5 mm in diameter by 2 mm thick, and sterilized with ethylene oxide. Cell seeding of PGA scaffolds was as previously described (33). In brief, scaffolds were prewetted in culture medium, threaded onto 4-in. long, 22 gauge needles and held in place with 3 mm long segments of silicone tubing. Three needles with one scaffold apiece were inserted into a silicone stopper that was in turn placed into the mouth of a spinner flask containing a magnetic stir bar and 110 ml of culture medium. The side arms of the flasks were loosened to permit gas exchange and the flasks placed on a magnetic stir plate at 80 rpm in a humidified 37°C/5% CO₂ incubator. After 16 h, flasks were inoculated with chondrocytes and cultured with mixing at 50 rpm, conditions previously shown to reproducibly seed essentially 100% of the cells throughout the scaffolds (33). After 2 days of seeding, cell-polymer constructs were cultivated in either Petri dishes or two types of bioreactors (spinner flasks and rotating vessels), as shown schematically in Fig. 1 (5, 30).

In the Petri dish studies, scaffolds initially seeded with 5 million cells apiece were subdivided into four experimental groups: (i) static dish (35 mm in diameter) with one construct and 6 ml of unsupplemented culture medium, (ii) static dish with one construct and 6 ml of medium supplemented with IGF-I (50 ng/ml), (iii) mixed dish (orbital shaker, 50 rpm) with one construct and 6 ml of unsupplemented medium, and (iv) mixed dish (orbital shaker, 50 rpm) with one construct and 6 ml of medium supplemented with IGF-I (50 ng/ml). In the bioreactor studies, scaffolds initially seeded with 3 million cells apiece were subdivided into six experimental groups: (i) static flask with 110 ml of unsupplemented medium, (ii) static flask with 110 ml of medium supplemented with IGF-I (50 ng/ml), (iii) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of unsupplemented medium, and (iv) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of medium supplemented with IGF-I (50 ng/ml). In the bioreactor studies, scaffolds initially seeded with 3 million cells apiece were subdivided into six experimental groups: (i) static flask with 110 ml of unsupplemented medium, (ii) static flask with 110 ml of medium supplemented with IGF-I (50 ng/ml), (iii) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of unsupplemented medium, and (iv) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of medium supplemented with IGF-I (50 ng/ml). In the bioreactor studies, scaffolds initially seeded with 3 million cells apiece were subdivided into six experimental groups: (i) static flask with 110 ml of unsupplemented medium, (ii) static flask with 110 ml of medium supplemented with IGF-I (50 ng/ml), (iii) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of unsupplemented medium, and (iv) mixed flask (orbital shaker, 50 rpm) with one construct and 6 ml of medium supplemented with IGF-I (50 ng/ml). The
speed of the rotating vessel was adjusted to maintain each settling construct at relatively steady position in the vessel (31). In all studies, culture medium was completely exchanged three times per week for 4 weeks.

Biochemical analysis of engineered tissues. Engineered tissues \((n = 2 \text{ or } 6 \text{ per group})\) were weighed, frozen, lyophilized, and digested with proteinase K at 60°C for 16 h \((1 \text{ mg/ml proteinase K in buffered solution, } 1 \text{ ml enzyme solution for up to } 20 \text{ mg dry weight sample})\) (34). Sulfated GAG content was determined spectrophotometrically at 525 nm after reaction with dimethylmethylene blue dye, using bovine chondroitin sulfate as standard (35). Hydroxyproline content was determined spectrophotometrically after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T (36), and the amount of total collagen was calculated using a 1:10 ratio of hydroxyproline to collagen (34). The number and mass of cells was assessed from the DNA content using a spectrofluorometer and conversion factors of 7.7 pg DNA per chondrocyte (37) and \(10^{2} \text{ g per chondrocyte}\) (32). Undegraded polymer was assumed not to account for a significant fraction of the wet weight of a 4-week construct (approximately 1 to 2%) (32).

Mechanical analysis of engineered tissues. From each of three randomly selected constructs in each of the four Petri dish study groups, a single disc \((2 \text{ mm thick and } 4.5 \text{ mm diameter})\) was cored from the center of each construct using a flat razor and a dermal punch. These discs represented between 6 and 34% of the total construct wet weight, depending on the experimental group. Equilibrium moduli were assessed using a custom-made uniaxial confined compression system (38). In brief, each disc was equilibrated at a tare strain of 4%, then subjected to five strain ramps, 3% each, and the equilibrium load measured after each step. The slope of the best-fit line through the stress-strain data provides the equilibrium modulus \((H_a)\). After mechanical testing, the weight and biochemical composition of the discs and the remainder of the construct were determined as described above and used to calculate the composition of the total construct.

Statistics. Statistical significance \((P < 0.05)\) was assessed using one-way analysis of variance (ANOVA) \((\alpha = 0.05)\) followed by Tukey’s HSD multiple comparisons procedure with the JMP IN statistical package.

RESULTS AND DISCUSSION

The interactive effects of mechanical and biochemical factors on tissue growth, structure, and function were investigated by culturing engineered cartilage constructs in different hydrodynamic environments with or without growth factor supplementation. In Petri dish studies, constructs seeded with 5 million chondrocytes apiece were cultured for 4 weeks either statically (static group) or on an orbital shaker at 50 rpm (mixed group) using medium that was either unsupplemented (control medium), or supplemented with 50 ng/ml IGF-I (IGF-I medium). In the static group, IGF-I medium yielded constructs with 2.3-fold higher wet weight (Fig. 2A), but lower weight percentages of cells, GAG, and collagen (Figs. 2B, 2C, and 2D, respectively), indicating that the beneficial ability of IGF-I to increase tissue growth rate was offset by detrimental effects on ECM content in static dishes. In control medium, mixed dishes yielded constructs with a 2.1-
fold higher weight percent of collagen (Fig. 2D) and comparable weight percentages of cells and GAG (i.e., Figs. 2B and 2C, respectively), but only 33% the total mass (Fig. 2A) as static dishes, indicating that although mixing increased the weight percentage of one biochemical component, it decreased the total mass of generated tissue. The combined use of mixed dishes and IGF medium yielded constructs that manifested the benefits that both biochemical and mechanical stimuli displayed individually (i.e., increased construct wet weight and percentage of collagen) (Figs. 2A and 2D, respectively).

The primary functions of cartilage in vivo are mechanical in nature, and the mechanical properties of cartilage are largely determined by the composition and amount of ECM components (39). To determine whether variations in construct weight percents of GAG and collagen correlated with variations in construct mechanical properties, discs of cartilage were cored from randomly selected constructs and subjected to uniaxial confined compression stress-relaxation tests. Construct weight percentages of GAG correlated positively with equilibrium modulus (Fig. 3), as observed in native (39) and engineered (5, 20) cartilage. Weight percentages of total collagen did not correlate with equilibrium modulus (data not shown). The percent GAG in the cored disc, although fairly consistent within a specific group (Fig. 3) did not correlate with the percent of GAG in the entire construct from which the disc was cored. This lack of correlation is not unreasonable since the wet weight of the mechanically tested discs represented only 6 to 34% that of the construct as a whole, and there is a well-described spatial distribution of GAG within the constructs (5, 30). Taken together, these data demonstrate that there is a correlation between the weight percent of GAG and mechanical properties of the tested discs of engineered cartilage but additional mechanical testing would be required to confirm the reasonable assertion that this correlation can be applied to the total constructs.

In bioreactor studies, constructs seeded with 3 million chondrocytes apiece were cultured for 4 weeks either in static flasks, mixed flasks (50 rpm), or rotating bioreactors (20 to 40 rpm) with medium that was either unsupplemented (control medium), or supplemented with 100 ng/ml IGF-I (IGF-I medium). Under all conditions investigated, the constructs developed over 4 weeks of in vitro cultivation into tissues that resembled native cartilage histologically and biochemically. Constructs grown statically had irregular shapes (Figs. 4A and 4D), whereas those grown in mixed flasks and rotating bioreactors had regular shapes (Figs. 4B, 4C, 4E, and 4F), consistent with previous studies (5). In all three mechanical environments, IGF-I medium yielded constructs with 2.5- to 3.7-fold higher wet weights than control medium (Figs. 4 and 5A), a finding consistent with previous studies carried out in Petri dishes (28).

IGF-I medium also increased construct absolute amounts of cells, GAG, and collagen, but to different extents in the three hydrodynamic environments, resulting in differences in construct weight percentages of cells (Figs. 5B, GAG (Fig. 5C), and total collagen (Fig. 5D). In static flasks, IGF-I medium reduced the weight percentage of collagen (Fig. 5D), but did not affect that of GAG (Fig. 5C). In contrast, under mixed conditions (flasks and rotating vessels), IGF-I medium increased the weight percentage of GAG (by 1.7-fold in both bioreactors) (Fig. 5C), but did not affect the weight percentage of collagen (Fig. 5D). The combined use of rotating bioreactors and IGF-I medium yielded constructs with the highest weight percentage of GAG (3.7%, Fig. 5C), demonstrating that the mechanical and biochemical stimuli can act synergistically to improve the quality of engineered cartilage.

IGF-I substantially enhanced tissue quality in both mechanically active environments, but the mechanisms by which this was achieved may have differed between mixed flasks and rotating vessels. In control medium, cell fractions were higher in mixed flasks than rotating vessels, a difference that was further increased by IGF-I medium (Fig. 5B). In mixed flasks, supplemental IGF-I increased the amount of GAG per cell by 1.5-fold but decreased the amount of collagen per cell by 15%, whereas in rotating vessels supplemental IGF-I increased the per cell amounts of both GAG and collagen by 2.9-fold and 1.6-fold, respectively (data not shown but can be calculated from presented data). Taken together, these data suggest that in mixed flasks, IGF-I may increase the construct GAG percentage by increasing both the cell percentage and the per cell GAG production, whereas in rotating ves-
sels IGF-I may increase the construct GAG and collagen percentages primarily by increasing the per cell production of each component.

Consistent with a recent report of IGF-I and dynamic compression synergistically increased collagen and GAG synthesis rates in explanted cartilage disks (40),
both sets of studies presented here revealed interactions between the mechanical and biochemical environments. Petri dish studies demonstrated that the ability of IGF-I to promote growth could combine additively with the ability of mixing to increase collagen, yielding larger constructs with higher weight percentages of collagen. In contrast to an additive effect, bioreactor studies demonstrated that the combination of mechanical and biochemical stimuli present in rotating vessels containing IGF-I medium substantially increased the construct weight percentage of GAG, even though neither rotating vessels or IGF-I medium alone increased this parameter. Two distinct, nonexclusive mechanisms may account for the strong influence of the mechanical environment on the effect of the growth factor: convection-enhanced transport and mechanical stimulation of the chondrocytes.

Laminar flow in rotating vessels (3, 31) and turbulent flow in mixed flasks (5, 30) may increase the transport of the IGF-I to and into the construct thereby increasing the pericellular concentration of the growth factor. Alternatively, convection-enhanced transport may have also modified the local cellular environment by increasing the concentration of nutrients while decreasing the concentration of metabolites so that the chondrocytes could more readily respond to a given concentration of the growth factor.

Rotating vessels, mixed flasks, and mixed Petri dishes each exposed the engineered cartilage to a different mechanically active environment (30, 31). Although forces experienced in vitro (5, 30, 31) are orders of magnitude smaller than the MPa-level stresses known to play an important role in the maintenance and remodeling of cartilage in vivo (12, 13), other investigators have shown that chondrocytes in vitro can respond to well-defined Pa-level hydrodynamic forces (41–43). Flow-induced shear stress at the surface of the engineered cartilage can potentially mechanically stimulate the chondrocytes to affect their proliferation, ECM production, or release of regulatory factors that may in turn affect chondrocytes within the construct. Consistent with this model are the observations that (a) at the outer surface of constructs exposed to turbulent flow in mixed flasks, the cells elongate and the cell density is increased relative to the interior regions of constructs grown in mixed cultures and to the outer surface of constructs cultured statically (5, 30) and (b) exposure of cultured chondrocytes to a laminar flow environment with a shear stress of 1.6 Pa causes the cells to elongate, increase GAG synthesis by twofold, increase prostaglandin E2 release by 10-fold, and increase mRNA levels for a tissue inhibitor of metalloproteinase by ninefold compared to static controls (42). The intracellular signal transduction pathways stimulated in chondrocytes by physical forces are just beginning to be elucidated, and similarities between mechanically induced and growth factor induced pathways are evident (43), as already noted in other cells types (e.g., reviewed in (13)). Regardless of the mechanism(s) by which the mechanical and biochemical environments interact, the present studies suggest that judiciously tailoring these stimuli can improve the size, structure, and function of in vitro engineered cartilage.

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REFERENCES


