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Growth factors for sequential cellular de- and re-differentiation in tissue engineering

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Abstract

A model system for the in vitro generation of cartilaginous constructs was used to study a tissue engineering paradigm whereby sequentially applied growth factors promoted chondrocytes to first de-differentiate into a proliferative state and then re-differentiate and undergo chondrogenesis. Early cultivation in medium with supplemental TGF-β1/FGF-2 doubled cell fractions in 2-week constructs compared to unsupplemented controls. Subsequent culture with supplemental IGF-I yielded large 4-week constructs with high fractions of cartilaginous extracellular matrix (ECM) and high compressive moduli, whereas prolonged culture with supplemental FGF-2 yielded small 4-week constructs with low ECM fractions and moduli. Sequential supplementation with TGF-β1/FGF-2 and then IGF-I yielded 4-week constructs with type-specific mRNA expression and protein levels that were high for type II and negligible for type I collagen, in contrast to other growth factor regimens studied. The data demonstrate that structural, functional, and molecular properties of engineered cartilage can be modulated by sequential application of growth factors. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Tissue engineering; Chondrocyte; Biomaterial; Bioreactor; TGF-β1; FGF-2; IGF-I

Advances in cell culture techniques allow the in vitro generation of functional cartilaginous tissue (e.g., [1]). Engineered cartilage implants have been used for articular cartilage repair in experimental animal models with some degree of success (e.g., [2]). The generation of autologous cartilage implants for clinical use would benefit from techniques that (i) decrease the initial number of cells required, thereby minimizing donor-site morbidity, and (ii) increase the rate of tissue growth, thereby shortening the in vitro culture period. It is logical that the above improvements can be achieved by the judicious application of growth factors, e.g., by intermittently supplementing the culture medium with growth factors (e.g., [3–5]) and through the use of cells genetically engineered to overexpress growth factors [6,7] or polymeric systems that provide controlled release of growth factors [8] or their genes [9].

Previous studies demonstrated that exposure to TGF-β1 and FGF-2 in vitro caused chondrocytes to proliferate and de-differentiate (i.e., stop producing cartilage-specific extracellular matrix (ECM) components including collagen type II and aggrecan) in a manner that could be reversed by transfer to high cell density conditions in vitro or in vivo [10–13]. The presence of certain growth factors during the expansion of chondrocytes in monolayers affected cell responsiveness to other growth factors upon transfer into three-dimensional (3D) culture systems [14,15]. An in vivo study demonstrated an anabolic effect of FGF-2 [16], whereas in vitro studies showed that FGF-2 inhibited chondrocyte differentiation [17] and had catabolic effects on cartilage explants [18,19].

Previous in vivo and in vitro studies consistently demonstrated anabolic effects of IGF-I on chondrocytes, engineered cartilage, and explanted or native cartilage (e.g., [3–5,7,18–23]). IGF-I increased synthesis and inhibited degradation of cartilaginous ECM and helped to maintain tissue electromechanical properties. IGF-I responsiveness was enhanced in mechanically

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active environments (i.e., cartilage constructs or explants exposed to dynamic laminar fluid flow [24] or cyclic compression [25]).

In the present study, culture medium supplementation with exogenous TGF-β1, FGF-2, and IGF-I in specific combinations and temporal sequences was explored as a means to promote chondrocytes to first de-differentiate into a proliferative state and then re-differentiate and undergo chondrogenesis in a model system for cartilage tissue engineering [26].

Materials and methods

Materials. Cell culture reagents including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were from Gibco (Grand Island, NY). Recombinant human IGF-I, porcine TGF-β1, and bovine brain FGF-2 were from R&D Systems (Minneapolis, MN). Other reagents were from Sigma (St. Louis, MO), unless otherwise specified. Rotating bioreactors were from Synthecon (RCCV-110, Houston, TX).

Cells. Chondrocytes were obtained from femoropatellar groove cartilage of 2–4-week-old bovine calves within 8 h of slaughter. Cells were isolated using 0.15% (w/v) type II collagenase (300 U/mg, Worthington, Freehold, NJ) in DMEM containing 10% FBS for 14 h at 37 °C with shaking (25 rpm) and resuspended in basal culture medium (DMEM with 10% FBS, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.4 mM proline, 50 mg/L L-ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml fungizone) as previously described [27,28]. Cartilage harvested from five different knee joints was used in two independent studies.

Tissue culture. Cell seeding of biomaterial scaffolds and cultivation of engineered cartilage constructs were carried out as previously described [26]. The scaffold was a non-woven mesh of 13 μm diameter polyglycolic acid (PGA) fibers with a porosity of 97% and known in vitro degradation kinetics [29]. Scaffolds were sterilized with ethylene oxide, aerated, wetted in 70% ethanol, rinsed in PBS, and threaded onto needles embedded in the stoppers of spinner flasks (four needles with three scaffolds apiece and 120 ml medium per flask), and incubated at 37 °C for 24 h in DMEM containing 10% FBS. Spinner flasks were inoculated with a suspension of freshly harvested chondrocytes (3.2 million cells per scaffold) and magnetically stirred (55 rpm) in a humidified 5% CO_2 incubator with loosened side caps to permit gas exchange. After 2 days, cell–polymer constructs were transferred into rotating bioreactors (15 constructs and 110 ml medium per vessel) and incubated with continuous gas exchange and solid body rotation of the vessel around its central axis. The rotation speed was increased from 15 to 40 rpm over the 4-week cultivation period to maintain the constructs freely suspended [26,30].

Growth factor supplementation. Basal medium was first supplemented or not with the combination of TGF-β1 (1 ng/ml) and FGF-2 (10 ng/ml) (TGF-β1/FGF-2) from day 3 to 10 and then supplemented or not with either FGF-2 (10 ng/ml) or IGF-I (100 ng/ml) from day 10 to 28, thereby forming six experimental groups. Culture medium was completely replaced every 2 or 3 days and constructs were sampled after 2 and 4 weeks.

Biochemical analyses. Representative constructs ($n = 3$ samples per data point) were weighed, frozen, lyophilized, and digested for 18 h at 60 °C with 125 μg/ml papain in buffer (100 mM phosphate, 10 mM EDTA, 10 mM cysteine, pH 6.5) using 1 ml enzyme per 5–10 mg dry sample [26]. Chondrocyte number was determined from the DNA content measured spectrofluorometrically using Hoechst 33258 dye and conversion factors of 7.7 pg DNA per chondrocyte [31] and 10 ng dry weight per chondrocyte [29]. Glycosaminoglycan (GAG) content was measured spectrophotometrically using dimethylmethylene blue dye [32] and bovine chondroitin sulfate as a standard. Total collagen content was determined from hydroxyproline content measured spectrophotometrically after acid hydrolysis of papain-digested constructs and reaction of *p*-dimethylaminobenzaldehyde and chloramine-T [33] using a hydroxyproline to collagen conversion factor of 1 to 10 [34].

Mechanical analysis. Compressive equilibrium moduli were determined in uniaxial stress-relaxation studies [35,36]. In brief, disks 3.25 mm in diameter and 1.1–2.4 mm thick were harvested from constructs ($n = 5$ independent samples per group) by punching out a central core and trimming its top and bottom surfaces until flat. Discs were equilibrated in PBS containing protease inhibitors, placed in a radially confining chamber filled with the same buffer, mounted in a servo-controlled Dynastat mechanical spectrometer (IMASS, Hingham, MA), and compressed using a porous polyethylene platen. The test sequence consisted of applying a tare load corresponding to a 5% strain followed by four consecutive stress relaxations each consisting of a load corresponding to a 2% strain. Data were recorded at a sampling rate of 10 points/s over a time increment of 480 s. Constructs were considered to fully relax during this increment based on a change in stress less than 0.006 MPa over the final 180 s. Equilibrium moduli were determined as the slopes of the best linear regression fits ($r^2 \geqslant 0.85$) of equilibrium stress versus applied strain. Moduli were within 10% of corresponding values obtained by fitting relaxation data obtained from 120 to 480 s to a simple exponential and extrapolating to $t \to \infty$.

Histological analyses. Representative constructs ($n = 3$ samples per data point) were fixed for 24 h at 4 °C in 4% paraformaldehyde in 0.1% diethyl pyrocarbonate (DEPC)-treated PBS (pH 7.4), paraffin embedded, and sectioned to 5 μm. Consecutive sections were stained with safranin-O/fast green for GAG or immunostained with monoclonal antibodies against bovine collagen type I (Sigma) and type II (NeoMarkers, Fremont, CA). Immunohistochemical sections were hydrated, incubated for 30 min at room temperature (RT) with 2 mg/ml testicular hyaluronidase in PBS (pH 5), rinsed with PBS, incubated for 30 min at RT with normal goat serum diluted at 1:10 in PBS and for 1 h at RT with the primary antibody, stained (Vectastain ABC, Burlingame, CA), and counterstained with hematoxylin.

In situ hybridization. Labeled cDNA probes for collagen type I and II mRNAs were obtained by random-primed labeling the dsDNA of two plasmids (Sp6α1(I), a 1.5 kb fragment of proα1(I) cDNA in a 3 kb SP6 vector, and pC3-1, a 1.2 kb fragment of proα1(II) cDNA in a 2.7 kb pUC19 vector) with digoxigenin (DIG) high primer reaction mix (Roche, Indianapolis, IN) followed by ethanol precipitation [37]. Histological sections were rehydrated, equilibrated in DEPC-treated PBS with 0.1 M glycine and 0.3% Triton X-100, digested for 30 min at 37 °C with proteinase K (10 μg/ml) in RNase-free 100 mM Tris–HCl with 50 mM EDTA (pH 8), fixed at 4 °C for 5 min in 4% paraformaldehyde, acetylated for 10 min in 0.1 M triethanolamine with 0.25% (v/v) acetic acid anhydride (pH 8.0), and incubated for 2 h at 37 °C in 4× SSC (0.6 M NaCl, 0.06 M sodium citrate) containing 50% (v/v) deionized formamide (pH 7). The hybridization solution was 4× SSC with 40% deionized formamide, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10 mg/ml bovine serum albumin), 10% dextran sulfate, 10 mM dithiothreitol (DTT), 1 mg/ml yeast tRNA, and 1 mg/ml denatured and sheared salmon sperm DNA, prepared immediately prior to use. Hybridization solution containing 5–10 ng of the appropriate DIG-labeled cDNA probe per section was heated to 100 °C for 5–10 min, cooled in ice water for 2 min, carefully placed on the section, and incubated overnight in a 42 °C humidified chamber. Hybridized sections were washed twice in 1× SSC at RT, 37 and 45 °C each for 15 min, rinsed in buffer 1 (100 mM Tris–HCl, 150 mM NaCl, pH 7.5), blocked for 30 min at RT in 0.1% Triton X-100 and 2% sheep serum in buffer 1, and incubated in a humidified container for 2 h at RT with alkaline phosphatase conjugated polyclonal sheep anti-DIG

Fab fragments (Roche) diluted 1:1000 in buffer 1 containing 0.1% Triton X-100 and 1% normal sheep serum. Sections were washed twice in buffer 1, once in buffer 2 (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and incubated for 2–5 h at RT in a light-tight box with 300 μl color solution. The color solution was made by adding 67 μl of 75 mg/ml nitroblue tetrazolium in 70% dimethylformamide (DMF), 53 μl of 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 100% DMF, and 3.6 mg of levamisole into 15 ml of buffer 2. Upon optimal color development, sections were immersed in 10 mM Tris–HCl with 1 mM EDTA (pH 8).

SDS–PAGE and Western blot. Representative samples ($n = 1$ specimen per data point) were diced, lyophilized, extracted with 4 M guanidinium hydrochloride (GuHCl) [38], washed, equilibrated with 0.5 M acetic acid, homogenized, partially digested with pepsin [39], and centrifuged at 48,000*g* for 1 h. A salt precipitate of the supernatant was obtained [40], lyophilized, resuspended in 75 mM Tris–HCl, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 2 mM PMSF, 5 mM benzamidine–HCl, and 5 mM NEM (pH 6.8) and aliquots containing 1.5 μg of total collagen, as determined from hydroxyproline content [33], were separated on a 7.5% acrylamide gel (Bio-Rad, Richmond, CA). Bands were stained with Coomassie blue or electroblotted at 14 V overnight at 4 °C onto polyvinylidene difluoride membranes using a mini-trans-blot electrophoretic transfer cell (Bio-Rad). Non-specific binding was blocked with 5% non-fat milk in TTBS (100 mM Tris–HCl, 0.9% NaCl, 1% Tween 20, pH 7.5) for 1 h. Membranes were incubated for 1 h at RT with primary monoclonal antibody to bovine collagen type I (Sigma) or II (NeoMarkers, Fremont, CA), then for 1 h at RT with horseradish peroxidase conjugated anti-mouse IgG (Promega, Madison, WI) diluted 1:7500 in TTBS. Bands were visualized using a chemiluminescent substrate (ECL-plus, Amersham, Piscataway, NJ) and photographic film (Hyperfilm-ECL).

Statistical analysis. Statistics were assessed by one-way analysis of variance (ANOVA); p values less than 0.05 were considered statistically significant.

Results and discussion

To promote chondrocyte proliferation, the construct culture medium was supplemented with a combination of TGF-β1 and FGF-2 from day 3 to 10, based on a pilot study done in the same model system in which constructs cultured for 2 weeks with TGF-β1/FGF-2 had wet weight (ww) cell fractions approximately twice those of unsupplemented constructs or those treated with either TGF-β1 or FGF-2 individually (data not shown). Early TGF-β1/FGF-2 supplementation yielded 2-week constructs with ww cell fractions 1.8–2-fold higher than untreated controls (Fig. 1A), consistent with previous studies (e.g., [12,15]). In 2-week constructs, the cell proliferation induced by TGF-β1/FGF-2 was associated with de-differentiation, as evidenced by construct ww fractions of GAG (Fig. 1B) and total collagen (Fig. 1C) that were only 40–55% as high as those in untreated controls.

To promote cellular re-differentiation and chondrogenesis, construct culture medium that already contained 10% FBS [3,26] was supplemented from day 10 to 28 with either FGF-2 or IGF-I, since the latter promotes construct growth [3], especially when used in rotating bioreactor cultures [24]. The biochemical composition (Figs. 1D–F), size (Fig. 1G), mechanical function (Fig. 1H), and molecular properties (Fig. 2) of 4-week constructs all depended on the growth factor regimen. Wet weights of 4-week constructs ranged from 0.09 to 0.28 g

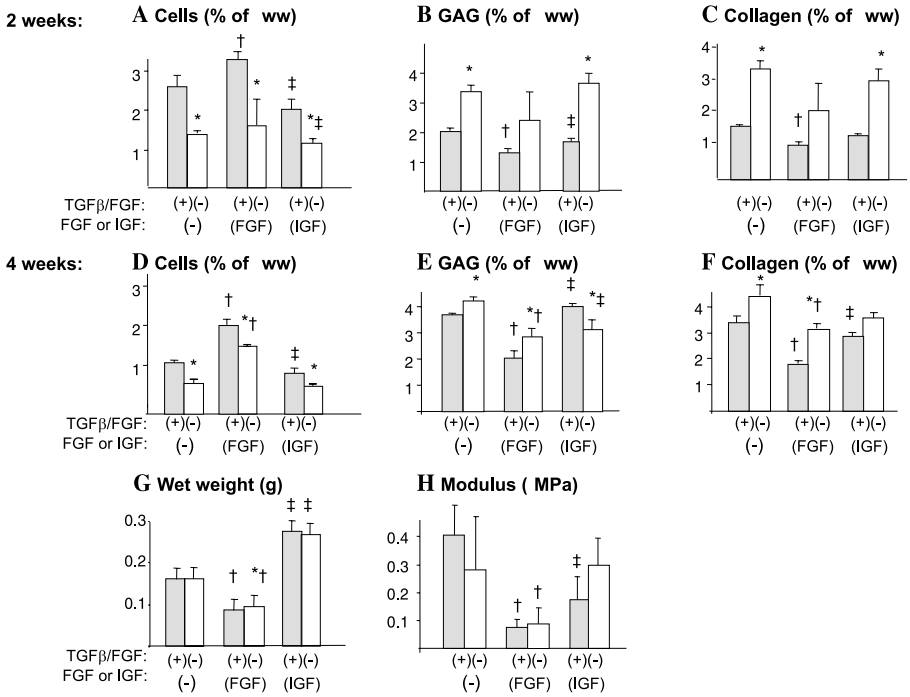

Fig. 1. Differential effects of growth factors on (A–C) 2-week and (D–H) 4-week constructs with respect to: (A, D) cells, (B, E) GAG, (C, F) total collagen, (G) wet weights, and (H) compressive moduli. Data are the average ± standard deviation of three and five independent samples for (A–G) and (H), respectively. (*) Significantly different from (+) TGF-β1/FGF-2; (†) significant effect of FGF-2; (‡) significant effect of IGF-I.

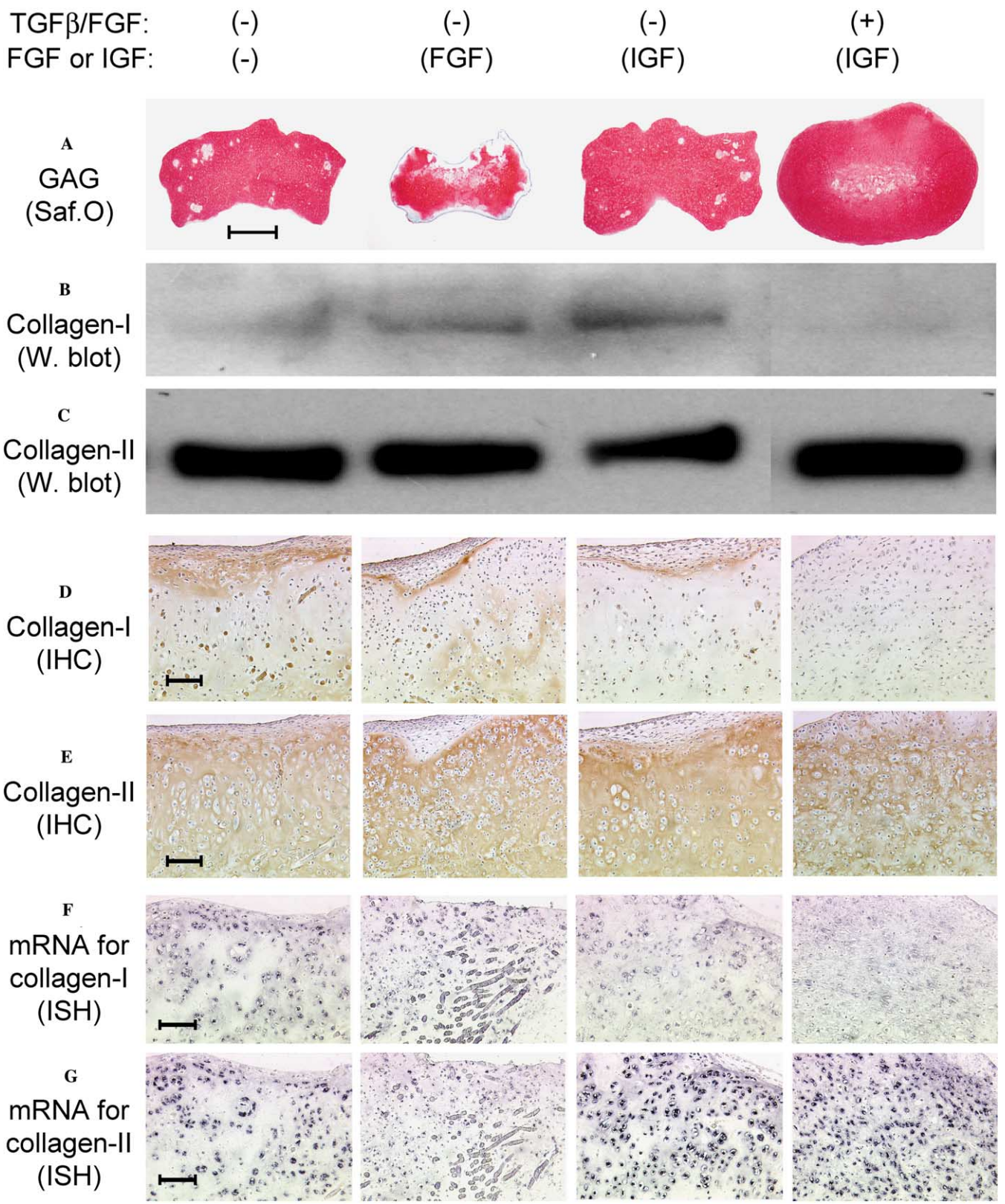

Fig. 2. Differential effects of growth factors on representative 4-week constructs with respect to: (A) GAG, (B, D) collagen type I protein, (C, E) collagen type II protein, and (F or G) collagen type I or type II mRNA, respectively, as assessed by: (A) safranin-O staining (Saf.O); full construct cross-sections are shown, (B, C) Western blot (W. blot); 116 kDa bands are shown, (D, E) immunohistochemistry (IHC), and (F, G) in situ hybridization (ISH). Scale bars are: (A) 2.0 mm and (D–G) 100 μm.

(Fig. 1G), which corresponded to construct diameters of 0.75–1.0 cm and thicknesses of 0.25–0.5 cm (Fig. 2A). Moduli of 4-week constructs ranged from 0.08 to 0.41 MPa (Fig. 1H); for comparison, moduli of native bovine cartilage ranged from 0.27 MPa at the articular surface to 0.71 MPa in the deep zone with a mean homogenous value of 0.47 MPa [28,41], whereas moduli of 3-day constructs which consisted mainly of cells and PGA mesh were previously found to be too fragile to allow measurement of mechanical properties [36].

By 4 weeks of cultivation, the effects of early TGF-β1/FGF-2 supplementation (i.e., enhanced chondrocyte proliferation and decreased ECM deposition) were still detectable but significantly offset (compare Figs. 1D–F with Figs. 1A–C), implying reversibility of the de-differentiated state as previously reported (e.g., [13–15]). Four-week constructs exhibited distribution patterns of GAG (Fig. 2A) and collagen type II protein (Fig. 2E) and mRNA (Fig. 2G) that were similar to one another, indicating coordinate transcription and translation of proteoglycans and collagen type II. Collagen type I protein, if and when present, was localized to a relatively thin layer ($\leqslant 100\,\mu m$) at the construct surfaces (Fig. 2D); collagen type I is known to be synthesized by chondroprogenitor cells in developing cartilage [42], but is generally not present in mature cartilage.

IGF-I supplementation during the latter part of the cultivation yielded 4-week constructs with high wws (1.6-fold higher than those grown in basal medium, Fig. 1G), moderately high compressive moduli (0.2–0.3 MPa, Fig. 1H), and high ww fractions of GAG (Fig. 1E) and total collagen (Fig. 1F) that was predominately type II and not type I (Figs. 2B–E). Supplemental IGF-I up-regulated collagen type II mRNA expression (Fig. 2G), consistent with previous studies (e.g., [4,43]).

FGF-2 supplementation yielded 4-week constructs with low wws (approximately 56% as high as those grown in basal medium, Fig. 1G), low compressive moduli (<0.08 MPa, Fig. 1H), and low ww fractions of GAG (Fig. 1E) and total collagen (Fig. 1F) that was predominately type II and not type I (Figs. 2B–E). Supplemental FGF-2 down-regulated collagen type II mRNA expression (Fig. 2G), consistent with a previous study [17].

Sequential supplementation with TGF-β1/FGF-2 and then IGF-I yielded 4-week constructs with thick exterior regions that stained strongly positive for GAG and central regions consisting of immature cartilaginous tissue ((+)/(IGF), Fig. 2A), a structure that provides an explanation for the relatively low moduli measured for disks harvested from the construct centers (Fig. 1H). Based on previous studies [1], the central regions of 4-week constructs can be expected to mature into cartilaginous tissue with further cultivation in the model system since chondrogenesis is typically initiated at the construct periphery and progress towards its center in a time-dependent manner [26]. Constructs sequentially supplemented with TGF-β1/FGF-2 and then IGF-I contained only collagen type II protein and negligible collagen type I, as shown by Western blot and immunohistochemistry (Figs. 2B–E), and expressed only collagen type II mRNA and negligible collagen type I mRNA, as shown by in situ hybridization (Figs. 2F–G), consistent with a previous study [44].

In summary, the culture medium in a cell–biomaterial–bioreactor system for cartilage tissue engineering was sequentially supplemented first with TGF-β1/FGF-2 to promote chondrocyte proliferation and then with IGF-I to promote rapid chondrogenesis and tissue growth. The data demonstrated that structural, functional, and molecular properties of engineered cartilage could be modulated by the judicious application of growth factors and support a general paradigm for tissue engineering whereby sequentially applied growth factors promote cells to first de-differentiate into a proliferative state and then re-differentiate and regenerate full tissues.

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