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Growth factor effects on costal chondrocytes for tissue engineering fibrocartilage

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Abstract

Tissue engineered fibrocartilage could become a feasible option for replacing tissues like the knee meniscus or temporomandibular joint disc. This study employed five growth factors insulin-like growth factor-I, transforming growth factor- β 1, epidermal growth factor, platelet-derived growth factor-BB, and basic fibroblast growth factor in a scaffoldless approach with costal chondrocytes, attempting to improve biochemical and mechanical properties of engineered constructs. Samples were quantitatively assessed for total collagen, glycosaminoglycans, collagen type I, collagen type II, cells, compressive properties, and tensile properties at two time points. Most treated constructs were worse than the no growth factor control, suggesting a detrimental effect, but the IGF treatment tended to improve the constructs. Additionally, the 6wk time point was consistently better than 3wks, with total collagen, glycosaminoglycans, and aggregate modulus doubling during this time. Further optimization of the time in culture and exogenous stimuli will be important in making a more functional replacement tissue.

Introduction

Tissue engineering seeks to create functional replacement tissue and often employs an autogenic cell source to avoid issues with immune rejection. Identifying an appropriate cell source can be particularly difficult, since cells from the tissue of interest are often scarce and/or already diseased. This is especially true for cartilage and fibrocartilage tissues. Fibrocartilage, like that seen in the knee meniscus or temporomandibular joint (TMJ) disc, is frequently injured or diseased and largely lacks the ability to repair itself.^{1,2} Unlike healthy, hyaline articular cartilage, fibrocartilage also contains collagen type I, and while cartilage functions primarily in compression, fibrocartilage has an important tensile component to its mechanical role.³ The biochemical and mechanical demands of this unique category of tissues require a highly productive cell type.

Costal chondrocytes (CCs) appear particularly well suited for the purposes of fibrocartilage tissue engineering due to similarities in their tissue characteristics. Native rib cartilage contains glycosaminoglycans (GAGs), primarily chondroitin sulfate and keratan sulfate,^{4–6} and collagen types I and II in a ratio of 1:5.⁷ Costal cartilage is hypocellular, containing 4–10 cells per 0.22mm² of tissue.⁸

While CCs have shown potential in cartilage tissue engineering,^{9–11} their ability to produce collagen I suggests they may function even more effectively in fibrocartilage tissue engineering. Indeed, CCs have shown potential to produce extracellular matrix (ECM) relevant

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for tissue engineering the TMJ disc; however, improved collagen content and mechanical properties are necessary before a functional replacement can be achieved.^{12,13}

The growth factors and concentrations were judiciously chosen based on their ability to improve ECM production in CCs or fibrochondrocytes. Transforming growth factor- β 1 (TGF), at 1ng/mL was shown to increase proline, thymidine, leucine, and sulphate incorporation in CCs.¹⁴ At higher concentrations TGF improved collagen production and mechanical properties in constructs made from TMJ disc fibrochondrocytes.¹⁵ The combination of fibroblast growth factor (FGF), platelet-derived growth factor-BB (PDGF), and TGF increased proliferation and collagen type I and elastin staining in CCs.¹⁶ PDGF also increased collagen production in TMJ disc cells.¹⁷ FGF also increased proline, leucine, and thymidine incorporation in CCs, but decreased the sulfate incorporation.¹⁴ FGF increased GAGs, collagen, and mechanical properties in TMJ disc cells.^{15,18} Epidermal growth factor (EGF) increased proliferation of growth plate CCs and promoted a more elongated morphology; the effects of proliferation were saturated at 30ng/mL.^{19,20} GAG was increased with insulin-like growth factor-I (IGF) up to 0.5 μ g/mL in CCs²¹ and collagen was increased in TMJ disc cells with 100ng/mL of IGF.¹⁸

This study attempts to improve biochemical and mechanical properties of tissue engineered constructs using CCs through the addition of growth factors to this *in vitro* approach. IGF, TGF, EGF, PDGF, and FGF were applied continuously to scaffoldless, tissue engineered constructs and examined for changes in ECM quantities and mechanical properties.

Materials and Methods

Cell preparation

Costal cartilage tissue was scraped from the non-floating ribs of three skeletally-mature, female goats obtained from a local abattoir. This tissue was minced into cubes approximately 1mm³ in size and digested overnight at 37°C with 0.2% type II collagenase (Worthington, Lakewood, NJ) in Dulbecco's modified eagle's medium (DMEM) (Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1% Penicillin-Streptomycin-Amphotericin B (PSF) (Cambrex, Walkersville, MD), 1% non essential amino acids (NEAA) (Gibco), and 25 μ L/mL L-ascorbic acid (Sigma, St. Louis, MO). Isolated cells were cultured on tissue culture-treated plastic until 70–90% confluent. They were then passaged with trypsin-EDTA (Gibco). After passage 1, cells were frozen in DMEM with 10% DMSO, 20% FBS, 1% PSF, and 1% NEAA. Upon thawing, cells were again cultured in monolayer until passage 3.

Construct preparation

Constructs were formed using a method modified from the self-assembly of articular chondrocytes.²² Agarose wells were formed from a mold, and 2M cells were seeded into each 5mm well. After 4hrs, additional construct medium was added: DMEM with 1% PSF, 1% NEAA, 1% ITS+ premix (BD Biosciences, San Jose, CA), 0.1 μ M dexamethasone, 40 μ g/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50 μ g/mL ascorbate 2-phosphate (Sigma), and 100 μ g/mL sodium pyruvate (Fisher). Five growth factors (in addition to a no treatment control—no GF) were used individually in the construct medium throughout the entire 6wk culture period: TGF (1ng/mL), IGF (100ng/mL), FGF (10ng/mL), EGF (30ng/mL), and PDGF (10ng/mL) (Peprotech, Rocky Hill, NJ). After 1wk, constructs were removed from the wells and cultured for the remaining time in unconfined, agarose-coated, 6-well plates. All groups were studied at 3 and 6wks for gross morphological, histological, biochemical, and mechanical changes.

Histology and immunohistochemistry

At least one construct per group was removed at each time point, frozen in HistoPrep™ (Fisher), and sectioned into 14µm slices. Slides for histology were fixed in formalin and stained with safranin-O/fast green for GAGs or picosirius red for collagen. Immunohistochemistry (IHC) was used to examine collagen types I and II, as described previously.¹³

Biochemistry and enzyme-linked immunosorbent assay (ELISA)

Six samples per group were examined at each time point for biochemical content. Samples were weighed before and after lyophilization to determine wet and dry weights. Dry samples were digested under constant mechanical agitation for 7 days at 4°C with a 125µg/mL papain (Sigma) solution containing N-acetyl-cysteine (Sigma) and EDTA. Elastase (Sigma) at 1mg/mL was added for another 2 days of digestion. Digested samples were stored at -20°C and used for biochemical assays and ELISAs.

DNA was quantified with a PicoGreen® (Molecular probes, Carlsbad, CA) reagent by comparing to calf thymus DNA (Sigma). Cell number was determined with a conversion factor of 7.7pg DNA/cell.²³ Sulfated GAGs were quantified with a Blyscan kit (Biocolor, Newtownabbey, Ireland) according to the manufacturer's protocol. Collagen content was measured with a modified colorimetric hydroxyproline assay.²⁴ Collagen types I and II were quantified with ELISAs as described previously.¹³

Mechanical testing

Five samples were cut in half through the circumference and tested with a creep indentation apparatus to determine compressive properties.²⁵ An indentation tip of 1mm was used to apply a tare load of 0.002N and a creep load of 0.007N. Loading occurred until the sample equilibrated (defined as deformation less than 10⁻⁶mm/s) or 10min passed (tare load) and 1hr passed (creep load). The step load was removed and recovery distance measured. Data were analyzed with a semi-analytic, semi-numeric model to determine the biphasic properties: aggregate modulus, permeability, and Poisson's ratio.²⁶

At least five samples per group were also tested in tension on a 5565 Instron (Norwood, MA). Constructs were cut in half through the circumference and then cut again to form a dog bone shape. Samples were glued to a paper frame and loaded at 10% strain/min until failure. Elastic modulus (E) and ultimate tensile strength (UTS) were calculated from the stress-strain data.

Statistics

All mechanical and biochemical data were analyzed with a two-way ANOVA. Factors were time and growth factor, which had two and six levels, respectively. An F-test was used to determine if a factor was significant ($p < 0.05$), and a Tukey's *post hoc* test was used to determine differences between the levels.

Results

Morphology and histology

Morphological differences among the treatment groups at 6wks are illustrated in Fig. 1. Control and IGF constructs retained their initial seeding diameter of 5mm, while other groups contracted into more spherical-shaped constructs. These spheres were actually fluid-filled structures. Staining of the inner fluid with trypan blue showed numerous dead cells (data not shown). At both time points, 100% of the PDGF, FGF, and TGF constructs appeared to have this cyst-like morphology. At 3wks, EGF, IGF, and controls were cyst-like in 80%, 54%, and 64% of the constructs, respectively. At 6wks, 100% of the EGF constructs appeared fluid-

filled. The cyst-like IGF constructs also increased to 80% at 6wks, but the control samples remained about the same with 60% forming cyst-like structures; these structures did not stain for any ECM (see Fig. 1). Collagen and GAG staining was seen throughout the outer region of the constructs. The collagen staining was particularly dense on the outermost surface of all the constructs. IHC staining was positive for both collagens type I and II for all constructs and regionally distributed in a similar manner to the picrosirius red stain.

Biochemistry and ELISA

Figure 2 illustrates cell number, GAG content, and total collagen content for all treatments and time points. Data are presented as per construct quantities, but wet and dry weights are shown in Table 1. IGF constructs had the largest wet and dry weights, and no GF constructs had a significantly larger dry weight than TGF, EGF, PDGF, and FGF constructs. The cell number (Fig. 2a) was greatest in IGF constructs, which was significantly more than control, TGF, and PDGF constructs. EGF and FGF groups had significantly more cells than the PDGF group; however, all groups remained near the initial seeding of 2M cells. The total GAG and collagen in the constructs (Fig. 2b and 2c), on the other hand, showed dramatic differences for the treatment groups. IGF and control groups had the greatest GAG and collagen, significantly more than any other group. TGF constructs contained significantly more GAG than the EGF, PDGF, and FGF constructs and more collagen than PDGF and FGF constructs. Collagen II quantities (Fig. 3a) had the same statistical differences as the GAG quantities, while collagen I content (Fig. 3b) in the EGF group was significantly greater than the control and FGF groups. Time was significant for all biochemical, ELISA, and weight metrics, with the 6wk time point having greater quantities than the 3wk time point.

Mechanical properties

Aggregate moduli (Fig. 4a) of control samples were significantly greater than EGF or FGF samples. Aggregate moduli also significantly increased from 3wks to 6wks. Permeability and Poisson's ratio were not significantly different between the experimental groups with ranges of 3.97×10^{-15} – $1.87 \times 10^{-13} \text{ m}^4/\text{N}\cdot\text{s}$ and 0.0108–0.368, respectively. Permeability was significantly greater at 3wks than 6wks. Tensile properties (Fig. 4b and 4c) were highest for the control group. Control constructs had a UTS significantly greater than EGF, PDGF, and FGF constructs. The control group also had a significantly greater elastic modulus than PDGF and FGF groups. The UTS values of the IGF samples were also significantly higher than the FGF samples. Time was not a significant factor in the tensile properties.

Discussion

This study examined the effects of various growth factors on scaffoldless, costal chondrocyte constructs for the purposes of fibrocartilage tissue engineering. IGF-treated constructs were equal to or better than control for all the metrics examined in this study. The increase in cell number with IGF treatment is consistent with previous work with CCs.²⁷ The increase in GAG seen previously with CCs²¹ or increase in collagen seen previously with TMJ fibrocartilage^{15,18} were not as prominent as expected, however the increasing trend was certainly apparent suggesting the benefits of IGF for fibrocartilage tissue engineering.

TGF, EGF, and PDGF had considerably less GAG, total collagen, and collagen type II, but more collagen type I, demonstrating the promotion of a more fibroblast-like phenotype. Previous work with CCs showed that EGF and FGF treatment led to a more elongated cell morphology.²⁰ This morphology is generally indicative of a cell phenotype which would produce more collagen type I and less collagen type II, as was seen in this study. While previous work supports many of the results seen here, more improvements in ECM content were expected for TGF,^{14,16,28,29} FGF,^{14,16,29} and PDGF.¹⁶ The limited improvements seen with

these growth factor treatments are likely due to the base medium used, which had no serum and contained additional additives that have been shown to promote chondrogenesis.³⁰ The previous cited work added 10% serum to the base medium, which contains small amounts of growth factors. Serum is often considered undesirable in tissue engineering, due to issues with immune response of serum from another species, but it may be necessary to promote growth factor effects. Alternatively, the presence one or two other growth factors may be sufficient to encourage the effects of a single growth factor. Future work may wish to consider a combination of a small number of growth factors in lieu of adding serum. Other studies may also wish to consider alternative growth factors with the base medium used here. The medium's chondrogenic ability appeared to decrease with the addition of TGF, EGF, PDGF and FGF, as was seen with the 10–60% decrease in GAG, total collagen, and collagen type II from the control. (IGF treatment was largely unchanged from the group without additional growth factors.) While these results were contrary to expectations, they are partially supported by current work with articular chondrocytes in a scaffoldless approach with serum-free medium, which showed a decrease in GAG with the addition of TGF- β 1.³¹ While fibrocartilage tissue engineering is still in its early stages, the field of cartilage tissue engineering has been explored in considerably more depth and established this base medium as better than FBS media for cartilage regeneration.^{32,33} However, it is apparent from this study that more work needs to be done, particularly for fibrocartilage tissue engineering, to determine growth factors that can further improve properties from the base medium rather than diminish them.

Regardless of the growth factor used, constructs consistently benefited from a longer time in culture. For all biochemical assays and ELISAs, quantities of ECM and cells increased temporally across all treatment groups. At 6wks, total collagen and GAG increased at least two fold over the values seen at 3wks for all the groups. Aggregate modulus also approximately doubled in most groups. Tensile properties, however, did not have a clear trend with time. Most groups remained the same from 3wks to 6wks; it was not considered a significant factor for either UTS or elastic modulus. This suggests that while collagen quantities are increasing in the constructs, the collagen is likely not being organized or packed appropriately to improve the tensile strength or stiffness. The application of a mechanical stimulus, like tension, could be important to the ECM organization, which would likely improve the mechanical properties. The temporal changes also suggest the need to examine the time factor in greater depth—exploring both longer and shorter time points to elicit an overall trend on the changes that occur with time.

Previous work using passaged chondrocytes in a scaffoldless approach have also observed the fluid-filled structure that formed in many of the constructs from this study.^{12,13,34} It is well accepted that chondrocytes prefer cell-cell interaction and retain their phenotype better in three-dimensional culture, while attachment to the tissue culture plastic alters their phenotype, promoting a more fibrochondrocytic phenotype.^{16,35} This phenotypic alteration may not occur uniformly, and it is very likely that the cell population after passaging is non-uniform. As discussed previously, in the absence of another surface on which to attach, similar cells attach to one another; this is referred to as the differential adhesion hypothesis.^{36–39} Considering these observations, it is reasonable to infer that the passaged CCs aggregate into distinct cell populations upon construct formation. Within the first 48hrs, formation of the fluid-filled structure becomes apparent, (data not shown). The ensuing death of the inner population of cells may be due to the outer cells forming a barrier for nutrient and/or waste transport. Additionally or alternatively, the inner region may be more amiable to substrate attachment versus cell attachment, causing them to die after three-dimensional seeding. This suggests an interest in determining the characteristics of these populations and purifying them prior to construct seeding. Interestingly, at the end of 6wks, all the growth factors appeared to promote this cyst-like structure either at 3wks or over time (like IGF treatment). This fascinating observation gives insight into the subpopulations of these cells. One could postulate that these

growth factors initially encouraged growth of the population of cells that formed in the center. Their subsequent death made the growth factors appear ineffective and even detrimental. If the methods used here were altered so that those cells did not die, the growth factors may have caused beneficial changes in the constructs. Further exploration of ways to eliminate this structure will likely be necessary. Altering the expansion conditions would certainly affect the cell characteristics and may also eliminate the “cyst” structure.

While there is considerable work that still needs to occur to optimize the constructs created here, the approach used is promising for the purposes of fibrocartilage tissue engineering. Comparing the construct properties to those seen in a porcine TMJ disc, it is apparent that compressive properties are nearly the same,⁴⁰ and that GAG content is actually greater than the native tissue.⁴¹ Collagen content was about ten times lower than native,⁴² and tensile modulus was 2–40 times lower.⁴³ The fibroblast-promoting growth factors, like TGF, PDGF, EGF and FGF, were not able to improve these properties from the control, but they were able to alter the ratio of collagen I to collagen II suggesting the potential of the growth factors to augment the construct for various types of fibrocartilages. Additionally, the IGF treated group was better than the other groups for biochemical and mechanical content. Future work examining a combination of growth factors, an optimal culture time, purification of the expanded cell population, or improved expansion conditions could further develop these constructs such that they are able to function as native fibrocartilage.

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Figure 1.

Gross morphological images from 6wks are shown in frames a–l. Spaces below the images are 1mm in length. Control and IGF groups retained the initial 5mm diameter, while the other groups contracted and rounded into more spherical morphologies. Frames m–r show picrosirius red staining for the constructs at 6wks. This stain clearly shows the collagen which stained consistently in an outer ring and densely on the very outer edge of the constructs. These images also illustrate the void structure that formed frequently, particularly in the growth factor-treated groups. The scale bar in frame m is 1 mm and applies for all picrosirius red staining micrographs. Frames s–x show safranin-O/fast green staining. Collagen types I and II IHC are seen in frames y–dd and ee–jj, respectively. All samples stained positive for GAGs and collagens types I and II. The scale bar in frame ee is 100 μ m and applies to the IHC and safranin-O/fast green stains.

Figure 2.

Cell (a), GAG (b), and collagen (c) content at both time points (mean + SD). Treatment groups with different letters are statistically significant ($p < 0.05$). For all the biochemical metrics, the 6wk time point was significantly greater than the 3wk time point as indicated by the *. While some statistical differences in the various growth factors were apparent, cell quantities were around 2M for all groups. GAG/construct and collagen/construct were significantly greatest in the control and IGF constructs. TGF-treated constructs had significantly more GAG content than EGF, PDGF, and FGF constructs and significantly more collagen than PDGF and FGF constructs.

Figure 3.

Collagen type II (a) and collagen type I (b) quantities for constructs at both time points (mean + SD). Treatment groups with different letters are statistically significant ($p < 0.05$). The 6wk time point was significantly greater than the 3wk time point in both collagen types as indicated by the *. Control and IGF samples had significantly more collagen type II than all other groups. TGF samples had significantly more collagen type II than EGF, PDGF, and FGF samples. Collagen type I was significantly greater in EGF constructs than in FGF or no GF constructs.

Figure 4.

Aggregate modulus (a), UTS (b), and elastic modulus (c) for all groups at both time points (mean + SD). Treatment groups with different letters are statistically significant ($p < 0.05$). The 6wk time point was significantly greater than the 3wk time point as indicated by the * for aggregate modulus. The no GF group had the highest aggregate modulus, which was significantly larger than EGF and FGF groups. The control group also had the highest tensile modulus and UTS—significantly greater than PDGF and FGF. Its UTS was also significantly higher than the EGF group. IGF constructs were significantly greater than FGF constructs for both E and UTS. The TGF group was significantly larger than the FGF group for UTS. Time was not a significant factor for either tensile measurement.

Table 1

Data are shown as (mean $\pm$ SD). Treatment groups with different letters are statistically significant ($p < 0.05$). Weights were significantly larger at 6wks as indicated by the *. IGF constructs had wet and dry weights significantly larger than any other group. No GF constructs also had significantly greater dry weight than the other groups.

Wet weight(mg)	3wk	6wk*	Dry weight (mg)	3wk	6wk*
No GF ^b	27.3 $\pm$ 4.42	36.0 $\pm$ 6.05	No GF ^b	2.97 $\pm$ 0.193	4.49 $\pm$ 0.366
IGF ^a	33.9 $\pm$ 6.10	46.1 $\pm$ 9.60	IGF ^a	3.33 $\pm$ 0.542	6.02 $\pm$ 0.272
TGF ^b	19.8 $\pm$ 1.51	28.6 $\pm$ 1.07	TGF ^c	2.22 $\pm$ 0.131	3.92 $\pm$ 0.378
EGF ^b	21.1 $\pm$ 3.82	35.7 $\pm$ 3.25	EGF ^c	2.00 $\pm$ 0.305	3.72 $\pm$ 0.379
PDGF ^b	24.9 $\pm$ 19.3	30.2 $\pm$ 5.90	PDGF ^c	1.91 $\pm$ 0.422	3.56 $\pm$ 0.501
FGF ^b	22.7 $\pm$ 1.72	33.6 $\pm$ 3.87	FGF ^c	2.24 $\pm$ 0.224	3.56 $\pm$ 0.302

Table 2

Ratios of collagen type I to collagen type II at both time points.

Data are shown as (mean $\pm$ SD). Treatment groups with different letters are statistically significant ($p < 0.05$).

Ratios were significantly larger at 3wks as indicated by the *. EGF and PDGF constructs had significantly higher ratios than IGF, TGF, or no GF treated constructs. The FGF ratio was also significantly greater than IGF and control groups.

Collagen I/Collagen II	3wk*	6wk
No GF ^d	2.33 $\pm$ 0.150	2.67 $\pm$ 0.206
IGF ^d	2.82 $\pm$ 0.552	2.61 $\pm$ 0.237
TGF ^{cd}	3.22 $\pm$ 0.265	3.13 $\pm$ 0.518
EGF ^a	6.35 $\pm$ 1.60	3.82 $\pm$ 0.180
PDGF ^{ab}	5.22 $\pm$ 0.815	3.42 $\pm$ 0.223
FGF ^{bc}	4.73 $\pm$ 0.572	3.33 $\pm$ 0.580