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REGULAR ARTICLE

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 such as the knee meniscus or temporomandibular joint disc. This study employed five growth factors (insulin-like growth factor-I, transforming growth factor-β1, 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 had lower biomechanical and biochemical properties than the controls with no growth factors, suggesting a detrimental effect, but the treatment with insulin-like growth factor-I tended to improve the constructs. Additionally, the 6-week time point was consistently better than that at 3 weeks, 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.

Keywords Fibrocartilage · Tissue engineering · Growth factors · Temporomandibular joint disc · Knee meniscus · Goat

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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 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 (Detamore and Athanasiou 2003a; Sweigart and Athanasiou 2001). Unlike healthy hyaline articular cartilage, fibrocartilage also contains collagen type I, and whereas cartilage functions primarily in compression, fibrocartilage has an important tensile component to its mechanical role (Almarza and Athanasiou 2004). The TMJ disc contains primarily collagen type I (Milam et al. 1991), whereas the knee meniscus is mostly collagen type I in the outer two-thirds and has a 3:2 ratio of type II:type I collagen in the inner one-third (Cheung 1987). 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 because of similarities in their tissue characteristics. Native rib cartilage contains glycosaminoglycans (GAGs), primarily chondroitin sulfate and keratan sulfate (Dearden et al. 1974; Pietila et al. 1999; Rosenberg et al. 1965), and collagen types I and II in a ratio of 1:5 (Safronova et al. 1991). Costal cartilage is hypocellular, containing 4–10 cells per 0.22 mm^2 of tissue (Stockwell 1967).

Although CCs have shown potential in cartilage tissue engineering (Johnson et al. 2004; Popko et al. 2003; Szeparowicz et al. 2004), their ability to produce collagen

I suggests that they may function even more effectively in fibrocartilage tissue engineering. Indeed, CCs have shown potential to produce extracellular matrix (ECM) relevant for tissue engineering the TMJ disc; however, improved collagen content and mechanical properties are necessary before a functional replacement can be achieved (Johns and Athanasiou 2008)

The growth factors and the concentrations thereof have been judiciously chosen for this study based on their ability to improve ECM production in CCs or fibrochondrocytes. Transforming growth factor-β1 (TGF), at 1 ng/ml, has been shown to increase proline, thymidine, leucine, and sulfate incorporation in CCs (Lee et al. 1997). At higher concentrations, TGF improves collagen production and mechanical properties in constructs made from TMJ disc fibrochondrocytes (Detamore and Athanasiou 2005). The combination of fibroblast growth factor (FGF), platelet-derived growth factor-BB (PDGF), and TGF increases proliferation and collagen type I and elastin staining in CCs (Tay et al. 2004). PDGF also increases collagen production in TMJ disc cells (Hanaoka et al. 2006). FGF improves proline, leucine, and thymidine incorporation in CCs but decreases sulfate incorporation (Lee et al. 1997). FGF increases the amounts of GAG and collagen and the mechanical properties of constructs made from TMJ disc cells (Detamore and Athanasiou 2004, 2005). Epidermal growth factor (EGF) increases the proliferation of growth plate CCs and promotes a more elongated morphology, the effects on proliferation being saturated at 30 ng/ml (Kato and Gospodarowicz 1984; Makower et al. 1989a). GAG is increased with insulin-like growth factor-I (IGF) up to 0.5 μg/ml in CCs (Takigawa et al. 1991), and collagen is increased in TMJ disc cells with 100 ng/ml IGF (Detamore and Athanasiou 2004).

This study attempts to improve the biochemical and mechanical properties of tissue-engineered constructs involving CCs by the addition of growth factors to this in vitro approach. IGF, TGF, EGF, PDGF, and FGF have been 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 of approximately 1 mm^3 in size and digested overnight at 37°C with 0.2% type II collagenase (Worthington, Lakewood, N.J.) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, Calif.) with 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, Calif.), 1% penicillin-streptomycin-amphotericin B (PSF; Cambrex, Walkersville, Md.), 1% non-essential amino acids (NEAA; Gibco), and 25 μl/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% dimethylsulfoxide, 20% FBS, 1% PSF, and 1% NEAA. Upon being thawed, cells were again cultured in monolayer until passage 3.

Construct preparation

Constructs were formed by using a method modified from the self-assembly of articular chondrocytes (Hu and Athanasiou 2006a). Agarose wells were formed by means of a mold, and 2 million cells were seeded into each 5-mm well. After 4 h, additional construct medium was added: DMEM with 1% PSF, 1% NEAA, 1% ITS+ premix (BD Biosciences, San Jose, Calif.), 0.1 μM dexamethasone, 40 μg/ml L-proline (EMD Chemicals, Gibbstown, N.J.), 50 μg/ml ascorbate 2-phosphate (Sigma), and 100 μg/ml sodium pyruvate (Fisher). Five growth factors (in addition to a non-treated control; no GF) were used individually in the construct medium throughout the entire 6 week culture period: TGF (1 ng/ml), IGF (100 ng/ml), FGF (10 ng/ml), EGF (30 ng/ml), and PDGF (10 ng/ml; Peprotech, Rocky Hill, N.J.). After 1 week, 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 6 weeks 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 14-μm slices. Slides for histology were fixed in formalin and stained with safranin-O/fast green for GAGs or picrosirius red for collagen. Immunohistochemistry (IHC) was used to examine collagen types I and II, as described previously (Detamore et al. 2005).

Biochemistry and enzyme-linked immunosorbent assay

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 125-μg/ml papain (Sigma) solution containing N-acetyl-cysteine (Sigma) and EDTA. Elastase (Sigma) at 1 mg/ml was added for another 2 days of

digestion. Digested samples were stored at -20°C and used for biochemical assays and enzyme-linked immunosorbent assays (ELISAs).

DNA was quantified with a PicoGreen reagent (Molecular probes, Carlsbad, Calif.) by comparison with calf thymus DNA (Sigma). Cell number was determined with a conversion factor of 7.7 pg DNA/cell (Kim et al. 1988). 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 (Woessner 1961). Collagen types I and II were quantified with ELISAs. Collagen type I content was determined with an indirect ELISA, as described previously (Darling and Athanasiou 2005a). Briefly, sample digests were incubated overnight, blocked with bovine serum albumin, and incubated with primary and secondary antibodies. Collagen type II was quantified with a Chondrex kit (Redmond, Wash.) according to the manufacture's protocol; this kit utilized a sandwich ELISA. Both ELISAs involved the use of a tetramethylbenzidine substrate for detection.

Mechanical testing

Five samples were cut in half through the center of the sample, and the remaining piece of neotissue was tested with a creep indentation apparatus to determine compressive properties (Athanasiou et al. 1994). An indentation tip of 1 mm was used to apply a tare load of 0.002 N and a creep load of 0.007 N. Loading occurred until the sample equilibrated (defined as deformation less than 10^{-6} mm/s) or 10 min passed (tare load) and 1 h passed (creep load). The step load was removed, and the recovery distance measured. Data were analyzed with a semi-analytic semi-numeric model to determine the following biphasic properties: aggregate modulus, permeability, and Poisson's ratio (Mow et al. 1989).

At least five samples per group were also tested in tension on a 5565 Instron (Norwood, Mass.). Constructs were cut in half through the circumference and then cut again to form a dog bone shape so that only a solid piece of neotissue remained. 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 analysis of variance. The factors were time and the growth factor, which had two and six levels, respectively. An F-test was used to determine whether a factor was significant ($P<0.05$), and a Tukey's *post hoc* test was used to determine differences between levels.

Results

Morphology and histology

Morphological differences among the treatment groups at 6 weeks are illustrated in Fig. 1. Control and IGF constructs retained their initial seeding diameter of 5 mm, whereas 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 3 weeks, EGF, IGF, and controls were cyst-like in 80%, 54%, and 64% of the constructs, respectively. At 6 weeks, 100% of the EGF constructs appeared fluid-filled. The cyst-like IGF constructs also increased to 80% at 6 weeks, 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 was regionally distributed in a similar manner to the picrosirius red stain. The morphology of the cells in the constructs was primarily rounded and chondrocyte-like.

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; 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 higher than that in control, TGF, and PDGF constructs. EGF and FGF groups had significantly more cells than the PDGF group; however, all groups resulted in cell quantities near the initial seeding of 2 million cells. The total GAG and collagen in the constructs (Fig. 2b,c), 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 the PDGF and FGF constructs. Collagen II quantities (Fig. 3a) had the

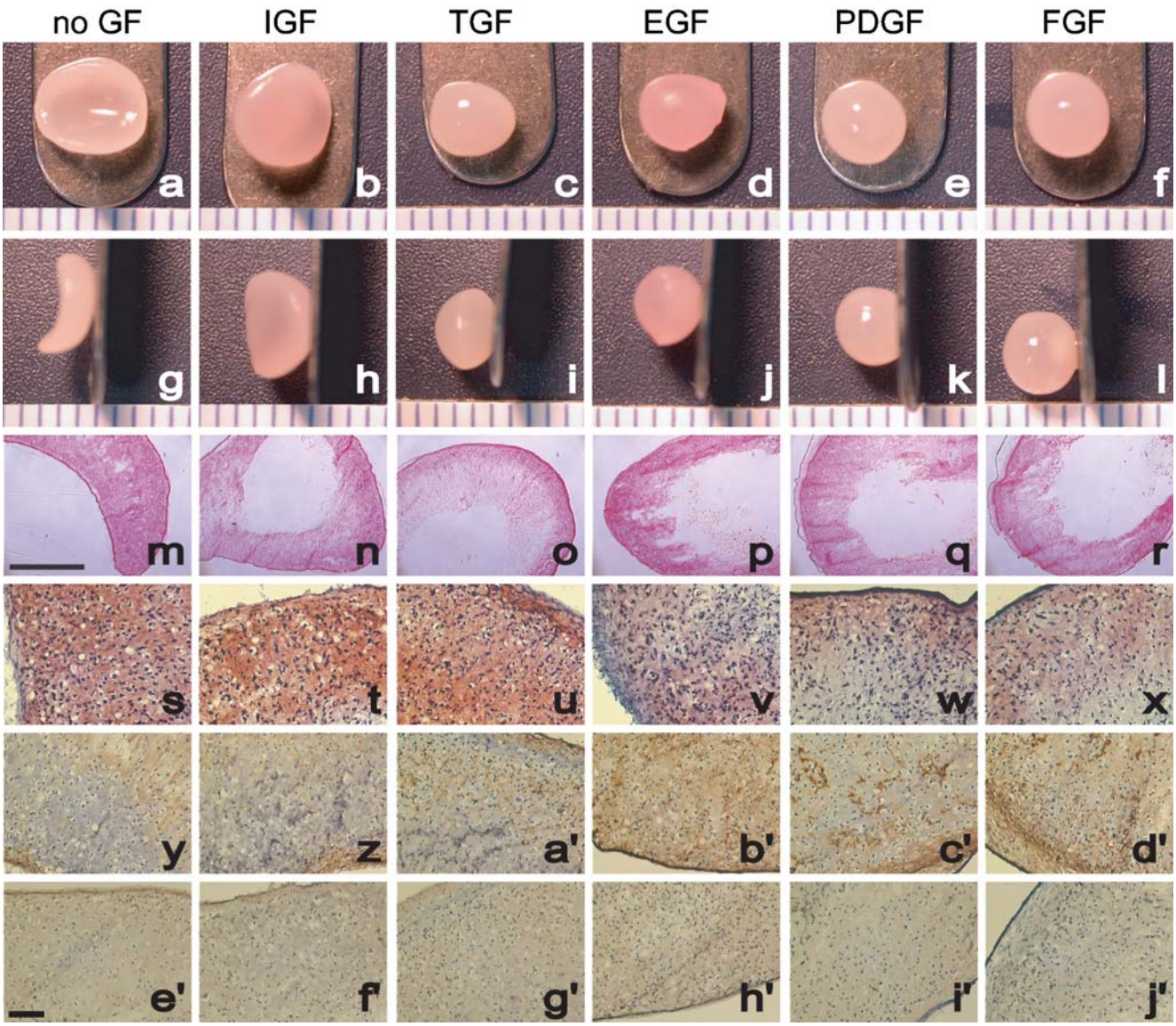

Fig. 1 **a–l** Gross morphological images from 6 weeks. Spacers *below* the images are 1 mm in length. Control (*no GF*) and insulin-like growth factor-I (*IGF*) groups retained the initial 5-mm diameter, whereas the other groups (*TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) contracted and became rounded into more spherical morphologies. **m–r** Picrosirius red staining for the constructs at 6 weeks. This stain clearly shows the collagen, which stains consistently in an outer ring and densely on the extreme outer edge of the constructs. These images also illustrate the void structure that formed frequently, particularly in the growth-factor-treated groups. *Bar* 1 mm (in **m**); applies for all picrosirius red staining micrographs. **s–x** Safranin-O/fast green staining. **y–d′** Collagen type I IHC. **e′–j′** Collagen type II IHC. All samples stained positively for glycosaminoglycans (GAGs) and collagens types I and II. *Bar* 100 μm (in **e′**); applies to the IHC and safranin-O/fast green stains

same statistical differences as the GAG quantities, whereas the collagen I content (Fig. 3b) in the EGF group was significantly greater than that in the control and FGF groups. The ratios of collagen type I to collagen type II are shown in Table 2. EGF constructs had significantly the greatest ratio, with EGF and PDGF being significantly greater than TGF, IGF, or control constructs. Time was significant for all biochemical, ELISA, and weight metrics, with the 6-week time point having greater quantities than the 3-week 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 3 weeks to

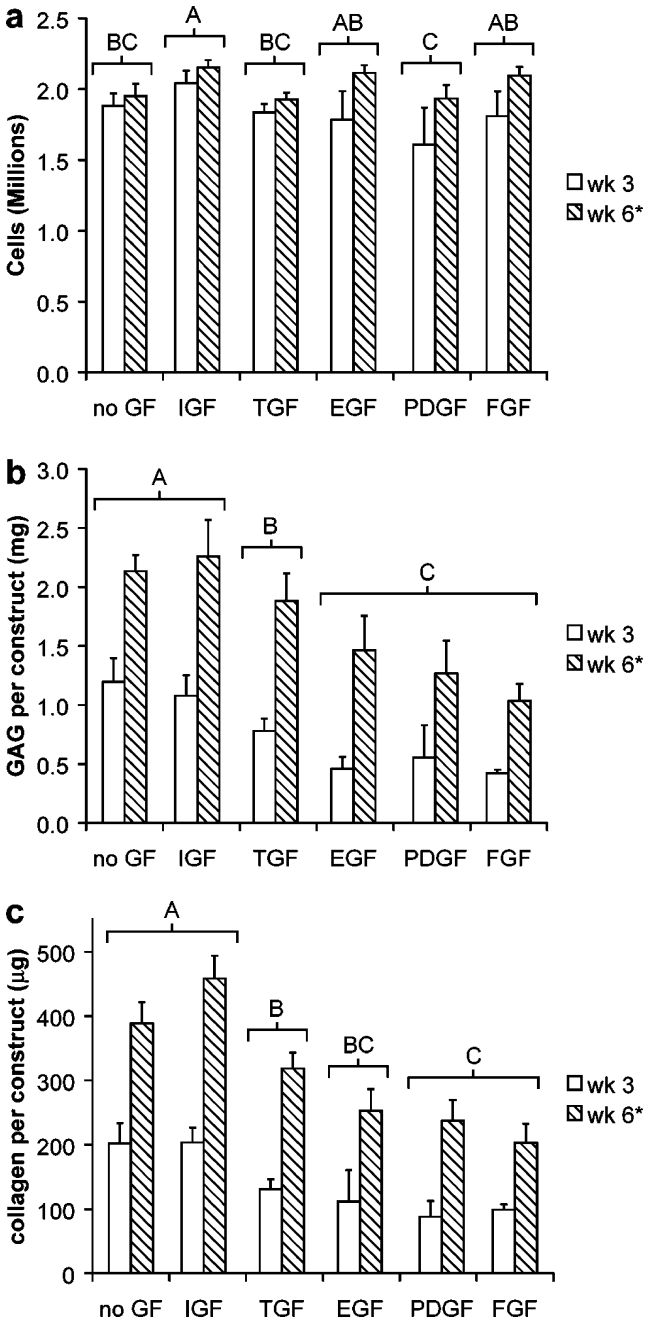

Fig. 2 Cell (**a**), glycosaminoglycan (*GAG*; **b**), and collagen (**c**) content at both time points (mean±SD). Treatment groups with *different capital letters* are statistically significant ($P<0.05$). For all the biochemical metrics, the 6-week (*wk 6*) time point was significantly greater than the 3-week (*wk 3*) time point (*asterisks*). Although some statistical differences in the various growth factors (*IGF* insulin-like growth factor-I, *TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) were apparent, cell quantities were around 2 million for all groups. The amount of GAG/construct and collagen/construct were significantly greatest in the control (*no GF*) 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

6 weeks. Permeability and Poisson's ratio were not significantly different between the experimental groups with ranges of $3.97*10^{-15}$–$1.87*10^{-13}$ $m^4/N*s$ and 0.0108–0.368, respectively. Permeability was significantly greater at 3 weeks than 6 weeks. Tensile properties (Fig. 4b,c) 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 the 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 has examined the effects of various growth factors on scaffoldless costal chondrocyte constructs for the purposes of fibrocartilage tissue engineering. IGF-treated constructs are equal to or better than the control for all the metrics examined in this study. The increase in cell number with IGF treatment is consistent with previous work with CCs (Makower et al. 1989b). The increase in GAG seen previously with CCs (Takigawa et al. 1991) or the increase in collagen seen previously with TMJ fibrocartilage (Detamore and Athanasiou 2004, 2005) are not as prominent as expected; however, the increasing trend is certainly apparent suggesting the benefits of IGF for fibrocartilage tissue engineering.

Treatment with TGF, EGF, and PDGF results in 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 has shown that EGF or FGF treatment leads to a more elongated cell morphology in monolayer (Makower et al. 1989a). This morphology is generally indicative of a cell phenotype that would produce more collagen type I and less collagen type II. Although dramatic differences in cell morphology have not been observed, and although most of the cells appear rounded in shape, the EGF and FGF groups produce larger ratios of collagen type I to type II than the control group. Whereas previous work supports many of the results seen here, more improvements in ECM content were expected for TGF (Hiraki et al. 1991; Iwasaki et al. 1995; Lee et al. 1997; Tay et al. 2004), FGF (Iwasaki et al. 1995; Lee et al. 1997), and PDGF (Tay et al. 2004). The limited improvements seen with these growth factor treatments are probably attributable to the base medium used, which has no serum and contains additional additives that have been shown to promote chondrogenesis (Mauck et al. 2006). The previous cited work involved the addition of 10% serum to the base medium, which contains small amounts of growth factors. Serum is often considered

Table 1 Wet and dry weights of constructs at two time points

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

Data are shown as means±SD. Treatment groups (*IGF* insulin-like growth factor-I, *TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) with *different letters* are statistically significant ($P<0.05$). Weights were significantly larger at 6 weeks (*asterisks*). IGF constructs had wet and dry weights significantly larger than any other group. Control (*No GF*) constructs also had significantly greater dry weights than the other groups

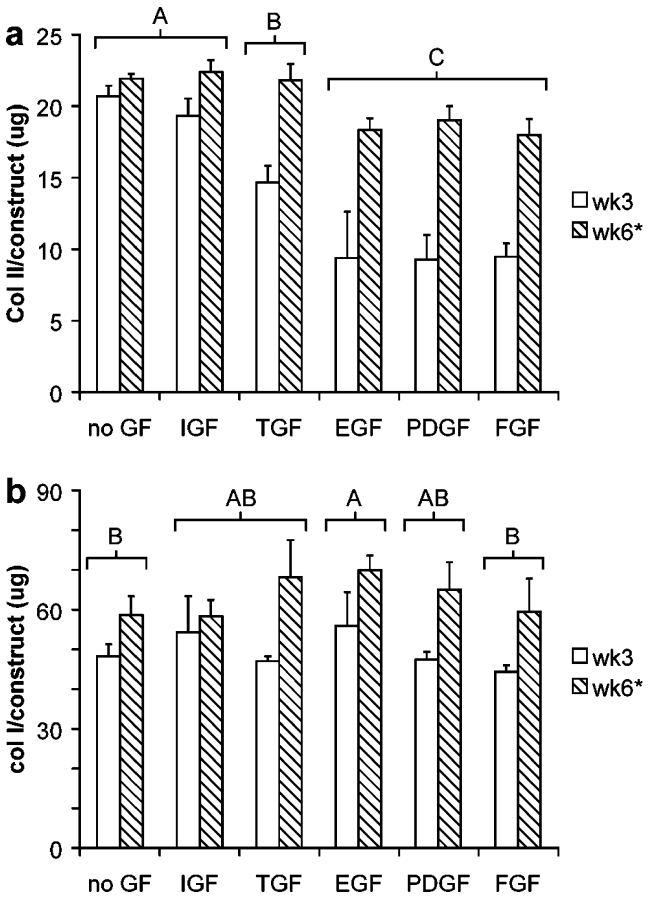

Fig. 3 Collagen type II (**a**) and collagen type I (**b**) quantities for constructs at both time points (mean±SD). Treatment groups (*IGF* insulin-like growth factor-I, *TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) with *different letters* are statistically significant ($P<0.05$). The 6-week (*wk6*) time point was significantly greater than the 3-week (*wk3*) time point in both collagen types (*asterisks*). Control (*no GF*) 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

undesirable in tissue engineering, because of 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. A combination of a small number of growth factors, in lieu of adding serum, should be considered for future work. Other studies might involve alternative growth factors with the base medium used here. The chondrogenic ability of the medium appears to decrease with the addition of TGF, EGF, PDGF, and FGF, as can be seen from the 10%–60% decrease in GAG, total collagen, and collagen type II from the control values. (The constructs after IGF treatment are largely similar to the group without additional growth factors.) Whereas these results are contrary to expectations, they are partially supported by current work with articular chondrocytes in a scaffoldless approach with serum-free medium, which has shown a decrease in GAG with the addition of TGF-β1 (Hoben and Athanasiou 2008). Although fibrocartilage tissue engineering is still in its early stages, the field of cartilage tissue engineering has been explored in considerably more depth and has established this base medium as being better than FBS media for cartilage regeneration (Kisiday et al. 2005). However, our study has revealed that more work needs to be done, particularly for fibrocartilage tissue engineering, in order 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 benefitted from a longer time in culture. As shown by all biochemical assays and ELISAs, the quantities of ECM and cells increased temporally across all treatment groups. At 6 weeks, total collagen and GAG increased at least two-fold over the values seen at 3 weeks for all the groups. The aggregate modulus also approximately doubled in most groups. Tensile properties, however, did not have a clear trend with time, most groups remaining the same from

Table 2 Ratios of collagen type I to collagen type II at two time points

Treatment	Collagen I:collagen II ratio	
	3 weeks*	6 weeks
No GF[d]	2.3±0.1	2.7±0.2
IGF[d]	2.8±0.6	2.6±0.2
TGF[cd]	3.2±0.3	3.1±0.5
EGF[a]	6.4±1.6	3.8±0.2
PDGF[ab]	5.2±0.8	3.4±0.2
FGF[bc]	4.7±0.6	3.3±0.6

Data are shown as means±SD. Treatment groups (*IGF* insulin-like growth factor-I, *TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) with *different letters* are statistically significant ($P<0.05$). Ratios were significantly larger at 3 weeks (*asterisk*). EGF and PDGF constructs had significantly higher ratios than IGF, TGF, or control (*No GF*) constructs. The FGF ratio was also significantly greater than that for the IGF and control groups

3 weeks to 6 weeks; time was not considered significant factor for either UTS or elastic modulus. This suggests that, while collagen quantities are increasing in the constructs, the collagen is probably not being organized or packed appropriately to improve the tensile strength or stiffness. The application of a mechanical stimulus, such as tension, could be important to the organization of the ECM and might improve the mechanical properties of the construct. The temporal changes also suggest the need to examine the time factor in greater depth by exploring both longer and shorter time points to elicit an overall trend in the changes that occur with time.

Previous work on passaged chondrocytes in a scaffold-less approach have also observed the fluid-filled structures that formed in many of the constructs in our study (Hu and Athanasiou 2006b). Whereas the number of cells at 3 and 6 weeks were near 2 million, the outer region of cells may have proliferated, with cell death occurring in the central region. Chondrocytes are known to prefer cell-cell interaction and retain their phenotype better in three-dimensional culture, whereas attachment to the tissue culture plastic alters their phenotype, promoting a more fibrochondrocytic phenotype (Darling and Athanasiou 2005b; Tay et al. 2004). This phenotypic alteration may not occur uniformly, and the cell population after passaging is most probably 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 (Foty et al. 1996; Foty and Steinberg 2005; Napolitano et al. 2007; Yoon et al. 2002). Considering these observations, we can reasonably infer that the passaged CCs aggregate into distinct cell populations upon construct formation. Within the first 48 h, formation of the fluid-filled

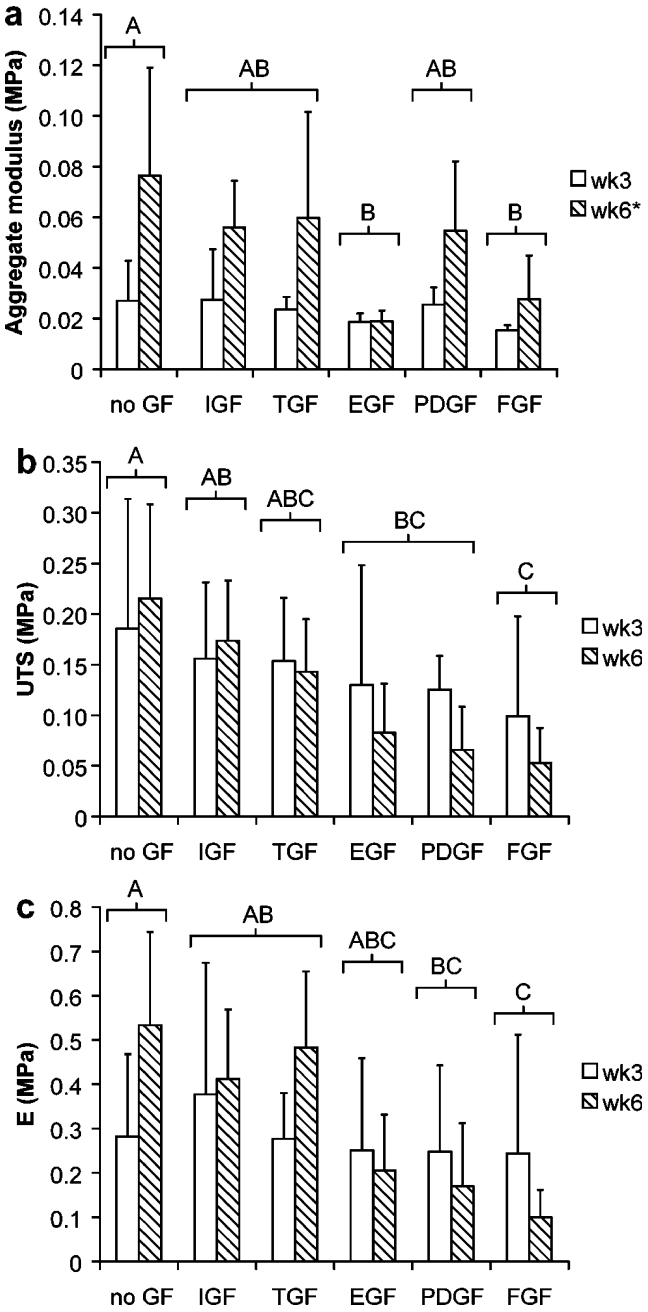

Fig. 4 Aggregate modulus (**a**), UTS (**b**), and elastic modulus (*E*; **c**) for all groups at both time points (means±SD). Treatment groups (*IGF* insulin-like growth factor-I, *TGF* transforming growth factor-β1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor-BB, *FGF* fibroblast growth factor) with *different letters* are statistically significant ($P<0.05$). The 6-week (*wk6*) time point was significantly greater than the 3-week (*wk3*) time point (*asterisk* in **a**). The control (*no GF*) group had the highest aggregate modulus, which was significantly larger than in the EGF and FGF groups. The control group also had the highest tensile modulus and UTS, being significantly greater than PDGF and FGF. Its UTS was also significantly higher than that of 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

structure becomes apparent (data not shown). The ensuing death of the inner population of cells may be attributable 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 that the characteristics of these populations should be determined, and that the cells should be purified prior to construct seeding. Interestingly, at the end of 6 weeks, all the growth factors appeared to have promoted this cyst-like structure, either at 3 weeks or over time (as for IGF treatment). This fascinating observation gives insight into the subpopulations of these cells. One could postulate that these growth factors initially encourage the growth of the population of cells that form in the center. Their subsequent death makes the growth factors appear ineffective and even detrimental. If the methods used here had been 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 probably be necessary. Alterations in the expansion conditions would certainly affect the cell characteristics and may also eliminate the "cyst" structure.

Although considerable work still needs to be carried out to optimize the constructs created here, the approach used is promising for the purposes of fibrocartilage tissue engineering. Using methods similar to those used here, Kim et al. (2003) have found a compressive aggregate modulus for the porcine TMJ disc around 20 kPa, which is less than or equal to the moduli seen for the engineered constructs. The GAG content of the constructs is greater than that of the native tissue (Detamore et al. 2005). Comparing the construct properties with those seen in the porcine TMJ disc, the tensile modulus is twice to 40 times lower than that in the native state, measuring between 1–20 MPa (Detamore and Athanasiou 2003b). The collagen content is about ten times lower than the native value (Almarza et al. 2006). The fibroblast-promoting growth factors, such as TGF, PDGF, EGF, and FGF, are not able to improve these properties above those of the control, but they are 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 is more advantageously affected than the other groups with respect to biochemical content and mechanical properties. Future work examining a combination of growth factors, the optimal culture time, purification of the expanded cell population, or improved expansion conditions might further develop these constructs such that they are able to function as native fibrocartilage.

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