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K.D. Allen and K.A. Athanasiou*

Department of Bioengineering, Rice University, MS-142, PO Box 1892, Houston, TX 77251-1892, USA; *corresponding author, athanasiou@rice.edu

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Scaffold and Growth Factor Selection in Temporomandibular Joint Disc Engineering

ABSTRACT

Temporomandibular joint disc tissue-engineering studies commonly fail to produce significant matrix before construct contraction. We hypothesized that poly-L-lactic acid (PLLA) non-woven meshes would limit contraction, allow for comprehensive mechanical evaluation, and maintain viability relative to polyglycolic acid (PGA) non-woven mesh controls. Additionally, we proposed that growth factor stimulation, while limiting contraction, would increase construct properties relative to previous reports. After 4 wks, cell proliferation and matrix deposition were similar between the two meshes, but PGA constructs had contracted significantly. Furthermore, only PLLA constructs could be tested in tension and compression. Additional PLLA constructs were formed, then treated with insulin-like growth factor-1 (10 ng/mL), transforming growth factor-beta 1 (5 ng/mL), or transforming growth factor-beta 3 (5 ng/mL). Transforming growth factor-beta 1 yielded the most cells, collagen, and glycosaminoglycans at 6 wks; these constructs also demonstrated improved mechanics. Analysis of these data demonstrated significant temporomandibular joint disc-engineering potential for PLLA and transforming growth factor-beta 1.

KEY WORDS: Temporomandibular joint, tissue engineering, scaffolds, growth factors.

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INTRODUCTION

The temporomandibular joint (TMJ) can experience degenerative episodes initiated by disease or trauma (Wilkes, 1989; Puzas *et al.*, 2001; Stegenga, 2001). Tissue engineering offers potential to regenerate tissue otherwise unable to heal (Vacanti and Vacanti, 1994; Terada *et al.*, 2000). TMJ engineering focuses on regenerating bony structures (Hollister *et al.*, 2000, 2002; Alhadlaq and Mao, 2003), articular cartilage (Alhadlaq and Mao, 2003), and fibrocartilage (Allen and Athanasiou, 2006b).

Compared with hyaline cartilage, the fibrocartilaginous TMJ disc has lower glycosaminoglycan (GAG) content and contains primarily collagen type I, not collagen type II. Additionally, the chondrocytic phenotype is overshadowed by the fibroblastic phenotype in TMJ disc cells. Mechanically, the disc must function under significant tension, compression, and shear. These characteristics create unique criteria for tissue engineers (Tanaka and van Eijden, 2003; Almarza and Athanasiou, 2004a; Allen and Athanasiou, 2006b).

Seeding method, scaffold architecture, and culture environment have been investigated in previous TMJ disc studies; however, a common trend is construct contraction prior to significant matrix deposition. Historically, this has been observed on polyglycolic acid (PGA) non-woven meshes (Allen and Athanasiou, 2006b). This scaffold selection is the result of TMJ disc cells' preference for mesh-like architectures (Almarza and Athanasiou, 2004b). However, since PGA degrades rapidly, these constructs reduce in volume by over 50% in 4-6 wks (Allen and Athanasiou, 2006b). Attempts to accelerate matrix production *via* exogenous growth factors have failed to pace construct contraction (Detamore and Athanasiou, 2005; Almarza and Athanasiou, 2006).

Toward resolving this issue, we hypothesized that a slow-degrading mesh would decelerate construct contraction, allow for tensile and compressive testing, and have no adverse effects on cell proliferation or matrix production. We tested this hypothesis by comparing poly-L-lactic acid (PLLA) non-woven meshes with PGA. Second, we proposed that, by increasing relative construct volume over culture time, growth factor stimulation would improve matrix production on the slow-degrading mesh relative to previous reports. To test this hypothesis, we applied growth factors investigated in previous PGA work to constructs formed with PLLA. Thus, this report both describes the potential of PLLA scaffolds and provides valuable insight into growth factor effects for TMJ disc tissue engineering.

MATERIALS & METHODS

Eighteen mature P.I.C. Genetic Breed hogs were obtained from an abattoir *post mortem*. TMJs were removed, capsule intact, within 8 hrs of each animal's death. Discs were dissected in sterile hoods and digested overnight in collagenase type II (1 mg/mL, Worthington, Lakewood, NJ, USA). Cells were

isolated *via* centrifugation and cryopreserved in Dulbecco's Modified Eagle's Medium (DMEM, Cambrex, East Rutherford, NJ, USA) with 20% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% dimethylsulfoxide. Cryopreservation was conducted in Nalgene cryo 1° C freezing containers (Thermo Fisher, Waltham, MA, USA), according to manufacturer protocols. Preserved cells were then transferred to liquid nitrogen cell storage containers. For seeding, cells were thawed in 37° C water baths, then transferred to culture medium (DMEM with 10% FBS, 1% non-essential amino acids, 1% penicillin-streptomycin-fungizone, 1% L-glutamine, and 25 μg/mL ascorbic acid; Gibco, Carlsbad, CA, USA). Cell yield and viability were verified *via* trypan blue staining before and after cryopreservation.

Cylindrical scaffolds were cut from non-woven PLLA or PGA meshes (5 mm diameter, 2 mm thick). Both meshes were approximately 95% porous and 45-55% crystalline. Average fiber diameter was 25 μm for PLLA (Biomedical Structures, Warwick, RI, USA) and 13 μm for PGA (Albany International, Albany, NY, USA). Scaffolds were sterilized with ethylene oxide and prepped for spinner flask seeding (Almarza and Athanasiou, 2004b). To compare PGA and PLLA, we seeded 3.75 million cells *per* mL scaffold. We chose this density, well below saturation, to discern cell attachment and proliferation differences. To compare growth factors, we seeded 7.5 million cells *per* mL scaffold volume. This density, also below saturation, was selected from previous work based on similar growth factors and concentrations on PGA constructs (Detamore and Athanasiou, 2005).

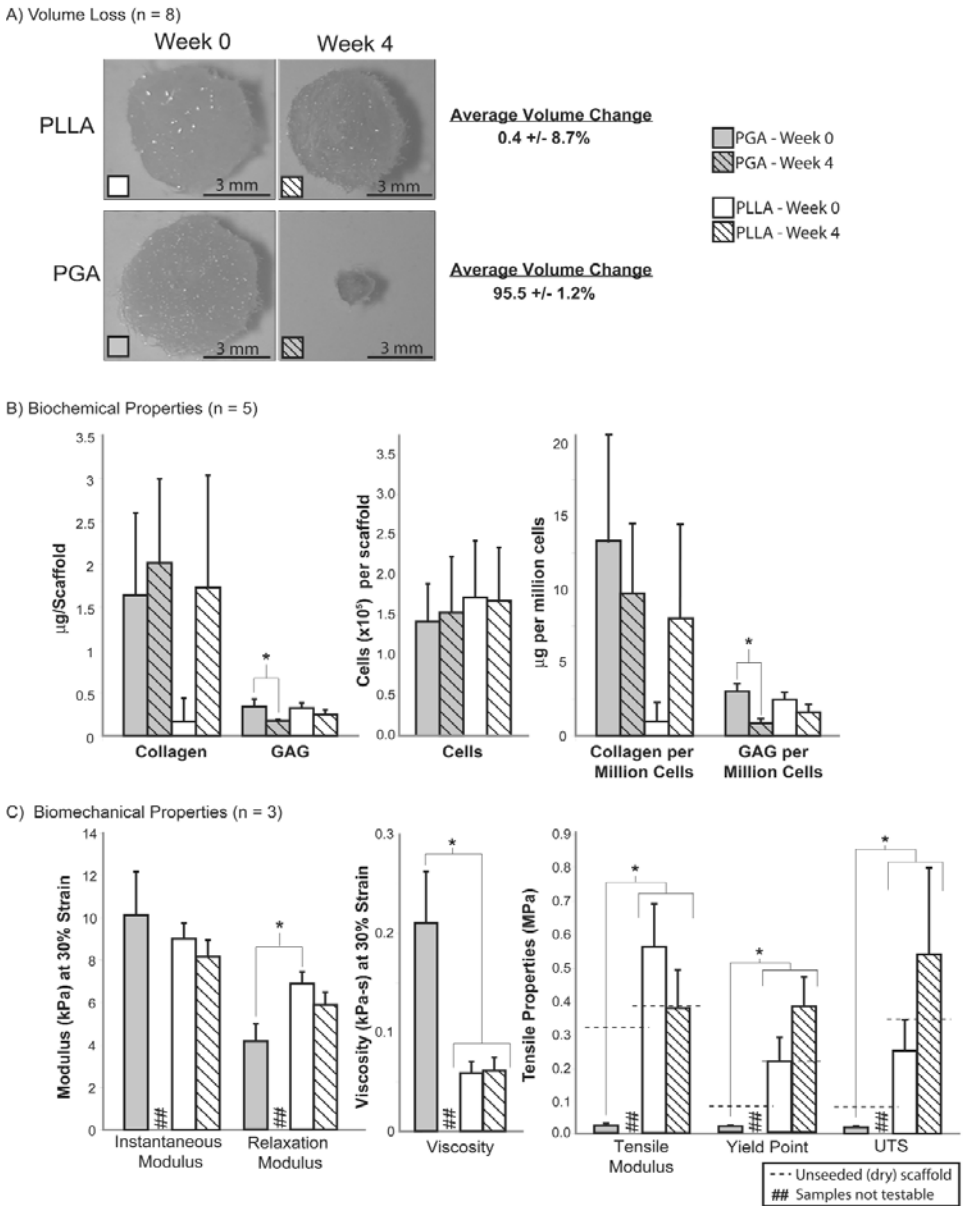

Figure 1. Comparisons of PLLA and PGA scaffolds for TMJ disc tissue engineering. Approximately 150,000 cells were seeded to either poly-L-lactic acid (PLLA) or polyglycolic acid (PGA) scaffolds and cultured for 4 wks. Weeks 0 and 4 properties are displayed in mean ± standard deviation form, and statistical differences are denoted by asterisks ($p < 0.05$). **(A)** PGA scaffolds contracted rapidly over 4 wks. By week 4, PGA constructs experienced at least a 90% reduction in volume; PLLA construct volume change was negligible. **(B)** Cellular, collagen, and glycosaminoglycan (GAG) properties of PLLA and PGA constructs were similar at weeks 0 and 4. GAG content of PGA constructs decreased from week 0 to week 4; this was also observed on a *per* cell basis. Collagen content at week 0 appeared higher on PGA; this difference was substantially less at week 4, since collagen *per* cell appeared to rise on PLLA. However, collagen differences were not statistically significant. **(C)** Under compression, week 0 PGA constructs had higher viscosity than PLLA constructs at either week 0 or week 4. PLLA constructs had larger relaxation moduli relative to PGA at week 0. Under tension, PLLA constructs were significantly stiffer and stronger than week 0 PGA constructs. Though PGA and PLLA entered seeding with similar tensile stiffness (unseeded, dry), PGA constructs lost considerable tensile stiffness during the seeding period. By week 4, PGA constructs were no longer testable under compression or tension, while PLLA constructs maintained testability and similar tensile properties.

After being seeded, PGA and PLLA constructs were transferred to agarose-coated six-well plates (5 mL media *per* well, 2.5 mL changed daily). Alternatively, growth-factor-treated constructs were transferred to deep Petri dishes with form-fit agarose wells (34 mL media in agarose-17 mL media above agarose, 17 mL changed daily). This practice eliminated media-growth factor concentration variations across wells, since constructs in respective treatments were exposed to the same media.

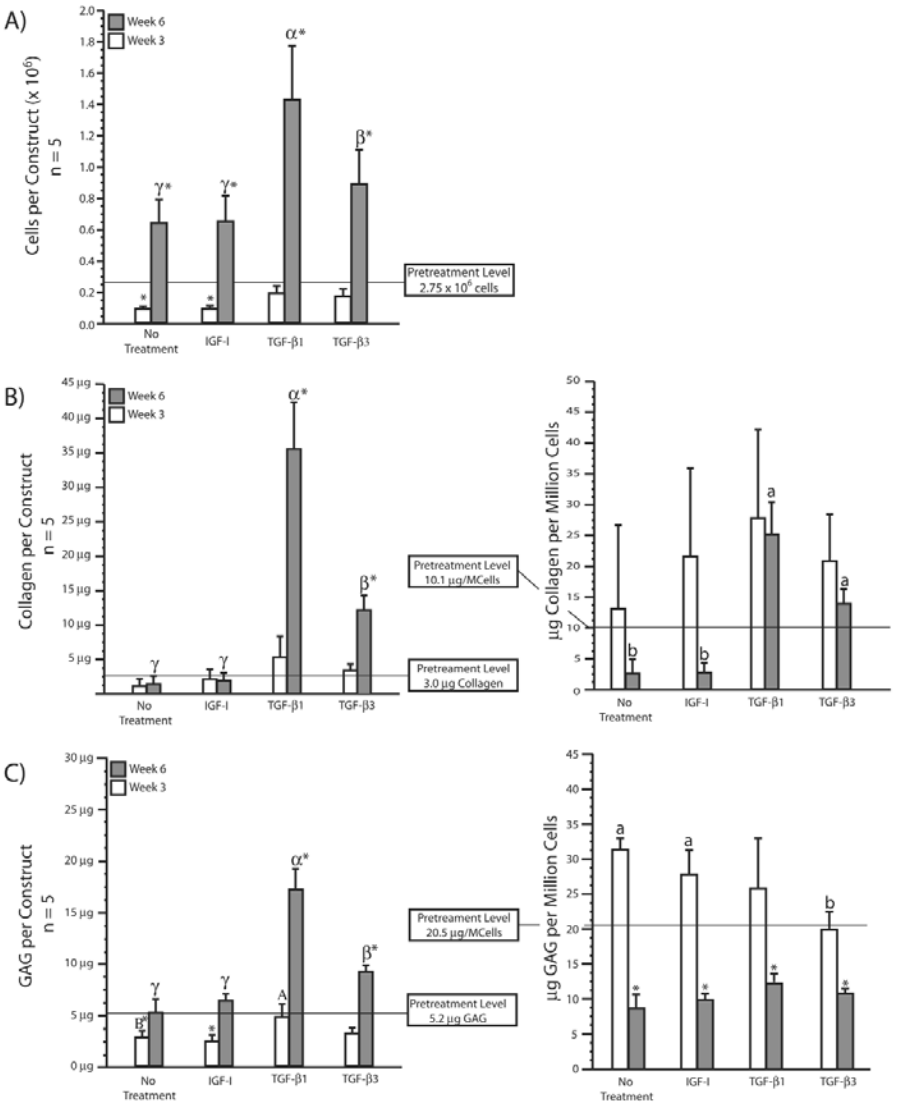

Figure 2. Biochemical properties of constructs exposed to IGF-I, TGF-β1, TGF-β3, or no additional growth factor. Construct biochemical properties are displayed in mean ± standard deviation form, and pre-treatment level is marked by a solid line (n = 5). Statistical differences between a treatment group and pre-treatment controls are marked by asterisks ($p < 0.05$). Differences among treatment groups at a specific time-point are denoted by letters. The absence of marking denotes that the treatment group did not statistically differ from other treatments. **(A)** Construct cellularity of 'no treatment' and IGF-I dropped from weeks 0 to 3. Week 3 TGF-β1 and TGF-β3 samples had cellularity similar to that of pre-treatment controls. At week 6, all constructs had higher cellularity relative to pre-treatment controls. TGF-β1 had the largest effect on cellularity at week 6 (α), statistically outperforming all other constructs. Week 6 TGF-β3 samples also showed large effects (β), statistically outperforming IGF-I and 'no treatment' samples (γ). **(B)** Collagen content was similar among all week 3 samples and pre-treatment controls. At week 6, samples treated with TGF-β1 (α) had more collagen deposition than all other samples. Week 6 TGF-β3 (β) samples had more collagen than week 6 IGF-I and 'no treatment' samples (γ). On a per cell basis, TGF-β1 and TGF-β3 (a) outperformed IGF-I and 'no treatment' (b) at week 6. TGF-β1 and TGF-β3 samples appeared to sustain collagen production levels per cell, while IGF-I and 'no treatment' samples appeared to drop below pre-treatment levels. However, this observation is not statistically significant. **(C)** Week 3 IGF-I and 'no treatment' samples had less GAG than pre-treatment controls; by week 6, IGF-I and 'no treatment' samples rebounded to pre-treatment levels. Week 3 TGF-β1 and TGF-β3 samples maintained similar GAG content relative to pre-treatment levels. At week 3, TGF-β1 samples (A) had significantly more GAG than IGF-I (B). At week 6, TGF-β1 (α) samples were outperforming all other treatments ($p < 0.05$). Week 6 TGF-β3 samples (β) had higher GAG content than week 6 IGF-I and 'no treatment' samples (γ). On a per cell basis, IGF-I and 'no treatment' samples (a) outperformed TGF-β3 (β) at week 3. By week 6, no differences existed among treatments on a per cell basis, though all were well below pre-treatment and week 3 levels.

Supplemented media contained either 10 ng/mL insulin-like growth factor I (IGF-I, PeproTech, Rocky Hill, NJ, USA), 5 ng/mL transforming growth factor-beta 1 (TGF-β1, PeproTech, Rocky Hill, NJ, USA), 5 ng/mL transforming growth factor-beta 3 (TGF-β3, EMD, San Diego, CA, USA), or no additional supplement; treatments began immediately following seeding (t = 0 days). IGF-I and TGF-β1 have previously demonstrated positive TMJ disc effects (Detamore and Athanasiou, 2005); TGF-β3 has demonstrated cartilage tissue engineering and stem cell differentiation potential (Mauck *et al.*, 2006; Baker and Mauck, 2007). Selected concentrations matched previous TMJ disc studies and were within each growth factor's working range (Detamore and Athanasiou, 2004, 2005; Allen and Athanasiou, 2006c).

To compare PGA and PLLA, we cultured the constructs for 4 wks, day 0 occurring immediately after construct seeding. The four-week period was selected due to rapid construct contraction. To compare growth factors, we extended culture to 0, 3, or 6 wks. These time-points matched previous work with similar growth factors (Detamore and Athanasiou, 2005; Almarza and Athanasiou, 2006). Histological samples were obtained at each time-point (n = 1), preserved in cryoembedding medium, sectioned on a cryotome (12 μm), and placed on adhesive slides (Instrumedics, St. Louis, MO, USA). We used picrosirius red and Safranin-0 staining to observe collagen and GAG content, respectively (Almarza and Athanasiou, 2004b). Biochemical content was analyzed quantitatively (n = 5). Samples were digested in papain (125 μg papain in 50 mM phosphate buffer containing 2 mM N-acetyl-cysteine and ethylene-diaminetetraacetic acid). Total collagen was detected by a modified hydroxy-proline assay (Woessner, 1961), total GAG by a 1,9-dimethylmethylene blue assay (Accurate Chemical, Westbury, NY, USA), and total DNA by a picogreen assay (Invitrogen, Carlsbad, CA, USA). Protocols are available in previous publications or from the manufacturer.

Mechanical properties were evaluated after seeding and final time-point (n = 3). Construct dimensions were measured with calipers prior to testing. For volume and stress calculations, constructs were assumed to be cylindrical. Samples were compressed, at

10% strain *per* second, to 10%, 20%, and 30% strain. Testing was conducted in a PBS bath (pre-load 0.2% strain, increments held for 10 min). Viscoelastic compressive properties were calculated by fitting the equation below to stress relaxation curves (details available in Allen and Athanasiou, 2005).

$$\sigma(t) = \frac{3}{2}\frac{E_r(u_{10\%} - u_{0\%})}{z}\left\{1 + \left(\frac{\tau_\sigma}{\tau_\varepsilon} - 1\right)e^{\left(\frac{-(t-t_{10\%})}{\tau_\varepsilon}\right)}\right\} \quad @10\% \text{ strain}$$

$$\sigma(t) = \sum_{i=0\%}^{20\%}\left[\frac{3}{2}\frac{E_r(u_i - u_{i-1})}{z}\left\{1 + \left(\frac{\tau_\sigma}{\tau_\varepsilon} - 1\right)e^{\left(\frac{-(t-t_i)}{\tau_\varepsilon}\right)}\right\}\right] \quad @\ 20\% \text{ strain}$$

$$\sigma(t) = \sum_{i=0\%}^{30\%}\left[\frac{3}{2}\frac{E_r(u_i - u_{i-1})}{z}\left\{1 + \left(\frac{\tau_\sigma}{\tau_\varepsilon} - 1\right)e^{\left(\frac{-(t-t_i)}{\tau_\varepsilon}\right)}\right\}\right] \quad @\ 30\% \text{ strain}$$

Specimen height (z) and time of strain event (t_i) were determined *a priori*. Deformation (u), time (t), and stress (σ) were recorded during testing. Relaxation modulus (E_r), relaxation time constant (τ_ε), and creep time constant (τ_σ) could be approximated from model fits, then converted into relaxation modulus, instantaneous modulus, and coefficient of viscosity equivalents (Allen and Athanasiou, 2006a). After compressive testing, samples were cut into rectangular bars (4 mm by 2 mm) and pulled continuously at 1% strain *per* sec (Sweigart and Athanasiou, 2005). Tensile modulus, yield point, and ultimate tensile stress (UTS) were determined from stress-strain curves.

Statistical Methods

Data were compared *via* analysis of variance (ANOVA), followed by Tukey's highly significant difference *post hoc* test ($\alpha = 0.05$). Two-factor ANOVAs with interaction were used; time-point and scaffold/growth factor type were statistical factors.

RESULTS

By 4 wks, PGA construct diameters reduced from 5 mm to 2 mm and thicknesses from 2 mm to 1 mm, representing volume losses above 90%. PLLA construct contraction was negligible (Fig. 1A). Volume differences did not translate to biochemical differences between materials, and equal cell numbers attached to each (Fig. 1B). Only PLLA constructs could be tested mechanically at 4 wks; PGA constructs lacked integrity and size. PGA constructs also lost significant tensile stiffness during seeding; PLLA constructs did not. Compressively, day 0 PLLA constructs had larger relaxation moduli; PGA constructs had larger viscosities (Fig. 1C).

At 3 wks, TGF-β1- and TGF-β3-treated constructs maintained cellularity relative to pre-treatment; IGF-I and 'no treatment' constructs experienced cellularity reductions. At week 6, construct cellularity was greater than pre-treatment in all groups. TGF-β1 treatment resulted in the largest cellularity. TGF-β3 outperformed IGF-I and 'no treatment'. IGF-I treatment did not differ from 'no treatment' (Fig. 2A). Collagen *per* construct did not differ at 3 wks. By week 6, TGF-β1 treatment was outperforming all treatments and pre-treatment. TGF-β3 treatment outperformed IGF-I, no treatment, and pre-treatment (Fig. 2B). At 3 wks, GAG *per* construct decreased in IGF-I and 'no treatment' relative to pre-treatment. At week 6, this loss was recovered. TGF-β1 treatment maintained GAG levels over 3 wks—outperforming IGF-I treatment at week 3—

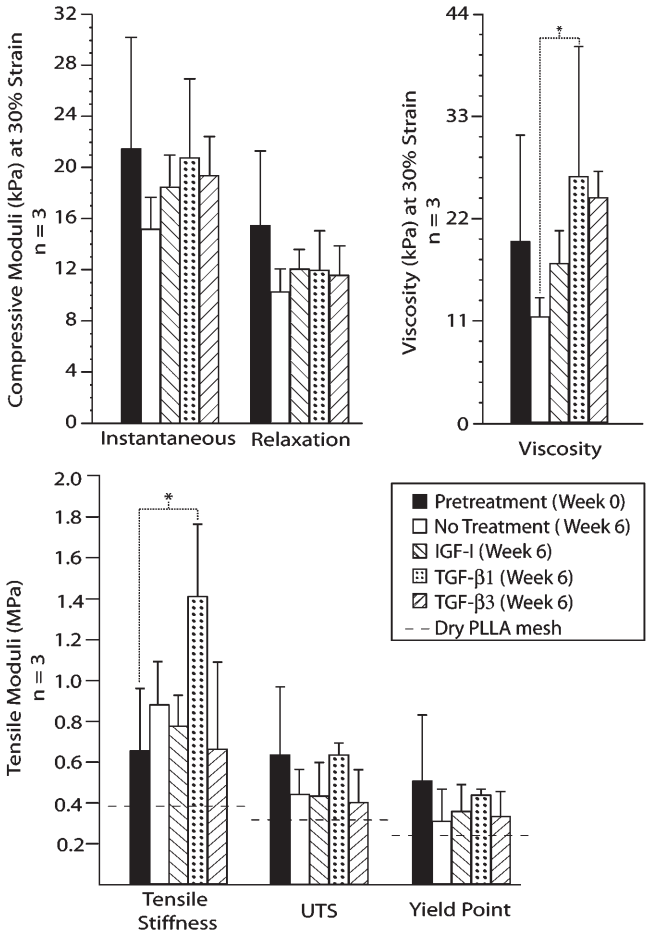

Figure 3. Biomechanical properties of constructs exposed to IGF-I, TGF-β1, TGF-β3, or no additional growth factor. Construct mechanical properties are displayed in mean ± standard deviation form, and statistical differences are denoted by an asterisk ($p < 0.05$). Statistical differences in compressive properties were limited to the coefficient of viscosity. Week 6 TGF-β1 samples had significantly larger coefficients of viscosity than week 6 'no treatment' samples, but did not differ from other week 6 treatments or pre-treatment levels. Statistical differences in tensile properties were limited to the tensile modulus. The tensile stiffness increased in TGF-β1-treated constructs, with week 6 TGF-β1 constructs having significantly larger moduli than pre-treatment controls. All week 6 samples appeared to increase slightly in stiffness relative to unseeded, non-wetted mesh.

and was above all other treatments and pre-treatment at week 6. TGF-β3 treatment also outperformed IGF-I, no treatment, and pre-treatment in GAG content at week 6 (Fig. 2C).

Growth factor treatments did not affect construct yield point, UTS, or compressive moduli. By week 6, TGF-β1-treated constructs had a larger tensile modulus than pre-treatment controls, but did not differ from other week 6 samples. Under compression, coefficient of viscosity for TGF-β1 constructs was larger than in 'no treatment' samples at week 6 (Fig. 3).

Histology supports the quantitative findings. At 3 wks, matrix staining differences were minimal. At week 6, the TGF-β1 construct appeared the most dense, followed by the TGF-β3 construct. Collagen staining appears to verify this observation (Fig. 4). It should be noted that no construct possessed dense matrix throughout. Instead, constructs were better described by

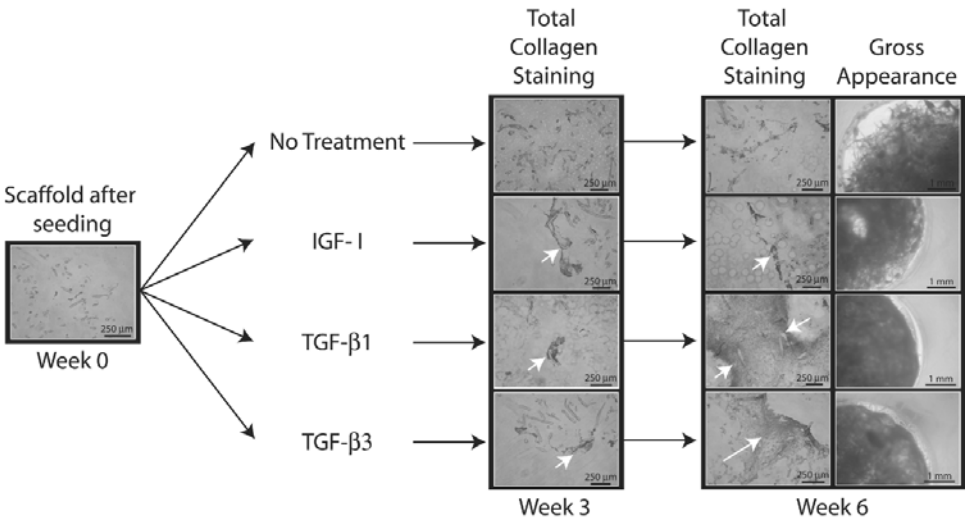

Figure 4. Total collagen staining and gross appearance of constructs exposed to IGF-I, TGF-β1, TGF-β3, or no additional growth factor. Collagen staining (marked by white arrows) was faint and sparse at week 0 (**10x photo**). Significant improvement in total collagen deposition was not observed in any treatment at week 3 (10x photo). Staining remained sparse and faint, generally located around the periphery of the scaffold. At week 6, constructs treated with TGF-β1 or TGF-β3 appeared to be denser (gross appearance – **4x photo**). Collagen staining verified this observation. TGF-β1-treated constructs showed significant staining for collagen relative to other treatments (10x photos). Staining was darkest around the periphery of the scaffold, followed by the pin-sized hole near the center of the scaffold created by the spinner flask mounting wire. Collagen staining in the interior of the constructs was less intense and non-continuous. TGF-β1- and TGF-β3-treated constructs were the only samples to exhibit any staining in the interior portions of the scaffold.

matrix pockets preferentially located near the construct periphery. Only week 6 TGF-β1 and TGF-β3 constructs demonstrated any matrix formation near the construct interior.

DISCUSSION

PGA, the most common TMJ disc tissue-engineering scaffold, contracts rapidly. Previous studies have used growth factors and high seeding densities to accelerate construct development. These efforts reduced construct contraction, but never sufficiently eliminated concerns (Allen and Athanasiou, 2006b). Moreover, high seeding densities are impractical without multiple cell passages, and passages result in phenotypic shifts and losses in matrix gene expression (Allen and Athanasiou, 2007). In the present study, primary cells were seeded an order of magnitude below scaffold saturation. PLLA constructs maintained ample volume, thereby providing additional volume and time for cellular, biochemical, and biomechanical development relative to PGA constructs. Still, TMJ disc cells developed constructs relatively slowly. The use of both growth factors and PLLA accelerated development. The increased volume for cell proliferation and biochemical deposition resulted in substantial histological and biomechanical changes. Significant collagen production and GAG deposition were observed with TGF-β1. To the best of our knowledge, this is the first TMJ disc tissue-engineering study to report construct tensile properties, maintain testable tensile integrity, and demonstrate statistical increases.

At 4 wks without exogenous growth factors, neither PGA nor PLLA demonstrated significant matrix deposition, yet PLLA maintained mechanical characteristics. Since PLLA degrades, it is reasonable to ask if the produced matrix was the primary contributor toward maintaining construct properties. However, it is unlikely that the scant matrix deposited had substantial mechanical contributions; instead, it is more likely that the polymeric scaffold itself provided strength, despite its molecular-weight degradation, since it is known that, in such polymers, strength loss occurs later than degradation. Both materials degraded *via* the hydrolytic scission of ester bonds. Initially, PLLA degradation products were too large to diffuse through the bulk material; only after sufficient reduction did they diffuse out, resulting in mass loss. Alternatively, PGA degradation products diffused rapidly (Athanasiou *et al.*, 1998). This phenomenon was manifested in construct weight: PLLA constructs were over 90% of the original dry weight at week 4, while PGA constructs were only 25%. Since no biochemical deposition differences appeared, the maintained mechanical integrity was primarily due to retained polymer. However, additional work is necessary to study this relationship.

We hypothesized that TMJ disc construct development would be improved by limiting contraction. PLLA meshes allowed us to investigate scaffolding material effects within this hypothesis. Certainly, other materials may offer additional advantages; PLLA was selected due to slower degradation rates and commercially available mesh-like architectures. There is a multitude of materials becoming available and a host of confounding factors. Elements such as local pH, degradation products, porosity, and nutrient transport can affect a material's success, and it is impossible to keep all factors constant. Ultimately, another material may prove more advantageous than PLLA for TMJ disc tissue engineering, but our results indicate that the slower-degrading PLLA offers significant advantages relative to PGA for *in vitro* tissue engineering.

Analysis of our data indicated powerful effects for TGF-β1. This result was somewhat surprising, since previous PGA studies showed strong responses from IGF-I (Detamore and Athanasiou, 2005). In the present study, TGF-β1 constructs had over twice the cellularity and GAG content and nearly 15 times the collagen content of IGF-I constructs at week 6. This disparity may originate from scaffold differences. Local pH may differ at early time-points, due to degradation rates; this may alter cellular responses to growth factors. Concurrently, cells may drive differences. Both studies used similar seeding densities, but cellular density as a function of time varies considerably, given the propensity of PGA to contract. Prior work utilized passaged cells, while present work used primary cells. Cell sensitivity to growth factors may be confounded by

cell passages, although previous results do not indicate these differences for the studied growth factors (Allen and Athanasiou, 2006c).

TMJ disc properties differ from those of our constructs, and while dense pockets of collagen exist in PLLA constructs, several voids persist. Mechanically, disc tensile moduli and UTS range from 0.5 to 32 MPa and 0.3 to 7.5 MPA, respectively, depending on fibril orientation (Detamore and Athanasiou, 2003). PLLA constructs reach the lower range, but these constructs are clearly not yet suitable for implantation. We are hopeful that mechanisms such as optimized biofactor concentrations, seeding phase growth factor treatments, improved cell sources, and longer culture times will lead to further improvements. We are also encouraged by mechanical property retention. Dry PLLA and PGA meshes share comparable properties, yet immediately after seeding, PGA shows mass and strength loss. With PLLA meshes, extensive mechanical stimuli may be explored, hopefully leading toward constructs which better approximate the TMJ disc.

Tissue engineering offers potential for the regeneration of tissues otherwise unable to self-repair. The challenge is significant, since several confounding factors are present. For TMJ disc tissue engineering, construct contraction has been a significant hurdle. Here, we have demonstrated the ability of PLLA meshes to maintain TMJ disc construct volume for 6 wks with no negative effect on biochemical composition relative to PGA controls. Furthermore, *via* TGF-β1 treatment, significant matrix production leading to biomechanical improvements was achieved. Since collagen production *per* cell did not increase, matrix production appeared driven by cell proliferation. However, altering growth factor concentrations during proliferation and synthesis phases may optimize the effects of TGF-β1. Overall, these findings represent an important step in TMJ disc tissue engineering; yet, further improvements and optimization are necessary. PLLA constructs do provide for more extensive studies, including mechanical stimulation and longer culture times.

ACKNOWLEDGMENTS

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