

Effects of Transforming Growth Factor β and Insulinlike Growth Factor 1 on the Biomechanical and Histologic Properties of Tissue-Engineered Cartilage

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Objective: To investigate the histologic and biomechanical properties of rabbit tissue-engineered cartilage exposed to insulinlike growth factor 1 and transforming growth factor β .

Design: Controlled study.

Subjects: New Zealand white rabbits aged 3 to 4 weeks.

Intervention: A mean of 3.42 million rabbit chondrocytes were placed onto 2 \times 1-cm polyglycolic/poly-L-lactic acid mesh templates. One group (n=21) was placed in complete medium for 4 days. The experimental group (n=19) was placed into complete medium with insulinlike growth factor 1 (50 ng/mL) and transforming growth factor β (1 ng/mL). After 96 hours the templates were removed and implanted into the dorsum

of the donor rabbit. The templates were harvested after 8 weeks and subjected to gross, histologic, and biomechanical testing.

Results: All samples showed histologic characteristics consistent with normal cartilage. No statistically significant differences were found with biomechanical testing between the control and experimental groups.

Conclusion: In spite of more promising results from earlier studies, these results do not support improved histologic features or mechanical performance with the addition of insulinlike growth factor 1 and transforming growth factor β to the chondrocyte/template complex.

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IN THE FIELD of facial plastic and reconstructive surgery, there has been tremendous need for implants to repair both congenital and acquired cartilaginous defects. Implant modalities have included autografts, homografts, and allografts. Autografts demonstrated significant limitations based on donor site morbidity, prolonged operating time, and scarcity of donor material.¹ Homografts avoided the problem of host immune reaction, but still were problematic secondary to long-term resorption

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of the implant. Allografts, while attractive because of their unlimited supply and shortened operative time, continue to show unacceptable levels of infection and extrusion.² As a result, a large amount of funding and effort has been devoted to the development of a model for both the in vitro and in vivo growth of a cartilaginous tissue construct.

Early studies showed the feasibility of using tissue engineering for cartilage neogenesis.³ Advances during the past decade have included the use of 3-dimensional scaffolding⁴ and the ability to grow tissue-engineered cartilage in an immunocompetent host.⁵ More recent work has focused on the ability to modulate both cellular expansion and matrix production in an attempt to duplicate native tissue. Benya and Chaffer⁶ found that dedifferentiated rabbit chondrocytes could be made to proliferate rapidly and then redifferentiate when grown in agarose. Buija et al⁷ demonstrated that chondrocytes grown in culture dedifferentiate, decrease production of type II collagen, and increase production of type I and type III collagen. They also confirmed that chondrocytes grown in 3-dimensional culture remained morphologically and functionally differentiated and produced a pericellular accumulation of matrix composed of type II collagen and proteoglycans.

Research during the past several years has explored numerous delivery options

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for the engineered chondrocytes. Polyglycolic acid polymer mesh, fibrin glue, collagen bilayers,⁸ and alginate beds⁹ are among some of the methods recently used. The focus of many recent studies has been on the use of growth factors such as transforming growth factor β (TGF- β), insulinlike growth factor 1 (IGF-1), and basic fibroblast growth factor (bFGF) to magnify both the chondrocyte proliferation and matrix synthesis and to produce a histologically and biomechanically normal cartilage construct for implantation.

Dunham and Koch¹⁰ have reported that bFGF combined with IGF-1 supported the growth and viability of human septal chondrocytes in short-term culture, with enhanced results after the addition of fetal calf serum. Insulinlike growth factor 1 alone has been shown to up-regulate synthesis of type II collagen and can prevent the dedifferentiation caused by bFGF.¹⁰ Buija et al¹¹ have reported a dose-dependent TGF- β stimulation of protein and glycosaminoglycan synthesis by human nasal chondrocytes cultured in monolayer and agar. Most of this stimulatory effect was found after 4 days of incubation with the growth factors.

When used in combination, IGF-1 and TGF- β induce type II collagen expression in dedifferentiated human articular chondrocytes.¹² Concentrations of 50 ng/mL of IGF-1 and 0.2-5 ng/mL of TGF- β were found to elicit the strongest responses. One interesting finding has been that the effect of the growth factors on the chondrocytes has been highly dependent to the source of origin for the cartilage cells. Lee et al¹³ specifically studied the effects of growth factors on cultured chondrocytes of different origins and reported that while TGF- β can induce proliferation in both rib and auricular cartilage, it does not increase protein and collagen synthesis in the auricular cartilage. Others demonstrated increased protein and collagen synthesis by nasal chondrocytes cultured with TGF- β .³ Nixon et al¹⁴ have shown that IGF-1 and TGF- β can independently increase both the size and amount of proteoglycan synthesized by equine articular chondrocytes grown in monolayer.

One additional variable present in earlier works is the disparity of initial seeding concentration. Some used as few as 2.2×10^4 cells/mL (10 studies), whereas others used seeding concentrations as high as 1.0×10^8 cells/mL (5 studies). This makes comparisons of different culture techniques, growth factor exposures, and template constructs difficult, if not impossible.

The goal of the present study was to examine the effects of IGF-1 and TGF- β on the histologic and biomechanical properties of cartilage produced after an 8-week implantation period in the autogenous rabbit model. Our previous work has demonstrated that current tissue engineering methods for *in vivo* growth of cartilage using a polyglycolic acid scaffold produces a product with reduced elasticity, toughness, and ultimate tensile strength (UTS) compared with controls.⁵ This may have been the result of reduced matrix formation coupled with fibrovascular infiltration, partially encouraged by the stunted matrix deposition. Our study examined whether supplementation with IGF-1 and TGF- β *in vitro* prior to implantation improved the intrinsic mechanical properties and histologic characteristics of the tissue-

engineered chondrocytes. The effect of template seeding concentration was also investigated.

METHODS

New Zealand white rabbits aged 3 to 4 weeks were obtained and allowed to acclimate for 7 to 14 days. The rabbits then received general anesthesia with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg) and local anesthesia with 1% lidocaine hydrochloride with 1:100000 epinephrine. The animals were prepared and draped in a sterile fashion. Heavy scissors were used to amputate both ears, leaving a 1-cm cuff of normal tissue, and the ears were placed into phosphate-buffered saline with 5% penicillin-streptomycin. The wound was soaked in povidone-iodine and sutured with 4-0 chromic gut suture. The auricular cartilage was then dissected from the overlying skin and perichondrium and diced into pieces no larger than 1×1 mm. The wet weight of the cartilage was then determined.

The cartilage was digested for 30 minutes in 20 mL of 0.05% hyaluronidase (Sigma, St Louis, Mo) and then for 2 hours in 4 mL of a 0.5% trypsin (Gibco Life Technologies, Grand Island, NY) and 0.5% collagenase solution (Worthington Biochemical, Freehold, NJ). The resulting suspension was filtered through a 153- μ m pore size sterile nylon mesh to remove undigested cartilage from the chondrocytes. The mesh was then washed twice with phosphate-buffered saline to ensure chondrocyte passage. The chondrocyte solution was centrifuged 3 times at 300g for 5 minutes, with aspiration of the supernatant and washing of the chondrocyte pellet with 5 mL of phosphate-buffered saline with penicillin-streptomycin. The isolated chondrocytes were then placed into suspension with "incomplete" medium (Dulbecco modified Eagle medium [Gibco Life Technologies] with HEPES buffer, without fetal calf serum). Determination of chondrocyte concentration was performed with a hemocytometer with cell viability determined by trypan blue exclusion. Solutions with a minimum concentration of 1.0×10^6 cells/mL and 75% viability were used in the study.

The templates were prepared by immersing polyglycolic acid mesh in poly-L-lactic acid solution (Polysciences Inc, Warrington, Pa). When dry, the polyglycolic/poly-L-lactic acid templates were cut into standardized 2×1 -cm rectangles and gas sterilized (100% ethylene oxide). The copolymer templates were placed in a polystyrene tissue culture dish for 30 minutes with 70% ethanol. The templates were then washed twice with "incomplete" medium, and immersed in incomplete medium for 2 hours. Prior to chondrocyte seeding, the excess medium was aspirated while avoiding desiccating the templates entirely.

The chondrocyte suspension was then divided equally for each rabbit donor to achieve a concentration of at least 1.0×10^6 cells/mL, with equal volume for each experimental and control group. A concentration of 2.5×10^6 cells/mL was assigned as a limit for assessing effect of concentration on cartilage templates. This is the mid-range of concentrations used in previously published studies. The chondrocyte-template constructs were placed into a 5% carbon dioxide incubator at 37°C for 4 hours to maximize chondrocyte adherence to the templates. They were then covered with "complete" medium (Dulbecco modified Eagle medium containing 4500 mg/L of glucose, 10% fetal calf serum, 10mM HEPES, 0.1mM nonessential amino acids, 0.4mM L-proline, 50 mg/L of L-ascorbic acid, 50 U/mL of penicillin, and 50 μ g/mL of streptomycin). The control group was incubated for 4 days in the complete medium. The experimental group was incubated in the complete medium combined with IGF-1 (50 ng/mL) and TGF- β (1 ng/mL) (Sigma, St Louis, Mo).

The templates were allowed to incubate without external perturbation for 96 hours. This is the same duration as de-

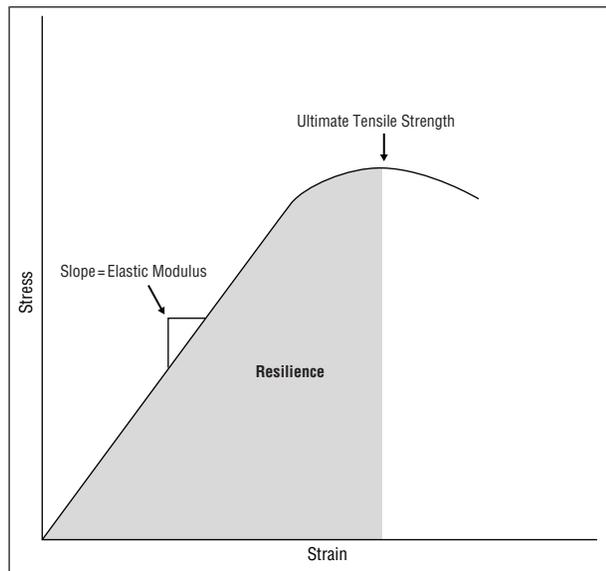


Figure 1. Stress/strain curve.

scribed in previously published work, allowing direct comparison of the results.⁵ At that time, the donor animals were again placed under a similar general anesthetic with local anesthesia. The rabbit flanks were shaved and pockets created beneath the panniculus carnosus. Experimental templates (those incubated with IGF-1 and TGF- β) were implanted on the animal's left side, with the control constructs implanted on the right side. The incisions were approximated with interrupted dermal 4-0 polyglactin 910 sutures (Vicryl; Ethicon EndoSurgery, Somerville, NJ).

After 8 weeks the animals were euthanized according to protocol created by the University of Virginia Care Advisory Committee. Gross examination, histologic testing, and biomechanical testing were then performed to determine the amount and quality of the obtained cartilage. Immunohistochemical analysis was performed by a senior pathologist (J.C.I.). Histologic features were evaluated by light microscopic examination of hematoxylin-eosin-stained tissue sections. Specific histochemical stains were performed to characterize constituent molecules within the cartilage matrix. Masson trichrome staining was used for collagen content and elastin van Gieson staining for elastic fibers. Biomechanical testing was performed with the assistance of the Materials Sciences Department at the University of Virginia, Charlottesville. Cartilage samples were cut into a "dog bone" shape so that applied stress would be focused to the central region of the sample and away from the grips. Prior to testing, the cross-sectional dimensions were measured to 0.05-mm accuracy using a micrometer. These dimensions were needed for subsequent stress calculations. The ends of the specimen were mounted in an ATS 900 (Applied Test Systems Inc, Butler, Pa) mechanical loading device using mechanical grips. The cartilage was then pulled in tension along the sample axis using a constant strain rate of 0.254 cm/min until the point of fracture. The applied load and the resulting elongation were recorded at 1-second intervals. Stress is defined as F/A_0 in which F is the instantaneous load and A_0 is the original cross-sectional area of the gauge length prior to loading. Strain, defined as $(l_i - l_0)/l_0$, is the instantaneous length (l_i) minus the original length (l_0), all divided by the original sample length (ie, prior to loading). The load and elongation data for each sample was normalized to stress and strain using the original sample cross-sectional dimension for each sample. A stress (ordinate) vs strain (abscissa) curve was subsequently produced for each specimen.

Using the stress-strain data for each sample, 3 fundamental material characteristics were quantified: the UTS, the resilience, and the elastic modulus. A material's UTS is the maximum tensile stress achieved by the sample without fracture. This would be represented by the peak stress value in an engineering stress-strain curve ("engineering" refers to the normalization of load and elongation to original values). Resilience is a measure of the capacity of a material to absorb energy when it is elastically deformed. Because the transition from elastic to plastic (ie, permanently deformed) behavior is not clearly delineated for these materials in the stress/strain data, this study quantified resilience as the area under the stress/strain curve bounded by strain=0 and the strain corresponding to the UTS. The elastic modulus, defined as the slope of the initial linear portion of the stress-strain curve, relates to the stiffness of the tested samples when deformation is totally elastic. The UTS, resilience, and elastic modulus are shown in **Figure 1**.

Statistical analysis was performed using S-Plus 2000 (Mathsoft Inc, Seattle, Wash). Owing to the small sample sizes, non-parametric data was assumed. A Wilcoxon rank sum test was applied with significance set at $P < .05$.

RESULTS

The histologic examination of both the complete medium alone and complete medium with growth factors demonstrated irregularly shaped islands of cartilage distributed within a fibrovascular stroma. In both groups, the cartilage was composed of viable-appearing chondrocytes within lacunae surrounded by matrix substance. The morphologic appearance of the chondrocytes in the 2 groups was identical and characterized by a relatively mature phenotype as shown in **Figure 2**. Hematoxylin-eosin staining demonstrated mature, phenotypically normal-appearing chondrocytes in both groups. There were no features of dedifferentiation identified in either sample population. While there was overlap in the relative chondrocyte density, overall chondrocyte density appeared somewhat greater in those grown in complete medium with growth factors. Fibrovascular ingrowth was present in both groups. Within the chondroid matrix, Masson trichrome staining demonstrated mature collagen. Elastin van Gieson staining confirmed the presence of elastic fibers in the lacunar territorial matrix. There was no apparent difference in the matrix constituents between the cartilage constructs. Although focal bone formation was seen in both populations, those samples grown in complete medium alone showed more extensive bone formation along with associated hematopoietic elements.

Ultimate tensile strength for both the complete medium with growth factors and the complete medium alone, along with controls, is shown in **Figure 3A**. Statistical significance was seen between the control group and the samples grown in complete medium ($P < .01$) and those grown in complete medium with growth factors ($P < .01$). Each group was further divided by template seeding concentration, one less than 2.5×10^6 cells/template and the other greater than 2.5×10^6 cells/template as shown in Figure 3B. No significance was found for UTS when comparing the complete medium with and without growth factors and a seeding concentration greater than 2.5×10^6 cells/template ($P < .47$) or less than 2.5×10^6 cells/template ($P < .48$).

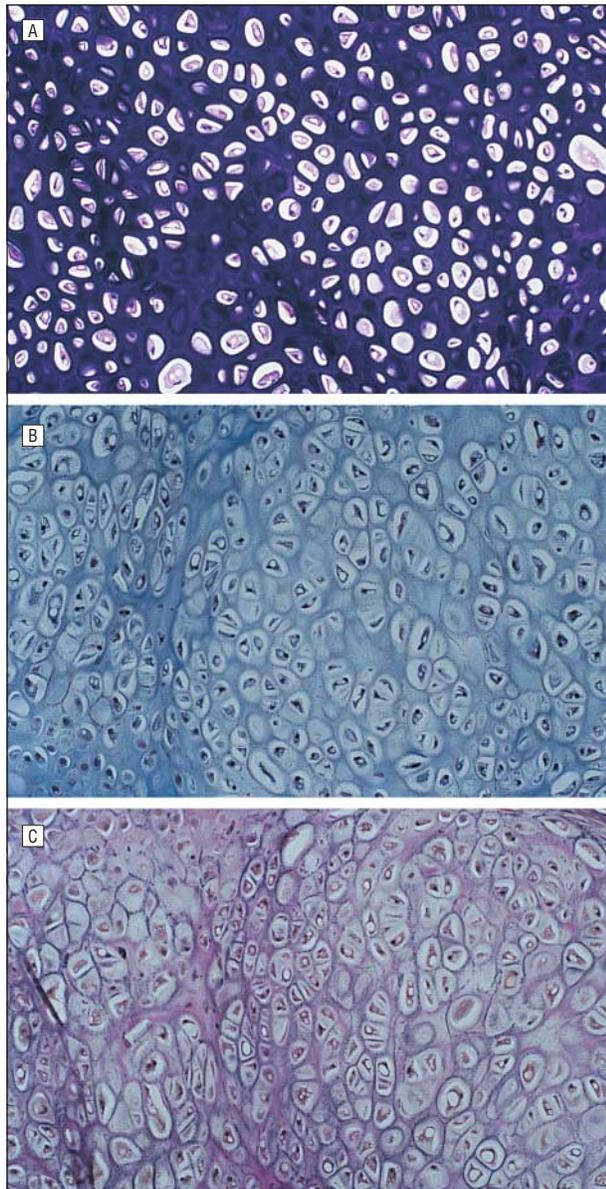


Figure 2. Histologic features of tissue-engineered cartilage exposed to complete medium with insulinlike growth factor 1 (50 ng/mL) and transforming growth factor β (1 ng/mL) for 96 hours. A, Hematoxylin-eosin stain (original magnification $\times 200$) demonstrating mature, phenotypically normal-appearing chondrocytes. B, Masson trichrome stain (original magnification $\times 200$) showing collagen within the chondroid matrix. C, Elastin van Gieson stain (original magnification $\times 200$) confirming the presence of elastic fibers in the lacunar territorial matrix.

The elastic modulus was then calculated for each group (**Figure 4A**). The control group was significantly greater than either the samples grown in complete medium ($P < .01$) or in complete medium with growth factors ($P < .01$). Figure 4B shows the elastic modulus when further divided by template seeding concentration. No statistical significance was found for elastic modulus between the complete medium with and without growth factors and a seeding concentration greater than 2.5×10^6 cells/template ($P < .48$) or less than 2.5×10^6 cells/template ($P < .38$).

The resilience of each sample was then established (**Figure 5A**). The resilience of the control group was

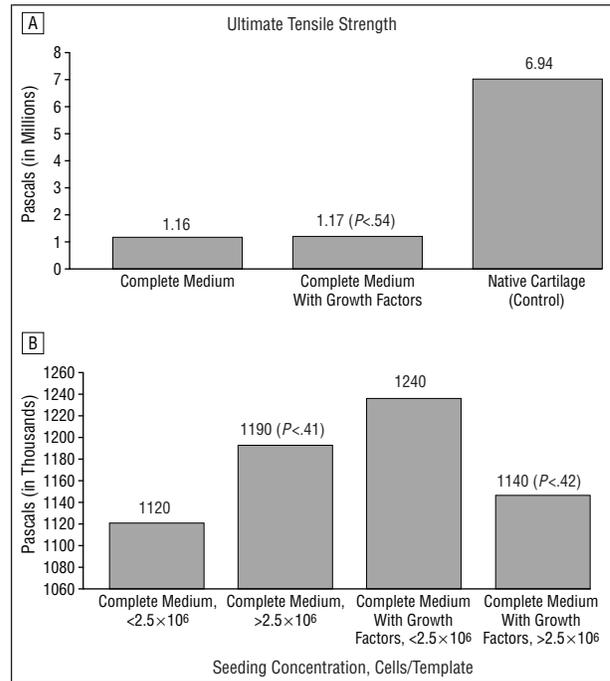


Figure 3. A, Ultimate tensile strength of tissue-engineered cartilage incubated with complete medium alone, complete medium with insulinlike growth factor 1/transforming growth factor β , and control. B, Ultimate tensile strength of tissue-engineered cartilage, with and without insulinlike growth factor 1/transforming growth factor β , with respect to initial seeding concentration.

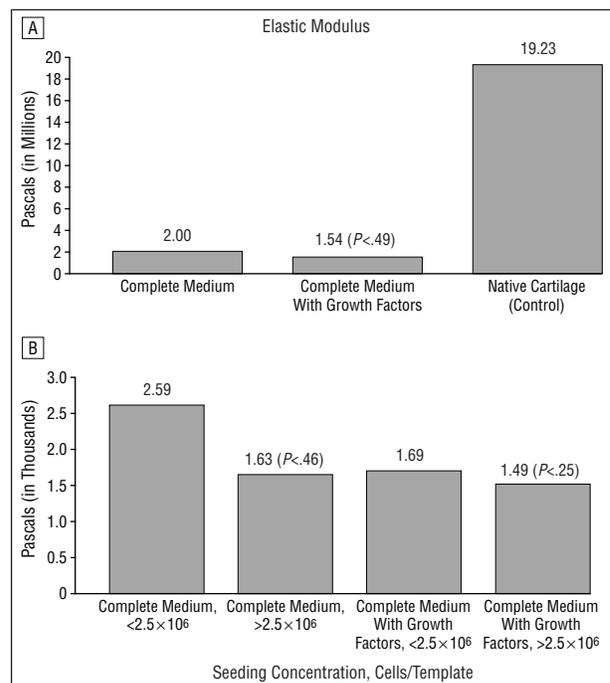


Figure 4. A, Elastic modulus of tissue-engineered cartilage incubated with complete medium alone, complete medium with insulinlike growth factor 1/transforming growth factor β , and control. B, Elastic modulus of tissue-engineered cartilage, with and without insulinlike growth factor 1/transforming growth factor β , with respect to initial seeding concentration.

not significantly greater than the complete medium ($P < .09$) or the growth factor groups ($P < .07$). Figure 5B shows the samples again divided by template seeding con-

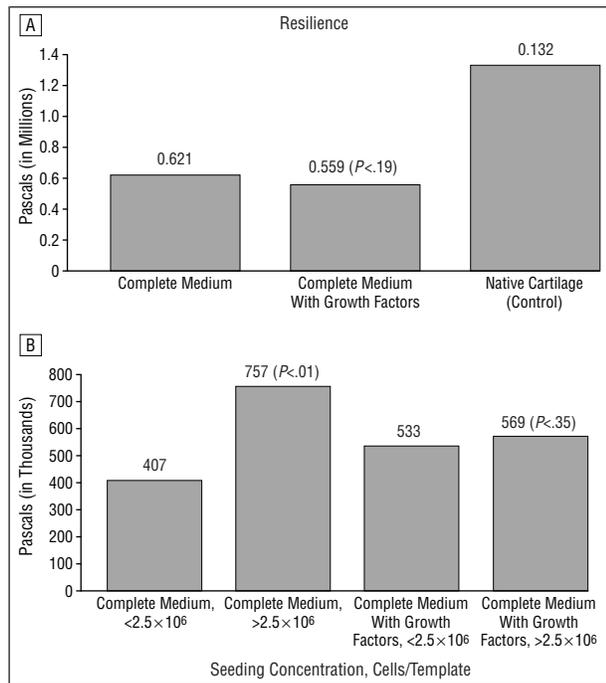


Figure 5. A, Resilience of tissue-engineered cartilage incubated with complete medium alone, complete medium with insulinlike growth factor 1/transforming growth factor β , and control. B, Resilience of tissue-engineered cartilage, with and without insulinlike growth factor 1/transforming growth factor β , with respect to initial seeding concentration.

centration. No statistically significant difference was found for resilience between the complete medium alone and the complete medium with growth factors with a seeding concentration of less than 2.5×10^6 cells/template ($P < .44$) or greater than 2.5×10^6 cells/template ($P < .19$).

COMMENT

The results of this study demonstrate the difficulties encountered when attempting to translate promising in vitro studies to potentially beneficial in vivo application. Previous studies have used various seeding concentrations, ranging from 2.2×10^4 to 1.0×10^8 cells/template. Much work has been accomplished with numerous chondrocyte delivery devices and their optimum seeding concentrations. These studies did not, however, subject the created tissue-engineered cartilage to biomechanical testing. This left an unanswered question regarding whether the gains made in culture techniques would be reflected in improved biomechanical properties of the tissue.

Earlier experiments in the literature have used toughness as a measure of the deformational capacity of the sample tested. After further analysis, it appears that resilience is a better indicator of the capacity of the tissue to absorb energy. This removes the variability inherent in the testing protocol after the initial fracture is noted in the sample. While some samples fracture completely with their first sign of failure, others take an extended period before their stress drops precipitously on the stress/strain curve. By using resilience, however, only the peak of the curve at the point of UTS is used to calculate the area under the curve. This yields a more reliable and re-

producible value and should be used for further biomechanical testing.

Although it was somewhat surprising that a larger influence for the addition of IGF-1 and TGF- β was not seen, several possible explanations exist. The first is that while 8 weeks of in vivo incubation was allowed, a longer period could be required to allow the development of a more mature and stable intercellular matrix. This improved matrix would theoretically lead to superior histologic and biomechanical properties. While impossible to definitively refute, earlier studies⁵ demonstrated no biomechanical advantage to 16 weeks vs 8 weeks of in vivo incubation. It is also possible that the effect of the cytokines might be amplified with longer-term ex vivo or in vivo culture or implantation.

One additional advantage to the use of growth factors is the potential for an increased density to the intercellular matrix. While not enough to show significant statistical benefit during our testing at 8 weeks, this small amplification in matrix density might prove important in lessening the amount of fibrovascular in-growth into tissue-engineered cartilage. This in-growth has been found in most engineered cartilage specimens and is believed to be important in the potential resorption of the implant. Further long-term studies will be required to answer this question.

The observed increase in density of chondrocytes seen on histologic analysis might also be responsible for the curious results obtained on UTS testing. Higher seeding concentration appeared to increase the calculated UTS for samples grown in complete medium alone. When exposed to growth factors, however, the higher seeding concentrations did not produce a significant difference in the mechanical properties. While we have seen this in other parameters, there are several possible hypotheses why this did not translate into a significant increase in UTS. The increased chondrocyte density will by definition increase the density of lacunae present. Since the UTS of the tissue is dictated by the amount and composition of intercellular matrix, one could envision an inverse relationship as the number of chondrocyte/lacunae complexes is increased. It is also possible that the lack of a statistically significant difference in UTS is simply an artifact created by the relatively small sample sizes, and with larger study populations a difference might disappear.

Variability between experimental samples continues to remain at unacceptable levels. Refinement of experimental technique has lessened the problem, but little variation can be tolerated if the procedure is to become clinically applicable. This same variability makes statistical analysis increasingly challenging. Constraints on large sample sizes make uniformity of chondrocyte models imperative for determining statistical significance. Small changes in chondrocyte viability, matrix production, and fibrovascular in-growth could demonstrate important long-term effects for graft survival. Only after this experimental variation is greatly reduced will these changes become evident.

It is clear that both the sample groups showed inferior UTS, elastic modulus, and resilience compared with controls. The question remains, however, as to what biomechanical properties are required to yield an accept-

able graft. It is quite possible that an implant with decreased strength and elasticity could yield acceptable results with respect to both cosmetics and durability.

When the samples were divided according to seeding concentration, an interesting pattern was noted when measuring the resilience of the sample. The samples placed in complete medium alone, with a seeding concentration of greater than 2.5×10^6 cells/template, had a statistically significant increase compared with those having a seeding concentration of less than 2.5×10^6 cells/template ($P < .01$). This difference was not seen, however, between the different seeding concentrations when placed in complete medium with IGF-1 and TGF- β . This demonstrates an important, although often overlooked, possible benefit for the use of growth factors. Most recent studies have attempted to show an increase in either the amount or quality of the tissue-engineered cartilage after the application of growth factors. An equally appealing, while less dramatic, finding is the ability to produce an equivalent graft material with a smaller initial seeding concentration. Our data demonstrates that with the use of conventional media, a statistically significant increase in resilience can be found with increasing the cell-seeding density. When IGF-1 and TGF- β are added, however, the resilience of the samples with lower seeding concentrations is equivalent to those using higher concentrations. This finding may one day allow smaller donor sites for chondrocyte harvest, with the inherent benefits of decreased morbidity and operating time.

CONCLUSIONS

The field of tissue engineering has made dramatic strides over the past several years. Current technology allows reliable production of tissue-engineered cartilage, though with histologic and biomechanical properties inferior to their native counterparts. This study demonstrated no statistical significance for UTS and elastic modulus after the addition of IGF-1 and TGF- β . Samples with higher initial seeding concentrations, exposed only to complete medium, showed a statistically significant increase in resilience. This significance was not seen when growth factors were present in the culture medium.

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