

Mechanical and Cellular Changes during Compaction of a Collagen-Sponge-Based Corneal Stromal Equivalent

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Abstract—The need for corneas suitable for transplantation, combined with the decreasing supply, has fueled interest in the development of a corneal replacement. In this study, a collagen-sponge-based stromal equivalent, consisting of human corneal fibroblasts cultured on a type I collagen sponge, was maintained in culture for up to 21 days and characterized with respect to mechanical properties and cellular behavior. The Young's modulus of the stromal equivalent varied from 95 to 370 Pa, and its permeability varied from 5.3×10^{-8} – 4.2×10^{-7} m⁴ N⁻¹ s⁻¹. The greatest changes occurred during the first few days in culture, but the mechanical properties continued to change during the entire 21 days. Cell traction stress, determined from sponge compaction and DNA count, decreased during the compaction process with the maximum traction value the initial value of $6.6 \pm 2.9 \times 10^{-3}$ Pa cm³ cell⁻¹. Microarray data showed that the expression level of fibronectin, decorin sulfate, collagenase, and gelatinase A was upregulated at day 14 in the sponge. This suggested that the repair fibroblast phenotype was being expressed by the fibroblasts. Additional analysis suggested that a subpopulation of cells expressed the myofibroblast phenotype.

Keywords—Tissue engineering, Wound healing, Young's modulus, Permeability, Myofibroblast phenotype, Repair fibroblast phenotype.

INTRODUCTION

Corneal transplantation remains the most commonly performed and successful transplantation procedure. The Eye Bank Association of America reports that in 2000 over 33,000 corneal transplants were performed in the United States.¹ Recent developments, however, may change significantly the supply of corneas available for transplantation.¹³ Specifically, corneas modified by any surgical procedure (including refractive surgery) are not considered suitable for use in transplantation.³⁹ Thus, the growth in refractive surgery may threaten the supply of corneas for transplantation. In addition to concerns over the availability of corneas

domestically, internationally approximately 11 million people suffer from diseases of the cornea resulting in blindness and the supply of tissue suitable for transplantation is a significant issue.¹³

One method of addressing concerns over the cornea supply involves the development of an engineered tissue replacement.²¹ The biomechanical characteristics of the cornea are central to the native tissue and any tissue equivalent developed. As described in a review by Buzard, "... the mechanical stability [of the cornea is] the basis of many refractive procedures... Although the ultrastructure of the cornea has been detailed in many publications, the structural role of these elements has not been conclusively examined."¹¹ Subsequent studies^{12,28,42,48,49} have developed theoretical models that predict geometric changes resulting from the refractive surgery for the native cornea based in biomechanical properties of the cornea. These models reinforce the role of the biomechanical properties of the cornea in normal refraction. As a connective tissue, the cornea also withstands significant loads *in vivo*. Specifically, intraocular pressure varies between 10 and 21 mm Hg for normal humans.⁴² The development of a functional corneal replacement must address biomechanical considerations.

Much work has been devoted to characterizing stromal fibroblast behavior *in vitro*. Monolayer culture, although providing valuable insight into the influence of cell density³¹ and soluble factors¹⁸ on stromal fibroblast phenotype, does not account for the three dimensionality of the native environment. Cells cultured in three-dimensional matrices demonstrate pronounced differences from monolayer cultures, including shape, migration, and communication patterns (reviewed in¹⁹). Collagen gel cultures provide a more tissue-like environment⁸ and have thus been suggested as a better model system. Studies on gels seeded with stromal fibroblasts have characterized the influences of coculture and soluble factors on contraction, transparency, and phenotype.^{9,25} Contractile forces exerted by single corneal fibroblasts cultured on top of gels have also been studied.^{44,45} Native cornea is much stiffer than collagen

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gels, however, and it has been shown that matrix compliance affects both the contractile properties and the phenotype of entrapped cells.^{4,40} Therefore, there is a need for a culture environment that maintains three-dimensional geometry with increased mechanical stiffness.

Our lab has developed and manufactured a three-dimensional fibrillar collagen sponge matrix from type I bovine dermal collagen as a better *in vitro* model and prototypical engineered corneal stroma. The compliance of the sponge can easily be altered by changing the crosslinking properties or by other modifications in composition and processing. The ability to control the initial properties of the matrix and mimic three-dimensional architecture provides a more realistic *in vitro* model for the cornea. Previous experiments in our lab have used the sponge matrix to study the behavior of human stromal fibroblasts alone and cocultured with epithelial and endothelial cells.³⁷ These studies also looked at the effects of matrix structure and composition on the transparency and contraction of this wound-healing model. We found that stromal fibroblasts are able to migrate throughout and populate the collagen matrix, as well as produce extracellular matrix components such as collagen and proteoglycans.³⁷ In general, these studies showed features of the normal wound healing response occurring within the sponge.

The research summarized above motivated two questions addressed in the current series of investigations. The first question is: *How do the biomechanical properties of the stromal equivalent evolve during culture?* This question is relevant both to corneal tissue engineering and to the stromal equivalent (SE) as an *in vitro* model system. Specifically, the modulus and the permeability of the SE were studied over time. The second question is: *How does cell behavior change over the course of the culture, and do observed cellular changes correlate to biomechanical changes?* This more subtle issue was probed by quantifying cell number and cell traction stress, and also by observing changes in mRNA expression during the course of the experiment.

MATERIALS AND METHODS—PREPARATION AND CULTURE

Cell Isolation

Human tissue unsuitable for transplantation was obtained from the Minnesota Lions Eye Bank with approval of the local Institutional Review Board and in conformance with the Declaration of Helsinki. Corneas were excised from whole globes and corneal cells were extracted according to published methods.³⁷ Stromal fibroblasts were isolated from corneas stripped of both endothelium and epithelium. The corneas were placed in a solution of collagenase (0.30 $\mu\text{g}/\text{ml}$, Sigma, St. Louis, MO) and incubated at 37°C overnight. The stromal fibroblasts were washed and resuspended in fibroblast culture medium con-

sisting of DMEM-F12/HAM basal medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and 1% penicillin/streptomycin (GibcoBRL, Grand Island, NY). Cells were plated in T150 tissue culture flasks at a seeding density of 9000 cells/cm² prior to culture on sponges and gels. The fibroblasts were fed 25 ml of fresh fibroblast culture medium every 2–3 days. The cells were passaged when they reached 70–90% confluency, and all cells used for experimentation were passage 1–4.

Collagen Matrix Preparation

Collagen sponges were prepared from bovine type I dermal collagen, as described in more detail previously.³⁷ Briefly, collagen dispersions of 0.5% w/v were prepared from the ground collagen by slow blending of the collagen/H₂O + HCl mixture (pH 3.0) at 4°C for 1 min followed by exhaustive deaeration. The dispersion was poured into a dish or pan, lyophilized at 30°C, and dehydrothermally (DHT) crosslinked in a vacuum oven at 110°C at a vacuum of 2.5 torr for 5 days. The sponges were sterilized using gamma irradiation (17,500 rads) prior to use in culture experiments.

Collagen gels were prepared from Vitrogen 100 (Collagen Biomedicals, Inc.). 10 \times phosphate-buffered saline solution and 0.1M NaOH were added to chilled Vitrogen 100 stock solution in a ratio of 1:8 and pH adjusted to 7.4. The neutralized solution was supplemented with stromal fibroblasts at a density of 5 \times 10⁴ cells/cm³, cast into wells of a 24-well plate at a thickness of 1.2 mm or 1.7 mm, and placed at 37°C to initiate gelation.

Cell Culture on Sponges and Gels

Stromal fibroblasts were cultured in 6-well culture plates (Costar, Cambridge, MA) on collagen sponges. Collagen sponge pieces approximately 14 mm in diameter and 1–2 mm thick and collagen gels were prepared as described above. All collagen sponges were hydrated with 150 μl of fibroblast culture medium prior to seeding of cells. The cells were seeded in the center of the porous side of the collagen sponge at a density of 5 \times 10⁴ cells/cm³. The sponges were allowed to sit at 37°C for 1–2 h to facilitate attachment of cells and were then supplemented with 1 ml of fibroblast culture medium. Collagen gel samples were transferred from 24-well plates to 6-well plates on day 2 of culture, and Transwell culture inserts (Corning Costar, Corning, NY) were added to all samples to reduce folding (of the samples).

All samples were fed with 4 ml of fresh medium every other day for the duration of culture. The stromal equivalents were removed from culture at specific time points throughout the period of culture to determine contraction, cell number, mechanical properties, and cell phenotype as discussed below.

MATERIALS AND METHODS—STROMAL EQUIVALENT CHARACTERIZATION

Quantification of Contraction

The radial contraction of the collagen sponge and gel samples seeded with stromal fibroblasts was measured under sterile culture conditions at various time points throughout the culture period. A Javelin Ultrachip Hi Res CCD camera was used to photograph the sample, and NIH Image was used to analyze the picture to determine the surface area of the sample as a measure of radial matrix contraction. The axial contraction of the sponge was measured manually, with calipers, after the sponge had been sacrificed. Statistical analysis of the data was performed using StatView (Cary, NC). Statistical differences in the end-point data were determined using an unpaired *t* test.

Quantification of Cell Density

The number of cells per sponge was estimated at different time points during the experiments using the DNeasy Tissue Kit from QIAGEN (Valencia, CA). The procedures provided in the DNeasy Tissue Kit Handbook were followed with the exception of using a longer time for lysis. Briefly, Buffer ATL (a lysis buffer) and proteinase K (600 mAU/ml solution) were added to the tissue sample. The sample was left in a 55°C shaking water bath overnight to ensure that the sponge was completely dissolved and that the cells were thoroughly lysed. The sample was then RNase treated to eliminate residual RNA. Next, the sample was bound to a spin column, washed, and eluted, as specified in the QIAGEN DNeasy handbook. The final output was the fibroblast DNA from the sponge sample in a buffer solution. The concentration of DNA in the sample was determined by measuring the absorbance of the sample at 260 nm with a spectrophotometer. An absorbance reading of 1 corresponds to 50 μg of DNA. A conversion of 6 mg of DNA per 1×10^6 cells was then used to find the final cell number, which was divided by the sponge volume to give cell density (cells/cm³).

Characterization of Sponge Biomechanics

Unconfined compression stress-relaxation tests were performed on sponge and gel samples with and without cells, using an 895 Micro-Bionix Test System (MTS Systems Corp., Eden Prairie, MN). The sample was placed between two impermeable plates and submerged in phosphate-buffered saline solution at room temperature. A compressive strain of 15% (engineering strain) was applied to the sample. The strain was maintained at 15% for 1 h, and the force exerted by the sample was recorded at 0.5-s intervals.

The data were analyzed using the analytical solution of Armstrong and colleagues³ for unconfined compression of a

linear poroelastic material. The mechanical parameters are the permeability k , Poisson's ratio ν , and the aggregate modulus H . The aggregate modulus is a mechanical stiffness measure related to Young's modulus E and Poisson's ratio by the formula $E(1 - \nu) = H(1 + \nu) \times (1 - 2\nu)$. Armstrong provides a series solution for the force on the piston as a function of time during an unconfined compression experiment, and the three parameters k , ν , and H were regressed to the data by minimizing sum of squared error between the calculated and measured values. The regression was performed using an *Excel* spreadsheet.

MATERIALS AND METHODS—CELLULAR RESPONSE

Determination of the Cell Traction Stress

Once the mechanical properties of the matrix had been determined, the traction stress was calculated from compaction data.⁵ The importance of permeability in the compaction process may be assessed using a dimensionless quantity,

$$N_{\text{perm}} = L^2/KEt$$

where L is the permeation length scale (in this case, the thickness of the sample, 2 mm), K is the hydraulic conductivity ($10^{-10} \text{ m}^2/(\text{Pa s})$), E is Young's modulus (300 Pa), and t is the time scale for the experiment (10^5 s). Substituting into the above equation, we get $N_{\text{perm}} \sim 10^{-3}$, indicating that the water permeation is very fast and may be neglected in the analysis. Thus, the system was treated as always at equilibrium between cell traction and mechanical stress in the matrix.

The stress exerted by the cells on the matrix (i.e. cell traction stress) was calculated from compaction data using methods previously applied in analysis of collagen gel compaction.^{5-7,35} As previously, the cell traction stress was assumed to be bilinear in cell concentration and collagen volume fraction—i.e., more cells per unit volume generate more stress, and more collagen per unit volume provides greater opportunity for cells to compact the matrix, corresponding to greater stress.³⁸ Contraction of the matrix was quantified by the engineering strain $\varepsilon = \Delta R/R_0$, where ΔR is the change in matrix radius and R_0 is the initial radius. We observed that the thickness and radius changed comparably during compaction, indicating homogeneous compaction and eliminating the need for anisotropic analysis. Homogeneous compaction also validates our assumption of fast permeation. In this preliminary analysis, it was assumed that the total amount of collagen in the sponge did not change during compaction. Based on the assumptions, the cell traction stress constant σ_c was calculated using the compaction data, the number of cells N , the volume of collagen V_c , the sample volume V , and the bulk modulus K , which was expressed in terms of the

Young's modulus and Poisson's ratio:

$$\begin{aligned}\sigma &= \frac{\sigma}{(N/V)(V_c/V)} = \frac{K\varepsilon}{(N/V)(V_c/V)} \\ &= \frac{3E(\Delta R/R_0)}{(N/V)(V_c/V)(1-2\nu)}\end{aligned}$$

In the final form, E (modulus) and ν (Poisson's Ratio) were known from the mechanical tests, ΔR , R_0 , and V were measured during compaction, V_c was known from the fabrication conditions, and N was known from the DNA counts. Thus, the stress per unit cell concentration and unit collagen volume fraction was determined as a function of time during the course of compaction.

Cell Phenotype Assessment

The U133A Human GeneChip[®] expression analysis probe array (Affymetrix, Inc., Santa Clara, CA) was used to quantify the amount and type of mRNA being expressed by fibroblasts from the same donor at day 14 in culture in monolayer culture and three-dimensional collagen sponges. The cells from the culture flask were approximately 80% confluent when they were removed for testing. Six sponge samples were combined in order to have an adequate amount of mRNA for testing.

Biotin-labeled cDNA fragments of the samples were created according to manufacturer's protocol to be hybridized to the probe array.⁴³ Briefly, total RNA was extracted from the cells grown in monolayers using QIAGEN's RNeasy Mini Kit (Valencia, CA). The cells were lysed and homogenized using buffers provided in the kit. The total RNA was isolated on a membrane and eluted. Total RNA was extracted from the sponge samples using TRIZOL (Invitrogen Life Technologies). The sample was first homogenized using a power homogenizer. Then, a phase lock gel (Ependorf, Germany) was used to separate the organic phase from the aqueous phase, which contains the total RNA. The RNA was precipitated and washed with ethanol. Final RNA products were resuspended in DEPC-treated water.

Next, double-stranded cDNA was synthesized from the total RNA using the SuperScript Choice System (Invitrogen Life Technologies) and a customized primer (Genset Oligos, La Jolla, CA). A phase lock gel-phenol/chloroform extraction was then used to clean up the double-stranded cDNA. The cDNA was turned into biotin-labeled cRNA using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Affymetrix Inc., Santa Clara, CA). QIAGEN's RNeasy columns were used to clean up the *in vitro* transcription products. Finally, the cRNA was fragmented and adjusted to a final concentration of 0.5–2.0 $\mu\text{g}/\mu\text{l}$.

The fragmented biotin-labeled cDNA samples were hybridized according to manufacturer's protocol. Once the samples were hybridized, they were washed, stained with streptavidin phycoerythrin conjugate, and scanned by the

GeneArray[®] Scanner at the excitation wavelength of 488 nm. The samples were scanned twice and an average intensity was calculated. This process helped to reduce background noise. The amount of light emitted at 570 nm was proportional to the bound target cDNA at each location on the probe array.

The expression levels of mRNA from the fibroblasts cultured in monolayer culture were compared to the expression levels of fibroblasts cultured in three-dimensional collagen sponges using the Affymetrix Microarray Suite. The Wilcoxon's signed-rank test was used to compare the results from the scaled and normalized intensities of the expression array. The p -values ranging from 0.0 to 1.0 were determined. Values close to 0.0 indicate likelihood for an increase in transcript expression. Values close to 1.0 indicate likelihood for a decrease in transcript expression level whereas values of 0.5 indicate a weak likelihood for change in either direction. The expression level changes were categorized as follows: Increase, Marginal Increase, No Change, Marginal Decrease, or Decrease. The relative changes of extracellular matrix components, proteoglycans, integrins, matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), and growth factors were chosen for further inspection.

RESULTS

Sponge Biomechanics

A typical stress versus time plot for the collagen sponge at an applied step strain of 15% is shown in Fig. 1. The stress increased sharply during the initial phase of the compression and then decayed steadily over 60 min, eventually reaching an equilibrium level. Also shown in Fig. 1 is a best fit of the Armstrong model. As one would expect for a dilute, highly compressible sponge, Poisson's ratio was very small. We found no significant change in the fit of the other model parameters for ν in the range (0,0.05) at all times. Estimates for the permeability (K) and aggregate modulus (H) for the collagen sponge as a function of time in culture were also obtained (Fig. 2). The permeability increased four orders of magnitude during the first two days of compaction from $4.58 \times 10^{-11} \pm 1.201 \times 10^{-11} \text{ m}^4/(\text{N} \times \text{s})$ to $4.19 \times 10^{-7} \pm 3.47 \times 10^{-7} \text{ m}^4/(\text{N} \times \text{s})$ and then decreased slowly over the duration of the 2-week experiment. The aggregate modulus decreased rapidly during the first two days from $359 \pm 57 \text{ Pa}$ to $115 \pm 73 \text{ Pa}$ and then rose steadily thereafter, but the effect was not nearly as dramatic as that observed in the permeability. The modulus and permeability for a control (acellular) collagen sponge cultured for 2 weeks did not vary significantly over the time period studied.

As noted qualitatively in our previous work,³⁶ the sponge was contracted by the stromal fibroblasts. The cross-sectional area of the collagen matrix was determined for

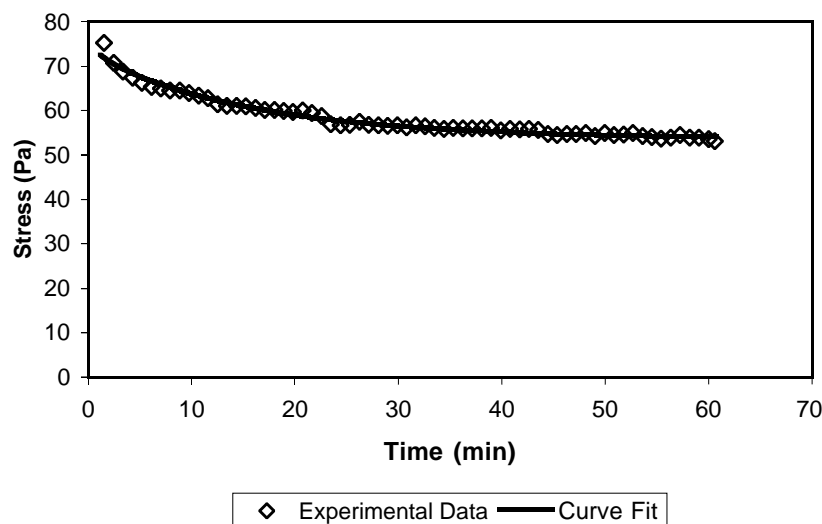


FIGURE 1. Stress versus time for unconfined compression testing of collagen sponge matrix without cells. Solid line indicates theoretical fit from Armstrong model.

14 days in culture and normalized with respect to initial area (Fig. 3). The cross-sectional area of the sponge decreased quickly during the initial 5 days in culture. After this initial period, the cross-sectional area decreased more gradually during the remainder of the 14 days in culture. Cell-free sponges did not compact significantly ($p > 0.005$).

Concurrent with these studies, the thickness of the sample was determined to evaluate our assumption of homogeneous matrix compaction. A plot of the diameter versus thickness of the compacting stromal equivalent is shown in Fig. 4. Changes in the thickness mirror changes in the diameter, providing macroscopic-scale verification of homogeneous compaction.

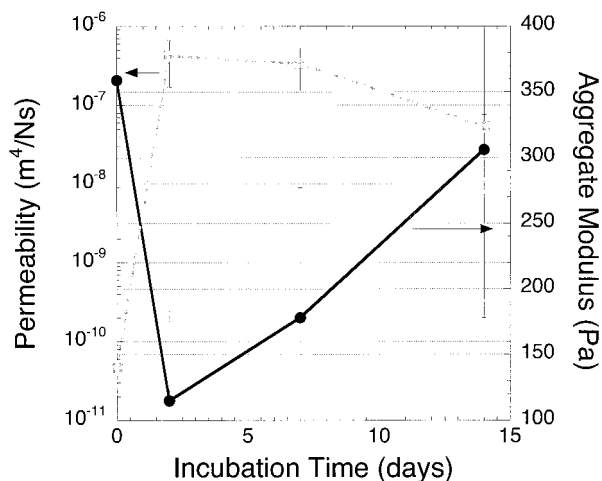


FIGURE 2. Aggregate modulus and permeability as a function of time in culture for stromal fibroblasts cultured in a collagen matrix.

Cell Traction Stress

Based on the compaction and modulus data, the cell traction stress was calculated at various time points during compaction (Fig. 5). The cell traction stress was highest ($6.6 \pm 2.9 \text{ mPa} \times \text{cm}^3/\text{cell}$) after day 1 and then decreased steadily to about 10% of that value after 21 days. Using an unpaired t test, the cell traction stress calculated at day 1 was statistically larger than that observed on day 2 and 7 ($p < 0.05$) with no statistical significance to differences between later time points.

Gene Expression/Cell Phenotype

The results described previously show changes in the mechanical traction force and biomechanical properties of the matrix with time in culture. We were also interested in gene expression/phenotype for the cells. The mRNA expression levels for stromal fibroblasts in three-dimensional collagen sponges at day 14 (see Table 1 for summary) were determined and compared to the gene expression of stromal fibroblasts grown to confluency in a tissue culture flask for reference. The mRNA levels of various extracellular matrix components were higher for cells cultured in a collagen matrix when compared to monolayer culture. These components included types VI, VII, XIII, XVI, XVIII, and XXI collagen, elastin, fibronectin, and multiple types of laminin. The production level of type XI and type XIV collagen decreased slightly for cells cultured in a collagen sponge when compared to monolayer culture. There was no difference in the production of type I collagen between cells cultured in the collagen matrix and monolayer culture. The mRNA levels for extracellular matrix molecules, specifically proteoglycans, also varied. The expression level of chondroitin sulfate proteoglycans 1 and 2 increased, while

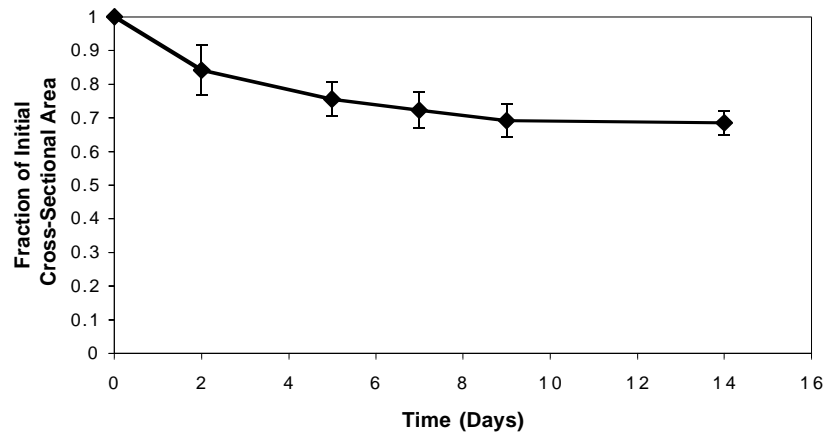


FIGURE 3. Normalized cross-sectional area of a collagen sponge seeded with stromal fibroblasts as a function of time in culture. Error bars indicate standard deviation and $n = 12$ for all time points.

the expression level of chondroitin sulfate proteoglycan 6 decreased. The production of heparan sulfate proteoglycan 1 decreased, while the production of heparan sulfate proteoglycan 3 increased. There was no change in the production of lumican between the two culture environments, and the production of decorin increased greatly.

The expression level of the alpha 4, alpha 6, and beta 3 integrins decreased for cells cultured in a collagen matrix when compared to monolayer cultures, while the expression level of the alpha 5 integrin increased. The alpha 4 integrin and the alpha 5 integrin can both form fibronectin receptors, and the alpha 6 integrin can form a laminin receptor by combining with beta integrins.

Because of their role in matrix remodeling, matrix metalloproteinases (MMPs) are of interest. The expression of interstitial collagenase (MMP 1) and gelatinase A (MMP 2) increased while the expression of stromelysin (MMP 3) decreased. The production of tissue inhibitors of matrix metalloproteinases (TIMPs) increased or decreased accordingly. The changes in expression levels of growth factors produced were also examined. A variety of growth factors in-

cluding epidermal growth factor (EGF), insulin-like growth factor (IGF), interleukin 1-alpha ($IL-1\alpha$), latent transforming growth factor beta ($TGF\beta$), vascular endothelial growth factor (VEGF), and vascular endothelial growth factor C (VEGFC) increased their expression when the fibroblasts were cultured in a three-dimensional matrix at day 14 when compared to monolayer culture. In contrast, the expression fibroblast growth factor 5 was lower for cells cultured in a collagen sponge compared to monolayer culture. Finally, it is important to note that there was no change in the expression level of alpha smooth muscle actin between the tissue culture flask and the collagen sponge.

DISCUSSION

Sponge Biomechanics

The unconfined stress-relaxation testing of the collagen matrix yielded plots of force versus time consistent with what was expected for a linear poroelastic material.³ As the strain was quickly applied to the sample, the force increased

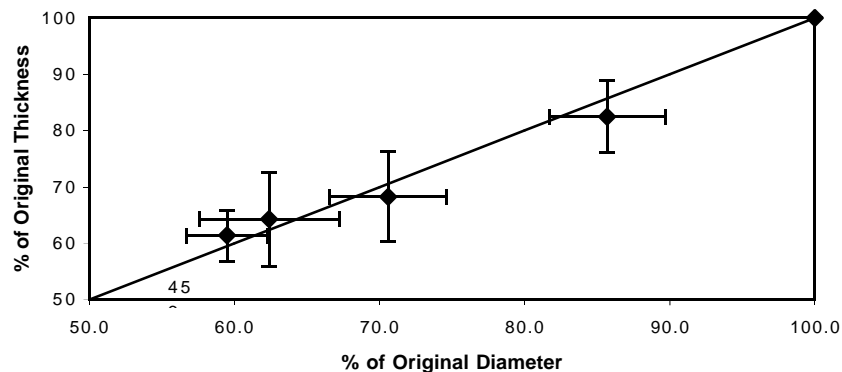


FIGURE 4. Longitudinal thickness and lateral compaction of the collagen sponge matrix seeded with stromal fibroblasts. Error bars indicate standard deviation and $n > 18$ per point.

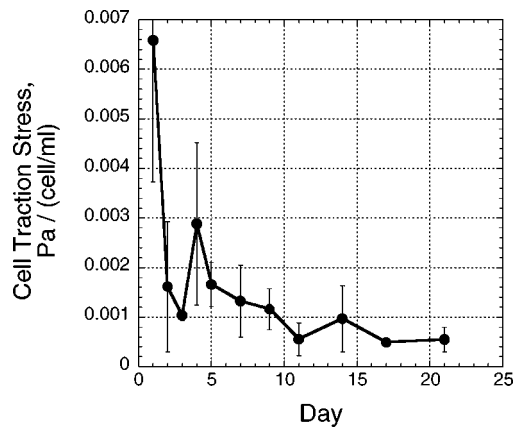


FIGURE 5. Cell traction stresses of corneal fibroblasts cultured in a collagen sponge as a function of day in culture. Error bars indicate standard deviation. $N > 6$ for day 2, 7, 14, 21. For all other time points, $N = 2$.

quickly due to compression of the matrix and pressurization of interstitial fluid. With time, the force decreased due to permeation effects and the viscoelasticity of the matrix, eventually reaching equilibrium.

The native cornea is extremely stiff, with a Young's modulus of the order of 1–10 MPa typically reported.^{26,46–48} The sponge modulus is roughly 300 Pa, some four orders of magnitude lower than native cornea, but two orders higher than the modulus of the collagen gels. The sponge, therefore, reflects more closely the *in vitro* environment for the study of cell behavior. Additional processing (e.g., crosslinking or compositional adjustment) may facilitate further stiffening of the sponge and thus further approach to a functional engineered cornea.

The permeability to water of the sponge, like the collagen gels,²⁹ is of the order of $10^{-7} \text{ m}^4/(\text{N}\cdot\text{s})$. This parameter is well below the native stromal permeability (see Table 2) of $4.9 \times 10^{-18} \text{ m}^4/(\text{N}\cdot\text{s})$.¹⁶ The similarity in permeability between the sponge and the gel is surprising because the density of collagen in a sponge is significantly higher than that of a gel. Microstructural differences between the sponge and the gel and the corresponding influence on permeability is an issue that merits further investigation, possibly by scanning electron microscopy.

Both the permeability and the aggregate modulus of the sponge vary with time in culture. These dynamic changes reflect the influence of (1) compaction of the matrix; (2) migration and proliferation of cells; and (3) remodeling of the extracellular matrix (degradation and/or deposition of new matrix). The significant changes in matrix behavior within the first 48 h after seeding of the matrix suggest that degradation of the matrix resulting from proteases is probably the dominant effect. This result is consistent with the upregulation of collagenase observed in this study. In a recent study, Daniels and colleagues¹⁴ demonstrated that MMP production over a 7-day period peaked at 24 h after seeding of a

TABLE 1. Comparison of mRNA levels for stromal fibroblasts cultured in a collagen sponge matrix after 14 days in culture when compared to monolayer cultures of stromal fibroblasts obtained from the same donor. The cells from culture flasks were approximately 80% confluent and less than passage 5.

Extracellular matrix components:	
Collagen—types VI, VII, XIII, XVI, XVIII, XXI	Increases
Elastin	Increases
<i>Fibronectin 1</i>	Increases
Laminin—alpha 2, alpha 3, beta 1, beta 2, beta 3, gamma 2	Increases
Collagen—types XI, XIV	Decreases
Proteoglycans:	
Chondroitin sulfate proteoglycan 1 (aggrecan 1)	Increases
Chondroitin sulfate proteoglycan 2 (versican)	Increases
Chondroitin sulfate proteoglycan 6 (bamacan)	Decreases
<i>Chondroitin sulfate/Dermatan sulphate proteoglycan (decorin)</i>	Increases
Heparan sulphate proteoglycan 1 (syndecan 2)	Decreases
Heparan sulphate proteoglycan 2 (perlecan)	Increases
<i>Keratan sulphate proteoglycan (lumican)</i>	No Change
Integrins:	
Alpha 4 integrin	Decreases
<i>Alpha 5 integrin (Fibronectin receptor)</i>	Increases
Alpha 6 integrin	Decreases
Beta 3 integrin	Decreases
MMPs & TIMPs:	
<i>MMP 1 (Interstitial collagenase)</i>	Increases
<i>MMP 2 (Gelatinase A)</i>	Increases
<i>MMP 3 (Stromelysin)</i>	Decreases
<i>TIMP 2 (Tissue inhibitor of MMP 2)</i>	Decreases
<i>TIMP 3 (Tissue inhibitor of MMP 3)</i>	Increases
Growth factors:	
Epidermal growth factor (EGF)	Increases
Fibroblast growth factor 5	Decreases
Insulin-like growth factor 1, 2 (IGF)	Increases
Interleukin 1-alpha (IL-1 α)	Increases
Latent transforming growth factor beta binding protein 1,2,3,4	Increases
Vascular endothelial growth factor (VEGF)	Increases
Vascular endothelial growth factor C (VEGFC)	Increases
<i>Alpha smooth muscle actin</i>	No Change

collagen gel with fibroblasts isolated from Tenon's capsule. These results also suggest that upregulation of MMPs may be involved in the decrease in modulus observed during early periods in culture.

The cell traction stresses produced by the corneal fibroblasts decreased throughout the duration of the experiment and ranged from 6.6 mPa/(cell/ml) to less than 1 mPa/(cell/ml). The cell traction stresses are of the same

TABLE 2. Permeability of native tissues and tissue equivalents.

	k (m ⁴ /Ns)	Reference
Native tissue		
Cartilage	1×10^{-15}	Armstrong, 1984
Corneal stroma	4.9×10^{-18}	Edwards, 1998
Intact cornea	2.2×10^{-18}	Edwards, 1998
Tissue equivalents		
Collagen gel	1.6×10^{-10}	Knapp, 1997
Collagen sponge with cells	$4.2\text{--}0.53 \times 10^{-6}$	This investigation
Unseeded collagen sponge	4.6×10^{-11}	This investigation

order of magnitude as stresses that were measured using human dermal fibroblasts in previous experiments.^{10,15} The contraction of the matrix is nearly complete by day 14, which is analogous to the period of curvature change after refractive surgery. During this time period after the initial stromal injury, the myofibroblast phenotype is expressed by the corneal fibroblasts and the cells are contracting the matrix.¹⁸ Most changes in the compliance of the matrix also occur during this time. Though the major changes in cell traction stress occur during this time period, the cell stresses continue to change to some degree after 14 days. This indicates ongoing changes on the microscopic level such as changes in extracellular fiber organization, cellular stress fiber formation, and phenotype.

Gene Expression/Cell Phenotype

Our analysis was limited to include four major groups: extracellular matrix molecules, MMPs, growth factors, and a limited list of integrins. Quantification of mRNA levels showed that many extracellular matrix and basement membrane components were upregulated for cells cultured in a collagen sponge at day 14. For example, laminin I and type VII collagen were observed to be higher for cells cultured in a collagen sponge. These components are also found in the injured cornea during the migration phase of the wound healing process.¹⁷ In addition, there was an increase in mRNA present for type VII collagen, a key component of Bowman's layer that is produced by fibroblasts,²⁰ which is consistent with a repair fibroblast phenotype.

The proteoglycan production rate of the fibroblasts was altered when they were cultured in a three-dimensional matrix. Specifically, message levels of decorin (chondroitin sulfate/dermatan sulfate proteoglycan) increased while the message of lumican (keratan sulfate proteoglycan) stayed the same. The increase in decorin message levels is consistent with observations of proteoglycan production in the healing cornea.¹⁸ A study by Hassell and colleagues showed that intact human corneas synthesize mainly decorin and lumican while fibroblasts cultured in a tissue flask synthesized mostly chondroitin and heparan sulfate proteoglycans.²⁴ Further studies will be needed to determine whether the

increase in proteoglycan production for stromal fibroblasts cultured in a collagen sponge when compared to cells in a monolayer may have contributed to the observed changes in permeability for the matrix.

Matrix metalloproteinases (MMPs) also play an important role in the healing process. MMPs are not found in uninjured native corneal tissue.²² In this study, however, mRNA for collagenase (MMP 1), gelatinase A (MMP 2), and stromelysin (MMP 3) was observed in both monolayer and collagen sponge cultures of stromal fibroblasts. The production of collagenase and gelatinase A was upregulated in the sponge, while the production of stromelysin decreased. Conversely, the production of the tissue inhibitor of gelatinase A (TIMP 2) decreased in the sponge, and the production of the tissue inhibitor of stromelysin (TIMP 3) increased. High levels of collagenase (MMP 1), gelatinase A (MMP 2), stromelysin (MMP 3), and gelatinase B (MMP 9) are associated with the repair fibroblast phenotype.³³ In this study, the production of collagenase in the sponge was nearly 30 times greater than the production by cells cultured in a monolayer. This agreed qualitatively with *in vivo* experiments of Girard and colleagues, who observed a large increase in the production of collagenase after a stromal wound in the native rabbit cornea for up to 9 months after injury.²²

Cytokines have been found to regulate the degradation of extracellular matrix molecules. Specifically, interleukin 1 (IL-1), an inflammatory cytokine, stimulates the degradation of extracellular collagen through the upregulation of collagenase activity.³³ The increase in message levels for IL-1 α and collagenase in the sponges is consistent with this behavior.

Many growth factors were upregulated when the corneal fibroblasts were cultured in the sponge matrix rather than in the two-dimensional tissue culture flask. These changes were similar to the changes that occur during the normal wound healing response in the cornea. The production of epidermal growth factor (EGF) increased when the cells were cultured on the sponge matrix, which may account for the proliferation of cells in the matrix³³ or when combined with insulin-like growth factor (IGF), enhance migration of stromal fibroblasts.² A third growth factor whose expression increased in the collagen sponge was the latent transforming growth factor beta binding protein (TGF β). When activated, TGF β plays a role in inducing the myofibroblast phenotype.^{18,27}

A corresponding increase in the expression of the $\alpha_5\beta_1$ fibronectin receptor and decrease in the expression of the $\alpha_4\beta_1$ fibronectin receptor in the sponge indicates that some cells in the three-dimensional sponge matrix are expressing the myofibroblast phenotype.³² This finding agrees with a study by Masur and colleagues that found the $\alpha_5\beta_1$ fibronectin receptor present in high levels in cultured corneal fibroblasts, but not in native corneal tissue.³⁰ A decrease in the amount of the α_6 integrin was also observed. This

indicates that cells in the sponge matrix have fewer laminin receptors than cells in the culture flask. The β_3 integrin, which was upregulated in the sponge, is not commonly found in the human cornea, but one study did observe some expression of either the β_3 or the β_6 receptor in freshly isolated, noncultured keratocytes.³⁰

It is important to note that the gene expression study performed in conjunction with this investigation is limited to message level, which may not correlate with expression at the protein or post-translational levels. Gene expression studies were performed at only two time points and additional studies performed at intermediate time points would facilitate determination of temporal trends in gene expression. Finally, the studies performed compared message levels using cells from the same donor but additional donors should be examined in paired studies to quantify donor-to-donor differences that may also arise. These studies would also facilitate quantification of changes in message levels.

Overall, corneal fibroblasts cultured in a three-dimensional sponge for 14 days exhibited different patterns of gene expression from those cultured in a monolayer. The pattern of expression of extracellular matrix, proteoglycans, integrins, MMPs, and growth factors were reminiscent of the normal *in vivo* corneal wound healing process. These findings agreed with previous studies that showed that cells cultured in a three-dimensional environment behaved more like cells *in vivo*.^{23,34} Initial microarray data indicated that fibronectin, decorin sulfate, collagenase, and gelatinase A were expressed at extremely high levels in three-dimensional culture, while the expression of alpha smooth muscle actin was the same as in two-dimensional culture. This would seem to indicate that many of the cells present at day 14 in the collagen sponge were expressing the repair fibroblast phenotype.¹⁸ The microarray data also showed increased levels of the α_5 integrin and decreased levels of the α_4 integrin. This indicates that at least some cells in the sponge are expressing the myofibroblast phenotype.

It has been hypothesized that functionally and phenotypically different subpopulations of fibroblasts exist during the wound healing process. The current study seems to provide preliminary data that populations of both repair fibroblasts and myofibroblasts are present in the collagen sponge tissue model at day 14 during the wound healing process. This hypothesis agrees with a study measuring the message levels of dermal fibroblasts during the wound healing process which showed that some of the fibroblasts are activated while others are quiescent.⁴¹ Further studies will need to be performed to quantify the relative contribution of the different subpopulations (repair fibroblast and myofibroblast) to the mechanical properties and gene expression profiles determined in this investigation.

The changes in biomechanical properties (aggregate modulus and hydraulic permeability) for the collagen sponge matrix containing stromal fibroblasts measured in this investigation, combined with the gene expression mea-

sured for the cells, illustrate the relationship between molecular level phenomena and macroscopic properties of the cells + matrix. This study also suggests that significant cell-matrix interactions are observed long after completion of the principal compaction process and reflect the interplay between matrix deposition and degradation. The sponge-based stromal equivalent may permit us to explore and manipulate the relationship between: (1) cellular environment and cell phenotype; and (2) molecular level expression with macroscopic properties of the cell populated matrix.

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