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Biomechanical and Optical Characteristics of a Corneal Stromal Equivalent[1]

Cell matrix interactions are important in understanding the healing characteristics of the cornea after refractive surgery or transplantation. The purpose of this study was to characterize in more detail the evolution of biomechanical and optical properties of a stromal equivalent (stromal fibroblasts cultured in a collagen matrix). Human corneal stromal fibroblasts were cultured in a collagen matrix. Compaction and modulus were determined for the stromal equivalent as a function of time in culture and matrix composition. The corneal stromal fibroblasts were stained for α-smooth muscle actin expression as an indicator of myofibroblast phenotype. The nominal modulus of the collagen matrix was 364±41 Pa initial and decreased initially with time in culture and then slowly increased to 177±75 Pa after 21 days. The addition of chondroitin sulfate decreased the contraction of the matrix and enhanced its transparency. Cell phenotype studies showed dynamic changes in the expression of α-smooth muscle actin with time in culture. These results indicate that the contractile behavior of corneal stromal cells can be influenced by both matrix composition and time in culture. Changes in contractile phenotype after completion of the contraction process also indicate that significant cellular changes persist beyond the initial matrix-remodeling phase. [DOI: 10.1115/1.1589773]

Introduction

Postoperative changes in corneal curvature and the development of corneal haze are the two principal problems associated with refractive surgery [1]. The wound healing response of the cornea is also a significant factor in the outcome of penetrating keratoplasty (corneal transplantation) [2]. A recent study determined that fibroblast-mediated growth of the stroma was responsible for the refractive instability observed after refractive surgery [1]. Specifically, stromal thickening within the photoablation center resulted in myopic regression. Other studies have determined that the haze observed after refractive surgery results from permanent phenotypic changes in the stromal fibroblasts [3–5]. As such, characterizing the wound healing response of the cornea, specifically, the contractile interactions between cell and matrix is important in developing new methods to deal with poor outcomes for these procedures.

A variety of different approaches have been pursued to model and characterize factors that influence the wound healing response for stromal fibroblasts. Specifically, factors that produce the phenotypic changes observed during wound healing are of interest. Masur and colleagues showed that the fraction of stromal fibroblasts expressing myofibroblast phenotype was inversely proportional to the density of cells in monolayer culture [6]. Other studies have examined the role of soluble factors such as TGF-β [7] and IL-1 [2] in inducing the myofibroblast phenotype for monolayer culture of stromal fibroblasts.

Other investigators have examined cell-matrix interactions in culture as a model of wound healing. Roy and colleagues [8] observed that the force exerted by corneal fibroblasts during migration across the surface of a collagen matrix was minimal and most of the force generation resulted from the contractility of non-mobile cells. Other investigators have examined compaction of the matrix resulting from stromal fibroblasts cultured in a collagen gel. Specifically, fetal calf serum was observed to enhance the contraction of the matrix [9–12]. Hepatocyte growth factor, platelet derived growth factor and epidermal growth factor are also associated with increasing contraction of the matrix for stromal fibroblasts cultured in a collagen gel [9,13].

Recent studies by Freyman and colleagues [14–16] showed that contractile force and cell phenotype are influenced by the compliance of the matrix. Therefore, it is critical to consider the mechanical properties of the matrix that is used for the *in vitro* study of corneal wound healing. Collagen gels are considerably more compliant than the native stromal matrix, and the initial compliance of the gels cannot easily be altered [17]. In addition, little has been done to characterize biomechanical and optical properties of these cell+matrix composites and connect those macroscopic properties to molecular level characteristics.

Previous experiments in our lab have used a native fibrillar collagen sponge matrix to study the behavior of human stromal fibroblasts alone and co-cultured with epithelial and endothelial cells [18]. 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. In general, these studies showed evidence of a normal wound healing response occurring within this three-dimensional collagen matrix. The current series of investigations provide more information on the biomechanical and optical characteristics of the stromal equivalent (cells+matrix). Specifically, these studies looked at the changes in the modulus and transparency of the stromal equivalent as a function of time in culture. Furthermore, we have correlated these macroscopic properties with the expression of α-smooth muscle actin as a marker for fibroblast phenotype. These properties are important because they allow: (1) the determination of the factors that influence the biomechanical and optical properties of the matrix; (2) direct comparison of the properties of the stromal equivalent with native tissue; and (3) a connection between the molecular and macroscopic properties in the stromal equivalent.

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Materials and Methods

Cell Isolation. Human tissue unsuitable for transplantation was obtained from the Minnesota Lions Eye Bank with approval of the local Institutional Review Board. Corneas were excised from whole globes and corneal cells extracted according to published methods [19]. Stromal fibroblasts were isolated from corneas stripped of both endothelium and epithelium. The corneas were placed in a solution of collagenase (0.30 mg/mL; Sigma; St. Louis, MO) overnight at 37°C. The stromal fibroblasts were washed and resuspended in fibroblast culture medium consisting 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 (Gibco; Grand Island, NY). Cells were plated in T150 tissue culture flasks at a seeding density of 8900 cells/cm^2 prior to culture on sponges and gels. The cells used for these studies were between passages 1 and 5.

Collagen Sponge Preparation. Collagen sponges were prepared from Bovine type-I dermal collagen, as described in more detail in a previous publication [18]. Collagen dispersions of 0.5% w/v were prepared from the ground collagen by slow blending of the collagen/H_2O+HCl mixture (pH 3.0) at 4°C for 1 min followed by exhaustive de-aeration. The dispersion was poured into a dish or pan, lyophilized at −30°C, and dehydrothermally (DHT) cross linked in a vacuum oven at 110°C in 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. Sponges prepared with the glycosaminoglycans, chondroitin sulfate (CS) (Sigma; St. Louis, MO) and hyaluronic acid (HA) (Lifecore Biomedical, Inc.; Minneapolis, MN), were added to the collagen matrix in a 1:10 weight ratio and 1:5 weight ratio, respectively, before being dispersed in the hydrochloric acid solution as per previous studies [20,21].

Cells Cultured on Collagen Sponges. Stromal fibroblasts were cultured in six-well culture plates (Costar, Cambridge, MA) on collagen sponges. Collagen sponge pieces of approximately 14 mm in diameter and 1.2 mm thick were prepared as described above and seeded at a density of 5×10^4 cells/cm^3. All collagen sponges were hydrated with 150 μL of fibroblast culture media prior to seeding of cells. The cells were seeded in the center of the porous side of the collagen sponge and allowed to sit at 37°C for 1–2 h to facilitate attachment of cells. Then the stromal equivalent was supplemented with 1 ml of fibroblast culture media. All cultures were fed with 4 mL of fresh media 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 transparency, contraction and cell phenotype as described below.

Cells Cultured in Collagen Gel. Collagen gels were prepared from Vitrogen 100 (Cohesion Technologies; Palo Alto, CA) with an initial collagen concentration of 3 mg/ml. A 10X 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 collagen solution was seeded with stromal fibroblasts at a density of 5×10^4 cells/cm^3, cast into wells of a 24-well plate at a thickness of 1.2 or 1.7 mm and placed at 37°C to initiate gelation.

Mechanical Testing. In order to determine the modulus, unconfined stress-relaxation tests were performed on the stromal equivalent using an 895 Micro-Bionix Test System (MTS Systems Corporation; Eden Prairie, MN). The sample was placed between two impermeable plates and submerged in phosphate buffered saline solution at room temperature. A small compressive strain of approximately 15% was quickly applied to the sample. This constant strain was maintained for no more than one hour while the force exerted by the sample was recorded.

An effective modulus for the samples was found by dividing the nominal stress by the nominal strain as shown in Eq. (1). The stress and strain values used for finding the modulus were taken at later time points when the system can be considered to be in an equilibrium mechanical state and all interstitial flow effects are gone. The collagen sponges reached a final equilibrium state by 50 min and the collagen gels reached a final equilibrium state by 5 min.

$$\text{Modulus (Pa)} = \frac{\sigma}{\varepsilon} = \frac{\text{Force}(N) \big/ \text{Area (m}^2\text{)}}{\Delta\text{Thickness (mm)} \big/ \text{Thickness (mm)}}. \tag{1}$$

Quantification of Contraction. Images of the stromal equivalent were taken at various time points during the 25-day culture period using a Javelin Ultrachip Hi Res charge-coupled device (CCD) camera while still in sterile culture. Images obtained were then analyzed using NIH Image to determine the projected surface area of the sample as a measure of lateral matrix contraction. Control studies showed that collagen gels or sponges without cells did not exhibit changes in cross-sectional area with time.

Quantification of Transparency. The fraction of light transmitted (F_T) through the cornea has traditionally been measured as a method for determining the amount of light scattering in the cornea. Previous studies had demonstrated that the fraction of light transmitted through a normal transparent cornea is a function of wavelength (0.98 for red light (700 nm) and 0.90 for blue light (400 nm)) [22–25]. We developed a system to measure light transmission through the cells+matrix while allowing the system to remain in a sterile culture environment. The samples were viewed through an inverted microscope (Nikon Eclipse T200) equipped with narrow band pass filters at 400±20 and 700±10 nm. Images obtained from a camera mounted on the microscope were analyzed with Image Pro Plus software to obtain the optical density of the sample. The index of light transmission was defined as the fraction of the optical density of the image through the sample divided by the optical density of the media alone.

Comparing the light transmission through two freshly isolated rabbit corneas validated this method of transparency measurement. All animals were treated according to the standard ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One cornea was placed in fibroblast culture media and the other in PBS. The fraction of light transmitted through these samples was compared to the values obtained by Farrell and his colleagues for wavelengths tested [22]. For the cornea suspended in media, the values obtained for light transmission at both 400 and 700 nm are not statistically different than the cited values ($p>0.1$). For the cornea suspended in PBS, the 700-nm value is not statistically different than the cited value ($p>0.1$); however, the 400 nm value is slightly lower ($p=0.01$).

Histology/Immunohistochemistry. The stromal equivalents were prepared by standard histology techniques [26]. Samples were first drop fixed in a glutaraldehyde fixative for less than 24 h (1% glutaraldehyde solution in distilled water buffered to pH7), rinsed thoroughly to remove all glutaraldehyde and stored in 10% neutral buffered formalin [27]. The samples were then embedded in paraffin, sectioned on an American Optical (Buffalo, NY) microtome at 6 μm, mounted on slides, and stained with Hematoxylin and Eosin (H&E), which stains the cell nuclei dark blue/purple and collagen pink.

For those fixed paraffin embedded sections stained for α-smooth muscle actin expression, sections were deparaffinized, rehydrated, placed in a citrate buffer (pH 6.0) for 20 min at 37°C and then permeabilized in 0.1% Triton X (Sigma; St. Louis, MO) in PBS. Samples were immersed in 10% normal sheep serum to block nonspecific binding and then incubated with the mouse monoclonal primary antibody to α-smooth muscle actin (Sigma; St. Louis, MO). Sections were then incubated with FITC-labeled sheep anti-mouse IgG (Sigma; St. Louis, MO), rinsed and cover slipped with anti-fade glycerol. Samples were photographed immediately with a SPOT camera (Diagnostic Instruments; Sterling Heights, MI). Rat aortic smooth muscle cells grown on cover slips were used as a positive control for α-smooth muscle actin, and cover slips stained with secondary antibody only were used as a negative control. A cell was specified as positive for α-smooth muscle actin if any stress fibers stained positive in the cell.

Results

Biomechanical Properties. The compressive stress as a function of time for a stromal equivalent showed an increase in stress applied with time followed by a decline over approximately 60 min. The modulus for the collagen matrix based on Eq. (1) was 364 ± 41 Pa. A similar behavior (stress as a function of time) was observed when the matrix was seeded with cells and cultured (Fig. 1). When analyzed for the modulus, a decrease in modulus was observed over the next 2 to 3 days (post-seeding) followed by a slow increase in the modulus over the 21-day time period studied (Fig. 2). The modulus at 21 days was approximately 177 ± 75 Pa, which is still considerably lower than the initial modulus measured prior to seeding of the matrix with cells.

Similar studies were performed on the stromal equivalents composed of collagen gel with stromal fibroblasts. A 15% strain value was applied but the total duration of the compression testing was approximately 10 min (in contrast to 60 min for the collagen sponge). Our initial studies indicated that collagen gels reached an

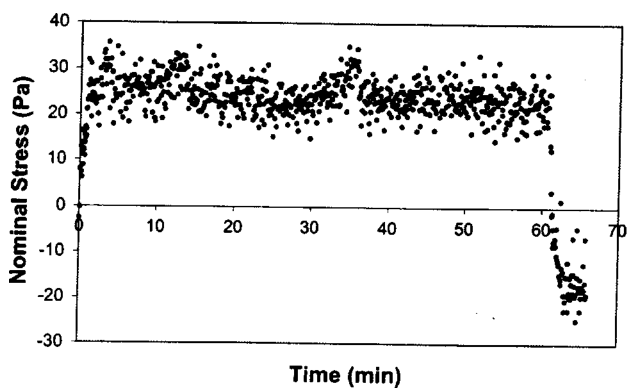

Fig. 1 Compressive stress as a function of time for a stromal equivalent (collagen sponge matrix with cells) after 7 days in culture. The sample was loaded at a 15% strain rate.

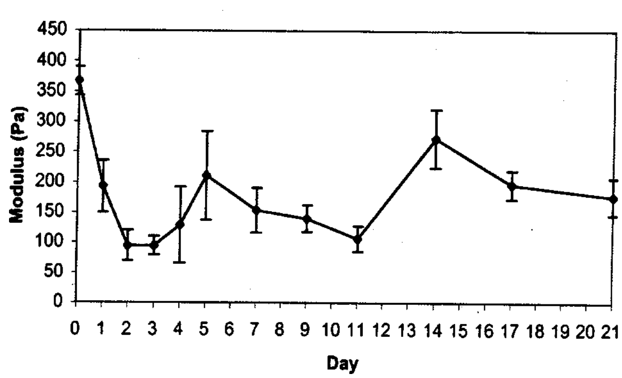

Fig. 2 Modulus as a function of time in culture for a stromal equivalent (collagen sponge matrix). *N*≥7 for day 0, 7, 14, and 21. For all other days, *n*=2.

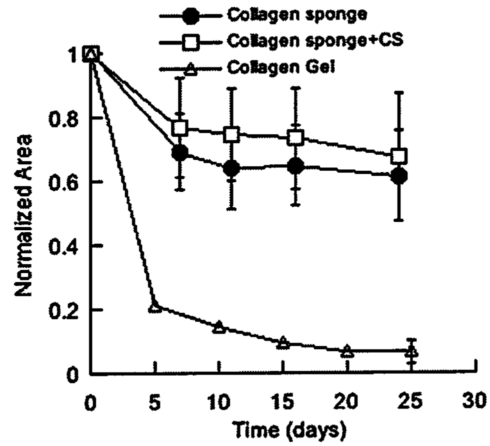

Fig. 3 Normalized projected area of stromal equivalents as a function of time and matrix composition (collagen sponge matrix). *N*≥6.

equilibrium state more rapidly than collagen sponges. The modulus for the stromal equivalent with collagen gel was roughly an order of magnitude smaller than those observed for the collagen sponge (10.3 ± 12.9 Pa at day 7 and 11.6 ± 14.7 Pa at day 14 in culture). The variability of the compressive strain tests for the stromal equivalent composed of a collagen gel was significantly higher than the stromal equivalent composed of the collagen sponge.

We were also interested in the compaction of the matrix with time as an indicator of matrix remodeling. The projected surface areas of the stromal equivalent decreased with time in culture (Fig. 3). Collagen gel samples contracted to 7 ± 2% of their initial area by approximately day 15 in culture. Collagen sponges contract to 63 ± 9% of their initial area by day 15 in culture, whereas collagen sponges augmented with chondroitin sulfate contracted to approximately 73 ± 7% of initial area in the same time period. Collagen gels contracted more than the collagen sponges with or without chondroitin sulfate ($p<0.0001$).

Optical Properties. Concurrent with the biomechanical characterization, we also measured its transparency. For stromal fibroblasts cultured in a collagen matrix, there was a decrease in the fraction of light transmitted as a function of time in culture (Fig. 4). The fraction of light transmitted through collagen gels is statistically significantly less than that through either the collagen sponge alone or the collagen sponge augmented with chondroitin sulfate at both 400 nm ($p=0.01$) and 700 nm ($p<0.005$). The collagen sponge augmented with chondroitin sulfate allows more light transmission than the collagen sponge alone at both 400 nm ($p=0.11$) and 700 nm ($p=0.06$). A comparison of light transmission values at day 24 in culture reveals that the addition of chondroitin sulfate or hyaluronic acid has a significant effect on the light transmission properties of the tissue (Table 1). Specifically, the collagen sponges augmented with proteoglycans increased the fraction of light transmitted to approximately 50% of a freshly excised rabbit cornea and ten times more than that through a collagen gel.

Cell Phenotype. As described in the Introduction, the phenotype of stromal fibroblasts in monolayer culture has been shown to be a function of the density of culture. As such, we were interested in examining the cell content of the stromal equivalent as a function of time in culture. The total number of cells seeded initially was approximately $1.72\times10^5\pm3.6\times10^4$ cells. The total DNA isolated from the stromal equivalent increased to almost $4.4\times10^5\pm3.5\times10^4$ cells after one week in culture. The total number of cells remained constant up to 21 days in culture (Fig. 5).

The fraction of cells expressing α-smooth muscle actin changed

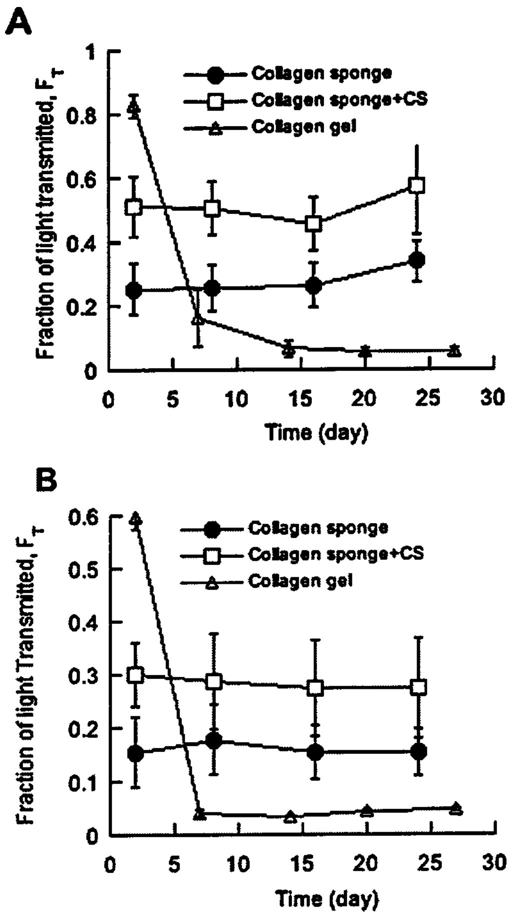

Fig. 4 (*a*) **Fraction of light transmitted, F_T, at 70 nm; and** (*b*) **fraction of light transmitted, F_T, at 400 nm as a function of time and matrix composition for the stromal equivalent** (**collagen sponge matrix**). $N \geq 6$.

Table 1 Transparency of stromal equivalent (**stromal fibroblasts in the specified matrix**) **at day 24 compared to excised rabbit cornea**

Matrix	F_T [a]
Excised rabbit cornea	0.997
Collagen sponge+chondroitin sulfate	0.570
Collagen sponge+hyaluronic acid	0.400
Collagen sponge	0.337
Gel	0.056 [b]

[a] Fraction of light transmitted
[b] Day 27

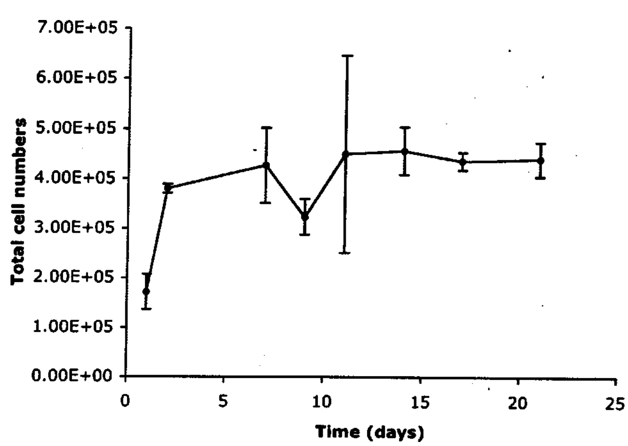

Fig. 5 DNA content of the stromal equivalent (**collagen sponge matrix**) **as a function of time.** $N=3$.

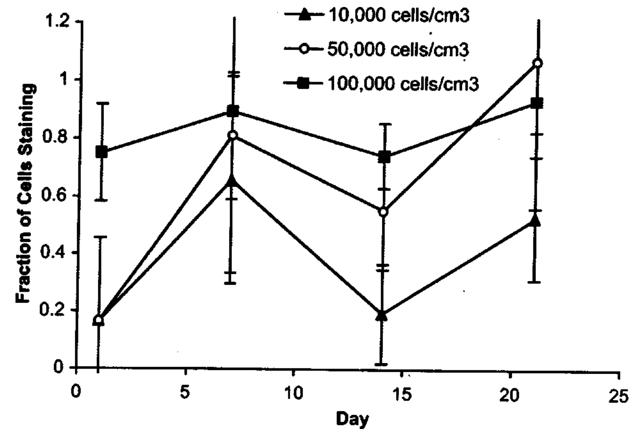

Fig. 6 Fraction of stromal fibroblasts expressing α-smooth muscle actin as a function time in culture for a stromal equivalent (**collagen sponge matrix**)**. Three different initial seeding densities were studied: 1×10^4, 5×10^4, and 1×10^5 cells/cm^3.** $N \geq 4$.

significantly with time in culture for the stromal equivalent (collagen sponge matrix) (Fig. 6). Prior to seeding in the collagen matrix, a small sample of the cells in monolayer culture was stained for the presence of α-smooth muscle actin. Approximately 3% of the cells stained positive for α-smooth muscle actin. After seeding and culture in the matrix, the fraction of cells staining positive for α-smooth muscle actin increased from day 0 to day 7 and then decreased again from day 7 to 14. The fraction of cells staining positive for α-smooth muscle actin was influenced as well by the initial seeding density of the cells. The percentage of cells expressing α-smooth muscle actin for an initial seeding density of 5×10^4 cells/cm^3 varies with time (day 1 versus day 7, 21, $p<0.04$ day 14 versus day 21, $p=0.03$) over the time period studied. For all initial seeding densities combined, the fraction of cells expressing α-smooth muscle actin at day 1 and day 7 ($p=0.03$), day 1 and day 21 ($p=0.02$), and day 14 and day 21 ($p=0.03$) are all distinctly different. At day 14 in culture, the fraction of cells that expressed α-smooth muscle actin was significantly higher for a seeding density of 1×10^5 when compared to 1×10^4 cells/cm^3 ($p=0.004$).

Discussion

Biomechanical Properties. The modulus of the stromal equivalent measured in this investigation ranged from 94 to 366 Pa depending upon the time in culture. The modulus of the collagen gel was one order of magnitude less and exhibited large variability. In contrast, the bulk modulus of the native cornea ranges from 5×10^5 Pa to 2.1×10^6 Pa [28,29]. Thus, the stromal equivalents studied in this investigation had a much lower modulus than native tissue although the stromal equivalent with a collagen sponge matrix was closer in its properties than the collagen gel. The rapid drop in the modulus after short periods of culture (2 to 3 days) must reflect a rapid degradation of the matrix resulting from interactions with the cells.

The compaction of the stromal equivalent as a function of time observed in this study is similar to that observed by other investigators [9–11,13]. The contraction of a collagen sponge was significantly less than a collagen gel for the same initial number of stromal fibroblasts. The measurements of both the modulus and matrix compaction provide insights into the matrix remodeling observed during *in vitro* culture of the cells. Specifically, the compaction of the matrix observed during roughly the first week of the experiment would seem to produce an increase in the modulus of the matrix resulting from an increase in local collagen concentration [10]. However, the actual modulus is observed to decrease over that same period of time followed by a slow increase. The transformation from a keratocyte to repair fibroblasts or myofibroblast is associated with an increase in production of matrix metalloproteinases (MMP's) [2]. Thus, proliferation and migration of stromal fibroblasts with a repair fibroblast phenotype may result in the observed decrease in modulus due to enhanced matrix degradation. The observation by Roy and colleagues that migrating cells exert minimal forces on the matrix during migration suggests that the compaction of the matrix observed resulted from contraction of stationary stromal fibroblasts exhibiting myofibroblast phenotype [8,30]. Thus, the interactions appear to be more complex than previously thought. Additional studies will be needed to examine in more detail the interplay between cellular proliferation, migration, matrix remodeling (deposition, degradation, and compaction) and the resulting biomechanical properties. This type of experimental system may provide an important opportunity to study not only the evolution of biomechanical properties but also the relationship between those properties and cellular behavior in an *in vitro* environment.

Transparency. The role of the cornea in the transmission of light to the anterior portion of the eye has led to interest in the optical properties of the stromal equivalent used in this investigation. There has been limited work quantifying transparency *in vitro* and the factors that influence the transparency of a corneal construct. A recent study by Ventura and colleagues described methods for quantifying transparency of native corneas *in vitro* [31]. Typically, investigators have described qualitatively differences in transparency of corneal cells cultured in a collagen matrix (for example, see Ref. [32]). The results of this investigation indicate that the transparency of the stromal equivalent with a collagen sponge matrix is more transparent than stromal equivalent with a collagen gel matrix. In addition, by supplementing the collagen sponge matrix with proteoglycans, we have shown that we can increase light transmission to about 50% of that exhibited by a freshly isolated rabbit cornea (Table 1). We found an inverse relationship between the contraction of the collagen matrix and the resultant light transmission properties of the matrix. We have also observed that the addition of a confluent layer of corneal epithelial cells improves the transparency of the cells cultured in a collagen sponge (data not shown). These results suggest that various methods can be used to modulate the optical properties of the stromal equivalent. Further work is needed however to determine the specific components of the transparency and the influence of changes in culture and matrix on transparency. For example, Jester and colleagues recently observed a significant cellular component to the transparency of the native cornea [3]. Specifically, they observed that specific ''crystallins'' produced in the cells were associated with higher transparency of stromal fibroblasts. Other investigators have looked at the role of matrix organization [23] on transparency of the matrix. These studies will be essential in the design of stromal equivalents with suitable transparency.

Phenotype. Masur and colleagues observed that when cultured under low density, a greater percentage of stromal fibroblasts expressed α-smooth muscle actin [6]. We observed a similar trend for cells in monolayer culture: an increasing percentage of cells expressing α-smooth muscle actin with decreasing density (data not shown). The relationship between cell density and α-smooth muscle actin expression did not change if the cells were grown on a collagen film. In contrast, when cultured in a three-dimensional matrix, this relationship did not hold true. A higher seeding density resulted in a higher percentage of cells expressing α-smooth muscle actin during the initial culture period. This outcome agrees at least qualitatively with Roy and colleagues who found that the contraction of the matrix did not result from migrating cells [8,30]. The higher initial cell density would imply that a smaller number of cells had to migrate in order to infiltrate the matrix. A larger percentage of cells would be involved in compacting the matrix, which requires a myofibroblast phenotype. The changes in the percentage of cells expressing α-smooth muscle actin with time in culture is consistent with the observation of ongoing matrix remodeling beyond the initial matrix compaction period. Specifically, the modulus continues to change beyond the initial compaction period. Taliana and colleagues [11,12] observed differences in the expression of α-smooth muscle actin for stromal fibroblasts cultured in a collagen gel during a 30 day culture period. One potential explanation may result from the upregulation of MMP production observed in wounded corneas [2]. If matrix degradation is accelerated in three-dimensional *in vitro* culture, the stromal fibroblasts may be degrading, depositing and then compacting the extracellular matrix on a cyclical basis after the initial compaction phase has been completed. Further studies may focus on a broader range of phenotypic markers and micromechanical environment as a function of time in culture and culture environment.

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