31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Characterizing the Effects of Aligned Collagen Fibers and Ascorbic Acid Derivatives on Behavior of Rabbit Corneal Fibroblasts

Donna Phu and Elizabeth. J. Orwin, *IEEE, Members*

***Abstract*—The cornea is responsible for functional optical activity of the mammalian eye, as it must remain transparent in order to focus light onto the retina. Corneal disease is the second leading cause worldwide of vision loss [1]. Human donor tissue transplantation in the cornea is associated with problems such as immunorejection and recurring graft failures [1]. Tissue engineering offers a promising alternative to using human donor tissues in treating corneal diseases. A viable tissue-engineered cornea must be mechanically resilient to protect the fragile intraocular components of the eye, and optically transparent to refract light onto the retina. In the native cornea, transparency is maintained by both the cells in the stromal layer and the high organization of the extracellular matrix (ECM). This study aims to combine the effects of aligned collagen fibers and ascorbic acid derivatives to control corneal fibroblast behavior to not only express the appropriate proteins, but also to deposit aligned, small diameter collagen fibers that resemble the highly organized structure of the natural ECM. Results from this study suggest that the combined effect of an aligned scaffolding material and ascorbic acid supplements can create a cell-matrix construct that both downregulates expression of the light scattering protein a-smooth muscle actin (α-sma) and supports an increased number of cell layers.**

I. Introduction

ANY injury or malady that causes opacification of the cornea can lead to visual impairment, or even blindness [2]. The only treatment for corneal diseases is limited because it requires the transplantation of substitute donor tissues, and human corneas are scarce [3]. In response to these limitations, many research groups worldwide have focused on developing a tissue-engineered (TE) corneal equivalent. A functional corneal equivalent must be mechanically resilient so that it can support cell growth and maintain its structure during transplantation. Most importantly, it must be able to control the cellular responses that are responsible for maintaining the optical transparency observed in the native human cornea.

The corneal stroma contains keratocytes that express corneal crystallins, such as aldehyde dehydrogenase class 1 (ALDH1) and transketolase (TKT), that help control the transparency of the cornea by matching the surrounding refractive index of the ECM [4] Upon injury, various paracrine cytokines stimulate the keratocytes to lose their quiescence and transition into fibroblastic repair phenotypes. Myofibroblasts are characterized by a decrease in levels of corneal crystallins and increased expression of the contractile protein, alpha-smooth muscle actin (α-sma) [5],[6]. An increased expression of α-sma decreases corneal transparency *in vivo* [5]. Therefore, it is essential that the cells in the cornea equivalent express the correct phenotype to reduce cell-induced optical haze.

Another key function of the corneal stromal keratocytes is the production and organization of the collagen fibers in the extracellular matrix (ECM) [7]. Proteoglycans are also known to control the organization of collagen fibers [8]. In the native cornea, the stroma is comprised mainly of small diameter collagen Type I nano-fibers that are arranged into sheets of aligned fibers stacked at various angles on top of one another [9]. The spatial arrangement of the fibers in the ECM is thought to contribute to the corneal transparency through destructive interference of the light passing through cornea [9].

Many studies have shown that culturing rabbit corneal fibroblasts (RCFs) in media supplemented with stable derivatives of ascorbic acid (AA) derivatives promotes greater cell stratification and proliferation, and collagen deposition [10]-[12]. Guo *et al*. found that culturing human corneal fibroblasts in glucopryranosyl-L-ascorbic acid (G-Asc) can stimulate both the stratification of corneal fibroblasts into multiple layers and the production of ECM, which are two desirable behaviors for creating thicker constructs [12].

This study looks at the combined effect of an aligned collagen substrate and media supplemented with AA to create a viable corneal construct that will not only support proliferation and stratification of cornea fibroblasts, but will also suppress the light-scattering phenotype. The results indicate that protein expression can be controlled with the alignment of collagen fibers and cell stratification and collagen deposition can be promoted with ascorbic acid.

II. Methodology

A. Preparation of Collagen Fiber Scaffolds

Aligned and unaligned collagen scaffolds were prepared via electrospinning, as described previously (Wray and Orwin, 2009). Briefly, collagen (type I acid soluble from calf skin, Elastin Products, Owensville, MO) was dissolved in acetic acid (>99%, EMD, San Diego, CA) at 5 wt% and stirred overnight at 25ºC. The solution was loaded into a 2 ml glass syringe. Applied voltages ranged from 6.0 kV to 10.0 kV at flow rates ranging from 0.06 – 0.20 ml/hr. Unaligned fibers were collected on 15 mm round glass coverslips (Warner Instruments, Hamden, CT) and aligned fibers were collected on a dual plate arrangement comprised of two copper strips attached to a quartz glass slide

Manuscript received April 7, 2009. This work was supported in part by Robert and Mary Jane Engman Family and Howard Hughes Medical Institute.

E. J. Orwin is with the Department of Engineering, Harvey Mudd College, Claremont, CA 91711 USA (phone: 909-621-8572; e-mail: orwin@hmc.edu).

D. Phu was with the Department of Biology, Harvey Mudd College. She is now with the Department of Biomedical Engineering, Duke University, Durham, NC 27705 USA. (e-mail: dp79@duke.edu).

(McMaster Carr, Santa Fe Spring, CA) with Gluseal (Glustitch, Gulf Road Point Roberts, WA). All electrospun scaffolds were crosslinked before cell seeding using methods described previously [13]. Scaffolds were placed in desiccator for 3 days with anhydrous calcium sulfate (Drierite; W.A. Hammond Drietie Company, Xenia, OH) and approximately 30 mL of 25% glutaraldehyde (EMD, Hitfield, PA). After 3 days, scaffolds were soaked in sterile 0.1% glutaraldehyde for one hour. To remove excess glutaraldehyde, scaffolds were soaked in 0.2 M sterile ethanolamine (Sigma; St. Louis, MO) solution for two hours. Each scaffold was rinsed 3 times with sterile milli-Q water and once with 1X PBS under UV light.

B. Cell Seeding and Culture

For this experiment, P3-P6 rabbit corneal fibroblasts (RCF) were seeded at a density of 110,000 cells/cm^2 and cultured for 2 weeks on scaffolds containing aligned and unaligned collagen fibers and standard tissue culture plates. Cells were fed with normal media (NM) (Ham's nutrient mixture F-12 (DMEM-F12; Sigma, St. Louis, MO), 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1% antibiotic/antimycotic (500 units penicillin, 0.5 µg streptomycin, 1.25 µg amphotericis B; JR Scientific, Woodland, CA) in Dulbecco's modified Eagle's medium) or NM supplemented with ascorbic acid (AA) (NM with 0.50 mM glucopryranosyl-L-ascorbic acid (Wako Chemicals, Richmond, VA)) or NM supplemented with transforming growth factor (TGF-β) every other day. TGF-β is used as a positive control for α-sma expression.

C. Confocal Immunofluoresence (IF) Microscopy Preparation and Imaging

After two weeks of culture, RCFs were sacrificed for immunoflourescence (IF) staining. Samples were fixed in 4% paraformaldehyde (VWR; West Chester, PA) for 20 minutes and permeabilized with 0.1% Triton-X (Sigma, St. Louis, MO) for 20 minutes. The samples were then incubated on a shaker in 10% goat serum (Sigma, St. Louis, MO) to block any nonspecific binding sites. The samples were then sequentially stained with a 1:200 dilution in 10 % goat serum of primary antibody monoclonal anti-human α-sma, mouse IgG1 isotype (Sigma, St. Louis, MO, a 1:400 dilution in 10% goat serum of secondary antibody rhodamine-conjugated goat anti-mouse IgG subclass 2a (Jackson Immuno Research, West Grove, PA), and a 1:2000 dilution in 10% goat serum of a nuclear stain Sytox Green, Invitrogen, Grand Island, NY). All samples were imaged with a Zeiss 510 confocal microscope (Harvey Mudd College, Biology Department). For each culture condition, three samples were imaged and three images were taken of each sample to obtain nine images per culture condition. The percentage of cells expressing α-sma was counted by three different individuals and all data was pooled together. A 3D scan of each cell construct was taken and the number of cell layers was counted. A two-way ANOVA was performed to assess significance of media type and scaffold type. Differences were considered significant for $p < 0.05$.

D. Scanning Electron Microscopy Sample Preparation and Imaging

After IF analysis, the samples were refixed in 10 % NBF overnight and further fixed in 2.5% glutaraldehyde for one hour and in 0.5% osmium tetroxide (2% aqueous solution; EMS; Hitfield, PA) for another hour. The samples were then dehydrated in increasing concentrations of ethanol from 60% to 100% ethanol in 1X PBS. After the dehydration process, the samples were allowed to dry overnight in hexammethyldislazane (HMDS) (EMS). After preparation, samples were mounted onto SEM stubs and sputter coated with a Cressington 108 Auto at 40 mA for 90 seconds. All samples were imaged with a Zeiss LEO 982 field emission scanning electron microscope (Pomona College, Claremont, CA) with an acceleration voltage of 5 kV.

III. RESULTS

A. Cell Stratification

All cell constructs cultured with AA supplemented media exhibited an increased number of cell layers over both constructs cultured in NM and TGF-β. The scaffold type had no effect on cell stratification ($p < 0.01$) (Fig 1). Constructs cultured with NM typically supported 3 cell layers, whereas those cultured in AA supported 4-5 cell layers.

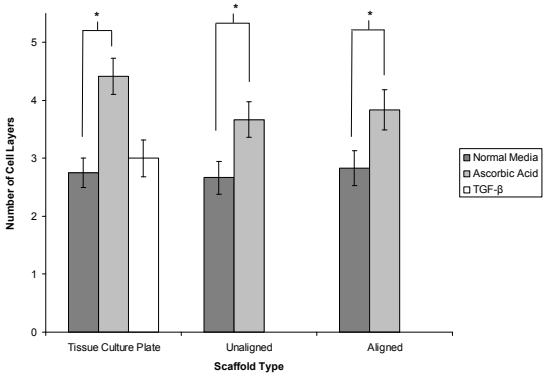

Figure 1. Number of cell layers for constructs grown on aligned, unaligned scaffolds and tissue culture plates with NM or AA assessed using confocal microscopy. Data represents mean ± standard error (*$p < 0.01$).

B. ECM Morphology

The morphology of the ECM deposited by the fibroblasts was assessed using scanning electron microscopy. Images revealed that AA induced cells to deposit more extracellular matrix content, but it was not as fibrous as the matrix laid down in NM samples. Instead, RCFs cultured with AA deposited a more sponge-like matrix. These sponge-like matrix components are indicated with a "ε" in Fig 2. Fibers with diameters ranging from 50-60 nm were observed in NM aligned and unaligned samples and are indicated with a "*" in Figure 2. However, these fibrous regions did not

appear to have any type of organized arrangement as it is found in the native cornea (Fig 2).

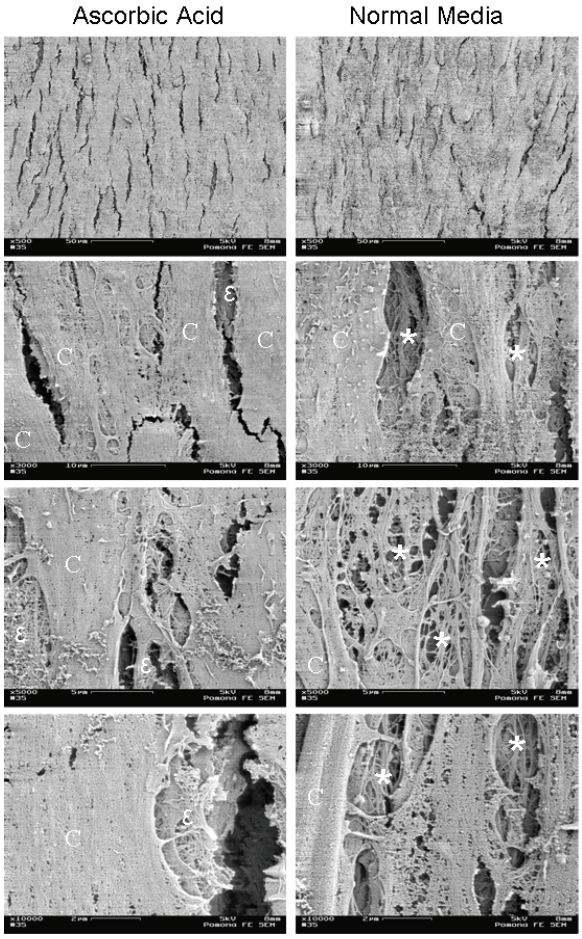

Figure 2. SEM images of fibroblasts cultured on aligned scaffolds cultured with NM (right column) and AA supplemented media (left column) at increasing resolutions. All cell bodies are labeled with "C", sponge-like matrix components are labeled with "ε" and 50-60 nm fibers are labeled with "*".

C. *Levels of α-sma Expression*

Analysis of the IF images revealed that the fibroblasts cultured on aligned fibers expressed significantly less α-sma than fibroblasts cultured on unaligned fibers and tissue culture plates relative to both media types ($p < 0.001$). However, the results revealed that AA upregulated α-sma expression for aligned and unaligned scaffolds ($p < 0.01$) (Fig 3). Representative IF images are shown in Fig 4.

IV. Discussion

A. *Effect of Alignment on Fibroblast Behavior*

As we have found in previous studies in our lab, the alignment of the scaffold significantly downregulates α-sma over unaligned scaffolds and normal tissue culture plates [13]. This is a promising finding as it suggests that an aligned template may provide a viable scaffold that suppress

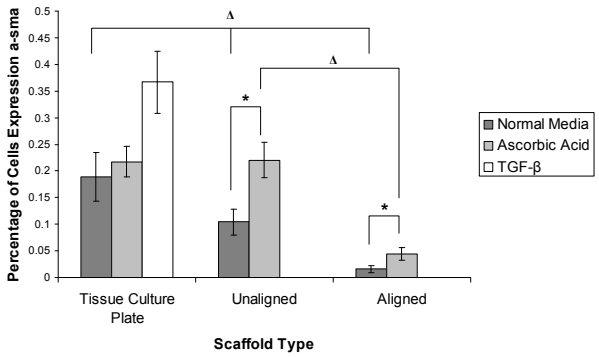

Figure 3. Percentage of cells expression α-sma for constructs cultured on aligned, unaligned scaffolds and tissue culture plates with NM or with AA supplemented media. Data represents mean ± standard error (*p <0.01 and Δp < 0.001).

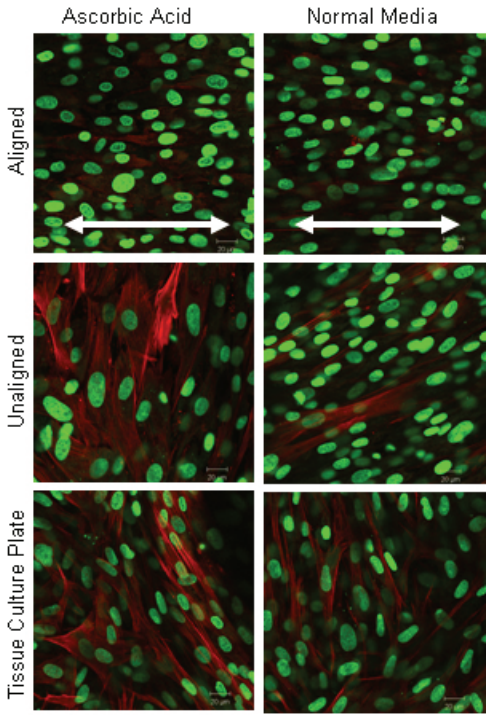

Figure 4. IF images of cell constructs cultured for 2 weeks on aligned (top row), unaligned (middle row), and tissue culture plates (bottom row) with NM (right column) and AA supplemented media (left column). Cell nuclei are stained green and α-sma are stained red. White arrows indicate direction of alignment.

the light-scattering phenotype of fibroblasts for a TE cornea. However, the alignment did not have an effect on promoting cell stratification or increasing matrix deposition. While it is important to control the protein expression of the fibroblasts, a viable cornea equivalent will need to contain cells that are also able to deposit an ECM that will help maintain a transparent phenotype. The highly-organized arrangement of aligned collagen nanofibers are believed to contribute to corneal transparency by reducing destructive interference of light given off of individual fibers, thereby maximizing the amount of light transmitted through the cornea [14]-[15]. In addition, we have shown that aligned fibers downregulate α-

sma, but we cannot conclude that the cells have reverted back to the quiescent phenotype. Therefore, it is necessary to quantify levels of expression of the cornea crystallins, ALDH1 and TKT, in all cell constructs to confirm that the cells have actually reverted back to a transparent phenotype.

B. Effect of Ascorbic Acid on Fibroblast Behavior

AA induced the cells to stratify into more cell layers and lay down more matrix components into the ECM. However, fibroblasts in AA media exhibited higher levels of α-sma expression and the ECM deposited by AA RCFs contained very few regions with smooth fibers of 50-60 nm. This suggests that AA may be able to promote RCFs to increase matrix production, but the RCFs are not able to organize the matrix into an arrangement that will minimize light scatter. This finding contrasts with the studies conducted by Guo *et al*, in which they used transmission electron microscopy (TEM) to show that human corneal fibroblasts were able to deposit parallel arrays of ECM fibrils when cultured with AA in transwells [12]. Future studies will be aimed at imaging cell constructs with a TEM to better assess cell layering and the arrangement of fibers in the ECM as we can only obtain topology information with the SEM.

Crabb *et al.* have shown that the microstructure of the matrix substrate can influence the type of ECM deposited by the fibroblasts [16]. We have started to show how the fibrous templates can promote the formation of a fibrous ECM, but that arrangement is lost when cultured with AA. The studies conducted by Crabb *et al*. identified the sponge-like ECM as fibronectin, which is a protein that is believed to assist in the normal wound healing process in the cornea [16]. This may explain why we observe more sponge-like ECM and higher levels of α-sma in the AA, as both are characteristic of a cornea tissue cell undergoing the wound-healing process. This information can assist in forming a strategy to build a viable TE cornea. First, we can use an aligned scaffold to downregulate α-sma, then culture with AA to increase ECM production, and then turn off the wound healing process with various cytokines to revert the fibroblasts back to the quiescent keratocytes after the constructs have reached a desired thickness [6]. In addition, future studies should incorporate proteoglycans into the cell culture, as these glycoproteins are thought to assist in the formation and organization of collagen fibers deposited by the fibroblasts.

V. CONCLUSION

Our investigations with ascorbic acid derivatives revealed that it was able to increase cell stratification, but it also increased the expression of contractile proteins, such as α-sma, that contribute to corneal haze. It should be noted that while AA did increase the expression of α-sma, the combined effects of AA and aligned scaffolds resulted in lower overall α-sma expression than on tissue culture plates (Figure 3). From our results, we conclude that culturing RCFs on aligned fibers with AA supplemented media provides a promising basis for a viable TE cornea, as the alignment can decrease α-sma and AA can promote cell stratification to expand from monolayer studies to 3-dimensional studies.

ACKNOWLEDGMENT

Thank you to the Harvey Mudd College Biology and Engineering departments. The authors would like to thank Jessica Wen for assisting in the protein quantification analysis.

REFERENCES

[1] Whitcher, J. P., Srinivasan, M., Upadhyay, M. P., 2001. Corneal blindness: a global perspective. Bull. World Health Organ. 79, 214–221.

[2] Ruberti, J. W. and J. D. Zieske. Prelude to corneal tissue engineering – gaining control of collagen organization. *Progress in Retinal and Eye Research*. 27: 547-577, 2008.

[3] Langer, R. and J. P. Vacanti. Tissue engineering. *Science*, New Series, Vol. 260, No. 5110 (May 14, 1993), pp. 920-926.

[4] Jester, J., *et al.* The cellular basis of corneal transparency: evidence for 'corneal crystallins'. *Journal of Cell Science*, 112: 613-622, 1999.

[5] Jester, J., *et al*. Expression of α-smooth muscle (α-SM) actin during corneal stromal woundh. *Investigative Ophthalmology & Visual Science*. 36(5), 1995.

[6] Fini, E. M. Keratocyte and fibroblast phenotypes in the repairing cornea. *Progress in the Retinal and Eye Research*, 18(4):529-551, 1999.

[7] Synder, M., C. Jan, P. G. Bergmanson and M. J. Doughty. Keratocytes: no more the quiet cells. *Journal of the American Optometric Association*, 69(3), 1998.

[8] Michelcacci, Y. M. Collagens and proteoglycans of the corneal extracellular matrix. *Brazilian Journal of Medical and Biological Research*. 36:1037-1046, 2003.

[9] Cen, L., *et al*. Collagen tissue engineering: development of novel biomaterials and applications. *Pediatric Research*, 63(5), 2008.

[10] Chan, D., *et al*. Regulation of procollagen synthesis and processing during ascorbate-induced extracellular matrix accumulation *in vitro*. *Journal of Biochemistry*, 269, 175-181, 1990.

[11] Saika, S. Ascorbic acid and proliferation of cultured rabbit keratocytes. *Cornea*, 12(3): 191-198, 1993.

[12] Guo, X., *et al*. Morphological characterization of organized extracellular matrix deposition by ascorbic acid-stimulated human corneal fibroblasts. *Investigative Ophthalmology & Visual Science,* 48(9), 2007.

[13] Wray, L. S. and E. J. Orwin. Recreating the Microenvironment of the Native Cornea for Tissue Engineering Applications. *Tissue Engineering.* Published online, ahead of print. 2009.

[14] Maurice, D.M. The structure and transparency of the cornea. *J. Physiol*. 136: 263–286, 1957.

[15] Benedek, G.B. Theory of the transparency of the eye. *Appl. Opt*. 10, 459–473, 1971.

[16] Crabb, R., E. P. Chau, M. C. Evans, V. H. Barocas, A. Hubel. Microstructural characteristics of extracellular matrix produced by stromal fibroblasts. *Journal of Biomedical Engineering,* 34(10):1615-1627, 2006.