Online Monitoring the Mechanical Remodeling of Hydrogels by Corneal Fibroblasts

M. Ahearne[1], Alicia J. El Haj[1], S. Rauz[2], and Ying Yang[1]

[1] Institute of Science and Technology in Medicine, Keele University, Stoke-on-Trent, United Kingdom
[2] Academic Unit of Ophthalmology, School of Immunity and Infection, University of Birmingham, Birmingham, United Kingdom

Abstract— **Cell seeded hydrogels provide a useful model for examining cell behavior and matrix remodeling in-vitro. Fibroblasts are capable of manipulating the mechanical behavior of tissues by attaching to their surroundings matrix and applying strain. Any resulting change in the mechanical properties of the matrix can in turn affect the cells behavior. In this study, a non-destructive spherical indentation technique was used to examine the change in mechanical properties of collagen hydrogels seeded with either human corneal fibroblasts or human keratocytes over prolonged culture periods. Cell seeded hydrogels were clamped between two transparent circular rings and a sphere was placed on top of them, causing them to deform. The deformation displacement was measured using a CCD camera system and applied to a theoretical model to calculate the mechanical properties of each hydrogel. The non-destructive on-line nature of this technique enables repeated measurements the same hydrogels at several different time-points thus enabling the change in mechanical behavior of the hydrogels resulting from corneal fibroblasts remodeling to be examined. In this particular study, the effect of different culture medium on the cell behavior was investigated. The spherical indentation technique has also been used to examine the effect that different drugs and chemicals have on the cells ability to remodel of the hydrogels including MMP and actin inhibitors.**

Keywords— **Collagen, Tissue engineering, Cornea, Modulus, Fibroblast.**

I. Introduction

Cell seeded collagen hydrogels have become popular for in vitro modeling of three-dimensional tissues. One of the most interesting applications for these hydrogels is to model wound healing behavior. In the cornea, keratocytes are known undergo phenotypic changes in response to injury. Keratocytes become fibroblastic leading to an increase in extracellular matrix production and matrix contraction. Identifying and understanding these changes has many applications including in examining the corneas response to surgical procedures, injury and disease. It is also important in development treatments to reduce scar formation which can impede vision.

We have developed a spherical indentation system for examining the mechanical characteristics of cell seeded hydrogels over prolonged culture periods [1]. This system allows non-destructive online measurement of the mechanical properties of hydrogels without risk of contamination to the sample or damage to the instrument. To date this system has been used to monitor the change in mechanical properties of collagen hydrogels seeded with human corneal fibroblasts which have undergone UVA-riboflavin treatment [2], been subjected to MMP inhibitors [3] and been manufactured using different collagen and cell concentrations [4]. Here the effect of different culture condition will be investigated to try and determine role cell phenotype plays in matrix remodeling and to investigate if there is a difference in the mechanical characteristics of collagen hydrogels seeded with corneal fibroblasts and those seeded with corneal keratocytes.

II. Materials and methods

A. Materials Preparation

All chemicals used were from Sigma-Aldrich unless otherwise stated. Human corneal tissue was obtained and used to extract corneal fibroblasts. The epithelial and endothelial layers of the cornea were physically removed using tweezers. The remaining corneal stromal tissue was placed into culture flasks from which cells were allowed to migrate onto the flask. Once a confluent layer of the cells was formed, these cells could be used. Collagen hydrogels were produced from rat-tail collagen type-1 (BD Bioscience, Erembodegem, Belgium) using as previously described [2, 3]. Human corneal fibroblasts were suspended inside the collagen hydrogel solution prior to gelation at a concentration of $5x10^5$ cells per hydrogel. Hydrogels without cells were used as a control. The hydrogels were cultured in six-well plates in one of two types of culture media. The first type of culture media promoted a fibroblastic phenotype and consisted of low glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented by 10% fetal calf serum, 1% L-glutamine and 1% antibiotic-antimycotic solution. Alternatively culture media which promoted a keratocyte phenotype [5] which consisted of DMEM/Ham F-12 supplemented with 1mM ascorbic acid, 0.1% insulin and 1% antibiotic-antimycotic solution was also used. The media was changed every 3 to 4 days. This research has received approval from Black Country Research Ethics Committee (06/Q2702/44).

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B. Instrumentation

A sample holder was constructed to suspend the hydrogels during measurement (Fig. 1A). Each hydrogels were clamped between two transparent plastic circular rings of inner diameter 20 mm. The rings were held in place using the stainless steel samples holder shown. Three hydrogels could be held in each sample holder at any one time. Each sample holders was assembled in phosphate buffered saline (PBS) within a large rectangular Petri dish in a Class II laminar flow hood. A PTFE sphere (Insley, Berks, UK) of diameter 4 mm was placed onto the centre of each hydrogel. The weight of the sphere caused the hydrogel to deform. The Petri dish was then placed into an incubator at 37ºC. A multi-LED lamp was placed in the incubator to illuminate the sample.

The resulting deformation on the hydrogel was captured using a home built image acquisition system which consisted of a long-working-distance objective microscope (Edmund Industrial Optics, York, UK) connected to a computer-linked CCD camera (XC-ST50CE, Sony, Japan) which we have previously described in more details [1]. The camera was linked to an image acquisition card (National Instruments, USA) which was used to acquire and process the images. LABView (National Instruments, USA) was used to write a program to acquire images from the camera. An example of a hydrogel deformation image acquired by this system is shown (Fig. 1B). This system allowed images to be recorded of the deformation profile of the hydrogel from outside the incubator through a glass window. The magnification of the system was calibrated with the computer-acquired images of a stage micrometer. Images of the spherically deformed hydrogels were recorded 10 minutes after the sphere was initially placed onto the hydrogel. This was to allow time-dependent deformation to reach equilibrium. The elastic modulus was measured one day after hydrogel formation and every 3 to 4 days subsequently for up to 24 days.

A home-built optical coherence tomography (OCT) system was used to examine the cross-sectional thickness of each specimen at the same examining frequency as the modulus measurement. OCT is a low coherence interferometric technique which measures backscattered light after the light source has passed through a material [6]. This technique allowed non-destructive imaging of the hydrogels cross-sectional thickness to be measured under sterile conditions [7]. A superluminescent diode with a central wavelength of 1310 nm and bandwidth of 52 nm was used as the source of the OCT. The system has an axial resolution of 14 μm in free space and penetration depth up to 2 mm.

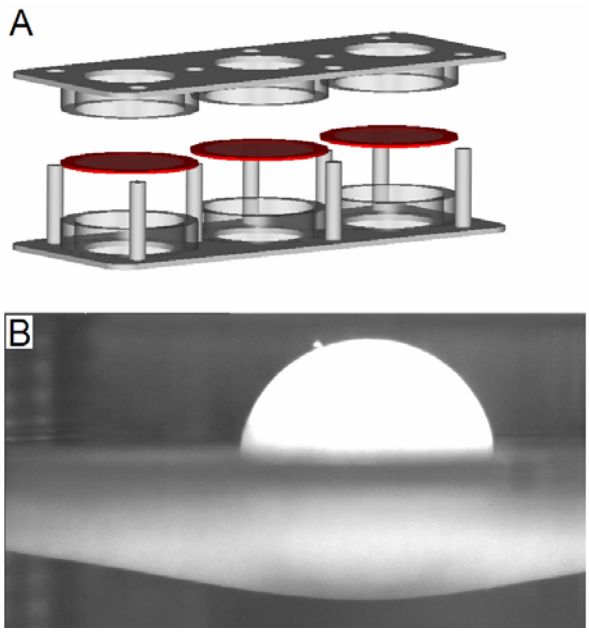

Fig. 1 (A) sample holder and hydrogels; (B) hydrogel deformed by PTFE sphere

C. Theoretical Modeling

The elastic modulus of the hydrogels was calculated from the images using a large deformation model [8]. From the acquired images, the central deformation (δ) of the hydrogel was calculated and substituted into equation 1 to find the elastic modulus (E);

$$6wr = Eh\,(0.075\delta^2 + 0.78r\delta) \quad (1)$$

where h is the hydrogel thickness, r is the radius of the sphere and w is the weight of the sphere. This equation is applicable when the ratio of $a/r = 5$ and $\delta/r \leq 1.7$, where a is the radius of the clamped portion of the hydrogel. This model also assumes that the ratio of thickness to the radius is low and the deformation is large, hence stretching of the membrane dominates over bending.

D. Immunofluorescent Staining

Cell viability was assessed using a live-dead cell double staining kit. The kit contained Calcein-AM and Propidium Iodide which fluorescently stain live cells green and dead cells red respectively. The presence of alpha smooth muscle actin in the cells was assessed using αSMA antibodies conjugated to FITC. The cells were examined using a FV300 confocal microscope (Olympus, Japan)

III. Results

The elastic modulus was measured over a 24 day culture period (Fig. 2). Hydrogels seeded with cells cultured in fibroblast media increased in modulus over the culture period. We have previously found this phenomena which is the result of contraction, remodeling and intrinsic strain applied by cells. Hydrogels seeded with cells cultured in keratocyte media showed little change in modulus over the culture period. Keratocytes have a quiescent phenotype in vivo which our results appear to mimic.

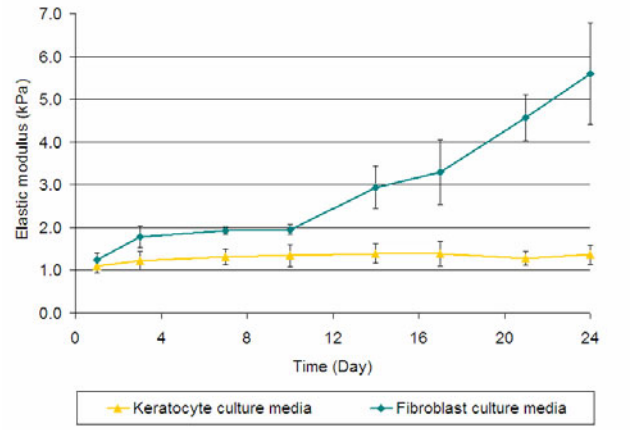

Fig. 2 Change in elastic modulus of cell seeded collagen hydrogels over a 24 day culture period

The change in thickness of the hydrogels was recorded using optical coherence tomography (Fig. 3). Cells in fibroblast media contracted the hydrogel making it thinner while those in the keratocyte media showed little or no change in thickness.

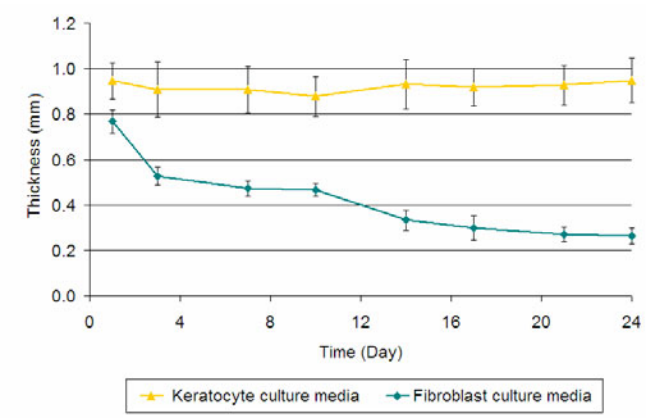

Fig. 3 Change in thickness of cell seeded collagen hydrogels over a 24 day culture period

To test the phenotype of the cells, they were stained for the presence of αSMA which has previously been shown to appear in corneal fibroblasts but not in keratocytes [9]. It can be seen that there was substantially more cells staining positive for αSMA in the hydrogels with fibroblast media compared to those with keratocyte (Fig. 4). This would appear to suggest that the culture media was able to dictate the cell phenotype.

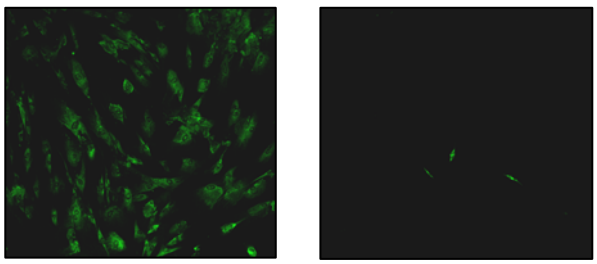

Fig. 4 Images of cells hydrogels cultured in fibroblast (left) and keratocyte (right) media and fluorescently stained for the presence of αSMA

IV. Discussion

It has been shown that altering the culture media in which corneal fibroblasts grow can affect the cell behavior. Cells in the fibroblast media displayed characteristics normally found in the cornea during wound healing such as matrix contraction and remodeling. These characteristics were removed when the cells were grown in keratocyte media. These cells are normally quiescent, which appears to correspond with our findings. The reduction in smooth muscle actin also appears to verify the phenotype change back into a keratocyte phenotype. However keratocytes in-vivo have a dendritic morphology which was not present in our cells. This might suggest that the cells do not fully return to a keratocyte phenotype but maintain some keratocyte and fibroblast characteristics. The matrix environment could also play a role in determining the cell morphology.

Previous studies have shown that altering the media conditions for corneal fibroblasts can results in different cell behaviors. Beales et al., (1999) showed that corneal cells retain their keratocyte characteristics in serum free media [10]. Berryhill et al., (2002) found that bovine corneal cells could partially return to a keratocyte phenotype after being transformed in fibroblasts in vitro [11]. Lakshman et al., 2010 found that by using different culture media, they could induce keratocyte or fibroblast phenotypes from rabbit corneal cells which resulted in different morphologies and contractile behaviors [12]. These results show that cell phenotype can be dictated by culture media conditions.

V. Conclusions

We have demonstrated that corneal phenotype can dictate the mechanical remodeling behavior of corneal stromal cells in three-dimensional matrices. This in-vitro approach to examining cell activity could be useful in determining new methods of reducing or preventing the induction of keratocytes into fibroblast.

Acknowledgment

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References

1. Ahearne M, Yang Y, El Haj AJ et al. (2005) Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications. J R Soc Interface 2:455–463
2. Ahearne M, Yang Y, Then KY et al. (2008) Non-destructive mechanical characterization of UVA/riboflavin crosslinked collagen hydrogels. Br J Ophthalmol 92:268–271
3. Ahearne M, Liu KK, El Haj AJ et al. (2010) Online monitoring of the mechanical behaviour of collagen hydrogels: Influence of corneal fibroblasts on elastic modulus. Tissue Eng C 16:319-327
4. Ahearne M, Wilson S, Liu KK et al. (2009) Influence of cell number and collagen concentration on the mechanical behaviour of collagen hydrogel constructs. Euro Cells Mater J 18:21
5. Musselmann K (2006) Developing culture conditions to study keratocyte phenotypes in vitro. PhD Thesis.
6. Bagnaninchi PO, Yang Y, Zghoul N, et al. (2007) Chitosan microchannel scaffolds for tendon tissue engineering characterized using optical coherence tomography. Tissue Eng 13:323-331
7. Ahearne M, Bagnaninchi PO, Yang Y et al. (2008) Online monitoring of collagen fibre alignment in tissue-engineered tendon by PSOCT. J Tissue Eng Regen Med 2:521-524
8. Ju BF, Liu KK, (20020 Characterizing viscoelastic properties of thin elastomeric membrane. Mech Mater 34:485-491
9. Jester JV, Petroll WM, Barry PA, et al. (1995) Expression of α-smooth muscle (α-SM) actin during corneal stromal wound healing. Invest Ophthalmol Vis Sci 36:809-819
10. Beales MP, Funderburgh JL, Jester JV et al. (1999) Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: Maintenance of the keratocyte phenotype in culture. Invest Ophthalmol Vis Sci 40: 1658-1663
11. Berryhill BL, Kader R, Kane B et al. (2002) Partial restoration of the keratocyte phenotype to bovine keratocytes meda fibroblastic by serum. Invest Ophthalmol Vis Sci 43:3416-3421
12. Lakshman N, Kim A, Petroll MW (2010) Characterization of corneal keratocyte morphology and mechanical activity within 3-D collagen matrices. Exp Eye Res 90:350-359

Mark Ahearne
Keele University
Thornburrow Drive
Stoke-on-Trent
United Kingdom
m.j.ahearne@istm.keele.ac.uk