Substrate Stiffness Influences the Time Dependence of CTGF Protein Expression in Müller Cells

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Abstract

Following ocular trauma and retinal detachment, gliotic changes in the retina may develop over the subsequent month, a process known as PVR (proliferative vitreoretinopathy). There have been no successful therapeutic interventions to inhibit PVR. The protein CTGF (Connective Tissue Growth Factor) has been associated with retinal PVR and other fibrotic diseases of the retina in clinical studies but the mechanistic link between different pathologies and retinal gliosis has not been determined. In addition, CTGF has been previously noted to be associated, in some cases, with YAP/TAZ (Yes-associated protein and Tafazzin protein complex), transcriptional regulatory proteins that change subcellular localization in response to mechanical cues, such as the stiffness of the underlying material. We have previously shown that the mRNA for CTGF is markedly (100-fold) upregulated in retinal Müller cells grown on soft substrates.

In order to evaluate if the mechanism by which mechanotransduction modulating CTGF production in retinal Müller cells involves the YAP/TAZ complex, this study tests the influence of substrate stiffness on the time dependence of CTGF protein expression, as well as subcellular localization of YAP/TAZ using a conditionally-immortalized mouse retinal Müller cell line plated on laminin-coated, polyacrylamide substrates of varying elastic modulus. Changes were assayed using immunohistochemistry and ELISA (Enzyme-Linked ImmunoSorbent Assay).

In retinal Müller cells, the relationship between elastic modulus and the pattern of CTGF protein expression was bimodal, with CTGF levels rising more rapidly for cells on hard substrates and more slowly for cells grown on soft substrates. In addition, nuclear localization of YAP/TAZ corresponded directly to the maximum CTGF expression.

Introduction

Extracellular matrix stiffness plays a critical role in influencing the morphology, gene expression, and differentiation of a wide variety of cell types[1-6]. The elastic modulus of the cells in adult mammalian retina varies between 200 Pa and 1000 Pa, and many pathologic processes of the retina lead to local changes in stiffness of retinal tissue[7]. For example, Bruch's membrane increases in stiffness by an order of magnitude with age[8]. Retinal detachments and laser photocoagulation can also strongly affect the stiffness of the retina[16].

At the molecular level, Connective Tissue Growth Factor (CTGF) expression is directly regulated by Yes-associated protein and Tafazzin protein (YAP/TAZ) complexes[9,10]. Previously, substrate stiffness was shown to alter the nuclear vs. cytoplasmic localization of the YAP/TAZ protein complex leading to the hypothesis that this complex may function to mediate mechanotransduction through a currently undefined pathway[11]. Expression of the protein CTGF was found to be strongly correlated with the localization of the YAP/TAZ protein complex in mesenchymal stem cells and endothelial cells[11]. As CTGF regulates multiple cellular processes, and is markedly upregulated in blinding conditions that involve retinal gliosis, such as proliferative vitreoretinopathy (PVR, retinal scarring)[12], age-related macular degeneration[13,14], and diabetic retinopathy[14,15], from a clinical and translational standpoint, understanding the mechanical influence of expression of CTGF will be helpful in developing novel therapies for retinal disease.

Previously, we demonstrated that Müller cells increase in spread area and change levels of mRNA transcription of CTGF with increased substrate stiffness[16]. Based upon these observations, we used the same conditionally immortalized Müller cell line[17] to determine the changes in YAP/TAZ complex localization and CTGF protein expression of as a function of time and substrate stiffness.

The goal of the present study was to understand how substrate stiffness influenced the time course of CTGF expression and YAP/TAZ localization in a conditionally immortalized Müller glial cell line[17].

Materials and Methods

Cell Culture

Conditionally immortalized Müller cells (ImM10)[17] were plated on laminin-coated polyacrylamide gels[18,19] or glass substrates and maintained in cell culture at 5.5% CO_2. After plating, adherent cells were maintained for 24 days at 39ºC in growth medium under nonimmortalizing conditions [Neurobasal media with 2% FBS, 1x B27 supplement, 2 mM L glutamine, 5 U/ml penicillin, and 5 ug/ml streptomycin (Gibco/Invitrogen)] with media changes at 3-day intervals. For growth under immortalizing conditions, media was supplemented with mouse recombinant IFN-γ (PreproTech; Rocky Hill, NJ) at 50 U/mL and cells were maintained at 33ºC. For maintenance and subsequent analyses, cells were grown on uncoated tissue culture dishes under immortalizing conditions, unless otherwise stated.

Substrate preparation

Glass coverslips were coated with a drop of 3-aminopropyltrimethoxysilane spread evenly on the surface. Each coverslip was then washed with deionized water, placed into foil packets, and autoclaved. Once autoclaved, the coverslips were transferred to a sterile petri dish containing 0.5% gluteraldehyde (70% stock solution, Sigma) in PBS and incubated for 30 minutes at room temperature. This was followed by 5, 10 minute washes in autoclaved deionized water. After the last wash, the coverslips were laid out onto a piece of autoclaved foil in a biosafety cabinet to dry.

Gel Formation

Three different stiffnesses of gel were prepared: 5000, 1000, and 500 Pa, using mixtures of acrylamide and bisacrylamide and elastic moduli were confirmed using atomic force microscopy and rheometry. Mixtures used to generate substrates of each stiffness were as follows: 5000 Pa: 7.5% acrylamide 0.5% bisacrylamide; 1000 Pa: 7.5% acrylamide 0.2% bisacrylamide; 500 Pa: 7.5% acrylamide 0.1% bisacrylamide. Following addition of TEMED, mixtures were sterilely filtered using a 0.22 micron syringe filter. Each mixture (5.9 µL) was placed onto an individual coverslip, which was then covered with a second sterile coverslip that had been pre-treated with Sigmacote (Sigma, St Louis, MO), according to the manufacturer's instructions, to ensure even spreading of the acrylamide. After polymerization, the overlying

coverslip was removed and gels, on their base coverslips, were placed in a 24-well plate, washed with HEPES and incubated in SULFO-SANPAH (0.5 mg/ml) (Sigma). The polymer surfaces, immersed in SULFO-SANPAH, were then activated under UV light for 10 minutes, followed by three more washes in HEPES buffer. Immediately after removal of the wash, 200 µL of laminin (10 µg/ml in HEPES) was added to each well to cover the acrylamide substrate and incubated at 37ºC (5.5% CO_2) for 4 hours. Subsequently, all liquid was removed and replaced with growth media and plates were returned to incubate at 37ºC for at least 15 minutes before cells were added.

Immunohistochemistry

For immunostaining, cells were fixed in 4% paraformaldehyde for 10 minutes at 37ºC. Samples were washed in PBS, incubated in PBS containing 0.5% Triton X-100 (10 minutes), and non-specific binding was blocked by incubating the samples in blocking buffer (PBS with 10% normal goat serum/0.5% Triton X-100/1% fish gelatin/5% bovine serum albumin) for 1 hour. Primary antibodies to YAP/TAZ (4912S, Cell Signaling Technology) were diluted 1:200 in blocking

buffer and applied for 2 hours at room temperature on a rotary platform. Secondary antibodies, conjugated to Alexa Fluor 555 (A21429, Gibco/Invitrogen) were diluted 1:500 and incubated with the samples for 2 hours at room temperature. Samples were then mounted using Slowfade Gold with DAPI (S36938, Invitrogen). Specificity of labeling was confirmed by omitting primary antibody or by substituting normal serum for the species used to generate the primary antibody. Immunostained cells were imaged with an inverted microscope (IX71; Olympus, Tokyo, Japan) with monochrome, cooled CCD digital camera (Rolera-XR; Q-Imaging, Surrey, BC, Canada). Three biological replicates were performed. All images were photographed at the same exposure time.

ELISA

ELISA was performed as a sandwich enzyme immunoassay using ELISA plates prepared with antibodies against mouse CTGF (SEA010Mu, USCN Life Sciences, Inc, Wuhan, China) and according to the manufacturer's instructions. ImM10 cells were cultured in 35mm dishes on the four different substrates for 24 days, with complete removal of the supernatant every 3 days for the first 12 days and then every 4 days until
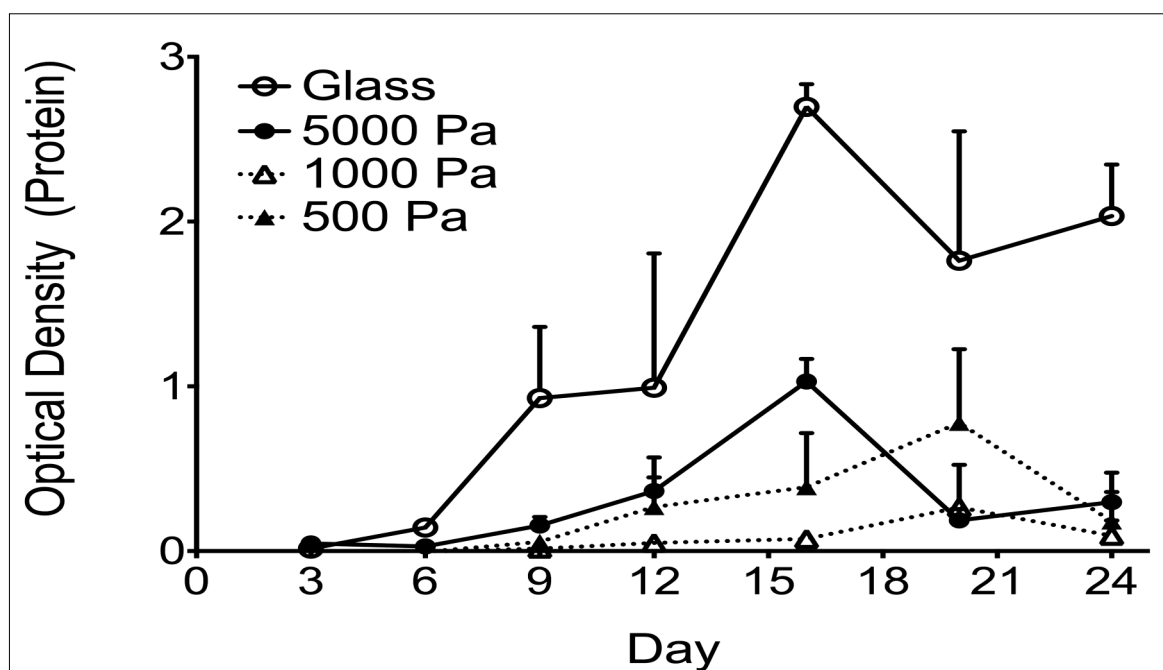

Figure 1. Graph showing ELISA quantification of CTGF protein as a function of both time and substrate stiffness. CTGF expression reaches a maximum earlier (16 days in culture) on firmer substrates relative to 20 days for the softer substrates. Each point shows the mean of 3 biological replicates. Error bars denote the SEM. This finding is significant (two-way ANOVA: p <0.0001).
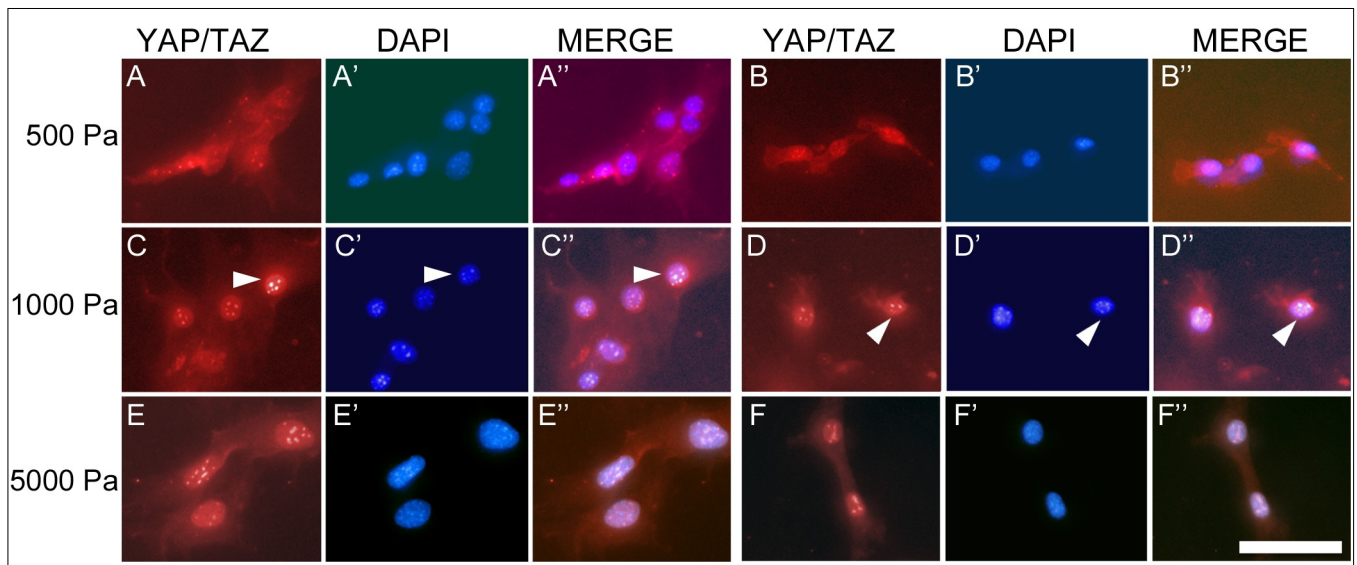
| | YAP/TAZ | DAPI | MERGE | YAP/TAZ | DAPI | MERGE |

Figure 2. (**A-F**) Immunohistochemistry for the YAP/TAZ complex in Müller cells at 16 days in culture. The YAP/TAZ complex is localized to the cytoplasm of cells grown on 500 Pa (**A, B**) and 1000 Pa (**C, D**) gels, as demonstrated by the corresponding DAPI staining of DNA (**A', B', C', D'**). The YAP/TAZ complex in cells grown on 5000 Pa gels (**E, F**) is localized to the nucleus, as shown by DAPI stain (**E', F'**). Scale bar: 50 microns.
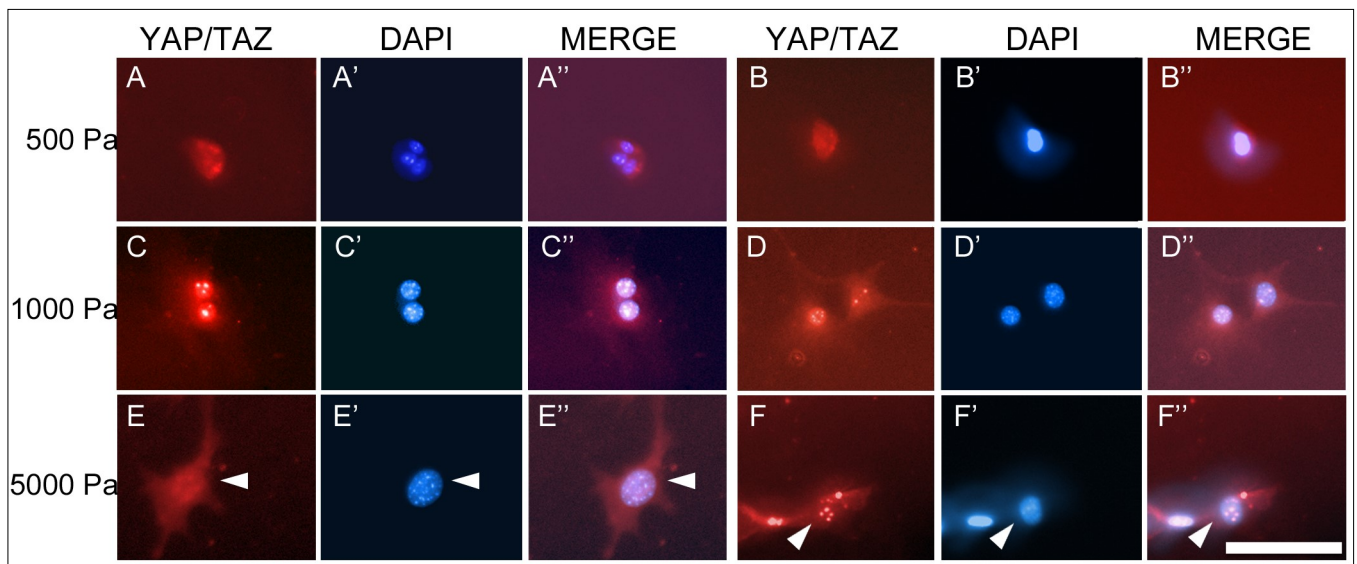

Figure 3. Immunohistochemistry for the YAP/TAZ complex in Müller cells at 20 days in culture. The YAP/TAZ complex is localized to the nucleus in cells grown on 500 Pa (**A,B**) and 1000 Pa (**C, D**) gels as shown by the corresponding DAPI staining of nuclear DNA (**A', B', C', D'**). The YAP/TAZ complex in cells grown on 5000 Pa gels (**E, F**) is primarily localized in the cytoplasm as demonstrated in the corresponding DAPI stain (**E', F'**). Scale bar: 50 microns.

day 24. Each supernatant sample was transferred to a microcentrifuge tube and frozen at -80ºC until all samples were collected. All samples were collected from three independent experiments and diluted 1:20 in deionized water before use in the ELISA assay. Prior to being transferred to the substrates of different elastic modulus, the cells were initially cultured, under non-immortalizing conditions, on 1000 Pa gels at 37ºC for 14 days.

Results

Müller cells cultured on the different substrates demonstrated two distinct temporal patterns of CTGF protein expression (Figure 1). On firmer substrates (glass and 5000 Pa gels), the maximal protein expression occurred at day 16 in culture, whereas on softer substrates (1000 Pa and 500 Pa), the maximal protein expression occurred at day 20. Although there were no statistically significant differences within groups (glass vs. 5000 Pa or 1000 Pa vs. 500 Pa), differences between the firmer and softer substrates were significant (2-way ANOVA, $p < 0.0001$). To further analyze these findings, and the relationship between CTGF levels and subcellular localization of the YAP/TAZ complex, immunohistochemistry for YAP/TAZ was performed on cells cultured for 16 (Figure 2) or 20 days (Figure 3) on 500 Pa, 1000 Pa, 5000 Pa, and laminin-coated glass substrates. The proteins YAP and TAZ were found to be predominantly nuclear only at the time of maximal CTGF protein expression.

Discussion

There is growing evidence[11] that cellular response to substrate stiffness is a bimodal function of stiffness, and that this bimodal response is at least in part a result of changes in the subcellular trafficking of YAP/TAZ complexes between the cytoplasm and nucleus. Previously, we noted that cortical actin stiffness, as measured by AFM, increased linearly up to a substrate stiffness of 2000 Pa, and then did not change further[16]. In addition, Müller cells on substrates of 1000 Pa or less proliferated at a slower rate than cells on firmer substrates[16]. Given that the normal, physiological stiffness of the retinal neurons that surround Müller cells is ~1000 Pa[7], these data suggest that the transition point between 'hard' and 'soft' for this cell type corresponds well to their natural environment. A small change in tissue stiffness away from equilibrium has the potential to induce substantial changes in cell behavior. This finding also suggests that CTGF and YAP/TAZ may play a role in tissue stiffness homeostasis through a feedback loop in which tissue that becomes overly soft will produce more CTGF, which will act to stiffen the tissue[16].

Our finding that the time-dependence of YAP/TAZ localization is influenced by substrate stiffness can account for the difference in time of maximal expression of CTGF in cells grown on substrates of different stiffness. Experimentally, our results suggest that authors who publish levels of gene expression of

Table 1. A review of similar mechanotransduction studies in which the investigators have determined a 'soft' and 'firm' regime experimentally. Note that the 'soft' regime is typically close to the normal, physiologic stiffness of the tissue that the cells encounter *in vivo* as well as the range of what cells consider a normal stiffness.

Cell Type	Physiologic	Soft	Firm
Astrocytes	0.33	<1	>2
Hepatic stellate cells	0.3-0.6	0.4-1[1]	1.75-12
Müller Cells[16,7]	1	<2	5
Trabecular Meshwork Cells	5	5	75
Corneal fibroblasts'	25	10	85

cells on different substrates should consider the time that the cells have been in culture.

If we compare other similar studies (see Table 1), we notice a trend in that 'soft' substrates are similar in stiffness to the cell's normal, physiologic environment, and that the physiological environment of different cells may differ by more than an order of magnitude. Together, these data suggest that each cell type responds similarly across a range of physiologically 'normal' elastic moduli, above which its environment is considered 'stiff' and the cellular response changes. This is consistent with observations from this and other studies[11,20] showing that cellular responses to changes in environmental stiffness tend to be bimodal rather than continuous. The dramatic differences in physiological stiffness between different tissues and the range of responses of different resident cell populations suggest that cells have intrinsic detection mechanisms and biomechanical responses that are likely determined during tissue development and maturation.

Although CTGF is one gene that is strongly regulated by the YAP/TAZ[11], Ankyrin repeat domain-containing protein 1 (ANKRD1), a transcription factor that may be involved in the myofibrillar stretch-sensor system, is also strongly regulated by YAP/TAZ[11], and future clinical studies might evaluate the role of this protein in retinal disease.

Conclusion

In summary, our findings suggest that changes in local elastic modulus can have significant, time-dependent influence upon gene expression. In particular, expression of CTGF, a protein that has been previously implicated in gliotic diseases of the retina, is strongly influenced by substrate stiffness. Consideration of the local elastic modulus may thus be critical in the design and long-term integration of retinal prosthetics and stem-cell based therapies. In particular, our results may help to explain the time course for the development of PVR/retinal gliosis in retinal detachment and suggest the duration over which therapies to prevent PVR should be administered after repair of a retinal detachment. Finally, prosthetics or matrices encapsulating tissues derived from stem-cells that possess non-physiologic stiffness for the local environment in which they are placed (typically the retina and/or RPE) may induce local cellular changes to their environment that may degrade their therapeutic effect.

Abbreviations

PVR = Proliferative Vitreoretinopathy

CTGF = Connective Tissue Growth Factor

YAP/TAZ = Yes-associated protein /transcriptional coactivator with PDZ-binding motif

ELISA = Enzyme Linked Immunosorbent Assay

Pa = Pascals

FBS = Fetal Bovine Serum

PBS = Phosphate-Buffered Saline

ANOVA = Analysis of Variance

AFM = Atomic Force Microscopy

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