Müller Cell Expression of Genes Implicated in Proliferative Vitreoretinopathy Is Influenced by Substrate Elastic Modulus

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PURPOSE. Matrix stiffness is recognized increasingly as a significant factor in cell and tissue function. To understand better the mechanosensitivity of Müller cells and its association with vitreoretinal disorders, we examined morphology, propagation, and expression of genes in Müller cells that were cultured on substrates of varying elastic moduli.

METHODS. A conditionally immortalized mouse Müller cell line was cultured on laminin-coated polyacrylamide substrates with calibrated Young's moduli. Glass was used as a control. Phase contrast, fluorescence, and atomic force microscopy were used to study cell morphology and propagation. Expression of extracellular matrix (ECM) genes was analyzed using quantitative reverse-transcription PCR.

RESULTS. The adherent area, stiffness, and propagation of Müller cells all are affected by matrix stiffness, but to different extents and with different ranges of sensitivity. Of 85 ECM genes tested 11 showed a continuous >4-fold increase or decrease in mRNA expression as a function of the substrate elastic modulus. The changes were statistically significant in four genes: connective tissue growth factor (*Ctgf*, $P = 0.04$), tenascin C (*Tnc*, $P = 0.035$), Collagen I α 1 (*Col1a1*, $P = 0.0001$), and Collagen IV α 3 (*Col4a3*, $P = 0.05$), with all showing increased expression on softer substrates.

CONCLUSIONS. There are significant changes in morphology, cytoskeletal integrity, and gene regulation in Müller cells as a function of the stiffness of the substrate. Changes in local tissue elastic modulus may have a role in vitreoretinal disorders. These findings also may have implications for strategies for improved integration of retinal prosthetics, and for stem cell therapies, particularly targeting the transcriptional regulators YAP and TAZ. (*Invest Ophthalmol Vis Sci.* 2012; 53:3014–3019) DOI:10.1167/iovs.11-8450

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The role of tissue stiffness recently has received increased recognition as a critical regulator of cell behavior.^{1–3} In some cases, such as development of liver fibrosis, changes in tissue stiffness precede and appear to contribute to subsequent dysfunction of hepatic cells.⁴ The elastic modulus of the cells in adult mammalian retina has been found to vary between 200 and 1000 Pascals (Pa), and many pathologic processes in the retina lead to changes in stiffness of the local retinal tissue.⁵ For example, it is well known that Bruch's membrane, immediately adjacent to the neurosensory retina, increases in stiffness by an order of magnitude with age.⁶ Laser photocoagulation, retinal detachment, and retinal gliosis are likely to alter markedly the local elastic modulus experienced by retinal cells. For this reason, we sought to determine the influence of substrate elastic modulus on Müller cells of the retina.

Müller cell morphology, propagation, and mRNA expression of select genes were studied using fluorescence and atomic force microscopy (AFM) and quantitative reverse transcription PCR (qRT-PCR) on a conditionally immortalized mouse Müller cell line (ImM10)⁷ that was cultured on laminin-coated polyacrylamide substrates of varying elastic moduli. Particular attention was paid to the influence of substrate elastic modulus on extracellular matrix (ECM) gene expression and an 85 ECM gene array (SABiosciences, Valencia, CA) was used to quantify gene expression.

METHODS

Cell culture substrates were prepared on German glass coverslips (15 mm, Carolina Biological Supply, Burlington, NC) as described previously^{1,8,9} according to the protocol of Pelham and Wang,^{10,11} with the following modifications. Gels were prepared with 7.5% acrylamide and bisacrylamide (bis) ranging from 0.02 to 1.0%. The elastic moduli of the gels were 500, 1000, and 5000 Pa, to model the elastic modulus range found normally in neural tissues.³ Laminin-coated glass¹² was used as a control to model typical cell culture conditions. To obtain a wider range of substrate stiffnesses in the evaluation of substrate stiffness versus cell stiffness, cells also were cultured on gels with a stiffness gradient, which ranged from 300 to 20,000 Pa.¹³ The strategy for creating stiffness gradients has been reported previously.¹³ Briefly, gradient generators, for production of gradient gels, made from polydimethyl siloxane (PDMS) microfluidic channels were fabricated using standard photolithography techniques.^{14,15} The stiffness of polyacrylamide gels was tuned by varying the concentration of bisacrylamide at a fixed acrylamide concentration.¹⁶ Three solutions with the same acrylamide (Bio-Rad, Hercules, CA) concentration but different N,N-methylene-bisacrylamide (Bio-Rad) concentrations were injected into the gradient generator. Each solution had an acrylamide concentration of 8% and a 2,2-diethoxyacetophenone (Sigma 227,102, St. Louis, MO) photoinitiator concentration of 0.5%. The bisacrylamide concentrations of the three inlets were: 0.02%,

0.02%, and 1%. During development of the technique, fluorescein (Sigma F6377) was added to the 0.02% bis-acrylamide solution to evaluate the gradient of bisacrylamide concentration upon polymerization. The solutions then were driven through the microfluidic channels by syringe pumps (Harvard Apparatus, Holliston, MA) at the same flow rate of 8 mL/hour. Once the flow in the outlet channel reached a steady state, a UV light was shined on the outlet region for 8 minutes. The syringe pumps were stopped after the outlet region was exposed to UV light for 10 seconds. Peeling off the PDMS gradient generator results in the gel being adherent to the activated coverslip. The resulting gel, 1.8 mm wide and 2 cm long, was immersed immediately in PBS buffer for 12 hours to remove unreacted photoinitiators. Once the gradient polyacrylamide gels were fabricated, the stiffness across the gel was characterized using AFM. The gradual transition in bis-acrylamide concentration, which correlates with gel stiffness, also was evaluated by fluorescence.

Final gel thickness after polymerization was $\approx 100 \mu\text{m}$. A bifunctional cross-linker (2 mM sulfo-SANPAH [Pierce, Rockford, IL] in 50 mM HEPES, pH 8.2) was used to ligate laminin or collagen to the polyacrylamide gels. Laminin (50 μg of 1 mg/mL natural mouse laminin in 5.95 mL HEPES buffer, Invitrogen, Grand Island, NY) or collagen (0.1 mg/mL rat-tail collagen, BD Bioscience, San Diego, CA) was applied to the gels and incubated for 2 hours at 37°C for cross-linking. Gels were rinsed once with Eagle's minimal essential medium (EMEM), and incubated overnight in EMEM in a humidified 37°C incubator. Three hours before plating the cells, EMEM was removed and replaced with culture medium.

The elastic modulus of each polyacrylamide mixture was confirmed using a Perkin Elmer DMA 7e dynamic mechanical analyzer. Each mixture was polymerized in the rheometer. The shear storage modulus G' , corresponding to the elastic resistance of the gels, was determined from the shear stress in phase with an oscillatory (1 rad/s) shear strain of 2% maximal amplitude, by standard techniques.

A conditionally immortalized mouse Müller cell line, ImM10⁷ was used in these experiments. The ImM10 cell line was isolated from the retinas of P10 mice that were heterozygous for the "immortomouse" transgene (H-2Kb-tsA58) that encodes an interferon gamma (IFN- γ) inducible, temperature sensitive SV40 large T antigen.¹⁷ When grown at 33°C , cells are immortalized by induction of the T antigen with IFN- γ at 50 U/mL final concentration in the media. Following withdrawal of IFN- γ , T antigen production is stopped, and shifting the cells to 39°C inactivates the remaining, temperature sensitive T antigen, thereby releasing cells from immortalization.

ImM10 cells were plated at 10,000 cells/well in a 24-well plate, and cultured at 5.5% CO_2 in growth medium composed of 500 mL Neurobasal media, 10 mL of B27 supplements, 5 mL of 200 mM L-glutamine, with 2% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin antibiotics (all from Invitrogen). When IFN- γ was required, 500 mg of mouse recombinant IFN- γ (Peprotech, Rocky Hill, NJ) was added to a final concentration of 50 U/mL. The cells were maintained with IFN- γ in the media, at 33°C . All experiments were performed in non-immortalizing conditions without IFN- γ , at 39°C . Cells were studied or RNA was harvested after 21 days. This length of time was selected to allow the cells to accommodate to the substrate upon which they were plated. It has been noted previously that Müller cells that had been cultured for at least 12 days modified their expression profile, up-regulating expression of GFAP, Sox2, CyclinD3, Ceruloplasmin, and Nestin, and down-regulating glutamine synthase, Kip1, Kir4.1, and Aquaporin-1.¹⁸ In addition, changes in contractility of Müller cells were noted previously on the time scale of 3 weeks.¹⁹ The cells were not confluent at the time of examination and were at passage 10 through 21.

AFM measurements were performed according to a published protocol.⁹ Briefly, Müller cells on substrates were rinsed with phosphate buffered saline (PBS) and placed in serum-free Dulbecco's modified Eagle's medium (DMEM). AFM measurements were done with a DAFM-2X Bioscope (Veeco, Woodbury, NY) mounted on an Axiovert 100 microscope (Zeiss, Thornwood, NY) using silicon nitride

cantilevers (196 μm long, 23 μm wide, 0.6 μm thick) with a pyramidal tip (when the cell is indented by 1 μm , the area of the indentation projected on the sample plane is 1.6 μm^2) for indentation. The spring constant of the cantilever, calibrated by resonance measurements, was typically 0.06 N/m (DNP; Veeco). To quantify cellular stiffness, the first 600 nm of tip deflection from the horizontal (Δd) were fit with the Hertz model modified for a cone:^{20,21}

$$\Delta d = \frac{k}{2A} + \Delta z - \frac{1}{2} \sqrt{\left(\frac{k}{A}\right)^2 + 4 \frac{k \Delta z}{A}} \quad \text{and} \quad A = \frac{2}{\pi} \frac{E}{1-\nu^2} \tan(\alpha)$$

where k and Δz are the bending rigidity and the vertical indentation of the cantilever, E is the Young's modulus, α is the cone tip angle, and ν the Poisson ratio. Young's modulus is the inverse ratio between the strain ($\delta z/z$) applied to the material and the resulting stress. The Poisson ratio is defined as the ratio of compressional strain in the direction normal to the applied stress and the extensional strain in the direction of the applied stress, and is taken to be 0.5 for all samples. To determine cell stiffness, AFM measurements were made on single cells by indenting on three positions of the peripheral cell body within a period of approximately 30 minutes. The average elastic modulus for each cell followed by averaging all obtained values. At very large indentation, it is possible that the tip can rupture the cell membrane. However, small indentations will not lead to cell rupture. For this reason, we only indented the Müller cells by approximately 1 μm , where the possibility of the tip penetrating the cell membrane is very low. Publications by the investigators and others previously have used this AFM system reproducibly to obtain data. Moreover, we also monitored the indentation process using an optical microscope, and did not observe changes in cell morphology after the AFM measurements.

It has been shown previously that AFM measurements will be influenced by the underlying substrate when the indentation is larger than 10% of the sample thickness.²² Therefore, it is likely that the stiffness will be influenced by the substrate stiffness if the AFM indentations were taken over the cell lamellipodia, whose thickness often is less than 1 μm . Being aware of this issue, and specifically seeking to avoid the influence of the substrate, we obtained measurements on the peri-nuclei regions of the cells. The cell thickness in these regions is on the order of a few micrometers, significantly larger than the thickness of a lamellipodium. To verify the appropriateness of this technique, we plotted AFM cantilever deflection as a function indentation as well as cell stiffness as the indentation depth increases from 200 nm to 1 μm . The cell stiffness values did not vary significantly with the indentation depth (see Supplementary Material, Figs. 1 and 2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8450/-/DCSupplemental>).

To evaluate cell morphology, the cells were fixed with 4% paraformaldehyde and prepared for dual immunofluorescence and epifluorescence microscopy. Cells undergoing rhodamine-phalloidin (Invitrogen) staining were treated according to a standard protocol²³ with nuclear counterstaining. Cells were imaged using an inverted Olympus microscope (IX71; Olympus, Tokyo, Japan) equipped with a monochrome, cooled CCD digital camera (Rolera-XR; Q-Imaging, Surrey, BC, Canada). Images were quantified for average spread area using NIH Image, with at least 10 cells per high-powered field analyzed. Propagation was measured in triplicate using a hemocytometer and Trypan blue stain. Less than 1% of cells counted were non-viable.

Gene expression was analyzed by quantitative qRT-PCR using the Stratagene MX3005P real-time PCR system, and the SABiosciences Extracellular Matrix and Adhesion Molecules real-time PCR array (which is composed of 85 genes important for cell-cell and cell-matrix interactions plus 5 housekeeping genes for normalization, controls for genomic DNA contamination, and positive and negative controls, Valencia, CA). Total RNA was isolated from cells using affinity columns (RNeasy; Qiagen, Valencia, CA). Briefly, cells were lysed in a guanidine hydrochloride buffer, loaded onto affinity columns, washed, and eluted

FIGURE 1. Photomicrographs of rhodamine-phalloidin stained Müller cells, with nuclear counterstaining, on laminin-coated substrates. Cells grown on laminin-coated glass (A) and 5000 Pa substrates (B) display prominent stress fibers. Cells on 1000 Pa (C) and 500 Pa substrates (D) demonstrate very minute levels of rhodamine-phalloidin staining as well as a more spherical morphology. Scale bar = 100 μ .

in RNase free water (RNeasy; Qiagen). Samples were quantified by 260 nm absorbance using spectrophotometry (Nanodrop, Wilmington, DE) and stored at -80°C . First strand cDNA was synthesized using reverse transcriptase (RT2 First Strand Kit; SABiosciences) according to the manufacturer's instructions. The RT2 kit uses a combination of random hexamers and oligo-dT to prime the reverse transcriptase reaction, and uses a proprietary procedure to eliminate any contaminating genomic DNA from the reactions. Master mix, containing 12.5 μL cDNA was added to each well of a PCR array (Extracellular Matrix and Adhesion Molecules PCR Array, PAMM-013; SABiosciences). Cycling conditions followed manufacturer's recommendations and performed on a real-time PCR instrument (Mx3005p; Stratagene, Santa Clara, CA). Web-based analysis software (SABiosciences) was used to analyze the results of the experiments. The proteins of greatest interest in this study are secreted and, thus, the protein cannot be obtained purely from cell lysates. Also, the proteins are known to degrade rapidly, producing broad bands on Western Blotting. For this reason, mRNA was quantified as an indirect measure of protein production.

Descriptive statistics, including mean, median, and standard deviation, were calculated. Linear regression was used to analyze

FIGURE 3. Müller cell spread area (square microns) plotted as a function of substrate stiffness ($P = 0.0428$) on collagen-coated substrates.

AFM elastic modulus measurements. ANOVA was used to assess the relationship between the genes expressed.

RESULTS

As Müller cells were grown on their separate substrates, morphological changes became readily apparent. Cells grown on the stiffer substrates, such as glass and 5000 Pa gels (Figs. 1A and B), had a much larger spread area ($P = 0.0428$) along with being more individuated than the corresponding cells on softer substrates (Figs. 1C and D). These changes are less apparent on collagen-coated substrates (Fig. 2), but still are present with an approximate 2-fold increase in spread area across the range tested (Fig. 3). Using phalloidin staining, prominent stress fibers are apparent in the larger cells, but not in cells with smaller adherent areas on softer gels. Cytoskeletal rigidity is shown by AFM measurements on the cortical actin in the cells, and demonstrates a linear increase in cell stiffness with respect to substrate stiffness up to 2000 Pa (Fig. 4), followed by an apparent saturation of cell stiffness as the matrix stiffness is increased further.

Propagation of glia may be important in proliferative vitreoretinopathy (PVR) because of the extensive gliosis that develops in this condition. Cells on harder substrates propagated more quickly than those on softer substrates, with cells on 1000 and 500 Pa being statistically indistinguishable (Fig. 5). The results for day 12 on the glass substrate do not demonstrate exponential growth because of near-confluence of the cells.

PCR results were obtained from preloaded plates for extracellular matrix proteins. From these results, it was

FIGURE 2. Images (from left to right) of Müller cells cultured on collagen-coated plastic, and collagen-coated gels of stiffness 1500 and 500 Pa. Cells were stained with Texas Red-Phalloidin with nuclear counterstaining. Note the transformation of cell shape from more polygonal to elongated with a decrease in the number of stress fibers, and that differences in basement membrane protein (laminin in Fig. 1 versus collagen in Fig. 2) significantly alter cell morphology. Scale bar = 200 μ .

FIGURE 4. AFM evaluation of the cortical actin elastic modulus as a function of substrate elastic modulus in cultured Müller cells. Below 2000 Pa (left), the cortical actin elastic modulus increases linearly with substrate elastic modulus. Above 2000 Pa (right), the cortical actin elastic modulus remains nearly constant (the horizontal line is included to guide the eye). Linear regression on data below 2000 Pa results in a best-fit slope of (0.933 ± 0.216) with a y -intercept of $(84.48 \pm 320, P = 0.0008, R^2 = 0.98)$.

observed that there is a large, nonlinear upregulation of connective tissue growth factor (*Ctgf*), as well as Tenascin C (*Tnc*, Fig. 6). *Ctgf* was shown to have a two order of magnitude increase on softer substrates, with respect to glass ($P = 0.05$) with Tenascin C having a 40-fold increase ($P = 0.035$). Similarly, both collagen type 4 alpha 3 and collagen type 1 alpha 1 increased with decreasing substrate elastic modulus ($P = 0.05$ and $P = 0.001$, respectively, 500 Pa vs. glass). In contrast to these changes in extracellular matrix components, no significant trend was observed in matrix metalloproteinase expression (Fig. 7).

DISCUSSION

The effective stiffness of the retina can be altered through a variety of pathological processes. Stiffening of the retina occurs with age⁶ and retinal gliosis. The local elastic modulus in the retina is likely to be decreased in conditions, such as retinal photocoagulation with coagulative necrosis and retinal detachment. In particular, it is known from thermodynamic calculations, that supported membranes (i.e., attached retina) have a markedly greater stiffness than unsupported membranes.²⁴

Prior studies, using similar techniques on different cell types,^{1,3,4} report changes in morphology that are consistent with the current findings, in a wide variety of cell types. This

suggests that changes in gene expression may be influenced by changes in cell shape and morphology, as has been found in many cell types.^{1,3,14}

Connective tissue growth factor (CTGF), the product of the gene most strongly affected by substrate stiffness, plays numerous roles in pathologic conditions of the retina, including PVR,²⁵ age-related macular degeneration,^{26,27} and diabetic retinopathy.^{27,28} Not only is CTGF thought to be the major mediator of retinal fibrosis in the presence of TGF- β ,²⁵ which is present at significant levels in the vitreous fluid, it is necessary for diabetes-induced basal lamina thickening.²⁸

Histologically, the immunoreactivity of PVR membranes to CTGF is known to increase with the stage of PVR.²⁹ CTGF induces fibronectin, laminin, and matrix metalloproteinase-2 (MMP-2) expression in retinal pigment epithelium (RPE) cells, is present strongly in Bruch's membrane, including basal deposits and drusen,²⁶ and stimulates migration of RPE cells.²⁷ Finally, CTGF stimulates the synthesis of fibronectin by hyalocytes and RPE cells,³⁰ and hypoxia is known to increase the expression of CTGF, collagen IV, and fibronectin.³¹

Based upon the prior literature reviewed in our study, the cultures were performed for 21 days before analysis. For this reason, there is much greater heterogeneity in the distribution of the cells. Cells that become confluent may respond

FIGURE 5. Müller cells, quantified using a hemocytometer, increased in number more rapidly on firmer substrates. There is a transition point between 1000 and 5000 Pa, such that cells on firmer substrates propagate more rapidly. The results for day 12 on the glass substrate do not demonstrate exponential growth because of near-confluence of the cells.

FIGURE 6. mRNA expression, quantified using real-time PCR arrays on cells grown on substrates of different elastic modulus. Note the orders-of-magnitude change in expression as a function of substrate elastic modulus. Genes plotted with solid lines demonstrate statistically significant changes with regression analysis (*Col1a1*, $P = 0.001$; *Col4a3*, $P = 0.05$; *Ctgf*, $P = 0.05$; *Tnc*, $P = 0.035$). Genes plotted with dashed lines were not found to have statistically significant changes.

FIGURE 7. Matrix metalloproteases gene expression using real-time PCR arrays on cells grown on substrates of different elastic modulus. There does not appear to be any dependence of MMP expression on elastic modulus, but a baseline expression is present.

differently to mechanical stress than non-confluent cells. For this reason, analysis of confluent monolayers was avoided.

Our finding that expression of *Ctgf* by Müller cells is increased markedly on soft substrates, thus, is notable because it provides a complementary, physical mechanism whereby CTGF can be regulated in pathological conditions. For example, it might be expected that, after cryotherapy or photocoagulation and resulting tissue necrosis, nearby Müller cells would upregulate their expression of CTGF. In addition, when embedded in a detached (and, thus, soft) retina, with no underlying substrate, Müller cells would be expected to upregulate their expression of CTGF.

Recently, the activity of the transcription regulators YAP/TAZ (Yes-associated protein and transcriptional coactivator with PDZ-binding motif, also known as WWTR1) has been found to be regulated by substrate stiffness to a degree comparable to short interfering RNA (siRNA) mediated YAP/TAZ depletion.³² YAP/TAZ was found to be predominately nuclear in spread cells and on hard substrates, while this pair was predominately cytoplasmic on soft substrates or when cells were confined to small islands. In a bioinformatic analysis on genes expressed differentially in mammary epithelial cell (MECs) grown on substrates of high versus low stiffness, *Ctgf* was found to be one of the genes regulated most strongly by YAP/TAZ.³³ This finding was confirmed in a variety of cell types, including MDB-MB-231 and HeLa cells. The change in nuclear localization was found to be related to cell geometry and cytoskeletal tension, independent of cell-cell contact, and independent of the Hippo pathway, within which YAP and TAZ are the nuclear transducers. Our findings of differential regulation of *Ctgf* on substrates of varying elastic moduli suggest that YAP/TAZ may have a critical role in gliotic changes present commonly in vitreoretinal disease, and may provide therapeutic targets for these blinding conditions.

Tenascin C (TNC) is an extracellular protein that is known to modulate cell-matrix interactions,³⁴ and either can contribute to or inhibit³⁵ integrin-mediated spreading of cells. TNC has been noted to be regulated differentially in fibroblasts on matrixes of varying stiffness, and it has been hypothesized that its promoter contains a stretch-responsive enhancer element.³⁶ In vivo, at the myotendinous junction, its expression has been found to be regulated strongly by mechanical loading.³⁷

Consistent with our findings, *Tnc* has been found previously to be non-linearly and markedly upregulated with injury in a rat model of elevated intraocular pressure,³⁸ and also has been identified³⁹ in the schisis cavities of patients with congenital x-linked retinoschisis. Given our results, these prior findings are

likely to be related to changes in the mechanical stiffness of optic nerves that have been injured by an acute episode of glaucoma, and of retinas that contain a schisis cavity.

Expression of multiple collagen genes can be regulated by mechanical stress, with the particular collagen involved determined by cell type.³⁶ Thus, it is not surprising that we find the expression of collagens (*Col1a1* and *Col4a3*) influenced by stiffness. Matrix metalloproteinases are expressed in Müller cells, but do not depend upon substrate modulus.

Weaknesses of this study include the use of a cell line that may not be representative of in vivo conditions. In addition, for many of the measurements, evaluation of the cells was performed at one time point, a time at which Müller cells were known to have changed their expression profile. This does not allow evaluation of more transient changes in the Müller cells as a function of substrate stiffness. Such evaluation would likely require “equilibration” of the cells, either in vivo (for primary cells) or at a physiological substrate stiffness (such as a 1000 Pa).

In summary, many surgical interventions and pathologic conditions in retinal disease can result in changes in the elastic modulus of ocular tissue. Examples include photocoagulation and cryosurgery, which can result in initial necrosis and decrease in retinal elastic modulus, and aging and traction, which can result in increases in retinal and basement membrane elastic modulus. Necrotic tissue, immediately after coagulative necrosis, will be soft, given the aggregation of cytoskeletal elements and the well-known formation of blebs in the plasma membrane,⁴⁰ while the subsequent longer-term tissue stiffness currently is a subject of study.

These physical changes in tissue can have marked and direct influence upon genes, such as *Ctgf* and *Tnc*, which are known to have a key role in pathologic conditions, such as PVR. Our findings suggest that the Yorkie-homologues, and transcriptional regulators YAP and TAZ may provide a therapeutic target for the treatment and prevention of gliotic diseases of the retina and optic nerve.

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