Published in final edited form as:

Exp Eye Res. 2009 September ; 89(3): 344–357. doi:10.1016/j.exer.2009.03.022.

Aberrant basal fiber end migration underlies structural malformations in a Streptozotocin-induced diabetic rat model

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Abstract

This study characterized early structural changes at posterior fiber ends in the crystalline lens after diabetic induction. Wistar rats (n=49), randomized into one naïve control group and four experimental groups, were rendered diabetic via streptozotocin injection. Animals were euthanized at 1 week intervals, blood glucose levels recorded and lenses were evaluated grossly, by SEM and by confocal microscopy. Indices were developed to assess structural alterations and for statistical correlations between the scores and the duration of hyperglycemic exposure as well as blood glucose levels. Average blood glucose levels increased progressively from 98.5 mg/dL (controls) to 331.4 mg/dL (4 weeks). Diabetic lenses displayed abnormal suture sub-branches and opacity formation beginning late in the first week post-injection and rapidly progressing in severity through four weeks. SEM analyses showed gradual elongation of fiber ends and filopodia which comprised a disorganized configuration and a loss of recognizable migration patterns. Structural alterations culminated in foci of fiber degeneration by the third to fourth weeks. The F-actin distribution at basal fiber ends was significantly altered as compared to naïve controls. Cadherin distribution was altered as compared to controls, but largely at later time points. The grading system clearly shows increased structural compromise with elevated blood glucose levels in streptozotocin-induced diabetes. Further, although the initial rise in blood glucose levels was associated with pathological changes, their progression depended to a larger extent on continued hyperglycemic exposure. The data suggests that hyperglycemia initially disrupts fiber end migration, resulting in structural alterations and eventual fiber degeneration.

Keywords

Crystalline lens; migration; diabetes; Streptozotocin; ultrastructure; sutures; basal membrane complex; immunofluorescence

1. Introduction

Ocular complications of Type 1 diabetes mellitus includes among other conditions, the formation of opacities and cataracts in the cortical lens fibers resulting in clouding and loss of

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vision. Although diabetes-related opacities can form in any area of the lens (Al Ghouli and Costello, 1993;Costello et al., 1993;Al-Ghouli et al., 1996), the posterior surface seems to be particularly vulnerable (Creighton, 1978;Fisher, 1985;Dickey and Daily, 1993;Kuszak et al., 2000a). This is not surprising because the posterior subcapsular region is adversely affected by numerous systemic and ocular diseases that alter the microenvironment of the lens (Black et al., 1960;Greiner and Chylack, 1979;Eshagian, 1982;Fraunfelder and Meyer, 1990;Al Ghouli and Costello, 1993;Costello et al., 1993;Al Ghouli and Costello, 1996;Kuszak et al., 2000a).

It is well established that the orderly arrangement of the lens fibers contributes to its inherent transparency (Maisel et al., 1981;Kuszak et al., 1986;Kuszak and Brown, 1994;Sivak et al., 1994;Kuszak, 1995). At the posterior surface, this order is established and maintained by the organized migration of the elongating fibers and subsequent suture formation. The basal ends of elongating fibers form a critical interface along the capsule to which they are attached, as they migrate towards their sutural destinations. The interaction between the fiber ends and the capsule is mediated via the basal membrane complex (BMC) through its integral membrane and cytoskeletal proteins (Bassnett et al., 1999;Lu et al., 2008). The dependence of fiber organization on the migratory process led to the hypothesis that faulty fiber migration is responsible for compromised lens transparency and eventual formation of posterior subcapsular cataracts (PSCs). Therefore, this study was conducted to address the following questions: What are the structural changes that characterize the migrating basal fiber ends in the diabetic lens? Do these structural changes occur only as a result of increased levels of blood glucose or are they dependent on the duration of hyperglycemic exposure?

Utilizing the Streptozotocin-induced diabetic rat model, this study characterized the initial morphological changes at the posterior fiber ends during diabetes. Additionally, the distribution of two components of the BMC, actin and cadherin, were assessed and the structural changes were correlated with blood glucose levels, as well as the duration of time that the lenses were exposed to a hyperglycemic environment. The results indicate that the progression of diabetic changes in the rat lens is not entirely dependent on blood glucose levels. The dynamic process of posterior suture formation is extremely sensitive to changes that cause the fiber ends to deviate from their predetermined migratory path, resulting in a loss of orderly arrangement of the lens fibers. It appears that hyperglycemic changes adversely affect fiber end morphology and migration patterns, resulting in a loss of organization at the posterior surface.

2. Materials and Methods

2.1 Animals

This investigation utilized 49 male Wistar rats (Harlan Sprague Dawley, Inc, Indianapolis, IN and Charles River Laboratories, Wilmington, MA) weighing approximately 100–150 g on arrival. The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all experimental techniques were performed under aseptic and sterile conditions as approved by the Institutional Animal Care and Use Committee (IACUC) of Rush University Medical Center, Chicago, IL. The animals were randomized into five groups (naïve control, 1 week, 2 weeks, 3 weeks & 4 weeks) and animals in the four experimental groups received a single intravenous (tail vein) injection of Streptozotocin (STZ-75mg/kg b. w) to induce diabetes. The animals were housed in pairs throughout the duration of the experiment and monitored twice every week for changes in body weights. At the specific time points that were pre-determined, the animals were euthanized with an intraperitoneal dose of sodium pentobarbital (1ml @ 398 mg/ml).

2.2 Lenses

Immediately following euthanasia, the eyes were enucleated and blood glucose levels recorded using a commercial blood glucose meter (One Touch Basic Blood Glucose Monitoring System, Lifescan, Milpitas, CA). Lenses were dissected from the orbit under a Stereoscopic Zoom Microscope (Nikon SMZ1500) and photographed with a digital camera (Nikon DIX, Nikon Inc., Melville, NY) operated by the Q Capture Pro Software (QImaging Corporation, Surrey, Canada) on a Pentium PC platform. Right eye (OD) lenses were processed for immunocytochemistry (ICC); left eye (OS) lenses were processed for scanning electron microscopy (SEM).

As illustrated in Figure 1A, the migrating posterior fiber ends can be located in one of four zones, namely, the equatorial zone, the lateral posterior zone, the peri-sutural zone and the sutural zone (Al-Ghoul et al., 2003). This study assessed migrating lens fiber ends that were located within a latitudinal ring that encompassed distal portions of the lateral posterior zone, the peri-sutural zone and the sutural zone (Fig. 1B).

2.3 SEM

Prior to fixation, all OS lenses were decapsulated beginning from the anterior side in order to expose the posterior fiber ends (Al-Ghoul et al., 2003). Decapsulated lenses were fixed in 2.5% glutaraldehyde in 0.07M cacodylate buffer (pH 7.2) for 3 days, with fresh fixative changes daily. Lenses were then washed in 0.1M cacodylate buffer, post-fixed in 1% aqueous osmium tetroxide at 4°C, washed in buffer and dehydrated through a graded ethanol series. Following dehydration with 100% ethanol, the alcohol was replaced with a graded ethanol/Freon 113 series. The lenses were then critical-point dried in 100% Freon 23 (DuPont, Wilmington, DE) in a Balzers CPD 020 (Balzers, Hudson, NH), secured on aluminum stubs with silver paint, sputter coated with gold and examined under a scanning electron microscope (Hitachi S-3000N, Hitachi America Ltd., Brisbane, CA). Digital electron micrographic magnification series were acquired from 50X–4000X for each specimen.

2.4 Laser Scanning Confocal Microscopy (LSCM)

All OD lenses were processed for ICC and LSCM visualization in the following manner: After lenses were dissected from the eyes, they were fixed in 3% paraformaldehyde for 2 hours. Fixed lenses were positioned posterior side up, secured with cyanoacrylate adhesive on specimen mounting blocks of a vibrating knife microtome (Vibratome Series 3000 Plus-Tissue Sectioning System), embedded in 3% agarose gel and sectioned at a thickness of 100µm. Sections were fixed for an additional 30 minutes in 3% paraformaldehyde and then permeabilized with 0.2% Triton X-100 for 30 minutes. Permeabilized sections were labeled for either F-actin or cadherin family proteins.

For F-actin labeling, sections were washed in 0.07M phosphate buffered saline (PBS), incubated in Phalloidin-FITC at 1:50 dilution of a methanolic stock (Sigma, Saint Louis, MO) and thoroughly re-washed in 0.07M PBS. The labeled sections were mounted on glass cover slips (Fisherbrand, Fisher Scientific, Pittsburgh, PA) with Vectashield mounting medium (Vector Laboratories Inc, Burlington, CA) to prevent photobleaching. The cover slips were sealed to glass slides using a commercially available lacquer.

For cadherin labeling, sections were incubated in blocking solution for 1 hr to block non-specific staining, then incubated with primary antibody at 1:200 dilution (monoclonal mouse anti-pancadherin; Sigma, Saint Louis, MO) overnight at 4° C. Sections were then thoroughly washed in blocking solution and incubated with secondary antibody at 1:100 dilution (rabbit-anti-mouse IgG FITC conjugate, Sigma, Saint Louis, MO) for 2 hours. After secondary

antibody incubation, sections were re-washed and mounted between glass cover slips and slides as specified above.

All specimens were examined using a Zeiss LSM5 laser scanning confocal microscope (Zeiss USA, New York, NY).

2.5 Indices

In order to quantify the results, two indices were devised to assess suture pattern and opacity in the lens. Both indices were assigned a linear numerical score with 0 being normal and subsequent higher scores increasing with severity. The lenses were assessed using photographs of fresh unfixed lenses directly after dissection from the globes.

To assign a suture pattern score, parameters such as the variation in suture pattern, the number of peripheral and central suture sub-branches and widening of the sutures were all taken into consideration. Scores ranged from 0 to 9 (Table 1) and were categorized into Normal (Score 0), Mildly Variant from Normal (Scores 1 to 3), Moderately Variant from Normal (Scores 4 to 6) and Severely Variant from Normal (Scores 7 to 9).

To assign a posterior surface opacity score, the presence, distribution and size of opacities on the lens were used (Table 2). Score 0 was assigned to a normal, transparent lens. Lenses with varying degrees of opacity received scores between 1 and 4, with lenses showing a frank posterior cataract being assigned a score of 4.

Although both indices can be used to record the degree of sutural variations and opacities on both anterior and posterior surfaces, this study focused only on the posterior surface of the lens.

2.6 Statistical Analyses

The blood glucose levels exhibited a normal distribution and therefore a one-way Analysis of Variance was carried out, followed by the Bonferroni's Multiple Comparison Test to determine the difference between the different groups. Since the suture pattern and opacity scores did not follow a normal distribution, the non-parametric Kruskal-Wallis Test was run on the data, followed by a post-hoc Dunn's Multiple Comparison Test to determine the difference between groups. The Spearman's Coefficients of Rank Correlation (r_s) were calculated for the index scores (suture pattern score and opacity score) versus the duration of hyperglycemic exposure and for scores versus blood glucose levels using a commercially available statistical package.

3. Results

3.1 Suture Pattern Variation in the Diabetic Lens

Anterior and posterior lens surfaces in rodents typically exhibit upright Y-shaped and inverted Y-shaped sutures (Fig. 2A) respectively, that are comprised of three branches oriented at 120° angles to each other (and offset by 60° from anterior to posterior). Deviations from this three-branched regular pattern can be an ocular consequence of systemic pathology (Kuszak et al., 2000b). Diabetic lenses were evaluated at 1, 2, 3 and 4 weeks post-induction for changes in suture pattern. As expected, variations were evident in the first week and these changes increased temporally. The first noticeable change was the formation of one or more sub-branches at the proximal ends of the sutures (peripheral sub-branches; Fig. 2B) and some lenses showed these sub-branches originating towards the center of the suture (central sub-branches; Fig. 2C). Eventually the sub-branches progressed to form a 'double Y' shaped suture (Fig. 2D). By three weeks, the peripheral and central sub-branches began to occur in combination (Fig. 2E). Sutural widening began to be seen at 3 weeks post-induction along the sutures and at the

confluence of the sutures by 4 weeks post-induction (Fig. 2F). This widening was often accompanied by peripheral and/or central sub-branch formation.

3.2 Opacity Formation in the Diabetic Lens

Cataract and opacity formation are common diabetic complications affecting the crystalline lens. All lenses at the end of the first week and most lenses after the second week post-induction did not reveal loss of transparency and diabetic lenses were comparable to the normal controls with respect to opacity (data not shown). Some lenses in the second week post-induction revealed the presence of a few scattered opaque flecks (Fig. 3A). By the third week post-induction, the number of opacities increased, several of the opaque flecks coalesced to form larger opaque areas and there were distinct opacities along the sutures (Fig. 3B). Late in the third week and early in the fourth week, sutural cataracts were often visible (Fig. 3C). All the changes in lens transparency that were evident by the third week were more pronounced in the fourth week (Fig. 3D) and almost all lenses showed nascent PSCs (Fig. 3: E, F).

3.3 Posterior Fiber Ends

As expected, the posterior fiber ends from the control lenses were irregularly spheroidal to ovoid in shape with an ordered, tightly packed arrangement as previously shown (Al-Ghoul et al., 2003). They exhibited specific directionality towards the suture branches, that is, in the direction of fiber end migration (Fig. 4: A, B, C). Filopodia were also present extending in the general direction of migration of the fiber ends towards the sutures (Fig. 4: A', B', C'). During the second week post-induction, the fiber ends exhibited various degrees of lengthening to take on a somewhat elongated shape. The tight packing was still evident in most of the lenses (Fig. 5: A, A'). By the third week post-induction, elongation of fiber ends was more pronounced (Fig. 5: B, B') and some filopodia were lengthened. Groups of elongated fiber ends appeared to be arranged in curved, wavy or 'swirled' patterns (Fig 5: B, B', C, C'). Disruptions of the tight packing seen in control lenses were present (Fig 5: B'-star). In general, changes that began in the second week were exaggerated and more severe in the third and fourth weeks post-induction. These changes were also accompanied by localized areas of fiber breakdown (Fig. 5: C, C'-stars). By four weeks post-induction, basal fiber ends were extremely attenuated. Similarly, filopodia were also extremely elongated and additionally had lost the uniform directionality that was characteristic in the naïve controls.

3.4 Actin Distribution

The actin cytoskeleton is involved in the maintenance of cell shape, cell motility and migration. Regulation of this protein plays an important role in fiber end migration in the lens. This study characterizes the actin cytoskeletal arrangement in the fiber ends of normal and diabetic rat lenses. In the naïve controls, the posterior fiber ends were clearly delineated by F-actin labeling at the fiber-capsule interface. The typical ovoid shape of the fiber ends was evident, which correlates with the results obtained from our SEM studies (Fig. 6: A, B, C-C"). Most one week lenses showed F-actin labeling comparable to the naïve controls (Fig. 6: D, E), with the ends clearly outlined by F-actin and exhibiting a cohesive arrangement. By the second week post-induction, some fiber end profiles lost their ovoid shape and appeared to be slightly elongated (Fig. 6: F, G-stars). In lenses from animals diabetic for three weeks, there was evidence of fragmented material positive for F-actin within the irregular fiber end profiles (Fig. 7: A, C). There were also areas wherein the F-actin was arranged in patterns consistent with actin stress fiber formation (Fig. 7: A, B). The arrangement of the fiber ends also showed the 'swirling' pattern that was seen on SEM analyses (Fig. 7: D, E). By the fourth week post-induction, the actin distribution that was exclusive to the periphery of the fiber end profiles in normal lenses was altered, and was seen both around the periphery and within the BMC of the fiber ends in diabetic lenses. This seems to be a reflection of the changes associated with the hyperglycemic

microenvironment that the lenses are exposed to. Some of the fiber ends in less compromised areas began to take on a 'stellate' appearance because of the extension of processes in several directions (Fig. 7E). F-actin within the fiber ends of severely compromised lenses exhibited more intense stress fiber formation and the loss of orderly arrangement was also clearly evident (Fig. 7: G, H). There were also large areas with no labeling indicating either zones of extracellular space (ECS) dilations or areas of structure loss. The fourth week also saw the clumping of F-actin around the perimeter of the fiber ends, as opposed to the relatively uniform distribution of F-actin around the ends in normal lenses (Fig. 7I). The F-actin arrangement had a cloudy appearance at the BMC and fully elongated fiber profiles deep to the BMC were excessively enlarged (Fig. 7: Series J). F-actin positive fragments within the fiber ends were also seen four weeks post-induction of diabetes.

3.5 Cadherin Distribution

Cadherins, the calcium-dependent adhesion molecules, are typically found in the adherens junctions of cells. We examined the cadherin distribution in the basal fiber ends of naïve control and diabetic lenses. Control lenses showed characteristic cadherin labeling around the perimeter of the posterior fiber ends revealing a regular shape and arrangement (Fig. 8A). Cadherin labeling was also visible along the lateral membranes of the fibers (Fig. 8B) as has been demonstrated by others. In the first week post-induction, the cadherin labeling still delineated the fiber ends and the lateral membranes of the fibers. The shape and arrangement of the ends were comparable to the naïve controls (Fig. 8C). In the second week post-induction, although the cadherin labeling intensity had not changed drastically from the previous week, the ends had begun to take on the elongated shape seen in the SEM and actin data (Fig. 8D). A diffuse cytoplasmic appearance of the cadherin which was not apparent earlier was also noticed (Fig. 8: E-stars). By the third and fourth weeks post-induction, the structural breakdown of the posterior fiber ends (Fig. 8: G, H, I-asterisks) was very clearly highlighted by the cadherin labeling around the elongated and misshapen ends. Although the lateral membrane labeling remained comparable to the controls, the fiber ends and posterior segments of the fibers showed an increased cadherin labeling in the cytoplasm (Fig. 8: F, G, H, I). The 'swirling' arrangement of the fiber ends was evident which is consistent with the SEM and actin data. Large unstained areas, representing either dilations of the ECS or enlarged fiber ends were also present (Fig. 8: G, H, I).

3.6 Scoring Indices and Statistical Analyses

Observation of the data revealed an emerging pattern with regard to structure and pathology and hence, scoring indices were developed to evaluate the lenses with respect to Suture Pattern and Opacity in STZ-diabetic rats. Using the indices as a guide, each lens was given a numerical score on a linear scale (Tables 2 and 3). There was a significant difference in the blood glucose levels (Fig. 9A) between the naïve controls and each of the four experimental groups ($p < 0.001$). A student t-test also showed a significant difference between the blood glucose levels in the 1 week post-injection group and the 4 weeks post-injection group ($p < 0.001$).

The suture pattern scores exhibited significant difference between the naïve controls and the 4 weeks post-induction group as well as between the 1 week post-injection and the 4 weeks post-induction groups ($p < 0.001$). Significance at $p < 0.05$ was present between the naïve controls vs. the 3 weeks post-induction group and between the 2 weeks post-induction group vs. the 4 weeks post-induction group (Fig. 9B). The opacity scores also exhibited significant difference at $p < 0.001$ for controls vs. 3 weeks and 4 weeks post-induction (Fig. 9C).

Each of the score sets (Suture Pattern and Opacity) was evaluated against the blood glucose levels and the duration of hyperglycemic exposure to obtain Spearman's Coefficients of Rank Correlation. Spearman's Coefficients (r values) of suture pattern scores vs. blood glucose levels

was 0.3854 ($p < 0.05$) and suture pattern scores vs. duration of hyperglycemic exposure was 0.8293 ($p < 0.0001$). Opacity scores vs. blood glucose levels gave an $r_s = 0.4092$ ($p < 0.05$) and $r_s = 0.8255$ ($p < 0.0001$) for opacity scores vs. duration of hyperglycemic exposure (Fig. 10).

4. Discussion

This study established a correlation between hyperglycemia and early structural changes at the posterior ends of elongating fibers. Direct visual observation of diabetic rat lenses indicates that the first sign of pathology is the generation of altered suture patterns, followed by opacity formation. The normal 'typical' suture pattern in the rodent lens is three-branched with an upright 'Y' anteriorly and an inverted 'Y' posteriorly (Kuszak et al., 1984; Kuszak, 1995; Kuszak et al., 2004; Kuszak et al., 2006). Recent evidence (Donohue and Al-Ghoul, 2008) has revealed that most rats develop one or more secondary suture branches as a consequence of the normal ageing process which form slowly over a period of several months. These additional branches represent a second generation of suture branches similar to the successive generations of suture patterns in primate lenses (Kuszak and Brown, 1994). In contrast, in the present study, the rapid formation of suture sub-branches in diabetic rodent lenses occurred within weeks or even a few days. Multiple sub-branches often formed simultaneously and their location was unpredictable, i.e. they initiated from either the proximal suture ends or at any point along the lateral aspect of primary suture branches. The sub-branches that formed in diabetic lenses can also be distinguished from secondary suture branches in that they never progressed to form complete branches. All of the above underscore the differences between secondary suture branch formation in a growing lens and the aberrant sub-branch formation in hyperglycemic lenses. The number and rate of occurrence of suture sub-branches in lenses from diabetic animals were greatly accelerated as compared to lenses from naïve controls. This suggests that the changes in the overall suture pattern are a result of the underlying pathology.

One possible explanation for aberrant sub-branch formation in the present study is accelerated growth of the diabetic lens coupled with a rapid shift in migration patterns. Accelerated growth has been shown to occur in both diabetic lenses and in PSC associated with topical steroid therapy (Brown and Hungerford, 1982; Shun-Shin et al., 1991). In the present study, accelerated growth may have occurred in the diabetic rodent lenses resulting in the observed changes. Another possible explanation is that dynamic rearrangement of superficial fiber ends occurred, resulting in the formation of sub-branches and altered suture patterns. The second explanation implies a certain degree of plasticity and mobility of the fiber ends even after fiber elongation and migration is completed. Although dynamic rearrangement of fiber ends has not been conclusively shown, the lack of junctional apparatus across sutures (Lu et al., 2008) certainly leaves this possibility open for future exploration.

One of the hypotheses of this study was that the duration of exposure to elevated levels of glucose was more critical to the structural changes in the diabetic lens, than the exact levels themselves. We observed that the suture pattern varied rapidly with the initial rise in blood glucose levels and by one week post-induction, the lenses exhibited peripheral sub-branches. Over time, the variation in the pattern was more evident and by two weeks post-induction, the peripheral sub-branches were longer and there was initiation of central sub-branches. The temporal progression of suture pattern variation often resulted in a 'Double-Y' shaped suture. Although there was no significant difference in the levels of blood glucose between the first and second weeks post-induction of diabetes (268.6 mg/dL and 300.9 mg/dL, respectively), the variation in suture pattern was quite remarkable. This indicates that the dynamics of posterior suture pattern formation were affected to a larger extent by the length of time spent in a hyperglycemic environment than the levels themselves. At three and four weeks post-induction of diabetes, similar changes in suture pattern variation were seen. By three weeks,

lenses manifested with a combination of peripheral and central sub-branches and by the fourth week post-induction, these sub-branches were accompanied by sutural widening. Although there was no significant difference in the levels of blood glucose between the first and third/fourth weeks post-induction of diabetes (268.6 mg/dL vs. 311.6 mg/dL and 331.4 mg/dL), the variation in suture patterns (as evidenced by the increased suture pattern scores) was significant. This indicates that the duration of hyperglycemic exposure was critical for the rapid changes in suture pattern.

The importance of the hyperglycemic period in relation to the structural changes was even more evident with respect to opacity formation within the superficial posterior cortex of the lens. The development of opacities and cataracts in the lens cortex is an established ocular complication of diabetes and is associated with structural changes such as, areas of lens fiber breakdown, ECS dilations and sutural abnormalities (Kuwabara et al., 1969; Sakuragawa et al., 1975; Creighton, 1978; Unakar et al., 1978; Costello et al., 1993; Lu et al., 1993; Mackic et al., 1994; Bond et al., 1996; Kuszak et al., 2000a). It is interesting to note that opacity formation was not seen immediately after the initial surge in blood glucose levels during the first week post-induction. Once the levels of hyperglycemia were established and the lens had spent a considerable amount of time exposed to these conditions (in this case almost 2 weeks), scattered opacities across the posterior surface of the lens began to form. Specifically, over the span of three weeks, the flecks coalesced to form larger opaque areas, sutural cataracts formed and then eventually progressed to a posterior subcapsular cataract (PSC) by the fourth week post-induction of diabetes. The development of discrete opacities on the posterior surface of the lens appeared to be a result of the hyperglycemia, but the progression and eventual formation of a PSC correlated to the length of time that the lens was exposed to elevated glucose levels.

SEM analyses of control Wistar rat lenses demonstrated ultrastructure of basal fiber ends consistent with a prior investigation on Sprague Dawley rats (Al-Ghoul et al., 2003), indicating that normal lens fibers in Wistar rats have a comparable organization and morphology. Lenses from the experimental animals showed a vast array of differences in structural arrangement and organization with increasing blood glucose levels and extended duration of hyperglycemic exposure. This included the rapid loss of cohesion and orderly migration, the alteration in size and shape of the fiber ends, the excessively long, disordered filopodia and the absence of the tight packing of fiber ends coupled with disruption of structure. The greatly elongated fiber ends took on a 'wavy' or 'swirled' pattern of arrangement indicating that the normal expected migration paths were not being followed. It is well established that cell migration requires a coordinated sequence of events including, cell polarization, membrane extensions at the leading edge, adhesion, translocation and retraction of the trailing edge. In the present study, the disordered filopodial extensions and extremely elongated fiber ends suggest that one or more of the migratory events had been disrupted. In particular, controlled filopodial extension towards the sutures and retraction of the trailing edges appear to be adversely affected in hyperglycemic states. A sustained adhesion of the trailing end of a cell coupled with forward translocation of the leading edge can result in elongation (Schwarzbauer, 1997; Horwitz and Parsons, 1999; Webb et al., 2002). Our SEM analyses of the posterior fiber ends suggest that a similar situation presents itself in the diabetic rat lens.

Cellular movement and migration, change in cell shape and cell adhesive properties are mediated by the cytoskeleton through its myriad interactions, in which actin plays a leading role. The various actin configurations that can be seen in cell migration include: actin network at the leading edge, actin bundles beneath the cell membrane and actin fibers within the cytoplasm (Ridley et al., 2003). In the lens, actin plays a vital role in maintaining cellular structure during lens development and growth (Rafferty, 1985; Lo et al., 1997; Lee et al., 2000; Lo et al., 2000; Beebe et al., 2001). In the present study, control lenses showed the BMC clearly delineated by F-actin and the irregularly ovoid shape of the fiber ends could be

visualized, consistent with the morphology seen by SEM and in prior studies (Bassnett et al., 1999; Al-Ghoul et al., 2003; Lu et al., 2008). Changes in the actin configuration were apparent only by the second week post-induction, congruent with the observation that the duration of hyperglycemic exposure was responsible for the progression of diabetic changes in the lens. Atypical F-actin labeling configurations that were observed in diabetic lenses included; the presence of F-actin positive fragments within the fiber end profiles, distinct actin stress fiber formation and irregular clumping of actin around the BMC periphery in some fiber ends. The F-actin material within the fiber ends could indicate membrane fragments from regions of fiber cell breakdown. The membrane is closely linked to cytoskeletal actin, and breakdown of the membrane as a consequence of fiber degeneration during hyperglycemia may account for the F-actin positive membrane fragments. Actin forms stable stress fibers when a non-motile cell adheres to a substrate and dynamic stress fibers during retraction of the 'trailing edge' of migrating cells (Cramer, 1997). In the diabetic lenses we examined, the presence of actin stress fibers contrasts with the actin distribution in naïve controls and may indicate that the fiber ends have reduced motility and increased adhesion. This suggests that the retraction of the trailing edges may be delayed or impaired. Actin clumping is one of the cytoskeletal changes that has been seen in ageing cells (Maisel, 1984; Gourlay et al., 2004). The F-actin clumping seen in STZ-induced diabetic rat lenses seems to precede fiber cell breakdown and could be attributed to an accelerated senescent change.

The structural link between actin and cadherin strengthens cell-cell adhesions and specifically, cadherin-cytoskeletal interactions play an important role in the lens (Ferreira-Cornwell et al., 2000). In order for cadherin to form stable junctions, it must be linked to the cytoskeleton. Several studies have indicated that this linkage is crucial to optimal cadherin function (Ferreira-Cornwell et al., 2000). The present study elucidated the distribution and arrangement of cadherin in the BMC as well as in the lateral membranes of posterior fiber segments in order to determine any changes in the configuration associated with hyperglycemia. The cadherin labeling of the fiber ends corroborates the SEM findings and the F-actin data with respect to shape changes of the fiber ends in diabetic lenses over time. The changes that are observed in the F-actin configuration could be driving the changes in the cadherin distribution, in effect, disrupting the intricate cadherin-cytoskeletal interaction. A consequence of this could be the loss of adhesion between the fiber cells resulting in the well characterized ECS distensions (Bond et al., 1996; Costello et al., 1993) and also impacting the morphology and arrangement of migrating fiber ends. Another interesting change that was seen was the diffuse cytoplasmic appearance of cadherin labeling by the second week post-induction. Cadherin is a calcium-dependent membrane linked protein, which plays a key role in intercellular adhesion. The presence of cadherin immunofluorescence within the cytoplasmic domain could indicate an alteration of adhesion dynamics (Pavalko and Otey, 1994; Ferreira-Cornwell et al., 2000), which could further explain the change in the paths of the migrating fiber ends.

A closer look at the morphology of whole lenses, the suture pattern variations, the formation of opacities, F-actin and cadherin distributions revealed a distinct pattern of observations, which prompted us to devise two grading indices. The linear scoring pattern and the defined criteria helped elucidate the progressive severity of structural damage in the diabetic lens. Assigning numerical scores also facilitated statistical inferences regarding the correlation between severity of structural defects versus hyperglycemic levels and exposure time. The correlation coefficients clearly underscore the increased severity of diabetes-related structural damage as dependent on the time period spent in a hyperglycemic environment. The evidence garnered from observations of lens ultrastructure and immunohistochemistry are corroborated by the statistical analyses.

In conclusion, although the levels of blood glucose trigger the initiation of diabetic changes in the lens, the progression and severity of these changes are more critically dependent on the

duration of hyperglycemic exposure. Posterior fiber end migration is a dynamic process that involves an intricate interplay between the BMC and the cytoskeletal components of the lens fibers. Conditions that interfere with this synchrony result in aberrant migratory paths of the fibers, with a consequent loss of lens transparency and formation of PSCs.

Acknowledgments

Portions of this work were presented at the 2007 and 2008 Annual Meetings of the Association for Research in Vision and Ophthalmology, Ft. Lauderdale, FL. This work was supported by NIH NEI Grant EY14902 (KJA) and by The Doctor Bernard and Jennie M. Nelson Fund, Chicago, IL.

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Fig. 1. Illustrations of the posterior fiber ends depicting the regions of fiber end migration and the area that was imaged

A: Migrating fiber ends on the posterior surface of the lens are defined as being located within one of four regions: (1) the equatorial zone, (2) the lateral-posterior zone, (3) the peri-sutural zone and (4) the sutural zone. The zones are delineated in red, the fiber ends are in blue and the suture is shown in gray.

B: The region of the posterior lens surface that was imaged is delineated by a green circle. Comparison with panel A shows that this latitudinal ring includes migrating fiber ends from three zones, namely, distal portions of the lateral-posterior zone, the peri-sutural zone and the sutural zone. The fiber ends are in blue and the suture is shown in gray.

Fig. 2. Photographs of suture patterns in control and STZ-induced diabetic rat lenses

A: Posterior surface of the lens from a naïve control shows the characteristic inverted-Y suture. Index Score = 0.

B–F: Posterior surface of the lens from STZ-induced diabetic rats. B: Single peripheral sub-branch (arrow) seen as early as one week post-induction of diabetes. Index Score = 1. Double arrowheads depict the pupillary margin as seen through the lens. C: Progressive formation of peripheral sub-branches (arrows) and a single central sub-branch (arrowhead). Index Score = 3. This pattern was seen between 1 and 2 weeks post-induction of diabetes. D: Sub-branches progressed to the sutural confluence to form the ‘Double-Y’ shaped suture; this pattern was seen as early as the second week. Index Score = 3. E: Suture patterns at three and four weeks post-induction shows a combination of peripheral (arrows) and central (arrowheads) sub-branches. Index Score = 5. F: Sutural widening accompanied by peripheral (arrow) and/or central (arrowhead) sub-branches was seen four weeks post-induction. Index Score = 8. All photographs were taken at the same magnification. Scale bar = 0.3 mm.

Fig. 3. Photographs of opacities in STZ-induced diabetic rat lenses

A: Scattered opaque flecks on the posterior surface (arrows), seen as early as the second week post-induction in some lenses. Index Score = 1. Double arrowheads depict the pupillary margin as seen through the lens. B: Distinct opacity along one suture branch (arrow), seen in the second to third week post-induction of diabetes. Index Score = 2. C: Sutural/stellate opacity which was very distinct during the third week post induction (arrow). Index Score = 3. D: Opacities along all three sutures (arrows) and scattered opaque flecks. Index Score = 3. E: Opacity along the suture (arrows) and a nascent PSC (arrowhead) which was evident in the fourth week post-induction of diabetes. Index Score = 4. F: Nascent PSC (arrowhead) seen four weeks post-induction along with discrete opacities (arrows). Index Score = 4. All photographs were taken at the same magnification. Scale bar = 0.3 mm.

Fig. 4. SEMs of posterior fiber end morphology in normal rat lenses

Each set of panels represents low and high magnifications of the same specimen. A, B, C: Posterior fiber ends of naïve control rat lenses exhibit a spheroidal to ovoid shape. The ends are tightly packed together and directed towards the suture branches (A: double-headed arrow). A', B', C': Most filopodia (arrows) extend in the general direction of fiber end migration and are distinct at higher magnifications.

Fig. 5. SEMs of posterior fiber end morphology in STZ-induced diabetic rat lenses

Each set of panels represents low and high magnifications of the same specimen. A-A': Fiber ends in the second week post-induction look slightly elongated in shape as compared to controls. The tight packing seen in the controls is still evident. B-B': By the third week post-induction, a 'swirling' pattern of distorted fiber ends can be visualized, as well as the initiation of fiber end breakdown (B'-star). Higher magnification shows severely elongated fiber ends and lengthened filopodia. C-C': Changes seen in the third week are more severely manifested in the fourth week post-induction. These features are coupled with areas of structural breakdown of the fiber ends (stars). The extremely elongated filopodia extend in multiple directions and overly the attenuated fiber ends.

Fig. 6. Confocal micrographs of F-actin labeling at posterior fiber ends in naïve controls and STZ-induced diabetic rat lenses (1 and 2 weeks post-induction)

A-A': Paired fluorescent and DIC images of the posterior sutural confluence of naïve control lenses clearly shows F-actin outlining the fiber ends at the capsule-fiber interface. B: At higher magnification, the F-actin around the periphery of the fiber end profiles of a control lens is seen to clearly delineate their ovoid shape. C-C'': Z-series of fiber ends from a control lens taken in 1μm intervals. The capsule-fiber interface is the reference for $z=0$. The z-series beginning at 1μm deep to the capsule-fiber interface and extending up to a depth of 3μm shows the regular shape and arrangement of normal migrating fiber ends. D-D': Paired fluorescent and DIC images of the posterior fiber ends in the first week post-induction shows F-actin

labeling comparable to that seen in the naïve controls. E: At higher magnification, the fiber end profiles seem to maintain their ovoid shape and regular arrangement. F-F': During the second week post-induction, fiber ends slowly start to lose their regular shape and begin to take on a slightly elongated shape (stars). These elongated profiles continue to be clearly outlined by F-actin. G: At higher magnification, few elongated fiber end profiles (stars) can be discerned between the ovoid fiber ends.

Fig. 7. Confocal micrographs of F-actin labeling at posterior fiber ends in STZ-induced diabetic rat lenses (3 and 4 weeks post-induction)

A: During the third week post-induction, fragmented material positive for F-actin is present within the highly irregular fiber profiles (arrow). F-actin is arranged in patterns consistent with actin stress fibers (boxed area). B: Higher magnification of the boxed area shown in panel A demonstrates the actin stress fiber arrangement (arrowheads) within the fiber end profiles. The profiles themselves no longer maintain the ovoid shape typical in the controls. C: Higher magnification showing the F-actin positive fragments within the irregular fiber ends. D and E: Fluorescent images of third week post-induction lenses show the fiber ends arranged in 'swirling' patterns consistent with the SEM findings. The regular configuration seen in the controls has been lost. Actin stress fiber formation is also evident within the fiber ends. F: In less compromised areas from the four weeks post-induction group, some profiles are slightly elongated (dotted outlines) while others exhibit a 'stellate' shape (bold outlines). G: Four weeks post-induction, lenses typically reveal ECS dilations between their lateral membranes as well as enlarged and irregular fiber ends. H: Higher magnification of the boxed area in panel G shows dense arrays of actin stress fibers within the fiber ends (arrowhead). I: Four weeks post-

induction, some lenses present with F-actin clumping around the perimeter of the irregularly shaped fiber ends. The complete loss of regular fiber end arrangement is evident. This correlates with the SEM data which shows a similar disarray of fiber arrangement and structural breakdown J-J': Z-series of fiber ends from a diabetic lens taken in 1 μ m intervals. The capsule-fiber interface is the reference for z=0. The z-series beginning at 1.5 μ m deep to the capsule-fiber interface and extending up to a depth of 3.5 μ m, shows intense clumping of F-actin around the fiber end profiles. The profiles are enlarged and also have F-actin positive fragments within them.

Fig. 8. Confocal micrographs of cadherin immunolabeling in control and STZ-induced diabetic rat lenses

A & B: In naïve controls, fiber ends and lateral membranes are clearly delineated by cadherin immunofluorescence. C: In the first week post-induction, posterior fiber end shape and the distribution of cadherin are comparable to the controls. Strong cadherin labeling continues to be seen outlining the ends as well as the lateral fiber membranes. D & E: By the second week post-induction, the fiber ends are slightly elongated and closely resembles the configuration seen with SEM and F-actin labeling. Cadherin labeling along the lateral membranes compares with the controls. Cadherin outlines the fiber end profiles, but a slight cytoplasmic labeling (stars) is present. F: During the third week post-induction, the fiber ends have lost their regular

shape and arrangement. Cadherin labeling is still evident, although the clarity of demarcation of the membranes is diminished. G, H & I: Cadherin labeling of lenses in the fourth week post-induction shows structural details consistent with SEM and F-actin data, including, areas of fiber structure breakdown (asterisks) and a complete loss of cohesive fiber end arrangement. The fibers surrounding the areas of degeneration seem to be 'pushed aside'. Although cadherin immunofluorescence is present along the lateral fiber membranes, it is relatively diminished around the fiber ends.

Fig. 9. Graphical representation depicting the relationship between blood glucose levels, suture pattern scores and opacity scores vs. time

A: Bar graph showing the gradual increase of blood glucose levels over time. Data are shown as mean $\pm$ sem. B: Bar graph showing the gradual increase of suture pattern scores over time. C: Bar graph showing the gradual increase of opacity scores over time. Data in panels B and C are shown as medians $\pm$ range. * $p < 0.001$, ** $p < 0.05$ and *** $p < 0.1$ between the selected groups in all panels.

Fig. 10. Graphical representation showing the relationship between the grading scores (suture pattern and opacity scores) and hyperglycemia (duration of hyperglycemic exposure and blood glucose levels)

A: Box plot of suture pattern scores against the duration of hyperglycemic exposure. Spearman's Correlation gave a positive r_s of 0.8293 ($p < 0.0001$). B: Box plot of opacity scores against the duration of hyperglycemic exposure. Spearman's Correlation gave a positive r_s of 0.8255 ($p < 0.0001$). Data in panels A and B are plotted as medians with range; + indicates the mean. C: Scatter plot of suture pattern scores against the levels of blood glucose. Spearman's Correlation gave a positive r_s of 0.3854 ($p < 0.05$). D: Scatter plot of opacity scores against the levels of blood glucose. Spearman's Correlation gave a positive r_s of 0.4092 ($p < 0.05$).

Table 1

Index for Scoring Suture Pattern Variations

PARAMETERS	SCORE
Y-shaped sutures with no sub-branches	0
Sutures with 1–2 peripheral sub-branches	1
Sutures with 1–2 central sub-branches	2
Sutures with a combination of 1–2 peripheral and central sub-branches	3
Sutures with a combination of 3–5 peripheral and central sub-branches OR widening along one suture	4
Sutures with a combination of 3–5 peripheral and central sub-branches OR widening along more than one suture	5
Sutures with a combination of 3–5 peripheral and central sub-branches accompanied by widening along one or more sutures	6
Sutures with more than 5 peripheral and/or central sub-branches accompanied by widening along one or more sutures	7
Sutures with a central widening pattern (may be accompanied by a varied number of suture sub-branches)	8
Indistinct suture pattern	9

Table 2

Index for Scoring Opacities

PARAMETERS	SCORE
No opacity present	0
Opacity present along any one of the sutures and/or the presence of scattered flecks	1
Opacity present along more than one suture and the presence of scattered opaque flecks and/or the coalescence of flecks into 1–2 opaque areas	2
Sutural/stellate opacity and the presence of scattered opaque flecks and/or the coalescence of flecks into more than 2 opaque areas	3
Frank anterior or posterior cataract	4

Table 3
Data Table showing Blood Glucose Levels and Index Scores

Parameters	Naïve Controls	1 Week	2 Weeks	3 Weeks	4 Weeks
Total N=49	10	10	10	9	10
Mean	98.5	268.6	300.9	311.6	331.4
Blood Glucose Levels	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
Mean	1.3	2.6	3.4	5.6	7.5
Suture	(Normal To Mild)	(Mild)	(Mild)	(Moderate)	(Severe)
Pattern Score (0–9)					
Mean	0.8	1.7	2.3	3.6	3.8
Opacity Score(0–4)					