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MOLECULAR RESPONSES OF CHOROIDAL ENDOTHELIAL CELLS TO ELASTIN DERIVED PEPTIDES THROUGH THE ELASTIN BINDING PROTEIN (GLB1)

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Abstract

Purpose—Neovascular AMD involves the activation of choroidal endothelial cells to increase their inflammatory and angiogenic behaviors. Elastin derived peptides (EDPs) can elicit some of these phenotypic changes in endothelial cells. This investigation was performed to follow up on those findings by determining a receptor for these peptides in the human eye as well as evaluating the effects of elevated EDPs on choroidal cells *in vitro* and *in vivo*.

Methods—The expression of elastin receptor genes including GLB1 was analyzed using reverse transcription PCR. Migration of choroidal endothelial cells was quantified in the presence of inhibitors to different EDP binding proteins. C57BL6 mice were injected with EDPs and studied by electroretinography, transmission electron microscopy, and microarray analysis.

Results—An alternatively spliced form of beta-galactosidase (GLB1) is present on human choroidal endothelial cells and acts as a receptor for EDPs. Elevated levels of circulating EDPs do not affect retinal function in the mouse, but do increase the expression and deposition of collagen IV in the RPE/choroid complex.

Conclusions—EDPs may play a role in neovascular AMD by binding to and inducing neovascular phenotypes in choroidal endothelial cells through their receptor, GLB1. These peptides also cause an increased mRNA expression and deposition of collagen IV in the RPE/choroid, which may alter diffusion properties between the retina and choriocapillaris.

Keywords

elastin; macula; Bruch's membrane; choroid; macular degeneration

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in people over the age of 60 in the United States (Seddon et al., 2005). Visual acuity is lost in this condition due to degenerative changes in the macula, the region of the posterior eye responsible for detailed vision. The neovascular form of AMD is characterized by the abnormal growth of choroidal blood vessels (choroidal neovascular membranes, CNVMs). This process is thought to be initiated in part by breakdown of Bruch's membrane. When intact, Bruch's membrane prevents pathologic angiogenesis into the retina through its barrier function and by the sequestration of anti-angiogenic proteins, which are less abundant in eyes with AMD (Bhutto et al., 2008).

The neovascular process in AMD can result in serous detachment of the retinal pigmented epithelium (RPE) and/or retinal detachment, as well as fibrous disciform scarring beneath the retina, causing a catastrophic decrease in visual acuity (Green, 1999; Grossniklaus and Green, 1998; Grossniklaus et al., 1992; Spraul et al., 1999; Vine et al., 2005). Current treatments for neovascular AMD are focused primarily on targeting vascular endothelial growth factor (VEGF)-mediated processes (Stone, 2006). While VEGF is an extremely potent inducer of angiogenesis, understanding the role of additional angiogenic stimuli would be invaluable for both a better understanding of how the extracellular matrix affects the pathology of AMD and the development of improved treatments (Bird, 2010; Kinnunen and Yli-Herttuala, 2011; van Wijngaarden et al., 2005).

Elastin is a glycoprotein that provides support as well as elasticity, and it is an abundant component of the extra-cellular matrix (ECM) of arteries, lung, and skin (Hayden et al., 2005). Degradation of elastin results in the mechanical deterioration of these structures as well as the production of elastin subunits. Fragments of varying sizes of elastin are known as elastin derived peptides (EDPs), and these fragments have a variety of functions. We have previously shown that EDPs are capable of promoting angiogenic behaviors in choroidal endothelial cells, so it is plausible that EDPs may play a role in progression of exudative AMD (Skeie and Mullins, 2008).

It has been established that the majority of cellular signals induced by EDPs are transduced after their interaction with the cell surface-residing 67 kDa elastin binding protein (Hinek and Rabinovitch, 1994; Hinek et al., 1993). This protein, with an additional lectin-like carbohydrate-binding domain, was later identified as an alternatively spliced, enzymatically inactive form of beta-galactosidase, or GLB1 (Privitera et al., 1998). This peripheral membrane protein with a high affinity for EDPs associating with the 61 kDa plasma-membrane anchored protein, neuraminidase, as well as with a 55 kDa protein, cathepsin A forms the cell surface elastin receptor (Hinek, 1996; Robinet et al., 2005). Whereas the neuraminidase and cathepsin A moieties are necessary for localization to the membrane and signal transduction, the binding of EDPs to this complex occur through the hexapeptide sequence VGVAPG on the tropoelastin monomeric unit to a conserved sequence of GLB1 (Senior et al., 1984). This hexapeptide sequence is repeated several times within the tropoelastin monomer allowing for EDP hexpeptides to be potent in triggering neoangiogenesis in capillaries by promoting cell migration and tubulogenesis, possibly by activating the ERK1/2 cell signaling pathway (Duca et al., 2007; Duca et al., 2002; Duca et al., 2004; Robinet et al., 2005). Interestingly, it has been also established that GLB1 can also interact with other hexapeptide sequences having a XGXXPG consensus that are also present in laminin (LGTIPG) (Hinek, 1994; Mecham et al., 1989) and in collagen type IV (Hinek, 1996; Senior et al., 1989). This raises another attractive hypothesis that the degradation products of these two proteins, present in the basement membranes of the choriocapillaris and retinal pigment epithelium, may also induce angiogenic signals via their

interaction with the 67 kDa GLB1 isoform. Another domain of the C-terminus of tropoelastin, has been also implicated in interaction with integrin $\alpha_v\beta_3$ (Rodgers and Weiss, 2004, 2005), which is expressed by endothelial cells and plays a role in angiogenesis (Nangia-Makker et al., 2000). Since inhibiting the integrin heterodimer $\alpha_v\beta_3$ results in decreased retinal neovascularization in mouse studies (Friedlander et al., 1996), it is feasible that blocking the integrin heterodimer may reduce neovascularization by preventing elastin peptides from binding in other tissues.

A major goal of this study was to determine if the elastin binding protein(s) in the human choroid are responsible for increased migratory behaviors found in our previous studies. In this investigation, we also set out to study the effect of elevated serum elastin fragments in the mouse. Elevated elastin fragments are present in humans with increasing severity of AMD and abnormalities in skin elastin are also associated with AMD (Blumenkranz et al., 1986; Sivaprasad et al., 2005), suggesting abnormal systemic elastin physiology occurs in AMD. In attempts to model aspects of AMD, mouse models have provided many insights. Currently, there are several mouse models that recapitulate some of the characteristics of atrophic AMD in humans (Imamura et al., 2006; Malek et al., 2005), however, none of these models completely recapitulate the disease phenotypes. Determining the effects of elevated elastin fragments *in vivo* on angiogenesis and inflammation in the mouse eye will further our understanding about how extracellular matrix abnormalities may affect the pathogenesis of AMD.

RESULTS

GLB1 elastin-binding protein RT-PCR

In order to identify whether human choroidal endothelial cells *in vitro* and *in vivo* synthesize transcripts for the alternatively spliced, elastin binding form of GLB1, as well as its associated membrane proteins, CTSA and NEU1, RT-PCR was utilized. Allele specific PCR was used as depicted in Figure 1. Amplimers of *GLB1*, *CTSA*, and *NEU1* were all detected following RT-PCR of both human choroidal endothelial cells and human RPE/choroid. Separation of the amplimers on an agarose gel is shown in Figure 2. Sequencing of a *GLB1* PCR fragment showed that the alternatively spliced form of *GLB1* is expressed by these cells (data not shown).

Blocked elastin binding protein migration assay

EBP antagonists, anti- $\alpha_v\beta_3$ and lactose, used for the migration assay did not affect the viability of the cells in culture. Endothelial cells (Rf6a or human choroidal EC, see Experimental Procedures) were used in migration assays toward fetal bovine serum (FBS) or elastin, and were exposed to either an anti- $\alpha_v\beta_3$ integrin heterodimer antibody or to 200mM lactose, an inhibitor of elastin binding by GLB1 (Mecham et al., 1991). Cells treated with an antibody directed against $\alpha_v\beta_3$ integrin heterodimer had a significant reduction in their migratory response toward both EDPs and fetal bovine serum (FBS) (p-values < 0.01). Cells exposed to 200mM lactose showed a significant reduction in migration towards EDPs (p-value = 0.01), however did not have a significant change in migration towards FBS (p-value = 0.65) in comparison to cells not exposed to either inhibitor (Figure 3). Similar results to blocking peptides in FBS and EDP exposed human primary choroidal endothelial cells were observed (data not shown). Again, cells that were treated with the $\alpha_v\beta_3$ integrin heterodimer antibody had a significant reduction in their migratory response toward both EDPs and FBS (p-values < 0.02), while cells exposed to 200mM lactose showed a significant reduction in migration towards EDPs (p-value = 0.04), however did not have a significant change in migration towards FBS (p-value = 0.20).

EDP treated endothelial cell PCR array profiling

Human choroidal endothelial cells were exposed to elastin derived peptides in culture and then cell lysates were analyzed by quantitative PCR to determine if the peptides changed gene expression by the cells. The statistically significant findings from the EDP exposed and control human endothelial cell quantitative PCR array are summarized in Table 1. Out of 84 genes evaluated on the array (supplemental data), the expression of 6 genes (*MMP9*, *CCL2*, *BCL2A1*, *TNFSF10*, *NOS3* and *SELL*) had a greater than 2-fold increase in the EDP treated endothelial cells compared to the control cells (with p-values < 0.05), and ICAM1 showed a significantly decreased expression. The complete list of genes on the array, as well as p-values and fold changes, is shown in Supplemental Table 1.

Electroretinograms of injected and control mice

The a- and b-wave heights were within the normal range for all control and EDP injected mice (Figure 4). The average a-wave value for control mice was $-268 \mu\text{V}$ (standard deviation $52 \mu\text{V}$) and the average b-wave amplitude was $341 \mu\text{V}$ (standard deviation $83 \mu\text{V}$). The average a-wave amplitude for the EDP injected mice was $289 \mu\text{V}$ (standard deviation $77 \mu\text{V}$) and the average b-wave amplitude was $352 \mu\text{V}$ (standard deviation $106 \mu\text{V}$). There were no notable differences in the a-wave latency following stimulus.

Ultrastructural observations of retina and choroid

Transmission electron microscopy (TEM) was used to look for ultrastructural changes in the ocular tissues of experimental and control mice. All of the tissues in both EDP and saline injected mice had normal morphologies overall (Figure 5). The capillary endothelial cells did not show any sign of cell division, dropout or migration. Some cellular extensions were noted in larger vessels, extending into the lumens, but this observation was made in control samples as well as EDP injected samples. There were similar macrophage distributions in both groups of mice. The penta-laminar structure of Bruch's membrane appeared normal in all of the samples, with none of the layers missing or clearly altered. While in some EDP-injected mice Bruch's membrane appeared thicker with wider basal laminae than control mice, this difference was not consistently observed. The retina/RPE/Bruch's membrane/choroid complexes were intact overall and had no gross morphological changes.

Changes in gene expression in elastin fragment injected mice using microarray analysis

To assess molecular changes that may precede structural or functional changes, we performed microarray analysis on injected and control mouse eyes, and noted that eyes of mice injected with elastin fragments showed a greater than 50% increase in expression of 20 genes including *Ceacam10*, *Ces7*, *Hspb1*, *Pacs1n3*, and *Col4a2* (p-values < 0.01). The most significant changes (fold change greater than 1.5 with corresponding p-values) are summarized in Table 2. Although these genes code for many types of proteins, we were most interested in proteins associated with the extracellular matrix. Most notably, there was a 53% increased expression of *Col4a2* (p-value < 0.01), which could be associated with extracellular matrix synthesis or turnover. Focused evaluation of other basal lamina genes revealed that, in addition to *Col4a2*, *Col4a1*, *Col4a5*, *Nidogen1*, and *Lama1* are also significantly upregulated. Although all of these genes may provide insightful information about elevated elastin in the eye, we chose to investigate the role of *Col4a2* in greater detail.

Collagen IV densitometry in Bruch's membrane

In order to determine whether elastin injection affected the abundance of collagen type IV in Bruch's membrane, we measured the labeling intensity of collagen IV in saline and EDP injected mice. The densitometry of collagen IV immunoreactivity in the control mouse eyes compared to the EDP mouse eyes indicated that there is a modest but significant 4.6%

increased labeling intensity of collagen IV in Bruch's membrane of the EDP injected eyes (p-value <0.02).

DISCUSSION

Disruption of Bruch's membrane by disease or experimental intervention is associated with CNVM formation (Chong et al., 2005; Spraul et al., 1999; Yu et al., 2008). Recently, it has been shown that elastin within Bruch's membrane is abnormally metabolized in AMD (Booij et al., 2010; Chong et al., 2005; Spraul et al., 1999). In a previous study we sought to determine if, in addition to disruption of the physical barrier, elastolysis may affect the behavior of choroidal endothelial cells, and found that EDPs increase the migration of these cells (Skeie and Mullins, 2008).

During this investigation, we found that receptors for EDPs including the alternatively spliced form of GLB1, as well as CTSA, NEU1 and the integrin heterodimer- $\alpha_v\beta_3$ are expressed by human choroidal endothelial cells. Furthermore, choroidal endothelial cells incubated with inhibitors to the elastin binding proteins showed a decrease in migration. Decreased migration with the application of anti- $\alpha_v\beta_3$ was independent of the presence of EDPs as a chemoattractant, as anti- $\alpha_v\beta_3$ inhibited migration from multiple pro-migratory stimuli. In contrast, the addition of 200mM lactose impairs migration in cells migrating towards EDPs, but not in cells migrating toward FBS. These results show that GLB1 is an elastin binding protein on the surface of choroidal endothelial cells that promotes migration toward elastin fragments. We further verified that its alternatively spliced transcript is present in the human RPE-choroid *in vivo*.

In addition to their effects on migration, EDPs affect gene expression in choroidal EC. The findings of the endothelial cell PCR profiling array indicated that EDPs can cause an increase in inflammation as well as extracellular matrix turnover, as EDPs caused an increase in the expression of *CCL2*, *SELL*, and *MMP9*. Monocyte chemotactic protein 1 (MCP-1) is a small chemokine that attracts monocytes from the circulation to a given site (Deshmane et al., 2009). L-selectin (SELL) is classically reported as a surface protein on leukocytes that aids in their extravasation from blood into nearby tissues by binding ligands (e.g., CD34) found on the surface of endothelial cells. In some cases, SELL is also found on the surface of endothelial cells while CD34 is present on leukocytes (Makowska et al., 2008). Although this method of leukocyte tethering is not the most common via SELL, it is possible that the increased expression of SELL on the endothelial cell surface increases binding to antigens on the leukocytes cell surface, including CD34. Both MCP-1 and SELL could play a role in increasing local inflammation, a feature of early AMD (Anderson et al., 2002; Mullins et al., 2006). Matrix metalloproteinase-9 (MMP-9) is an enzyme that breaks down extracellular proteins, including elastin and collagens (Birkedal-Hansen, 1995). Increased synthesis of MMP-9 in the choroid could increase the breakdown of Bruch's membrane, decreasing its ability to serve as a barrier, possibly allowing for the growth of new vessels to grow into the retina. The degradation of Bruch's membrane may also lead to the creation of free peptides (products of laminin and collagen IV degradation) that may also interact with GLB1.

In studies using cell cultures, we have found that soluble elastin fragments can bind to and induce choroidal endothelial cells to become more angiogenic and that choroidal endothelial cells can bind to elastin fragments via GLB1 *in vivo*. Since there is a correlation between elevated serum concentrations of elastin fragments and AMD severity in humans (Sivaprasad et al., 2005), we sought to determine if we could observe any early signs of choroidal endothelial cell activation or dysfunction, or any phenotypic characteristics of AMD, in a mouse model of elevated serum elastin fragments.

Ultrastructurally, we did not observe any major changes in the eyes of injected animals in comparison to the control eyes. The ERG results indicate that the rod photoreceptors and 'on' bipolar cells were functioning normally in all of the mice without any discernable differences between the two experimental groups or major injury to photoreceptor cells.

Collagen IV is a major component of the RPE and choriocapillaris basal laminae (Chen et al., 2003; Marshall et al., 1994), which comprise two of the five layers within Bruch's membrane. The collagen IV helices link head-to-head and form disorganized sheets with many kinks, a characteristic that is unique to collagen IV in comparison to the other collagens. This suits collagen IV for basement membranes as it allows transfer of nutrients, waste, and cells. With increasing age, there is evidence suggesting dysregulation of collagen IV content within Bruch's membrane as well as its accumulation in basal laminar deposits (Beattie et al., 2010; Chen et al., 2003; Marshall et al., 1994; van der Schaft et al., 1994).

We found that the expression of mRNA for precursor alpha 2 for collagen IV was increased by 53%. In Bruch's membrane, a slight but significant increase of 4.6% anti-collagen IV intensity was noted using morphometric analysis. This increase is found over a short experimental period, which may be much larger over the long period of AMD development in humans. Collagen IV has been described in basal laminar deposits beneath the RPE and an increase in collagen IV deposition may trap waste products from the retina and RPE such as lipoproteins and affect diffusion between the neural retina and choriocapillaris (van der Schaft et al., 1993; van der Schaft et al., 1994). In AMD, material trapped within Bruch's membrane may promote drusen formation, ischemia, and tissue atrophy. Moreover, increased synthesis of collagen IV in response to elastin fragments might represent an attempt at reassembling Bruch's membrane by the RPE and/or choriocapillaris.

In summary, elastin degradation plays a role in both the weakening of the barrier to endothelial cell migration into Bruch's membrane as well as activating endothelial cells to migrate and form tubes (Robinet et al., 2005). We suggest that elastin degradation may play an important role in choroidal neovascularization during late stage AMD. Mitigating this breakdown of elastin or interfering with EDP signaling (and the EDP-derived increase in MMP9 expression) may suggest a target for slowing the progression of AMD towards neovascularization.

EXPERIMENTAL PROCEDURES

Detection of the elastin binding protein mRNA

To determine if endothelial cells from the choroid express mRNA transcripts for the alternatively spliced form of GLB1 and its associated transmembrane proteins, RT-PCR was employed. Total RNA was isolated from choroidal endothelial cells and frozen punches of human RPE-choroid using the RNeasy mini kit according to manufacturer's instructions (Qiagen; Valencia, CA). Punches of human RPE-choroid (average age = 84 years) were obtained as described previously and were snap frozen within 4.75 hr of death (Mullins et al., 2006). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems; Foster City, CA). Following reverse transcription, PCR was carried out using the BIOLASE DNA polymerase kit with the following conditions for 35 cycles: denaturing at 94°C for 45 seconds, annealing at 62°C for 30 seconds, and elongation at 72°C for 1 minute (Bioline; Taunton, MA). Primers were designed for the amplification of *GLB1*, *CTSA*, and *NEU1* (Integrated DNA Technologies; Coralville, IA). Since the 61 kDa elastin binding form of GLB1 is translated from an alternatively spliced transcript lacking exons 3, 4, and 6, the forward primer for *GLB1* was designed to span the boundary between exons 2 and 5 (Figure

1). Amplified PCR products were separated using agarose gel electrophoresis and visualized using ethidium bromide. Identities of amplified fragments of *GLB1* were determined by automated DNA sequencing. Primers used were: *GLB1* forward primer, 5'-GCC ATC CAG ACA TTA CCT GGC A -3', *GLB1* reverse primer, 5'-TGT CCG GTA CAG CAC AAA CCC ATA -3', *CTSA* forward primer, 5'-GTG CCC AGC CAT TTT AGG TA -3', *CTSA* reverse primer, 5'-TTC TGG TTG AGG GAA TCC AC -3', *NEU1* forward primer, 5'-GAA CCT TGG GGC AGT AGT GA -3', *NEU1* reverse primer, 5'-CTC ATA GGG CTG GCA TTC AT -3'.

Elastin-mediated endothelial cell migration

Migration assays were performed using the sterilized culture plate inserts with 8 μ m diameter pores (Cat. # PI8P01250, Millipore; Billerica, MA), as described previously (Skeie and Mullins, 2008). Cells used in these studies were either human choroidal endothelial cells, with a cobblestone morphology and positive for UEA-I lectin and anti-PECAM1, described and characterized previously (Skeie and Mullins, 2008), or the chorioretinal endothelial cell line Rf/6a (from ATCC, Manassas, VA). Briefly, cells were added to the upper well of a culture insert placed in a 12 well culture plate. F12 medium (with 100 Units/mL penicillin/ 100 μ g/mL streptomycin) containing 100 μ g/mL EDPs, (Elastin Products Company; Owensville, MO) was added to the well of the 12 well culture plate (i.e., the bottom surface of the insert). An equal volume of HBSS or 20% FBS in medium were used as controls. A mouse antibody to integrin heterodimer $\alpha_v\beta_3$ (R&D; Minneapolis, MN) at a concentration of 5 μ g/mL or 200 mM lactose (Fisher Scientific; Fair Lawn, NJ) was added to the upper chamber with the cells. The antibody and lactose were added to competitively inhibit the binding sites of two possible elastin fragment receptors, $\alpha_v\beta_3$ and GLB1, respectively (Hinek et al., 1993; Robinet et al., 2007; Rodgers and Weiss, 2004). Following 8 hours of incubation, the lower surfaces of the membranes were observed using a scanning electron microscope (SEM; Hitachi S-3400N) at the University of Iowa Central Microscopy Facility. The number of migrated cells on the lower surface of the membrane were quantified using SEM as described previously (Skeie and Mullins, 2008). To ensure that the antibody did not alter migration due to changes in cell death or proliferation, cells were grown in the presence of the proteins, or HBSS as a control. Cell viability was tested after 48 hours using a LDH assay according to manufacturer's instructions (Roche; Madison, WI). Methods were repeated using a primary culture of human choroidal endothelial cells instead of Rf/6a cells (Skeie and Mullins, 2008). All experiments were performed with at least 3 replicates.

EDP treated endothelial cell PCR array profiling

To determine the changes in choroidal endothelial cell gene expression due to elevated levels of EDPs compared to controls, quantitative PCR arrays were used. Primary cultures of human choroidal endothelial cells at the second passage were plated into the wells of a 6-well culture plate and treated with either 100 μ g/mL EDPs or an equal volume of HBSS for 48 hours. Total RNA was extracted and reverse transcribed as described above, and quantitative PCR was performed using PCR arrays containing 84 probe sets for endothelial cell associated genes (Endothelial Cell Biology PCR Array, SABiosciences product # PAHS-015A; Frederick, MD). These arrays are designed to interrogate genes involved in multiple EC associated pathways, including vascular tone, angiogenesis, EC activation. The complete list of EC genes is available as Supplemental Table 1. Experiments were performed in triplicate, and values were normalized to 6 housekeeping genes on the PCR array as described by the manufacturer.

Mouse injections of elastin derived peptides

To elevate the level of circulating EDPs within the serum of mice, a series of EDP injections were given. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Animal Care and Use Committee at the University of Iowa. C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME) were given intraperitoneal injections of 200 ng etna-elastin (Elastin Products Company) in phosphate buffered saline (PBS) (concentration = 1 ng/ μ L) (n = 7) or an equal volume of Hank's buffered saline solution (HBSS) in PBS (n=8). Each condition was performed in triplicate. Injections were performed 3 $\times$ weekly for 4 weeks beginning when the mice were 7 months old. This method of administration was used because it has been previously demonstrated by other investigators that rats will metabolize roughly 60% of orally administered elastin within the first 22 hours, however, elastin remains in the system at elevated levels for more than 2 weeks after intraperitoneal injection (Yu et al., 1979; Yu and Yoshida, 1977); we used these studies to guide the timecourse of our protocol. Following four weeks of injections, the mice were evaluated by electroretinography (below), and then sacrificed. The eyes were enucleated and fixed in 4% paraformaldehyde (pH 7.4) for 4 hours (n= 7 EDP, n= 8 control), fixed in 1/2 strength Karnovsky's fixative (2% formaldehyde and 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, containing 0.025% CaCl₂) for greater than 12 hours (n= 4 EDP, n= 4 control), or flash frozen in liquid nitrogen (n= 3 EDP, n=4 control).

Electrophysiology of mouse eyes

At the end of the EDP injection series, we sought to determine if the EDP injections affected the physiological function of the retina. This was evaluated using electroretinography as described previously (Satz et al., 2009). Briefly, mice were dark adapted overnight and then were anesthetized with 1.75/0.25 mg/kg Ketamine/Xylazine intraperitoneally. Eyes were dilated with 0.5% Tropicamide (one drop per eye) and gold ring electrodes were placed on each eye. The reference eye was covered completely to prevent stimulus from reaching the retina. A ground wire was placed in the tail (Pinto et al., 2007; Saszik et al., 2002). A 525 nm light stimulus was applied to the measured eye. The mouse, lead wires and light stimulus were fully contained within the dark. Data were collected using Labview software. ERG output curves were analyzed for the heights of the a- and b-waves and compared between EDP (n = 4) and buffer (n = 4) injected animals.

Ultrastructural analyses using transmission electron microscopy

To determine whether EDP treatment results in ultrastructural changes in the retina, RPE cells, Bruch's membrane, and the choroid, transmission electron microscopy (TEM) was employed. Posterior halves of mouse eyes were fixed in 1/2 strength Karnovsky's fixative and processed for TEM, and embedded in Spurr's medium (Mullins et al., 2007). Sections were cut at 90 nm with an ultra-microtome (Leica) and collected on copper slot grids coated with Formvar (0.5%). TEM image collection was performed as described previously (Mullins et al., 2007).

Mouse gene expression evaluation using microarrays

The effect of elevated circulating EDPs on gene expression in the mouse eye was determined using expression microarrays. Frozen whole eyes were bisected and immediately immersed in the RLT digestion buffer (RNeasy kit, Qiagen; Valencia, CA). RNA was then isolated with the same kit according to the manufacturer's instructions. Microarray analysis was performed by the DNA facility at the University of Iowa, Iowa City, Iowa using Affymetrix mouse gene chip 430 2.0 array (Affymetrix; Santa Clara, CA) using methods described previously (Scheetz et al., 2006). These arrays have over 39,000 gene-level

probesets. Data were normalized using the Genomics Suite software (Partek, Inc. St. Louis, MO) and RMA-normalized data were imported into File Maker Pro (FileMaker, Santa Clara, CA). Genes were ranked according to fold change and p-value in order to find the most significant changes. Probesets with >1.5 fold increased or decreased expression, with a p-value of less than 0.01, are displayed in Table 2.

Bruch's membrane collagen IV densitometry

Morphological analysis was used to quantify differences in collagen IV protein presence in Bruch's membrane between EDP injected (n = 2) and control mice (n = 3). Cryosections of sucrose embedded mouse eyes were collected at a thickness of 7 μ m. Immunohistochemistry was performed as described previously, using 4.0 μ g/mL of goat anti-human collagen IV antibody (Santa Cruz Biotechnology Inc.; Santa Cruz, CA) and the Vectastain Elite ABC horse radish peroxidase kit (Vector Laboratories, Burlingame, CA) (Mullins et al., 2006). Briefly, sections were blocked 20 min with horse serum (1%), were rinsed, and were incubated 1 hr in collagen IV antibody. The secondary antibody used was a biotinylated anti-goat antibody, used at a concentration of 5 μ g/mL (Vector Laboratories). All sections were developed for 3.5 minutes using the Vector VIP peroxidase development kit (Vector Laboratories) which forms a purple reaction product, rinsed in distilled water, dehydrated in alcohol and Clear Rite (Richard Allan; Kalamazoo, MI). The time of development was consistent between samples and was within the linear region of peroxidase development for this antibody to avoid any saturation of sections. Slides were coverslipped with Permount (Fisher Scientific; Pittsburgh, PA) and images were collected using a BX-41 Olympus microscope equipped with a 100x objective lens and a SPOT-RT camera. Five images were randomly collected along the length of the choroid for each eye using identical exposure conditions in a masked fashion. These images were all converted to 8-bit grayscale and inverted so that black pixels had a value of 0 and white pixels had a value of 255 on the pixel intensity scale. The average anti-collagen IV intensity along the entire length of Bruch's membrane was measured by tracing a line along the membrane using the polygon tracing tool in ImageJ for each image. This line for each image gives a resultant average pixel intensity for the membrane. The higher the average pixel value, the higher the immunoreactivity in that image. Bruch's membrane is very thin in the mouse, so the line tracing for densitometric analysis included both RPE and choriocapillary basal laminae even though the choriocapillary basement membrane side of Bruch's membrane is where deposition of collagen IV occurs in aging humans (Marshall et al., 1994). In order to determine the average difference, the mean values for all EDP injected mouse images were averaged together, as were the values for the control images. Comparisons of the 20 measurements per condition were analyzed using a two-tailed paired Student's t-test.

Highlights

The mechanism of choroidal endothelial cell activation by elastin was explored. The alternatively spliced form of beta-galactosidase (GLB1) is present on human choroidal endothelial cells and functions as a receptor for elastin peptides. Elevated circulating elastin peptides alter gene expression of ECM proteins in the eye and may be related to ECM changes in macular degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLB1 **Alternatively Spliced Form of GLB1****Figure 1. *GLB1* primer design**

The gene for *GLB1* has 15 exons. The alternatively spliced form of *GLB1* results in the binding protein for soluble elastin fragments. In order to determine if cells expressed the mRNA for the alternatively spliced form of *GLB1*, primers were designed to amplify the alternatively spliced form, as shown.

Figure 2. Expression of *GLB1* in the human choroid

Transcripts for the peripheral elastin fragment binding protein, *GLB1*, and its two associated trans-membrane proteins are present in human RPE/choroid *in vivo*. The smaller (expected size) band for *GLB1* was confirmed by sequencing.

Figure 3. Endothelial cell migration assay

To block the possible binding sites for the EDPs, an antibody directed against $\alpha_v\beta_3$ at a concentration of 5 $\mu\text{g/mL}$ or 0.2 M lactose (to inactivate GLB1) was added to the upper chamber with the seeded cells. EDPs (100 $\mu\text{g/mL}$), saline, or 20% FBS were added to the lower chamber. The addition of the antibody directed against $\alpha_v\beta_3$ decreased the migratory response toward both EDPs and FBS significantly. The addition of 200 mM lactose only significantly reduced the migratory response induced by EDPs, not the response induced by FBS. (**) indicates a p-value < 0.01, (*) indicates a p-value = 0.01, and (NS) indicates a p-value < 0.5, which is non-significant. Error bars = SEM.

Figure 4. Mouse ERGs

An example of an ERG from one of the four mice in each experimental group is shown. The a-wave is measured from baseline to the bottom of the deepest trough (approximately 300 μ V in these graphs) and the b-wave is measured from the bottom of the a-wave to the highest peak (approximately 700 μ V for these graphs). All eight ERGs had similar forms and intensities.

Figure 5. Transmission electron micrographs of the eyes of EDP- and buffer-injected mice Although many regions were evaluated, the major area of interest was the RPE/Bruch's membrane/choroid complex, as shown. Ultrastructurally, there were no major, consistent differences detected between HBSS (left) and EDP injected mice (right), indicating that EDPs do not cause major morphological changes in the retina or choroid. A trend toward increasing basal lamina thickness was observed in some EDP treated eyes, but this was not consistently observed. RPE indicates the RPE basal infoldings; RPEBL, the RPE basal lamina; CCBL, choriocapillaris basal lamina; CCL, choriocapillaris lumen. Scale bar = 200nm.

Table 1

EDP exposed human choroidal endothelial cell gene expression.

Gene	Fold Change	P-value
<i>BCL2A1</i>	4.77	<0.01
<i>CCL2</i>	2.34	0.04
<i>ICAM1</i>	0.53	0.03
<i>LSEL</i>	4.80	0.04
<i>NOS3</i>	5.65	0.04
<i>MMP9</i>	4.30	0.04
<i>TNFSF10</i>	3.09	0.04

Table 2

EDP injected mouse gene microarray results. Genes with ≥ 1.5 fold increased expression in EDP injected mice compared to control mice (p-value ≤ 0.01).

Gene	Fold Change (base 10)	P-value
<i>Ptpru</i>	1.88	0.005
<i>Ces7</i>	2.02	0.008
<i>Plekha4</i>	1.66	0.006
<i>Pacsin3</i>	1.52	0.006
<i>Emid1</i>	1.56	0.010
<i>2210019G1Rik</i>	1.74	0.001
<i>Cr1</i>	1.79	0.010
<i>Ceacam10</i>	1.91	0.009
<i>Rspo1</i>	1.73	0.005
<i>Tomm40</i>	1.53	0.007
<i>Mypn</i>	1.59	0.001
<i>C030030A07Rik</i>	1.62	0.009
<i>LOC100046788</i>	1.97	0.008
<i>Clic5</i>	1.57	0.007
<i>Ankrd24</i>	1.77	0.006
<i>Dnase2b</i>	2.01	0.010
<i>LOC100048029</i>	1.79	0.010
<i>Col4a2</i>	1.54	0.008
<i>Hspb1</i>	1.52	0.003