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Diabetes-induced morphological, biomechanical, and compositional changes in ocular basement membranes

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ABSTRACT

The current study investigates the structural and compositional changes of ocular basement membranes (BMs) during long-term diabetes. By comparing retinal vascular BMs and the inner limiting membrane (ILM) from diabetic and non-diabetic human eyes by light and transmission electron microscopy (TEM), a massive, diabetes-related increase in the thickness of these BMs was detected. The increase in ILM thickness was confirmed by atomic force microscopy (AFM) on native ILM flat-mount preparations. AFM also detected a diabetes-induced increase in ILM stiffness. The changes in BM morphology and biophysical properties were accompanied by partial changes in the biochemical composition as shown by immunocytochemistry and western blots: agrin, fibronectin and tenascin underwent relative increases in concentration in diabetic BMs as compared to non-diabetic BMs. Fibronectin and tenascin were particularly high in the BMs of outlining microvascular aneurisms. The present data showed that retinal vascular BMs and the ILM undergo morphological, biomechanical and compositional changes during long-term diabetes. The increase in BM thickness not only resulted from an up-regulation of the standard BM proteins, but also from the expression of diabetes-specific extracellular matrix proteins that are not normally found in retinal BMs.

1. Introduction

As of 2012, diabetes affects more than 300 million people worldwide and its incidence and prevalence continues to increase (Sherwin and Jastreboff, 2012). While there is existing medication to successfully treat hyperglycemia and ketoacidosis, long term, chronic conditions, like diabetic nephropathy, neuropathy, and delayed wound healing are not well controlled. Diabetic retinopathy affects nearly 40% of individuals with type II diabetes, and 86% of individuals with type I diabetes in the United States alone (Cheung et al., 2010). Given its prevalence, it remains the leading cause of preventable blindness in individuals under the age of 50 (Frank, 2004; Fong et al., 2004; Kempen et al., 2004).

One of the hallmarks of long-term diabetes is a massive thickening of basement membranes (BMs) that has been particularly well documented for retinal vascular BMs (Ashton, 1974; Roy et al., 2010; Osterby, 1990; Tsilibary, 2003). BMs are thin sheets of extracellular matrix that are important for structural integrity of epithelia, muscle fibers, blood vessels and peripheral nerves (Yurchenco and Patton, 2009; Halfter et al., 2013a). Major BM proteins include type IV collagen, laminin, nidogen, and several members of the proteoglycan family, including perlecan, agrin, and collagen XVIII. Proteoglycans are the prime water-binding constituents in the highly hydrated BMs (Erickson and Couchman, 2000; Timpl and Brown, 1996). Abnormalities in the regulation of the expression of these BM components and in the expression of growth factors in the diabetic eye may play an important role in BM thickening and disease development in diabetic retinopathy (King, 2001; Tsilibary, 2003). It has been postulated that BM thickening may be linked to

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Table 1
Diabetic eyes. Overview of basic eye donor characteristics for the diabetic patients.

#	Age	Gender	Race	Cause of death	Med history
1	38	m	w	MI	IDDM; Type Ix30y
2	52	m	w	Peritonitis	IDDM; type IIx17y
3	54	f	w	ICH	IDDM; Type IIx30y
4	61	f	w	MI	IDDM; type IIx15y
5	62	m	w	MI	IDDM; type IIx25y
6	79	m	w	MI	NIDDM; type IIx10y
7	80	f	w	CA	NIDDM; type IIx42; IDDMx10y
8	81	m	w	MI	IDDM; type IIx10y
9	83	m	b	CHF	NIDDM; type IIx15y
10	83	f	w	Accident	NIDDM; type IIx10y
11	83	m	w	MI	IDDM; type IIx20y
12	85	f	w	CHF	IDDM type IIx10y
13	87	m	w	MI	NIDDM; type IIx3y

Type II or I: diabetes type II; or I.
x30y: duration of the diabetic condition for 30 years.
CVA = Cerebrovascular accident.
ICH = Intracerebral hemorrhage.
MI = Myocardial infarction.
MVA = motor vehicle accident.
CA = cancer.
CHF = congestive heart failure.

the formation of "advanced glycation end products" of BM proteins in hyperglycemia (Degenhardt et al., 1998; King, 2001; Stitt et al., 1997; Zhang et al., 2009), excessive cross-linking and a decreased rate of BM protein degradation, and an up-regulation of BM protein synthesis (Kuiper et al., 2008; Roy et al., 2010).

It is still unresolved why and how BMs become thicker and why this thickening leads to chronic disease conditions associated with diabetes, like diabetic retinopathy. It is reasonable to postulate that excessively thick BMs impede the passage of oxygen, proteins and immune cells and thereby contribute to hypoxia-induced uncontrolled angiogenesis in the retina and vitreous, to peripheral nerve damage, and to delayed wound healing (Kuiper et al., 2008). BM thickening may also result in a loss of vascular elasticity, thereby contributing to elevated blood pressure (Curtis et al., 2009; Durham and Herman, 2011; Zatz and Brenner, 1986).

Previous investigations have shown that human BMs undergo morphological, biophysical and compositional changes with advancing age (Candiello et al., 2010). In a comparative study of these changes in diabetic and non-diabetic ocular BMs using transmission electron microscopy we show that the thickness of BMs from diabetic patients is massively increased compared to age-matched non-diabetic patients. TEM uses sample preparations requiring dehydration that normally leads to shrinkage of tissues. To this end, we also investigated the age-related morphological changes of the ILM in fully hydrated conditions using Atomic Force Microscopy (AFM). AFM also provided data on diabetes-induced biomechanical changes. To explore the compositional changes in BMs between age-matched diabetic and non-diabetic patients, we used immunocytochemistry and western blotting.

The present study shows that the BMs of the human eye undergo diabetes-dependent alterations that include an increase in thickness and stiffness and in diabetes-related changes in its biochemical composition. We propose that BMs from other tissues undergo similar changes.

2. Methods

2.1. Human eyes and antibodies

Adult human eyes were obtained from CORE, the "Center of Organ Recovery and Education" of the University of Pittsburgh. The use of the human eyes was approved by the Institutional Review Board of the University of Pittsburgh under the IRB Exempt Protocol number #0312072. The clinical data for the diabetic eyes are listed in Table 1. The clinical data for the non-diabetic eyes was previously listed (Halfter et al., 2013b). Polyclonal antisera to collagen IV, laminin-1 were obtained from Rockland Immunochemicals (Gilbertsville, PA), Sigma (St. Louis, MO), Invitrogen (Carlsbad, CA), and Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal rabbit anti-human agrin antiserum was raised against a fusion protein from the N-terminal part of human agrin (Cotman et al., 2000). A monoclonal antibody to cellular fibronectin (Mab 1940) was purchased from Millipore, Temecula CA. Monoclonal antibodies to tenascin C (Mab RR1) and to the 7S domain of collagen IV a3/4/5 (Mab J3-2; SundarRaj and Wilson, 1982) were kindly provided by Dr. Ruth Chiquet-Ehrisman, Friedrich Miescher Institute Basel, Switzerland and Dr. Nirmala SundarRaj, University of Pittsburgh. SytoxGreen (Molecular Probes, Eugene, OR) was used as a nuclear counter stain.

2.2. Histology

For immunocytochemistry, fetal eyes or adult retinal samples were fixed in 4% paraformaldehyde and processed for cryostat-sectioning as described (Candiello et al., 2010). The sections were mounted in 90% glycerol and examined with an epifluorescence or a confocal microscope (Flowview, Olympus). For transmission electron microscopy, samples from the dorso-central retina were fixed in 2.5% glutaraldehyde, 2.5% paraformaldehyde overnight. The samples were osmicated and embedded in EPON according to standard procedures. Thin sections were examined by a JEOL electron microscope at 25,000×. TEM measurements of ILM thickness are based on the analysis of 27 pairs of non-diabetic and 11 diabetic human eyes. The eyes were enucleated from donors less than 30 h after expiration with expiration-enucleation intervals of approximately 10 h. Retinal samples were taken from both eyes, and two thin sections per retina sample were surveyed at 25,000×. At least twenty ILM measurements per retinal sample were taken at random, and the average value ±standard deviation was calculated for the pairs of eyes.

2.3. ILM and vascular BM preparation and western blotting

ILMs and vascular BMs sheets were prepared by incubating segments of adult human retina overnight in 2% Triton-X-100 in water. The detergent-insoluble BMs were transferred with a Pasteur pipette under a dissecting microscope and under dark-field illumination into new Triton-X-100 and 1% deoxycholate (Duhamel et al., 1983; Candiello et al., 2010; Halfter et al., 2013b). The transfer was repeated 3-times. The washed BMs were pelleted by centrifugation at 10,000 rpm. To facilitate the solubilization of the ILM proteins for SDS PAGE, the ILM pellets were incubated with 100 μL of each 0.1 μU/μl flavium, heparinum, heparitinase 1 and chondroitinase ABC for 1 h (Seikagaku Corporation, Japan). The deglycosylation step of the sample was omitted for the detection of the GAG-glycosylated agrin. 10× SDS sample buffer and solid urea is added to a final concentration of 1× and 8 M, respectively. The samples were boiled for 10 min, centrifuged and loaded onto 3.5–15% SDS gradient gels. The blotting was performed using a semi-dry blotting device (Biorad, Hercules, CA). The blots were blocked in 2% dry milk followed by primary antibodies at a dilution of 1:1000 for 4 h. Alkaline-phophatase-labeled antibodies (1:1000 for 4 h; Jackson ImmunoResearch) followed by NBT/BCIP (Roche, Indianapolis, IN) staining were used to visualize the protein in the blots.