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 tenasin 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.

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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 perlecain, 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
Organ Recovery and Education

2.1. Human eyes and antibodies

Methods

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2. Methods

undergo similar changes.

biochemical composition. We propose that BMs from other tissues

d徘徊 diabetes-related changes in its

dler diabetes-dependent alterations that include an increase in

the ILM in fully hydrated conditions using Atomic Force

tical, biophysical and compositional changes with

and Herman, 2011; Zatz and Brenner, 1986).

thickening may also result in a loss of vascular elasticity, thereby

disease conditions associated with diabetes, like diabetic retinopathy. It is reasonable to postulate that

this thickening leads to chronic disease conditions associated with

is excitotoxicity and to delayed wound healing (Kuiper et al., 2008). BM

damage, and to delayed wound healing (Kuiper et al., 2008; Roy et al., 2010).

It is still unresolved why and how BMs become thicker and why

these changes in diabetic and non-diabetic ocular BMs using

transmission electron microscopy we show that the thickness of

BM proteins in hyperglycemia (Degenhardt et al., 1998; King, 2001; Stitt et al.,

and chondroitinase ABC for 1 h (Seikagaku Corporation, Japan). The sections were

sectioning as described (Candiello et al., 2010). The sections were

and 8 M, respectively. The samples were boiled for 10 min, centrifuged and

for the pairs of eyes.

The transfer was repeated 3-times. The washed BMs were pelleted

and 8 M, SDS sample buffer

was raised against a fusion protein from the N-terminal

water. The detergent-insoluble BMs were transferred with a Pas-

chondroitinase ABC for 1 h (Seikagaku Corporation, Japan). The sections were

and examined with an epifluorescence or a confocal microscope (Flowview, Olympus). For transmission

dye-agglutination reactions. GAGs were identified by incubating the sections with 100

mu; medium 199, 10% FBS, and 1% streptomycin-penicillin. The samples were incubated for 1 h

with 1% SDS, 10% glycerol, and 0.1% Triton X-100 followed by 1% SDS, 30% glycerol,

The blots were blocked in 2% dry milk followed by primary antibodies at a dilution of 1:1000 for 6 h. Alkaline-phosphatase-labeled

the protein in the blots.

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 anti-

serum 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 75 domain of collagen IV a3/4/5 (Mab J3-2; Sundaraj and

Wilson, 1982) were kindly provided by Dr. Ruth Chiquet-

Ehrisman, Friedrich Miescher Institute Basel, Switzerland and

Dr. Nirmala Sundaraj, 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 JEO

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-enculeation 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 Pas-
teur 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 µL/mL 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

load 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-phosphatase-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.

<table>
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</table>

Type II or I: diabetes type II; or I.

x30y: duration of the diabetic condition for 30 years.

CVA – Cerebrovascular accident.

ICH – Intraocular 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).

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

BM proteins 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

Electron Microscopy we show that the thickness of

BM proteins 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.

The present study shows that the BMs of the human eye un-

dergo 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.