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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.

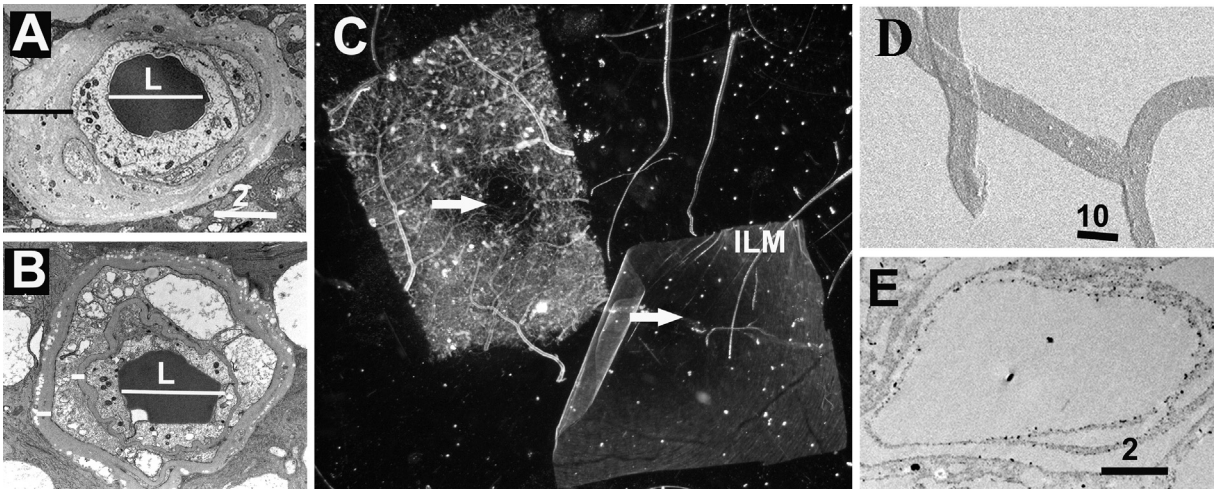

Fig. 1. Ultrastructure of diabetic and non-diabetic human retinal vascular BMs. Representative TEM micrographs show cross sections of similar-sized retinal capillaries from a 62-year old diabetic (A) and a 74-year old non-diabetic patient (B). While the lumen of both vessels (L) are similar in size, the associated vascular BM is much wider in the diabetic eye (A; black bar) than in the non-diabetic eye (white bars; B). Dark-field image showing the two types of BM sheets isolated from adult human retina as they appear under a dissecting microscope: to the left, a sheet of vascular BMs, to the right, the ILM. Both BM sheets were isolated from the foveal area of a diabetic retina. The foveal avascular zone (FAZ, arrow) is clearly visible in the vascular BM sheet (C). The foveal area is also recognizable in the ILM by its darker appearance (arrow C). An SEM (D) and a TEM image (E) of isolated vascular BMs sheets showed clean extracellular matrix tubes that are not contaminated by cellular debris. Scale bar: A, B, E: 2 μm; D: 10 μm.

2.4. Atomic force microscopy (AFM) of the ILM

ILM flat mounts for AFM probing were prepared in the following way: glass slides were coated with 10 μg/ml polylysine (P-1524; Sigma). The coated area of the slide was encircled with a PAP-Pen (Research Products International, Mt. Prospect, IL) to contain the droplet of PBS that holds the BM samples. Segments of ILMs were transferred into the PBS droplet, excessive PBS was removed, and the slides were centrifuged at 1000 rpm for 5 min to firmly attach the ILMs to the poly-lysine-coated slides (Candiello et al., 2010). The immobilized BMs were washed 3 times in PBS and immuno-stained for laminin or collagen IV for better visualization. The imaging and thickness measurements were done by an MFP-3D AFM (Asylum Research, Santa Barbara, CA) mounted on top of the Olympus IX-71 fluorescence microscope (Olympus, Tokyo, Japan) as described previously (Candiello et al., 2007, 2010). For all experiments, 100 mm long silicon-nitride triangular cantilevers with pyramidal tips were used (Veeco, Inc, Santa Barbara, CA) that have a nominal spring constant of ~0.8 N/m. The spring constant was individually calculated for each cantilever prior to experimentation using the thermal fluctuation method (Hutter and Bechhoefer, 1993). For thickness measurements 25 measurements were taken at 4 randomly selected locations. Average values with ±standard deviation were calculated. Statistical analysis was performed using Student's *T*-test.

All nanostiffness measurements were done using ARTIDIS AFM system (Nanosurf AG, Liestal, Switzerland) as described before (Henrich et al., 2012). Briefly, we used triangular cantilever with a nominal spring constant of 0.081 N/m (HYDRA6V-200WG, AppNano, Santa Clara, USA) and nominal tip radius of 6 nm. In total, we measured 12 samples (6 from diabetic and 6 from non-diabetic patients) and each sample on at least 10 different areas. Stiffness was measured in Force Spectroscopy mode using a force of 1.8 nN and over an area of 25 μm^2 where for each 100 force curves were collected. Using ARTIDIS analysis software, we calculated the dynamic elastic modulus based on the Oliver and Pharr theory.

3. Results

3.1. Diabetes-related morphological changes of the retinal vascular BMs

Diabetes-related changes of retinal vascular BMs have traditionally been recorded by using transmission electron microscopy (TEM) imaging of human retinal samples (Ashton, 1974; Osterby, 1990; Roy et al., 2010; Tsilibary, 2003). Consistent with earlier results, we found that of the BM walls of similar-caliber capillaries of diabetic eyes were consistently thicker than from similar aged non-diabetic eyes (Fig. 1A, B). Problematic in quantifying the thickness differences was the selection of capillaries to be compared and the fact that the capillaries were not consistently sectioned perpendicularly to their long axis. We therefore isolated the vascular BM sheets from adult human diabetic and non-diabetic eyes (Fig. 1C). SEM and TEM of the isolated BMs showed that the isolated BM sheets are not contaminated with cellular debris or organelles (Fig. 1D, E). The vascular BM sheets were prepared as flat-mounts and stained with anti-collagen IV for whole mount evaluation and capillary diameter measurements (Fig. 2A, B). We assumed that the thin capillary BM tubes in diabetic and non-diabetic eyes have a similar-diameter inner lumen that allows the passage of individual red blood cells and that an increase in the outer diameter of capillary BM tubes of diabetic relative to similar aged non-diabetic eyes is predominantly due in an increase in the thickness of the BMs outlining the vessels. An increase in the diameter of a capillary BM tubes was, therefore, considered a measure for an increase in the thickness of the vascular BMs. For consistency, we exclusively measured the caliber of the capillary tubes that outline the foveal avascular zone (black star; FAZ; Fig. 2A, B). Microvascular aneurisms that are regularly and frequently seen in BM preparations from diabetic but not in preparations from non-diabetic eyes (Fig. 2B, red arrow, 2C, white stars) were excluded from the measurements. Results showed that the outer diameter of capillary tubes from diabetic eyes were consistently thicker than that from non-diabetic eyes (Fig. 2D). The data were plotted according to age to account for the slight age-dependent increase in the capillary

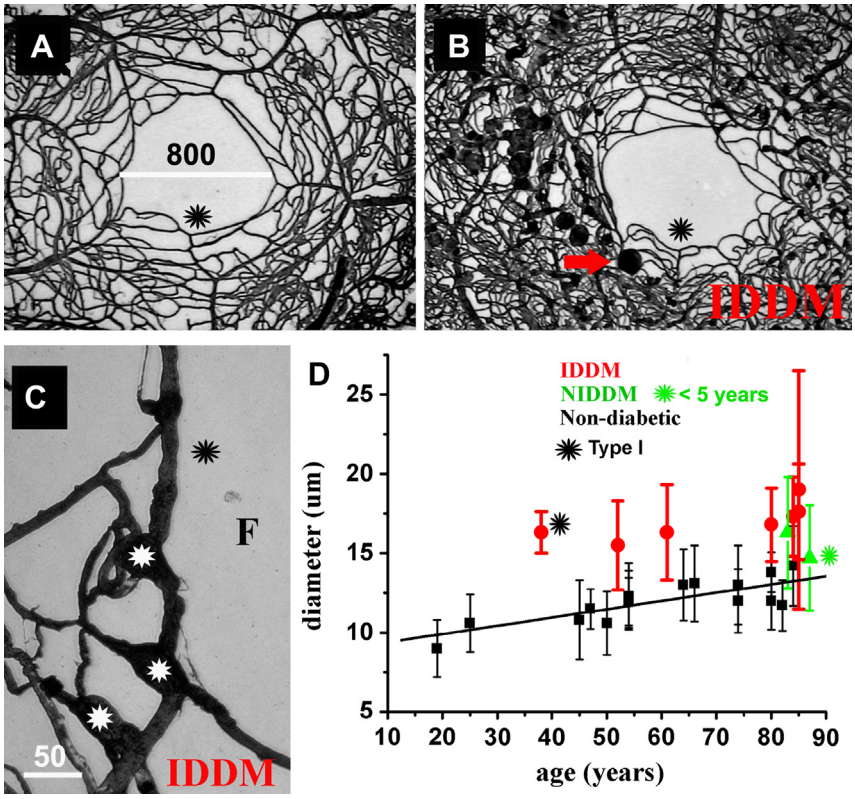

Fig. 2. Sheets of retinal vascular BM from a non-diabetic (A) and a diabetic eye (B). The sheets, stained for collagen IV, are from the foveal area with the foveal avascular zone (FAZ) in the center. Note the presence of microvascular aneurisms at low magnification (B; red arrow) and at high power (C, white stars). The outer diameter of the capillaries that outline the FAZ were measured and plotted against age. The plot shows that the diameter of the capillaries from non-diabetic eyes slightly increases with age. The plot also shows that the diameter of the capillaries from diabetic eyes is approximately 5 μm greater than the diameter of capillaries from non-diabetic eyes when diabetes is longer than 10 years. Bars: A, B: 800 μm; C: 50 μm.

diameter. The increase in capillary diameter was detected in vascular BM preparations from eyes of type I, type II, insulin-dependent and non-insulin dependent patients (Fig. 2D). However, the duration of the diabetic condition mattered, as capillary diameter increases were minor with diabetes durations of less than 5 years. The diameter differences between vascular BMs from diabetic with a diabetes ≥10 years and non-diabetic eyes were on average 5 μm. Since measurements of the capillary tube diameter from the whole mounts included the two sides of the BM walls we inferred that the increase in the thickness of an individual vascular BM wall with ≥10 years of diabetes relative to non-diabetic eyes is approximately 2.5 μm.

3.2. Diabetes-related morphological changes of the inner limiting membrane

The inner limiting membrane (ILM) was the second BM that was investigated for this study. The ILM is a BM that is located at the vitreal border of the retina (Fig. 3A, B). Measurements of ILM thickness by TEM were straightforward and reliable as the angle of sectioning was perpendicular to the retinal surface and a defined area of the retina was selected (Candiello et al., 2010). After examining only a few samples, it became obvious that the thickness of ILMs from diabetic eyes (Fig. 3A) was much greater than the thickness of the ILMs from similar aged non-diabetic retinas (Fig. 3B). Subsequent systematic measurements were based on TEM imaging of 27 non-diabetic and 11 diabetic retinas. Consistent with earlier studies (Candiello et al., 2010), data showed a natural, age-dependent increase in ILM thickness in non-diabetic eyes (Fig. 3C). Further, the ILMs from diabetic eyes were consistently thicker than the ILM from non-diabetic eyes of similar age (Fig. 3C). This applied equally to eyes from type I, type II, non-insulin and insulin-dependent diabetic patients (Fig. 3C). The duration of diabetes played, as in the vascular BMs, a major role, as the increase in ILM thickness was only minor with less than 5 years of diabetes.

TEM imaging of BMs requires chemical fixation and dehydration of the retinal samples. To determine the thickness of the ILMs under native conditions, we isolated ILMs from adult human diabetic and non-diabetic retinas (Fig. 1C) and used atomic force microscopy (AFM) to measure the thickness of ILMs. Previous TEM examination showed that the isolated ILMs were free of cellular debris and organelles (Candiello et al., 2010). For the AFM measurements, the ILMs were flat-mounted on glass slides with the vitreal side facing up (Fig. 4A). Thickness measurements were done along the edges of the ILM sheets or along scratches in the ILM to determine the height difference between the ILM and the adjacent the glass surface (Fig. 4A, B). AFM-based height measurements of the flat-mounted ILMs showed that the thickness of non-diabetic ILMs naturally increased with advancing age, consistent with previous AFM measurements (Fig. 4C; Candiello et al., 2010) and consistent with the age-dependent increase in ILM thickness recorded by TEM (Fig. 3C). Likewise, similar to the TEM-based measurements, the thickness of ILMs from diabetic eyes was greater than the thickness of ILMs from similar-aged non-diabetic eyes (Fig. 4C).

When TEM and AFM-based thickness measurements of ILMs from non-diabetic eyes were compared, the AFM thickness of an ILM of a given age was approximately twice the TEM-based thickness (Fig. 4D). This difference is due to the large quantities of water

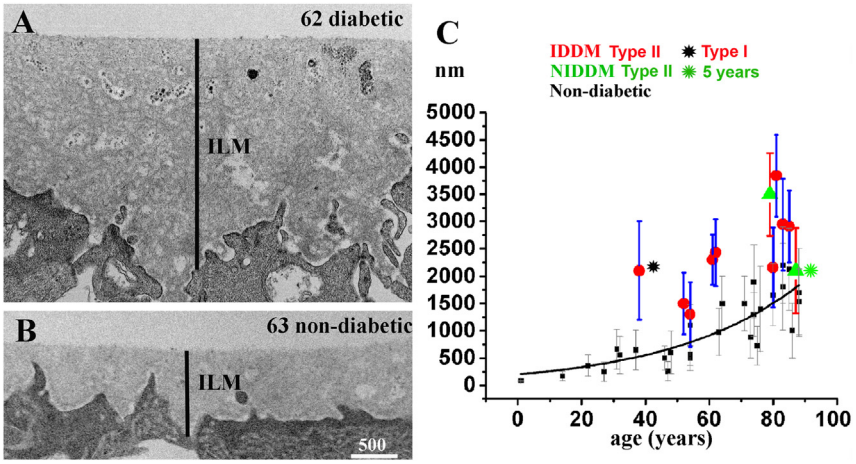

Fig. 3. Diabetic and non-diabetic ILMs. The TEM images in (A) and (B) show representative micrographs of ILMs from a 62-year old diabetic and a 63-year old non-diabetic patient. The graph in (C) shows that a) the thickness of ILMs increases with age, and b) the thickness of ILMs from diabetics is approximately doubled compared to ILMs from age-matched non-diabetic eyes. Except for the retinal sample marked by a black star, all samples came from patients with type II diabetes. The duration of diabetes was at least 10 years, except for the ILM marked by a green star (5 years). IDDM: insulin dependent diabetic; NIDDM: non-insulin dependent diabetic. Bar: A, B: 500 nm.

that is lost during the dehydration of the BMs samples for TEM but that is retained when using the AFM, since our AFM measurements were performed under aqueous conditions (Candiello et al., 2010). When we compared the AFM and TEM measurements of ILMs from diabetic eyes, we found that the AFM and TEM thickness data were similar, indicating that the water content of BMs from diabetic eyes is less than in BMs from non-diabetic BMs and suggests that BMs from diabetic eyes have a denser ECM and are, therefore, stiffer than normal (Fig. 4E).

To investigate the biomechanical qualities of ocular BMs, we compared the stiffness of flat-mounted ILMs from diabetic and non-diabetic eyes by AFM. Representative force curves from an ILM of a non-diabetic and a diabetic eye are shown in Fig. 4F. On average, force curves for ILMs from the diabetic eyes were steeper (i.e. stiffer ILMs) than the force curves of the ILMs from non-diabetic eyes suggesting a diabetes-induced increase in BM stiffness. The combined stiffness measurements from six diabetic and from six non-diabetic eyes are plotted in the summary graph in Fig. 4G. The graph confirmed that, on average, ILMs from diabetic eyes are stiffer than ILMs from non-diabetic eyes. The difference in stiffness varied between 20 and 60%.

3.3. *Changes in the protein composition of BMs from diabetic retinas*

Previous studies have shown that the protein composition of vascular BMs from diabetic eyes partially differs from the protein composition of vascular BMs from non-diabetic eyes (Ljubimov et al., 1996; Roy et al., 1996). Major candidates of diabetes-specific BM proteins were fibronectin and tenascin (Ljubimov et al., 1996; Roy et al., 1996). Both proteins are extracellular matrix proteins of the interstitial connective tissue and are not standard constituents of normal BMs.

Staining of retinal cross sections of diabetic and non-diabetic retinas confirmed the abundance of fibronectin in the ILM of diabetic eyes and the absence of this protein in the ILM of non-diabetic retinas (Fig. 5A–C). The abundance of fibronectin in diabetic retinas was further investigated in ILM whole mounts to localize the sidedness of fibronectin deposition in the ILM (Fig. 5D–G). Data showed that fibronectin is localized on the retinal side of the ILM (Fig. 5D, E) and undetectable in ILMs from non-diabetic eyes (Fig. 5F). The side-specific localization was confirmed based on co-localization with side-specific protein markers, such as laminin, which is localized on the retinal side and collagen IV 7S a3/4/5, which is localized on the vitreal side of the ILM (Fig. 5E, G; Halfter et al., 2013b).

The ILMs from diabetic eyes roll up similar to ILMs from non-diabetic eyes, whereby the retinal surface is facing outside and the vitreal side facing inside (Fig. 5H, Halfter et al., 2013b). Double-labeling of ILMs also showed a side-selective abundance of laminin on the retinal side and 7S domain of the collagen IV a3/4/5 on the vitreal side (Fig. 5I, J) in diabetic and non-diabetic eyes, indicating that the two-layered structure of the ILM from non-diabetic eyes is not disrupted in the ILMs of diabetic eyes.

Staining of vascular BM whole mounts showed that fibronectin is abundant in larger vessels and in the microvascular aneurisms of the retinal capillaries in the diabetic retinas (Fig. 6A, B) and undetectable in the vascular BMs of non-diabetic eyes (Fig. 6C). Microvascular aneurisms are regularly and frequently seen in vascular BMs in diabetic eyes (Fig. 2B, C) and never observed in non-diabetic eyes (see Fig. 2A).

Staining of cross section of diabetic and non-diabetic retinas showed that tenascin is up-regulated in the retinal vascular BMs (Fig. 6D, H). However, in difference to fibronectin, tenascin was not detected in the ILM of the diabetic retina (Fig. 6D, H). Staining of vascular whole mounts showed that tenascin is abundant in larger retinal vessels and in the microvascular aneurisms of the diabetic eyes (Fig. 6E). Very little tenascin labeling was detected in vascular whole mounts of non-diabetic eyes (Fig. 6F). Comparison of the distribution of fibronectin (Fig. 6G), tenascin (Fig. 6H) and collagen IV (Fig. 6I) in retinal microvascular aneurisms showed that the bulk of protein in these aneurisms was collagen IV, and that fibronectin and tenascin are added prominently as an outer ring to the collagen IV-dominated BM core.

Changes in the protein composition and relative abundance of laminin, collagen IV, agrin, fibronectin and tenascin in vascular BMs was determined by SDS PAGE and western blotting (Fig. 7). SDS PAGE followed by Coomassie-staining revealed only slight differences in the protein banding patterns between vascular BMs samples from non-diabetic and diabetic eyes (Fig. 7, lanes 1, 2). To compare the relative abundance of specific proteins by western blotting, the concentrations of solubilized vascular BM proteins from diabetic and non-diabetic were normalized. Western blots stained with polyclonal antisera to laminin (Fig. 7, lanes 3, 4), to

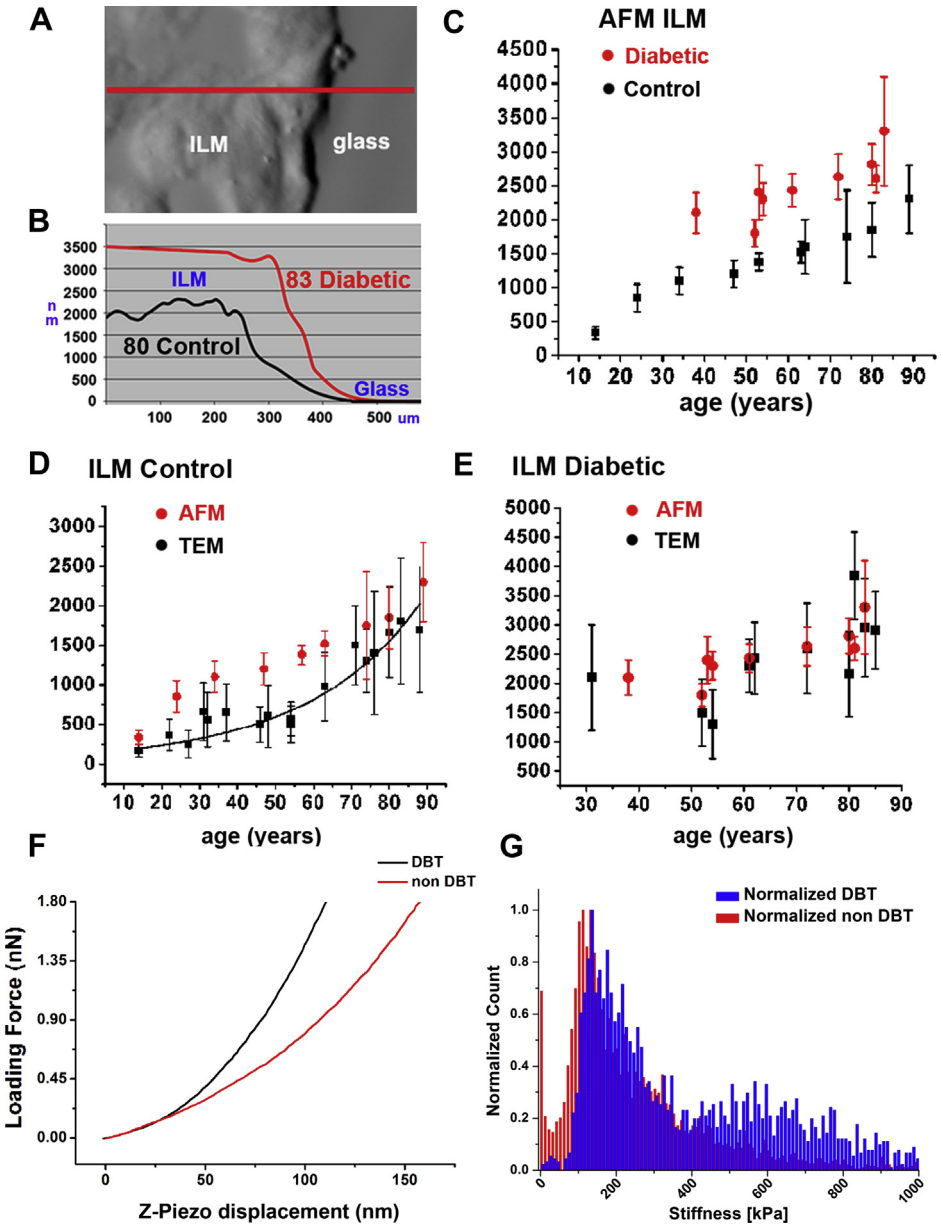

Fig. 4. The thickness of diabetic and non-diabetic ILMs as recorded by Atomic force microscopy (AFM): an AFM-generated image of the edge of an ILM is shown in (A). The ILM had been flat-mounted on a glass slide. The glass surface served as a reference for height measurements. The red line indicates a trace of the AFM probe to determine the thickness of the sample. Representative height traces of a diabetic and a non-diabetic ILM is shown in (B). AFM-based thickness measurements of non-diabetic and diabetic ILMs are plotted in (C). It shows that a) ILMs from non-diabetic eyes increase in thickness with advancing age, and b) that ILMs from diabetic eyes are on average twice thicker than similar aged-non-diabetic ILMs. When the TEM and AFM thickness data of ILMs from non-diabetic control eyes are compared, the AFM thickness measurements are approximately twice greater than TEM-based thickness measurements (D). In contrast, AFM and TEM thickness measurements of ILMs from diabetic eyes are similar and less age-dependent (E). This implies that the diabetes-induced increase in ILM thickness results in a denser, less hydrated BM. This was confirmed by AFM-based stiffness measurements: representative force curves from a diabetic ILM (DBT; 83 year of age) and a non-diabetic eye (F; non-DBT; 81 years of age) showed a steeper slope for the ILM from the diabetic eye than the force curve for the ILM from the non-diabetic eye. The summary of all stiffness measurements from six diabetic and six non-diabetic eyes and based on 6000 force curves showed that the stiffness of the ILMs from diabetic eyes is, on average, greater (37.9 ± 1.2 kPa) than the stiffness of ILMs from non-diabetic eyes (24.3 ± 1.2 kPa).

collagen IV (Fig. 7, lanes 5, 6), to agrin (Fig. 7, lanes 7–10) and monoclonal antibodies to fibronectin (Fig. 7, lanes 11, 12) and tenascin (Fig. 7, lanes 13, 14) showed that the relative concentrations of laminin and collagen IV in BMs from diabetic and non-diabetic eyes were similar, whereas the relative abundance of agrin, fibronectin and tenascin were higher in vascular BMs of diabetic than in non-diabetic eyes (Fig. 7). Agrin is a proteoglycan with long glycosaminoglycan side chains and runs as a smear around 400 kD (Fig. 7, lanes 7, 8). Deglycosylation of the samples reduced the 400 kD smear to the size of core protein of around 200kD (Fig. 7, lanes 9, 10). The western blots showed that both glycosylated agrin as well as the core protein of agrin are elevated in concentration in BMs of diabetic eyes (Fig. 7, lanes 7–10).

4. Discussion

4.1. Diabetes-induced changes in the morphology of the ocular BMs

A hallmark of long-term diabetes in the human eye is the thickening of retinal vascular BMs (Ashton, 1974). In addition to

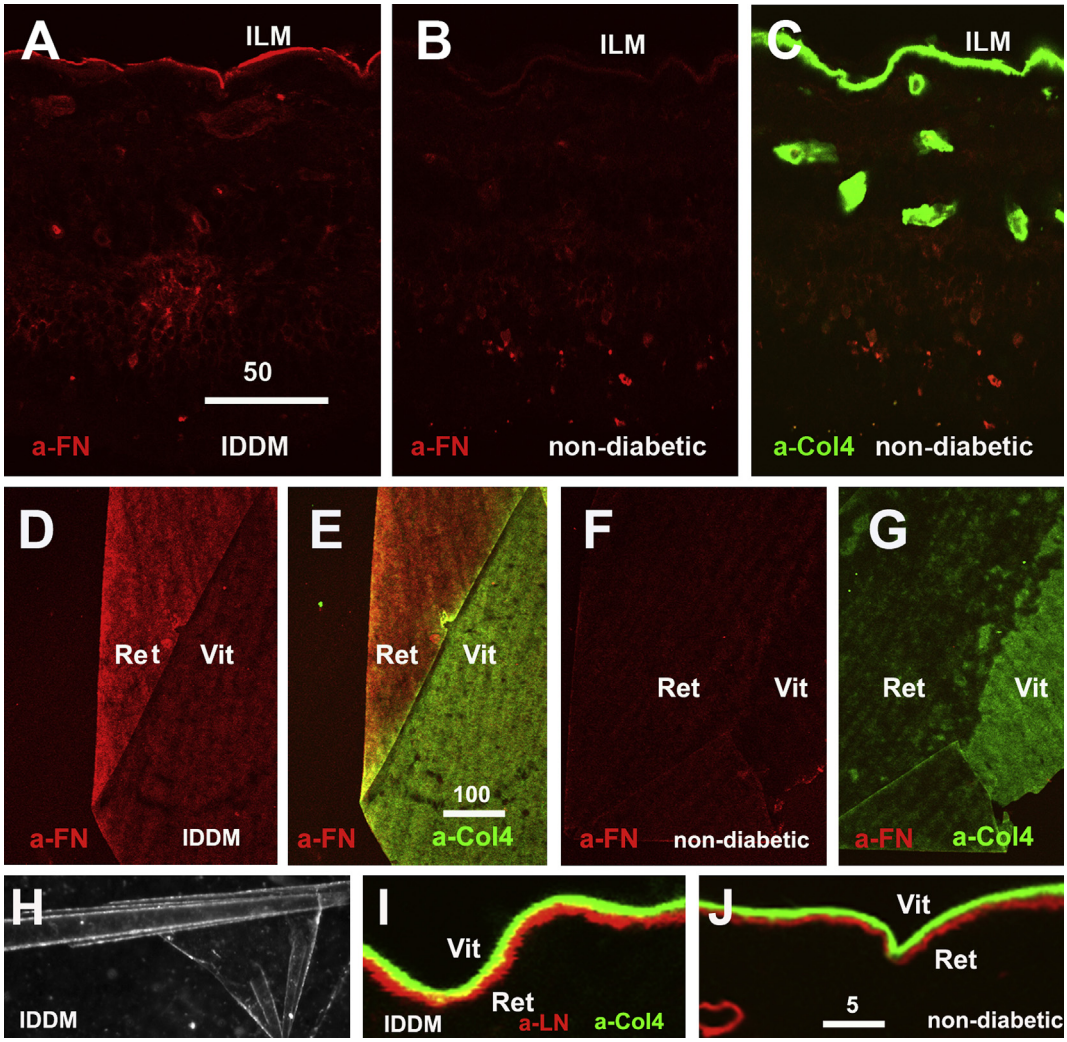

Fig. 5. Fibronectin is present in the ILMs of diabetic human retinas but not in ILMs from a non-diabetic retinas. Cross sections of human retinas were stained for fibronectin (a-FN; red; A, B). The section in panel (A) was from a 54-years old insulin-dependent diabetic patient and shows a prominent fibronectin-labeling of the ILM. The section in panels B and C were from a 66-year-old non-diabetic patient. FN was not detectable in the ILM of the non-diabetic eye (B). Double labeling for collagen IV (green; C) showed the presence of an intact ILM in this section (C). By staining ILM flat mounts with anti-fibronectin (D, F) or double stained with anti-fibronectin and anti-7S collagen IV (E, G), it was found that the diabetes-induced fibronectin is located at the retinal side (Ret) and not on the vitreal side (Vit) of the ILM. Anti-laminin staining serves as a marker for the retinal side, whereas anti-collagen IV 7S a3/4/5 staining serves as a marker labeled for the vitreal side of human ILMs. Isolated ILMs from diabetic eyes roll up like ILMs from non-diabetic eyes (H). The sidedness of the ILM from diabetic eyes and non-diabetic eyes is preserved as cross sections of retinas from diabetic (I) and from non-diabetic eyes (J) show a prominent laminin labeling on the retinal side (Ret) and a prominent 7S collagen IV-labeling of the vitreal side (Vit) of the ILMs. Scale Bar: A–C: 50 μm; D–G: 100 μm; I, J: 5 μm.

retinal vascular BMs, we found that the ILM, a BM that separates the retina from the adjacent vitreous, also undergoes a diabetes-induced thickness increase that is even more substantial than that of vascular BMs and suggests that BM thickening during diabetes is a systemic condition that affects many, if not all, BMs of the body. As shown previously, the ILM (Candiello et al., 2010), like many other BMs (Danysh and Duncan, 2008; Danysh et al., 2008; Murphy et al., 1984; Neuman et al., 2004; Vazquez et al., 1996; Xi et al., 1982), naturally increases in thickness with progressing age. Since the thickness increase of BMs during long-term diabetes is on average doubled as compared to age-matched controls, one is tempted to speculate that long-term diabetes causes an accelerated aging with respect to the production of BM extracellular matrix. Other studies hypothesized that chronic inflammation (Gardiner et al., 2003), a posttranslational glycation of proteins due to high serum glucose (Degenhardt et al., 1998; King, 2001; Stitt et al., 1997; Zhang et al., 2009) or an up-regulation of growth factors (Curtis et al., 2009; Durham and Herman, 2011; Kuiper et al., 2008; Zatz and Brenner, 1986) are the underlying cause for diabetes-related BM thickening.

As shown for the non-diabetic chick and human eye, the synthesis of ILM proteins is tightly regulated with a high synthesis during embryogenesis and early postnatal life followed by a dramatic down regulation with little BM protein synthesis during adult life (Halfter et al., 2005, 2008). The slow but steady increase in BM thickness during normal life is most likely due to the very long half-life of BM proteins combined with a continued yet very low rate of BM protein synthesis. The assumption of a long half-life of BM proteins is based on the fact that the half-life of collagens in vitreous and cartilage ranges between 11 and 100 years (Bishop et al., 2003; Maroudas et al., 1992; Verzijl et al., 2000, 2001).

Earlier studies showed a diabetes-induced up-regulation of collagen IV and fibronectin, indicating a switch in the translational control of BM protein synthesis (Roy et al., 1996). Since the regulatory process for BM protein synthesis is altered during diabetes, BM thickening is likely due to an inflammatory/fibrosis condition

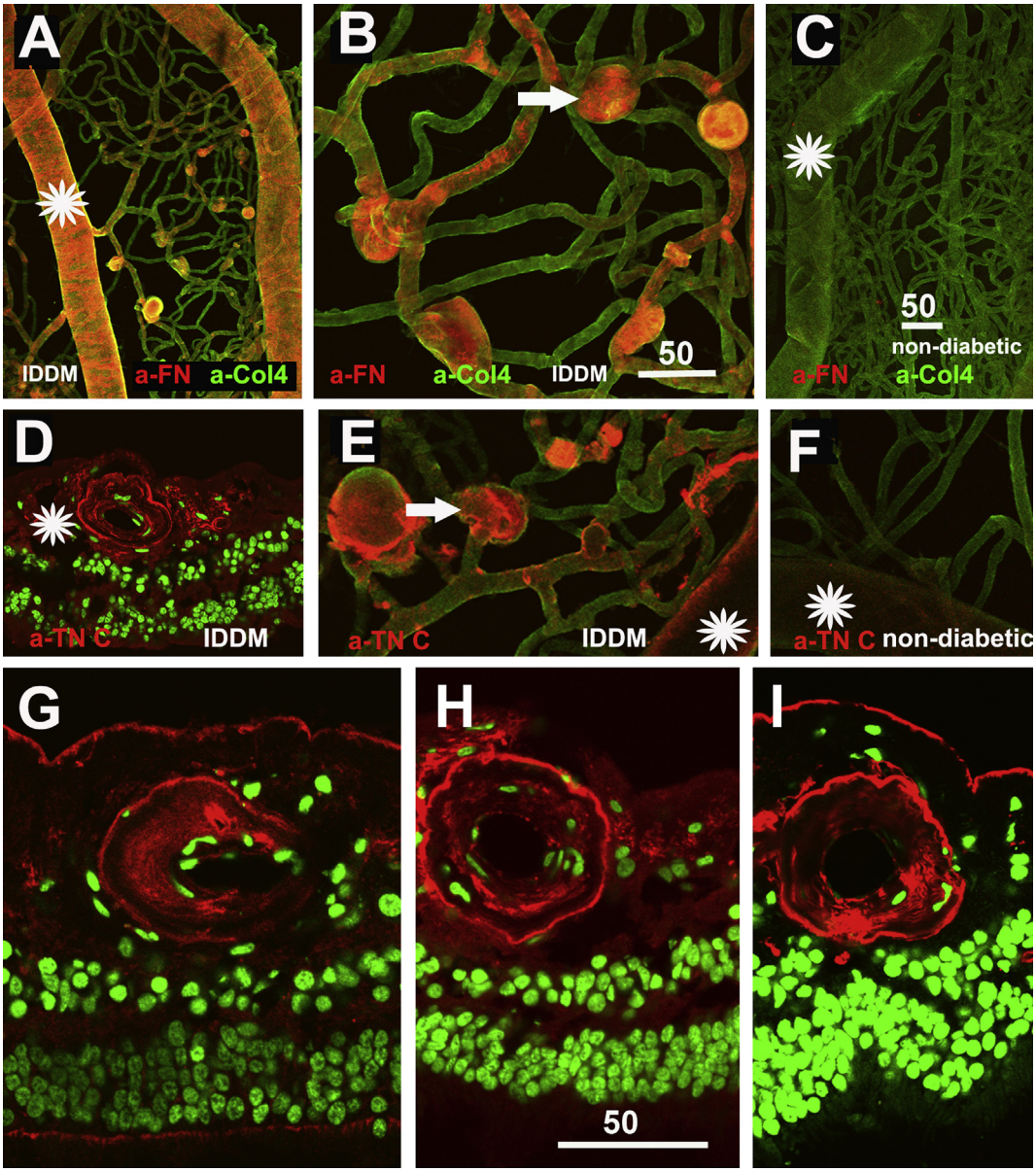

Fig. 6. Whole mount staining showing the diabetes-induced abundance of fibronectin and tenascin in vascular BMs of diabetic eyes (A). The staining of retinal vascular BM whole mounts demonstrates that fibronectin is particularly abundant in larger vessels (A, star) and in the vascular microvascular aneurisms of the retinal capillaries (A, B; arrow). Note, that fibronectin is all but absent in non-diabetic vascular BMs (C). Staining of retinal cross sections (D) and vascular whole mounts (E) with anti-tenascin showed that the protein was expressed in retinal vascular BMs of diabetic eyes with a high expression in large vessels (D, E; star) and in microvascular aneurisms (E, arrow) but undetectable in vascular BMs of non-diabetic eyes (F). Fibronectin (G) and tenascin (H) are particular high in the outer layer of the large vessels in retinas from diabetic eyes, whereas the bulk of the vessel BMs is provided by collagen IV (I). Bar: A, C: 50 μm; B, D, E, F: 50 μm. G–I: 50 μm.

with excessive production of BM protein, possibly following a cytokine-induced up-regulation of growth factors, such as TGFb, FGF-2 and CTF (Kuiper et al., 2008). What is currently unknown is whether the increase in BM thickness is mainly due to an elevated synthesis and deposition of the standard BM proteins, such as collagen IV and laminin, or due to the synthesis and deposition of diabetes-specific proteins that are normally not present in BMs. There are several findings arguing that BMs, at least in part, undergo a change in their protein composition during long-term diabetes.

4.2. Diabetes-induced compositional changes of BMs

The increase in ILM thickness, as detected by light and transmission electron microscopy, was confirmed by AFM, a high resolution imaging and probing method that allows investigation of unfixed and fully hydrated samples. Consistent with earlier AFM data, we found that the thickness of adult human and embryonic chick ILMs, when measured by AFM, was at least twice greater than measured by TEM. The sample preparation for TEM requires dehydration that normally leads to shrinkage of tissues between 10 and 20%. However, the over 50% difference of AFM and TEM thickness measurements indicated that BMs contain a much greater amount of water than other tissues (Balasubramani et al., 2010; Candiello et al., 2010). BM proteoglycans, such as perlecan, agrin and collagen XVIII that are present in BMs, are the prime candidates for the water-binding constituents in BMs. Earlier experiments showed that when the GAG chains of ILM samples in human and chick ILM were enzymatically removed, the ILMs underwent a 50% shrinkage (Balasubramani et al., 2010; Candiello

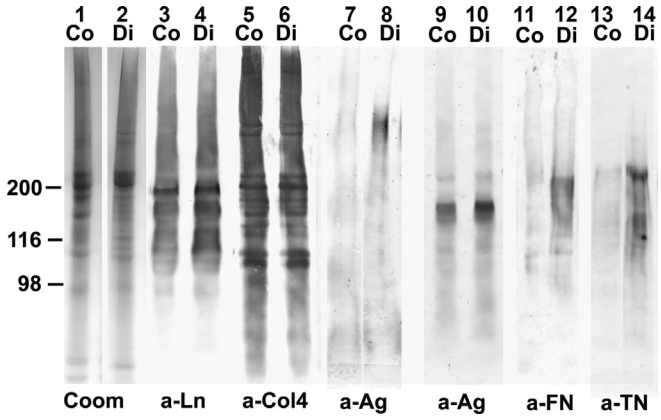

Fig. 7. Western blots comparing the protein-banding patterns and the abundance of laminin (LN), collagen IV (ColIV), agrin (Ag), fibronectin (FN) and tenascin (TN) in non-diabetic control (Co) and diabetic (Di) retinal vascular BMs. Vascular BMs from a non-diabetic control and a similar aged (72 year) diabetic retina were loaded for SDS PAGE. The samples had been normalized for identical protein concentrations. The protein banding patterns were detected by Coomassie blue staining (Coom, lanes 1, 2)) of the gel, and it showed a slightly different banding for diabetic versus non-diabetic BMs. Western blots were stained with antibodies to laminin (a-Ln, lanes 3, 4), collagen IV (a-ColIV, lanes 5, 6, agrin (a-Ag, lanes 7–10), fibronectin (a-FN, lanes 11, 12) and tenascin (a-TN, lanes 13, 14). While the relative abundance of laminin, and collagen IV was similar in non-diabetic and diabetic BMs, agrin, fibronectin and tenascin were more abundant in the diabetic BMs. For the detection of agrin, samples were loaded without deglycosylation of the BMs (lanes 7, 8) or after deglycosylation of the BMs (lanes 9, 10).

et al., 2010), demonstrating that water is the most prominent component of BMs and that this water is retained by the GAG side chains of proteoglycans. The finding that the difference of ILM thickness based on AFM measurements in diabetic eyes is minor, indicates a lower degree of hydration of the BMs following a loss of GAG side chains of the proteoglycans. It is conceivable that a lower hydration and higher compaction of BMs in diabetic vessels has physiological consequences, including a lower rate of diffusion and filtration through BMs, a loss of vascular elasticity that may contribute to chronic high blood pressure, and a higher incidence of aneurisms and rupture. Western blots showed a higher agrin concentration in diabetic as compared to non-diabetic retinal blood vessels. That appears to be in contrast to the lower water content that the AFM data suggest. Previous studies in chick ILM (Halfter et al., 2005) have shown that the GAG side chains for collagen XVIII decrease in length with advancing age, and we propose that the loss of glycosylation for collagen XVIII or perlecan could be responsible for the loss of water storage in diabetic BMs. Consistent with the current thickness data and consistent with our assumption of a higher compaction of BMs in diabetes patients, AFM studies showed that the ILMs from diabetic eyes are stiffer than ILMs from non-diabetic eyes. We propose that the higher density of protein and the lower hydration renders diabetic BMs more brittle and more prone to breaks. Indeed, isolating the Descemet's membrane from corneas of diabetic patients is complicated by the fact that the BMs break into small pieces, whereas the Descemet's membrane from non-diabetic patients can be regularly peeled off in one piece (W.H. personal observation).

Consistent with earlier studies (Ljubimov et al., 1996; Roy and Lorenzi, 1996), we found that fibronectin and tenascin, both of which are normally absent in the retinal BMs, are expressed in diabetic vascular BMs. The data also show that the diabetes-induced fibronectin is localized at the side of the ILM that faces the retinal neuroepithelium. We hypothesize that the fibronectin in the ILM of diabetic eyes originates from the Muller glia cells that have been shown to undergo a diabetes-induced activation (Coorey et al., 2012). Fibronectin and tenascin are considered typical interstitial extracellular matrix components and not standard BM proteins. Both proteins are up-regulated during wound healing (Pankov and Yamada, 2002; Mackie et al., 1988) and in the connective tissues surrounding tumors (Chiquet-Ehrismann and Tucker, 2004). Studies have shown that a variety of growth factors lead to a massive over-expression of both proteins (Caligliero et al., 1988; Chiquet-Ehrismann and Tucker, 2004), and the strong up-regulation of fibronectin and tenascin in diabetes argues in favor of a chronic and sustained inflammatory process that affects the cells that participate in the synthesis of BM proteins. Our vascular BM whole mount preparations revealed that both fibronectin and tenascin were particularly abundant in the microvascular aneurisms, indicating a focal up-regulation of both proteins at specific sites of the retinal vasculature. It as been well documented that long-term diabetes leads to the disappearance of pericytes and one is tempted to hypothesize that the vascular herniation are sites where pericytes might have died. However, the fact that BM thickening also affects the ILM, which is not in contact with pericytes, argues against a connection of BM thickening and pericyte loss. It is also possible that the aneurisms are sites of de-novo angiogenesis sprouts combined with a focal change in the protein composition, leading to a biomechanically altered, possibly weaker, segment in the vascular BM tubes.

Our studies also showed that agrin, both in the glycosylated form as well as the core protein, is more abundant in diabetic as compared to normal BMs of retinal vascular BMs, whereas the relative abundance of collagen IV and laminin, the dominant proteins of adult human BMs, was unchanged.

The current data indicate that long-term diabetes lead to a massive increase in the thickness of BMs possibly due to a chronic inflammation/fibrosis process that affects particularly the synthesis of BM proteins. We propose that this chronic condition is systemic and leads to the thickening and stiffening of possibly all BMs of the body. In addition to an up-regulation of dominant BM protein, such as collagen IV and agrin, diabetes also leads to the synthesis of proteins that are not normally present in BM, including fibronectin and tenascin. The deposition of diabetes-specific proteins may lead to the altered morphological and biomechanical changes of BMs and may contribute to the various pathologies in long-term diabetic patients.

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