Shaping vessels and microenvironment: adipose stromal cells in retinal-related diseases

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Chapter 6

Possible role of Fibulin-1 in the Extracellular Matrix Organization of the Retina and Adipose Tissue-derived Stromal Cells

Possible role of Fibulin-1 in the extracellular matrix organization of the retina and adipose-derived stromal cells

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In preparation

Abstract

Diabetic retinopathy is a multifactorial disease that affects several cellular populations of the retina. Pathological biochemical changes in the blood-retinal barrier lead to endothelial cells and pericytes apoptosis and migration respectively. Adipose-derived stromal cells (ASC) could replace pericytes and reestablish the physiological capillary bed damaged by diabetes. In this context, fibulin-1 is an important extracellular matrix organizer found in association with basement membranes and therefore important for vessels maintenance. In diabetic mice (ins2akita), fibulin-1 was downregulated in the retinas compared to healthy controls. ASC deposition of fibulin-1 and fibronectin fibrils arrangement was aligned. In diabetic ASC, fibulin-1 was upregulated as well as fibronectin. ASC fibulin-1 was also deposited in tunnels created by ASC cocultured with endothelial cells in a three-dimensional microenvironment, hinting to a functional role of fibulin-1 in guiding vessel-like tube formation. These data showed that fibulin-1 is a candidate for further investigations for understanding the interaction of ASC and extracellular matrix in the retinal pathological microenvironment.

Introduction

Diabetic retinopathy (DR) is a microvascular complication which, by different degrees, affects the majority of type 1 diabetes (T1D) patients [1]. More than 80% of insulin-treated (type 2 diabetes) T2D patients will develop DR. T2D patients who do not require insulin treatment has the 50% chance of developing DR [2,3]. Causalities that contribute to the insurgence of DR are to be attributed to long term exposure to hyperglycemia and hyperlipidemia [4,5]. The latter indirectly contribute to the progression of DR, which is characterized by permeability of the blood retinal barrier (BRB), microvascular endothelial cells damage, pericytes loss, capillaries occlusion and basement membrane (BM) thickening; followed by retinal neuronal and glia defects [6]. This reflects the non-proliferative phase (NPDR) of DR, whereas the proliferative phase of DR (PDR) represents the end stage of the disease. Among the numerous abnormalities observed during PDR, retinal fibrosis initiated by the progression of the diseases might be the ultimate trigger to retinal detachment and vision loss [7]. Cell migration, extracellular matrix (ECM) deposition and growth factors density and distribution are important components of the process of retinal fibrosis [8-10].

Fibulins represent a family of ECM proteins possessing domains that interact with and modulate key ECM proteins [11]. The first member, named fibulin-1 was identified by affinity chromatography on a synthetic peptide and, revealed a ~100 kb protein capable of interaction with the cytoplasmic domain of fibronectin receptor β subunit [12]. Several fibulin isoforms have been subsequently identified and they structurally share three domains, being the amino-terminal domain I and carboxyl-terminal domain III responsible of fibulins identity [13,14]. Four splice variants are found in fibulin-1 gene's products (fibulin 1A, B, C and D) and calcium-dependent binding to major components of basement membranes were identified [15]. Interestingly, fibulin-1 was also found in mouse serum (20 – 40 μg/ml) and in medium collected

from cells cultured *in vitro* [16]. Further examinations revealed fibulin-1 expression in a variety of tissue compartments i.e. brain, kidney and heart as well as cellular localization in adipocytes, fibroblasts and vascular smooth muscle cells [14,17]. To investigate the function of fibulin-1, transgenic mice lacking fibulin-1 were generated by ES cells homologous recombination and severe bleeding was observed with damaged endothelial compartments in several organs [18]. In the retina, fibulin-1 has been associated with axonal outgrowth in a genetic network extrapolated from gene variations in different mice strains [19]. In patients with PDR, fibulin-1 was elevated in plasma and, in the vitreous 37 extracellular matrix and adhesion proteins were differentially expressed in patients with PDR [20,21].

Cells of mesenchymal origin, such as adipose-derived stromal cells feature pericytic characteristics and have the possibility to be employed for vascular repair [22]. In general, cell therapy aims at compensating the pathological retinal microenvironment through secretion of trophic factors and through physical interaction with other cells (i.e. endothelial cells, glia cells and neurons) [23,24]. However, little is known about the ASC ECM remodeling during pathological conditions such as diabetes. Fibulin-1 in the retina might be responsible for maintaining physiological ECM composition. In this study, we measured fibulin-1 expression in the retina of diabetic mice. Subsequently, ASC and diabetic ASC fibulin-1 levels are also quantified. Given the fibulin-1 expression levels and function, feasibility for cell therapy is discussed.

Results and discussion

Vascular permeability, acellular capillaries and morphological alteration of microglia and astrocytes are characteristics of the first phase of retinopathy that can be induced in mice through an amino acid substitution in the insulin 2 gene [25]. In this model, fibulin-1 isoform was prevalently localized in the inner plexiform layer and in the inner nuclear layer, and to a lesser extent in the outer nuclear layer of healthy mice retinas (Fig. 1A). In Ins2akita mice a reduction in fibulin-1 deposition was measured (Fig. 1B, and 1D). Total retina extracts quantified by western blot showed significant lower deposition of fibulin-1 in the Ins2akita animal model ($P<0.05$) (Fig. 1E). Images acquired at high magnification displayed colocalization of fibulin-1 and capillaries stained with antibodies to detect endothelial cells adhesion molecule PECAM-1 (Fig. 2A). Interestingly, in ins2akita mice retinas, fibulin-1 was found in speckles in areas where alteration in the retinas layer occurred through extravasation and cellular agglomerates (Fig. 2B, white arrows). Comparative analysis of mouse fibulins isoforms demonstrated that elastic fibers are in strict contact with several fibulins domains and, importantly, these are associated with basement membrane components of blood vessels hinting at blood vessel maintenance and homeostasis [26]. To date, examples of fibulins associated with retina diseases are fibulin-3 (Efemp1) and Fibulin-6 (Hemicentin-1) with mutations in these genes reported to cause maculopathy [27,28]. Given the high similarity in fibulins structures and functions, it is likely that some overlap in functions occurs. Fibulin-1, however, holds a more supramolecular organization by interacting and organizing fibronectin, laminins and fibrinogen (more interactions are reviewed here [29].

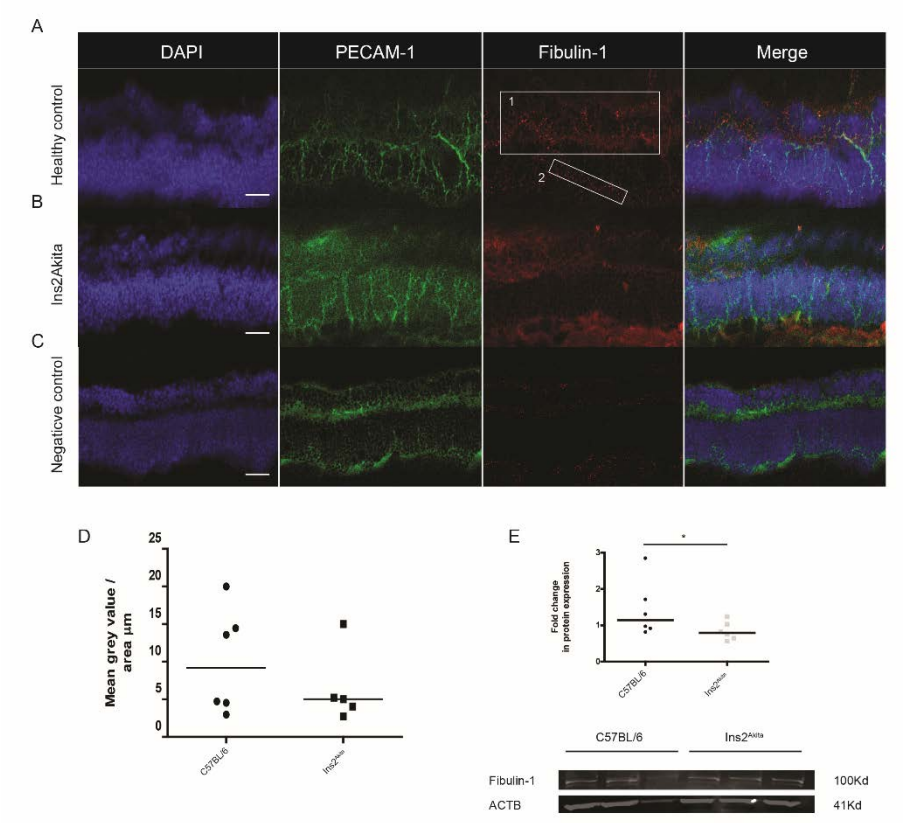

Figure 1. Fibulin-1 expression and localization in ins2^{akita} mice retinas. Retinas of Ins2^{akita} and C57BL/6 mice were stained with fibulin-1 (red) and PECAM-1 (green, capillaries) antibodies. (A) Retinas of controls showed fibulin-1 (red) expression in the inner plexiform layer (rectangle 1) and outer nuclear layer (rectangle 2). (B) Fibulin-1 localization in ins2^{akita} mice retina. (C) Negative control staining. (D) Quantification of Ins2^{akita} and C57BL/6 mice for fibulin-1. (E) Fibulin-1 quantification at protein level by western blot. PECAM-1. T-test, *, $p \leq 0.05$. Scale bars 50 µm.

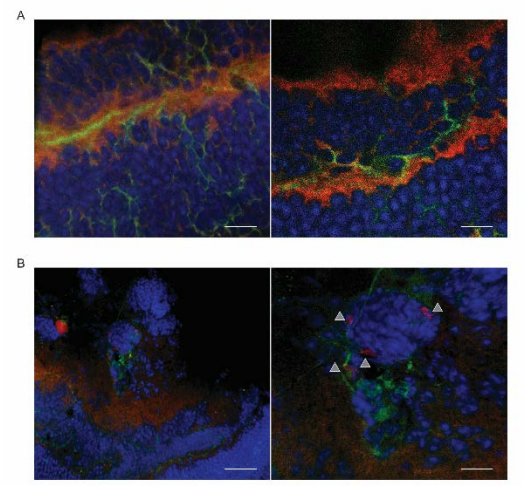

Figure 2. Fibulin-1 ins2akita mice retinas. High magnification of diabetic mice retinas and controls stained with fibulin-1 (red) and PECAM-1 (green) antibodies. (A) Retinas of controls showed fibulin-1 expression in the inner plexiform layer and outer nuclear layer. (B) Fibulin-1 localization at cellular agglomerates in diabetic retinas. Scale bars: (A, left) 100µm, (A, right) 50µm, (B, left) 200 µm, (B, right) 50 µm.

Pathological changes in cellular metabolism (i.e. ROS and AGEs), induce thickening of the vascular basement membranes in the retina. Collagens, fibronectin and laminins become increasingly deposited, whereas metalloproteinases and angiogenic growth factors secreted by the surrounding microenvironment enhance the burden of cells affected by hyperglycemia (reviewed here [30]). To date there are no studies that investigated the role of fibulins in diabetic retinopathy at histological level. However, increased blood hyperglycemia and fibulin-1 was reported to be upregulated in type 2 diabetic patients [31]. In arterial tissue from the same patients, fibulin-1 was upregulated in association with dysregulated arterial ECM. Another study reported similar findings, that is increased fibulin-1 deposition in atherosclerotic plaques. Fibrinogen that binds to fibulin-1, was also measured at high concentration in clots [32]. The authors argued that fibulin-1 accumulation could occur from circulating fibulin-1 and, in less amount, from secreted proteins in situ. Endothelial leakage might contribute to unwanted proteins deposition. Our data suggest that fibulin-1 deposition decreases in the vasoregression phase or retinopathy. It is plausible, that cell therapy could function as pericytes

replacement and to deposit functional ECM that is lost due to endothelial cells apoptosis and pericytes migration. To this extent, fibulin-1 was quantified in ASC. In this experiment, diabetic ASC were also considered. We found that hyperglycemia did not influence fibulin-1 deposition in heathy ASC. Diabetic derived ASC had a higher deposition of fibulin-1 in both normo- and hyperglycemia cultured ASC compared to healthy ASC (Fig. 3A, B) ($P<0.01$). Fibronectin is known to interact with fibulin-1 [12]. For this reason, fibronectin deposition was also measured in healthy and diabetic ASC. Reflecting fibulin-1 deposition, healthy ASC displayed the same pattern of deposition in normo- and hyperglycemia. In diabetic ASC, normoglycemia cultured diabetic ASC showed fibronectin upregulation compared to healthy ASC. Hyperglycemia-treated diabetic ASC, in contrast, had the highest fibronectin deposition compared to healthy and normoglycemia cultured ASC ($P<0.001$) (Fig. 3C, D).

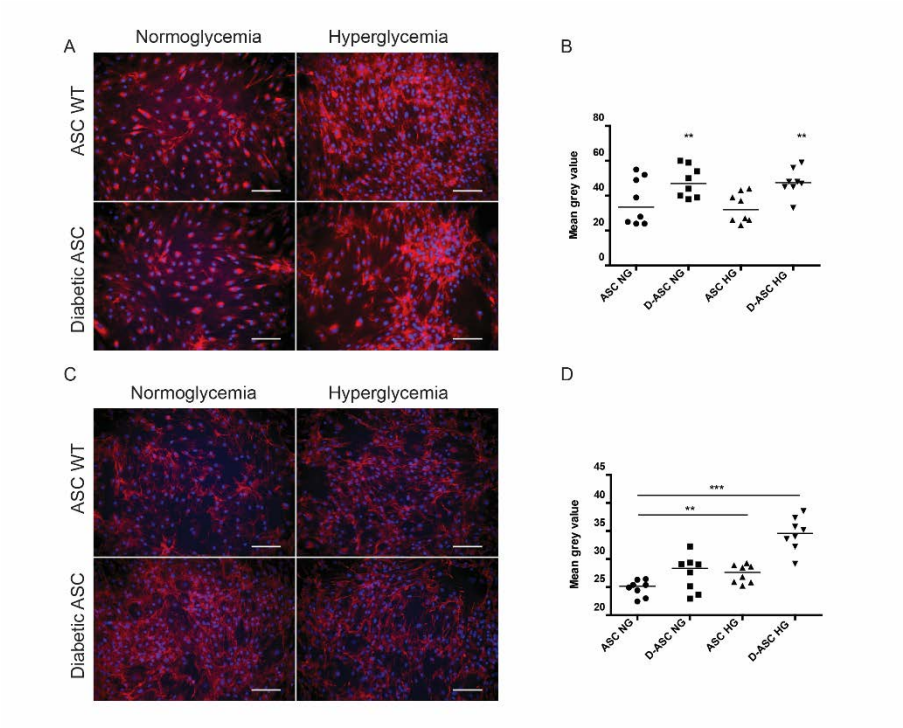

Figure 3. Fibulin-1 and fibronectin deposition is enhanced in diabetic ASC. ASC and diabetic ASC monolayer were cultured in normo- and hyperglycemia. (A) Representative images of ASC and diabetic ASC stained for fibulin-1 (red) in normo- and hyperglycemia. (B) Quantification of fibulin-1 expression measured as pixels/area. (C) Representative images of ASC and diabetic ASC stained for fibronectin (red) in normo- and hyperglycemia. (D) Quantification of fibronectin expression measured as pixels/area. *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$, one-way ANOVA Scale bars: 50µm.

The interaction between fibulin-1 and fibronectin investigated by Twal and co-workers elucidated important features translatable to cell therapy. In this study, fibulin-1 was responsible for cellular adhesion, motility, migration and invasiveness [33]. Interestingly, fibronectin binding to $\alpha_5\beta_1$ integrin was not compromised. Fibulin-1 was found to decrease the phosphorylation of myosin heavy chains. In a coculture of ASC and endothelial cells a in three-dimensional microenvironment, we found that fibulin was expressed on cells and strikingly, fibulin-1 was deposited along previous paths created by cells as they rearranged the substrate before communicating and forming vessel-like networks of ASC induced endothelial cells (Fig.4, white arrows heads).

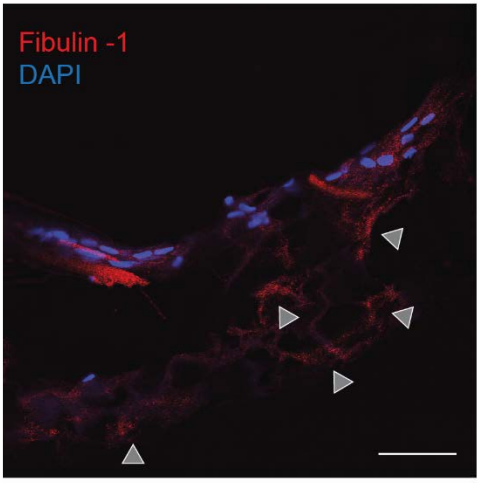

Figure 4. Fibulin-1 localization in three-dimensional cultured microenvironment with ASC and endothelial cells. ASC and HUVEC were embedded in matrigel and cultured in scaffolds. Fibulin-1 antibody was used for localization in the cocultured system. Fibulin-1 localization in tunnels created by prior cells activity (white arrow-heads). Nuclei were stained with DAPI (blue). Scale bars: 50µm.

Concluding remarks

We report that fibulin-1 is part of the ECM organization in mice retinas. Reduced deposition of fibulin-1 was, however, shown in an animal model featuring the first phases of retinopathy, namely ins2akita mice. In this model, fibulin-1 was detected in parts of the retinas where extravasation and cellular agglomerates occurred. In parallel, fibulin-1 was present in ASC. Diabetic ASC deposited higher amounts of fibulin-1 compared to healthy donors ASC. Fibronectin was found to overlap with fibulin-1 deposition. Interestingly, no difference was found when fibulin-1 deposition was measured in normo- and hyperglycemia treated cells in vitro. Finally, ASC and endothelial cells cocultured in three-dimensional environments showed fibulin-1 deposition in tunnels created by cells during migration. From these analyses it is clear that ECM deposition in the retina is altered in diabetic conditions. Because of neurodegeneration and blood-retinal barrier regression, the secretion of components such as fibulin-1 might be reduced due to cellular adaptation to the pathological microenvironment. However, more animal models featuring the retinal damage caused by diabetes are needed to confirm downregulation of ECM components. In terms of cell therapy, ASC need to be resilient to diabetes-driven biochemical changes, exert a supportive function by acting as pericytes and reestablish the blood-retinal barrier to confer protection from the systemic damaged caused by diabetes. However, we showed that ASC from diabetic patients had enhanced deposition of fibulin-1 and fibronectin. These characteristics suggest that autologous cells transplantation warrants a phenotypic characterization prior to injection and analysis of integration in the retinal microenvironment in animal models. In conclusion, fibulin-1 is likely involved in the retinal ECM organization and maintenance, ASC might contribute to control and induce a homeostatic microenvironment in part through the ECM deposition. However, experiments of loss of

gene function are needed to investigate further the fibulin-1 activity in DR context.

Methods

Animal model

C57BL/6J mice and spontaneous diabetic heterozygous Ins2Akita$^{+/-}$ mice (Jackson Laboratory, Charles River, Sulzfeld, Germany) were used for this study. All experimental procedures were performed according to the guidelines of the statement for animal experimentation issued by the Association for Research in Vision and Opthalmology and were approved by the local board for animal care (Medical Faculty Mannheim, Germany).

Immunofluorescence

Eyes of diabetic mice and controls were isolated and immediately stored at -80°C. Cross-section of 100µm in thickness were cut through the eyes and air-dried overnight. Subsequently, retinas were treated with 1% BSA and 0.5% Triton-X100 in PBS at room temperature for 1h. Overnight staining was with fibulin-1 (1:100, Abcam, ab211536) and PECAM-1 (1:100, Dako, Glostrup, Denmark). Samples were washed with PBS and incubated with the fluorescein-conjugated-donkey-anti-mouse-IgG (PECAM-1) (1:500, Jackson Immunoresearch, UK) and to fluorescein-conjugated-goat-anti-rabbit IgG (fibulin-1) (1:500, Jackson Immunoresearch, UK) in PBS containing DAPI. Confocal microscope (SP8) was used for images acquisition. Images quantification and analysis was performed by image J.

Immunoblotting

Retinal digests and endothelial cells were lysed in RIPA buffer (ThermoScientific, Waltham, MA) and protein concentration

determined by DC Protein Assays (BioRad, Hercules, CA) according to manufacturers' instructions. 20 µg of protein/lane was loaded on SDS-PAGE gel (10%) for electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated overnight with antibodies to fibulin-1 (1:1000, Abcam, ab211536), and b-actin (Cell Signaling, #4967, 1:5000). IRDye® labeled antibodies (1:10.000, Li-COR Biosciences, Lincoln, NE) were used for detection. Bands were visualized using the Odyssey® Infrared Imaging System (Li-COR Biosciences, NE, USA). Densitometry was performed using ImageJ. Protein expression levels were normalized to b-actin.

Primary cell isolation and cultures

ASC were isolated from lipoaspirates as described previously [34]. Anonymously donated samples were obtained with informed consent as approved by the ethical board of the University Medical Center Groningen following the guidelines for 'waste materials'. Propagation of ASC was in DMEM (BioWhittaker Walkersville, MD): 10% not heat inactivated fetal bovine serum (FBS), 1% l-Glutamine, 1% Penicillin/Streptomycin (P/S). Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Breda, The Netherlands) and the Endothelial Cell Facility of University Medical Center Groningen, The Netherlands. HUVEC were cultured in RPMI-1640 (Lonza Biowhittaker Verviers, Belgium), 10% heat inactivated FBS (Thermo Scientific, Helmel Hempstead, UK), 1% l-Glutamine, 1% Penicillin/Streptomycin (P/S), 2 mM L-glutamine (Lonza Biowhittaker Verviers, Belgium), 5U/ml heparin (Leo Pharma, The Netherlands) and 5µg/ml of ECGF growth factor). HUVEC were cultured on gelatin-coated tissue culture polystyrene (TCPS). Cells' medium was refreshed every three days. ASC and HUVEC were used between passage 3 and 5.

Primary cells immunofluorescence

Samples were fixed in 4% paraformaldehyde in PBS for 30min. Subsequently, cells were permeabilized with 1% BSA and 0.5% Triton-X100 in PBS at room temperature for 1h. After PBS washes, primary antibodies were incubated overnight: fibulin-1 (1:100, HPA001612, Sigma-Aldrich), rabbit-anti-human fibronectin (ab6584, Abcam, UK). Samples were washed with PBS and incubated with the fluorescein-conjugated-goat-anti-rabbit IgG (1:500, Jackson Immunoresearch, UK) in PBS containing DAPI. Images quantification and analysis was performed using image J.

3D printed scaffold design and cells culturing

3D scaffolds were printed with a commercially available 3D printer (Reprap Prusa i3, Anet 3D, China). Biodegradable material, polylactic acid (PLA), was used to print the scaffolds. 3D scaffolds were designed with SketchUp 2016 software. Three-dimensional co-cultures were achieved by embedding ASC in combination with HUVEC (1×10^5 ASC and 2×10^5 HUVEC, ratio 1:2) in 100µl of matrigel (Corning, growth factor reduced, New York, USA) accommodated in 3D printed scaffolds (volume: $60mm^3$) for 10 days. Immunostaining was performed as described above. After staining, the scaffolds were frozen with liquid nitrogen and sectioned for imaging. Confocal microscope (SP8, Leica) was used to acquire z-stack images at 63x. Post-processing for imaging was achieved using ImageJ software [35].

Statistical analysis

All the data are presented as a mean with either SEM or SD and were analyzed using GraphPad Prism 5 (GraphPad Software Inc.). Statistical significance was determined using Students t-test and one-way ANOVA analysis. Values of $p<0.05$ were considered statistically significant.

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