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Stimulatory effects of advanced glycation endproducts (AGEs) on fibronectin matrix assembly

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Abstract

Advanced glycation endproducts (AGEs) are a heterogeneous group of compounds that form via non-enzymatic glycation of proteins throughout our lifespan and at a higher rate in certain chronic diseases such as diabetes. AGEs contribute to the progression of fibrosis, in part by stimulating cellular pathways that affect gene expression. Long-lived ECM proteins are targets for non-enzymatic glycation but the question of whether the AGE-modified ECM leads to excess ECM accumulation and fibrosis remains unanswered. In this study, cellular changes due to AGE accretion in the ECM were investigated. Non-enzymatic glycation of proteins in a decellularized fibroblast ECM was achieved by incubating the ECM in a solution of methylglyoxal (MGO). Mass spectrometry of fibronectin (FN) isolated from the glycated matrix identified twenty-eight previously unidentified MGO-derived AGE modification sites including functional sites such as the RGD integrin-binding sequence. Mesangial cells grown on the glycated, decellularized matrix assembled increased amounts of FN matrix. Soluble AGE-modified bovine serum albumin (BSA) also stimulated FN matrix assembly and this effect was reduced by function-blocking antibodies against the receptor for AGE (RAGE). These results indicate that cells respond to AGEs by increasing matrix assembly and that RAGE is involved in this response. This raises the possibility that the accumulation of ECM during the progression of fibrosis may be enhanced by cell interactions with AGEs on a glycated ECM.

Keywords

Fibronectin; extracellular matrix; advanced glycation endproducts; RAGE; non-enzymatic glycation; mass spectrometry

1. Introduction

Non-enzymatic glycation of protein amino groups is a naturally occurring process that is accelerated under hyperglycemic conditions. A series of reactions lead to the formation of

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advanced glycation endproducts (AGEs) [1, 2] that modify arginine and lysine side chains as single adducts or as crosslinks between nearby residues [1]. AGEs accrue throughout the lifetime of an individual and have been linked to the overall aging of tissue proteins [3, 4]. AGEs are also associated with diseases including cancer [5], cardiovascular disease [6], neurodegenerative disorders [7], and a host of diabetic disorders. Complications from AGE exposure in diabetic individuals lead to retinopathy, neuropathy, vasculopathy, and nephropathy [1]. AGEs can also be exogenously absorbed through diet [1]. Diabetes and other chronic diseases are characterized by changes in the amount and organization of the extracellular matrix (ECM) [5, 8–11]. Therefore, in order to understand the progression of AGE-related disease in a global sense, it is essential to characterize the AGE-ing process within the ECM.

Normal developmental processes and tissue homeostasis rely on regulated assembly of a tissue-appropriate ECM. Dysregulation of ECM assembly, as occurs in many diseases, leads to fibrosis, which is characterized by an accumulation of disorganized ECM proteins and insoluble matrix fibrils. Fibronectin (FN) and collagens are major components of the ECM and are increased in fibrotic tissue [12]. FN matrix fibrils form a critical template for the incorporation of other ECM components into the matrix; both fibrillar and network-forming collagens depend on FN [13–16]. As a result, changes in FN matrix assembly can have wide-ranging effects on the overall organization and composition of the ECM.

Non-enzymatic glycation of ECM proteins can affect their functions. AGE-modification of collagens I and IV have been shown to affect interactions with other ECM components and the ability to support cell adhesion and migration [17–20]. Cooperative interactions between FN, collagen I or IV, and heparin were reduced by AGE-modification [21, 22]. Mass spectrometric analyses have identified a range of AGEs on proteins and have revealed preferential sites of non-enzymatic glycation on ribonuclease [23–25]. However, AGE modification sites and types have not yet been identified on FN. Additionally, AGE modifications can activate intracellular pathways through binding to their receptor RAGE [26]. Whether this AGE-RAGE axis has an effect on FN matrix assembly remains to be elucidated.

In the kidney, the mesangial cell ECM supports the organization and filtration activity of the capillary bed in the glomerulus. Poor glycemic control results in chronically high blood glucose levels, manifesting in metabolic changes that lead to increased mesangial ECM [27]. We recently showed that elevated glucose levels increase mesangial cell matrix assembly by stimulating the activation of $\alpha 5\beta 1$ integrin, the receptor responsible for FN matrix assembly [15]. This increase in FN matrix promoted deposition of type IV collagen. Non-enzymatic glycation is another complication of hyperglycemia, and AGEs have been implicated in the progression of fibrotic conditions in glomerulosclerosis [28], as well as atherosclerosis [29–32]. Since a major component of these lesions is FN [9, 33, 34], here we investigated the effects of a glycosylated FN matrix on mesangial cell-ECM interactions. Our results identify specific sites of AGE-modification within the matrix and show that an AGE-modified ECM stimulates mesangial cell matrix assembly.

2. Results

2.1 Identification of AGE modification sites on FN

Glucose is the most metabolically utilized sugar, but there are other more reactive sugars such as methylglyoxal (MGO), which is formed during anaerobic glycolysis and as an intermediate in lipid and protein decomposition [35, 36]. Although always present, MGO levels increase under hyperglycemic conditions [37]. Certain AGEs are fluorescent such as the MGO-arginine adduct argpyrimidine [38]. Monitoring increases in fluorescence provides a useful measure of the progress of MGO-mediated non-enzymatic glycation. A GST fusion protein containing the III₉₋₁₀ modules of FN, which includes the functionally relevant RGD and synergy sequences for integrin binding [39], was utilized to determine the relative rate of non-enzymatic glycation. The III₉₋₁₀ modules are rich in lysines and arginines, the primary amino acids targeted by glycation agents. The small size of GST-III₉₋₁₀ compared to full length FN is advantageous for identifying the types of AGEs that form during sugar incubations. This fusion protein was incubated in buffered 0.1 mM MGO or in buffer without MGO and the fluorescence was monitored at excitation/emission wavelengths of 335/385 nm over 14 days. Fluorescence levels in the MGO-treated sample continued to increase over time, reaching a maximum increase after 7 days (Figure 1A), so subsequent treatments were carried out for 7 days.

To identify the types of AGE modifications and the specific arginine and lysine residues susceptible to modification during MGO or glucose treatment, a mass spectrometry-based proteomic approach was performed. First, treated GST-III₉₋₁₀ was subjected to an in-solution chymotrypsin digestion. Then, the chymotryptic peptides were analyzed by nanoliquid chromatography coupled to tandem mass spectrometry to separate AGE-modified peptides and identify the specific AGE modifications and their respective residue. Although most global proteomics studies employ trypsin digestion, we selected chymotrypsin to digest FN for two reasons. First, unlike trypsin, chymotrypsin digestion would not be impaired at AGE-modified lysine and arginine sites [23]. Second, the theoretical chymotryptic digest of FN predicted adequate sequence coverage when using this digestion strategy. While early glycation products have the potential to mature into a wide variety of AGEs, different glycation agents can give rise to distinct types of AGEs [25]. Therefore, the primary goal in profiling the AGE modification status of the III₉₋₁₀ protein was to confirm that the glycation agents (glucose and MGO) induced the predicted AGE modifications and that the modifications could be identified by the proteomic and computational analysis. The predominant AGEs that can be derived from either glucose or MGO modification include [2]: N_ε-carboxymethyl lysine (CML), N_ε-carboxyethyl lysine (CEL), 3-deoxyglucosone hydroxyimidazolidine (3DG-H), N_ε-(5-hydro-4-imidazolone-2-yl)ornithine (G-H), hydroxyimidazolidine (MG-H) and dihydroxyimidazolidine (MG-DH), argpyrimidine, dihydropyrimidine, and tetrahydropyrimidine.

Using tandem mass spectrometry, peptides covering the entire III₉₋₁₀ sequence were detected, which allowed a reliable assessment of its AGE modification status. Eleven arginine and three lysine residues within the III₉₋₁₀ modules were modified with at least one type of AGE modification after treatment with glycation agents (Figure 1B). All 11 AGE-

modified arginine residues were identified with AGE modification in MGO-treated GST-III₉₋₁₀, while only seven of these were found upon incubation with glucose. In contrast, the three lysine residues were identified with AGE modifications in the glucose-treated but not MGO-treated GST-III₉₋₁₀ protein (Supplemental Table 1).

Overall, a total of nine different AGE species were detected and their prevalence varied with the treatment (Figure 1C; Supplemental Table 1). The mass spectrometry analysis provides information regarding the number of spectra obtained for each identified peptide, i.e. the number of times a unique amino acid sequence is identified during an LC-MS/MS acquisition. While spectral counting is not appropriate for comparing abundances between different sites, it can be used to evaluate the relative preference for specific types of AGE modifications at a given site as a function of the glycation agent. To visualize AGE preferences at each site, a heat map was constructed from the combined mass spectrometry spectral counting data for all three treatments (control, glucose, and MGO). To generate the heat map, the number of spectra identified for the AGE modification that was observed most frequently at that site was set to 1 (the darkest shade of red) (Figure 1C). The proportion of spectra for each of the other AGE modifications at that site was calculated relative to the most abundant one, and the fraction is illustrated in the heat map as a lighter gradation of red. As expected, the predominant type of AGE modification differed depending on the glycation agent (Figure 1C). MGO treatment generated MG-derived species, including MG-H, MG-DH, THP, and argpyrimidine, while the main AGE modification identified on the three lysine residues in glucose-treated GST-III₉₋₁₀ was the Amadori intermediate (Figure 1C). AGE modifications on the III₉₋₁₀ modules were also identified after incubation in buffer alone, but with less diverse AGE types and with many fewer spectra per AGE type (Figure 1C; Supplemental Table 1). It is likely that these glycation events occurred during expression and purification of the GST-III₉₋₁₀ fusion protein. All of the arginines and lysines in this domain are in solvent-accessible regions in the crystal structure of III₉₋₁₀ [40], so it was not surprising that 14 out of the 15 arginines and lysines in this domain were glycated during the 7 day incubation. In summary, the increased number of identified AGE-modified spectra and preferential identification of Amadori-derived and MGO-derived AGEs after treatment with glucose and MGO, respectively, reinforces the suitability of the treatment conditions and mass spectrometry-based proteomics strategy.

Identifying AGE modifications on FN within a cell-derived ECM will provide information about the availability of domains within the three-dimensional fibrillar and multi-component architecture of the matrix. To generate a glycated ECM, we decellularized a mouse NIH 3T3 fibroblast matrix following our established protocol [41] and then incubated it in a solution of glucose or MGO or in buffer alone. Matrices were solubilized, FN was resolved by SDS-PAGE, and the FN gel band was digested with chymotrypsin. After digestion and tandem mass spectrometry analysis of chymotryptic FN peptides, the AGE modification sites and overall sequence coverage were aligned with a diagram of a FN monomer (Figure 2A, B). As predicted by the *in silico* digest, relatively high FN sequence coverage (86 %, 2138/2477 amino acids) was obtained, as indicated by a schematic of colored lines for each peptide detected and by the plot of summed identified spectra along the length of FN (Figure 2B). AGE modifications were detected on amino acid residues spanning the length of FN (Figure 2A). Several MGO-AGE modification sites were clustered in the integrin-binding III₉₋₁₀

domain and in the III₁₃ heparin-binding module; these sites included the RGD arginine (R1614) and an arginine in the cationic cradle heparin-binding groove (R1925). Only four residues were glycosylated in the III₉₋₁₀ domain of matrix FN compared to 14 residues in GST-III₉₋₁₀ treated *in vitro*. It is possible that some of these residues are protected from reaction by interactions of FN within fibrils. This mechanism also may play a role more generally along the FN domain architecture, as there appears to be a selectivity in which residues were modified. While the majority of the FN sequence was detected (Figure 2B), AGE modifications were identified of only 28 of the 210 arginines and lysines. Interestingly, all 28 of these sites in FN were AGE modified under MGO treatment conditions, with fewer sites of modification identified in either buffer alone or glucose treatment (Figure 2C; Supplemental Table 2). Moreover, AGE modifications arising from MGO treatment were primarily in the form of MG-H and MG-DH (Figure 2C), consistent with the III₉₋₁₀ fragment experiments and the known chemistry of MGO. Since the cells were grown in glucose-containing medium, a basal level of glycation was expected in the control-treated decellularized matrix. The concomitant decrease in G-DH and increase in MGO-derived AGEs between control and MGO-treated matrix most likely is the result of the reversibility of the Amadori intermediate, as the more reactive MGO could replace the glucose-derived intermediates throughout the 7-day incubation. All of the sites with MG-H and MG-DH modifications were either identified exclusively or with greater numbers of spectra in the MGO-treated decellularized matrix compared to the control (Figure 2C; Supplemental Table 2). When examining the AGE modifications across all detected sites (representing > 95% of all detected modified spectra), the total of 87 modified spectra in the control increased to 154 in MGO-treated FN, supporting an elevated overall AGE-modification burden after MGO treatment (Supplemental Table 2). In summary, the mass spectrometry approach has provided information about the AGE modification sites on FN within an ECM, leading to the unambiguous identification of 28 residues available for modification. Since the relative levels of AGE modifications were higher in MGO-treated matrix compared to both the control and glucose-treated matrices, MGO was utilized as the glycation agent in subsequent experiments. Moreover, differences in accessibility of FN domains to modification when within a matrix may provide insights into the effects of AGEs on FN function.

2.2 Cell attachment on AGE-modified FN

The arginine in the RGD sequence was AGE-modified in both the GST-III₉₋₁₀ protein and in matrix FN. To test whether AGE modifications affect cell-FN interactions, MGO-treated GST-III₉₋₁₀ was used in a cell adhesion assay with mesangial cells. After 1 hour, approximately 50% fewer mesangial cells had attached to the MGO-treated GST-III₉₋₁₀ than to the control buffer-treated protein (Figure 3A). Mesangial cells also attached less readily to MGO-treated matrix initially. After 2 hours of adhesion, approximately 30% fewer mesangial cells had attached to the MGO-treated matrix than to the buffer-treated matrix (Figure 3B). This reduction in attachment was transient, and by 6 hours there was no difference between the numbers of cells attached on MGO versus buffer-treated matrices (Figure 3C). These results indicate that glycation reduces the total number of available cell binding sites which delays but does not prevent cell adhesion on the matrix.

2.3 Stimulation of FN matrix assembly by AGE-modified ECM

Accumulation of AGEs has been associated with the development of fibrotic conditions as in diabetic nephropathy [28], suggesting that an AGE-modified cellular environment might increase matrix protein deposition. To test whether cell-mediated matrix assembly is stimulated by AGEs, we glycated a decellularized ECM and used it as a substrate to culture mesangial cells. MGO-treated and buffer-treated decellularized matrices produced by mouse NIH 3T3 cells were visualized by staining in the absence of mesangial cells, which showed equivalent fibrillar networks independent of the treatment (Figure 4A). In order to follow new FN assembly over the background of the decellularized matrix, mesangial cells were cultured in the presence of exogenous rat FN and new FN fibril formation was detected both biochemically and microscopically using a rat FN-specific monoclonal antibody. With similar numbers of mesangial cells attached to the ECM (Figure 4A, phase), significantly more FN fibrils were assembled on MGO-treated compared to control-treated matrix (Figure 4A, Rat FN). Quantification of fluorescence in random fields revealed that the average fluorescence intensity was significantly higher on AGE-modified matrix compared to control-treated matrix (Figure 4A). Quantification of the deoxycholate (DOC)-insoluble FN, a biochemical readout of the irreversible maturation of FN matrix [42], shows that mesangial cells grown on MGO-treated matrix assemble about 5-fold more FN matrix than cells on control matrix (Figure 4B). Furthermore, FN mRNA levels increased 1.34-fold in mesangial cells grown on MGO-modified matrix as compared to the control-treated matrix (1.34 ± 0.06 ; $n = 3$). Collagen IV mRNA levels, however, were not significantly different when mesangial cells were grown on MGO-modified matrix ($n = 3$). Together these results clearly demonstrate that a glycated matrix stimulates cells to significantly increase FN matrix assembly.

2.4 Stimulation of FN matrix assembly by AGE-modified BSA

Although long-lived insoluble ECM proteins such as FN are ideal targets for non-enzymatic glycation, AGEs also form on soluble, shorter-lived proteins such as hemoglobin [43]. To determine whether the increase in matrix assembly results from AGE modifications on ECM specifically or whether any AGE-modified protein would have this effect, soluble AGE-modified BSA was added to the culture medium of mesangial cells growing on tissue culture plastic. After 24 hours, exogenous rat FN was added and matrix was analyzed after another 24 hours. There was a significant increase in FN fibrils assembled by cells treated with AGE-BSA as compared to BSA treatment as measured by average fluorescence intensities (1.15 ± 0.01 ; Figure 5A) and by quantitative immunoblotting of the DOC-insoluble FN matrix (Figure 5B). These data show that exposure of cells to a soluble AGE-modified protein has a similar effect on FN matrix assembly as adhesion on an AGE-ECM.

Cells recognize AGEs through the cell surface receptor for AGE (RAGE) [26]. RAGE is known to activate a variety of pathways that are implicated in changes in ECM expression levels [44]. In particular, the AGE MG-H found in MGO-treated decellularized matrix is known to bind RAGE [45]. Since mesangial cells express RAGE [46], a function-blocking anti-RAGE antibody was used to test whether RAGE might contribute to AGE-induced matrix assembly [47]. Mesangial cells were treated with AGE-BSA and the antibody was added along with rat FN for the final 24 hours of treatment. Matrix assembly was analyzed

by immunofluorescence staining and immunoblotting of DOC-insoluble matrix. With the addition of RAGE function-blocking antibody and AGE-BSA, there were fewer FN fibrils formed (Figure 6A) and the DOC-insoluble FN was decreased (Figure 6B) as compared to mesangial cells grown in the AGE-BSA control treatment. No change in DOC-insoluble FN was detected with a control antibody against lysyl oxidase (LOX) (Supplemental Figure 2). These data support the contention that RAGE plays a role in the effects of AGE-BSA on FN matrix assembly.

3. Discussion

Increases in non-enzymatic glycation and AGE modifications have been linked to changes in gene expression that can lead to fibrosis [26, 48]. Here we investigated whether AGEs also affect the assembly of the ECM. Our mass spectrometry analyses show that there are multiple sites of AGE modification on FN within matrix fibrils including functionally relevant sites such as the RGD arginine essential for integrin binding. Importantly, we found that mesangial cells are stimulated to assemble FN matrix when grown on an AGE-modified decellularized matrix. This increase in FN matrix appears to be an AGE-dependent response since soluble AGE-BSA also stimulates mesangial cell matrix assembly, at least in part through the AGE receptor RAGE. Together, these results suggest that pathways downstream of AGEs promote FN matrix assembly, which may help to explain the link between non-enzymatic glycation and fibrosis.

Non-enzymatic glycation is a relatively slow reaction that is dependent upon a multitude of factors, including the concentration and type of the glycating agent. In this study, MGO treatment increased the relative amounts of AGEs in FN from a decellularized matrix compared to both glucose and control treatments. The hydroimidazolones MG-H and MG-DH are common when MGO is the glycating agent [25], which were the primary types of AGEs identified following MGO treatment. MGO-derived AGEs are known to bind to RAGE [45], and RAGE activates intracellular pathways including ERK/MAP kinase [48], which regulates FN matrix assembly [49]. Furthermore, MGO-derived AGEs have been implicated in AGE diseases, as hydroimidazolones have been detected as the major AGEs on lens proteins and shown to contribute to cataract formation *in vivo* [50].

The location of AGEs on FN provides insights into the factors that determine whether a site is modified. Both the integrin and heparin-binding domains were targets for AGE modification, and these structural changes could have profound effects on FN function. The preferential clustering of non-enzymatic glycation in these regions may be based on solvent accessibility, as both the integrin-binding and heparin-binding sites are able to bind their partners from within fibrils [40, 51]. In contrast, the I₁₋₅ and the III₁₋₂ domains, the two major FN-FN binding sites, were very minimally modified. MGO incubation of a GST-III₁₋₂ fusion protein in solution modified 8 of 11 lysines and arginines, indicating that the sites can be modified (unpublished observations). The lack of glycation in I₁₋₅ and III₁₋₂ in matrix FN suggests that these domains have limited susceptibility to AGE modification most likely because they are involved in intermolecular interactions within fibrils. Besides solvent accessibility and the glycating agent, other factors may influence the probability that a site is

AGE-modified. For example, neighboring groups to lysine or arginine residues may impact their basicity and render these sites suboptimal for AGE modification [2].

Both AGE-modified ECM and soluble AGE-BSA affected cell-mediated matrix assembly in our in vitro system. In vivo, however, it is possible that AGE-induced changes in the structure or organization of the ECM itself may also affect cell-mediated matrix assembly. Since collagens and other ECM proteins depend on FN matrix for assembly [39], AGE modification of FN residues in binding sites for those proteins could affect their incorporation into the matrix thus changing ECM composition. Monoadduct AGEs on the RGD arginine and the heparin-binding cationic cradle could block integrin and syndecan binding, respectively, and by affecting cell-ECM interactions may lead to a compensatory increase in ECM protein expression or matrix reorganization by mesangial cells. Another potential effect of RGD modification might be an increase in unoccupied FN receptors, which could participate in and increase the assembly of unmodified protein. In addition, AGE modifications may change the conformation of FN to promote fibrillogenesis, as FN binding is enhanced when mechanically stretched [52]. Certain AGEs also form intra- or intermolecular crosslinks [2]. AGE-induced cross-linking has been shown to increase the stiffness of collagen [53], and chemically crosslinking FN matrix stabilizes it and reduces fibril breakage by shear forces [54]. Increased tissue stiffness also increased FN matrix assembly [55]. Therefore, AGE-induced crosslinking of FN within the matrix might be another mechanism to promote matrix accumulation. As mass spectrometry-based proteomic strategies continue to mature, the identification of crosslinked species should become more straightforward and aid in understanding their contribution to FN matrix assembly. Although the above discussion related to FN matrix assembly, the impact of AGEs on the ECM can also occur through modification of any of the many other components that form the ECM. From a technical perspective, the mass spectrometry-based proteomic approach we developed to identify AGE-modified FN sites could be readily adapted to other ECM proteins.

The AGE-RAGE axis has been linked to many intracellular signaling pathways [26, 48]. RAGE-dependent changes in gene expression can occur through NF- κ B, JAK/STAT, and MAP kinase pathways leading to increased levels of various cytokines and growth factors [48]. The accumulation of AGE-modified proteins has been shown to increase ECM protein expression [56]. In the glomerulus, the exogenous addition of AGEs into mice increases the expression of collagen IV and laminin [8], fitting with our observed increase of FN mRNA in mesangial cells on an MGO-treated matrix. Src kinase, TGF- β , and Rho GTPase/ROCK have been implicated in the stimulation of cell proliferation and migration downstream of RAGE [26, 48, 52, 57–60]. Each of these molecules has a role in the normal process of FN matrix assembly. Therefore, our results showing AGE stimulation of matrix assembly may result from a combinatorial effect on multiple pathways involved in this process.

The effect of AGEs on the amount of ECM suggests that, in addition to FN assembly induced in mesangial cells by high glucose levels [15], these modifications also contribute to the development of a fibrotic state. Furthermore, a fibrillar matrix environment on its own stimulates FN matrix assembly [41]. Therefore, glucose- and AGE-enhanced assembly

leading to build up of matrix may activate a cycle of assembly that provides a mechanism for the chronic progression of diabetes-related fibrosis.

4. Experimental Procedures

4.1 Fibronectin and antibodies

Fibronectin was purified from fresh frozen rat plasma by gelatin-Sepharose affinity chromatography [61]. The anti-FN antibodies used were: polyclonal antiserum R457 raised against the N-terminal 70-kDa fragment of rat FN [62] and rat-specific anti-FN monoclonal antibody IC3 from ascites fluid. An antibody against GAPDH (14C10, Cell Signaling, Danvers, MA, USA) was used as a loading control. Secondary antibodies used were horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG, HRP-goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL, USA) and Alexa Fluor 488 and 568-conjugated goat anti-mouse, goat anti-rabbit, and goat anti-rat IgG (Invitrogen, Eugene, OR, USA).

4.2 Cell culture

Conditionally immortalized mesangial cells were isolated from an Immortomouse as described previously [63] and were kindly provided by Drs. Ambra Pozzi and Roy Zent, Vanderbilt University Medical School. These cells express a temperature-sensitive SV40 large T antigen when grown in the presence of interferon- γ at 33 °C. Cells were maintained at 33 °C in the presence of 100 IU/ml interferon- γ (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (Hyclone, Logan, UT, USA) in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY, USA) with 1mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml Fungizone (Life Technologies, Grand Island, NY, USA). Cells were grown at 37 °C in the absence of interferon- γ for at least 4 days and one passage before use in experiments. These are conditions previously established to result in a phenotype similar to freshly primary mesangial cells [63]. Cells were propagated in the above medium containing 20 mM glucose and 10 mM mannitol. Cells were seeded for experiments in media containing DMEM with 5 mM glucose and 25 mM mannitol plus 10% serum, allowed 4 hours to adhere, and then serum-starved overnight in DMEM with 5 mM glucose and 25 mM mannitol. After 20 hours of starvation, medium was changed to DMEM with 5 mM glucose and 25 mM mannitol or 30 mM glucose, plus 10% fetal bovine serum and maintained 1–2 days before being seeded onto decellularized matrices, as indicated.

In experiments with exogenous AGE-BSA, 20 μ g/ml AGE-BSA (Millipore, Darmstadt, Germany) or fatty acid-free BSA (Sigma, St. Louis, MO, USA) was added to the media at 0 and 24 hours into a 48-hour time-course. 15 μ g/ml RAGE function blocking antibody (AF1179, R&D Systems, Minneapolis, MN) or lysyl oxidase (LOX) antibody (ab31238, Abcam, Cambridge, MA) was added for final 24 hours of 48 hour time-course where indicated.

NIH 3T3 fibroblasts were grown in DMEM with 10% bovine calf serum (Hyclone, Logan, UT, USA). Decellularized, three-dimensional (3D) matrices were prepared as previously described [41]. For DOC solubility and attachment assays, NIH 3T3 cells were seeded at a

density of 10^5 cells/cm² on gelatin-coated 6-well tissue culture plates and grown for 5 days. For immunofluorescence experiments NIH 3T3 fibroblasts were grown for 10 days on glass coverslips. For gelatin coating, wells were first coated with 0.1 mg/ml Poly-D-Lysine (Sigma, St. Louis, MO, USA) for 5 minutes at 37 °C, then removed and the wells allowed to dry for 30 minutes at 37 °C and finally coated with 0.1% gelatin (Millipore, Darmstadt, Germany) for 45 minutes at room temperature. For mass spectrometry preparations, 10 cm plates were seeded without gelatin coat.

4.3 Fluorimetry

200 µg/ml of FN fragment III₉₋₁₀ was incubated in EPPS buffer (50 mM EPPS, 150 mM NaCl, pH 8.5) and 3 mM NaAzide alone or 0.1 mM MGO in EPPS buffer at 37 °C over a 14 day time-course. An excitation/emission ratio of 335 nm/385 nm was used to measure autofluorescent AGE levels in a QuantaMaster 40 Fluorometer (Photon Technology International, Birmingham, NJ, USA). The fold changes in fluorescence were calculated for treated samples relative to control-treated protein.

4.4 Mass spectrometry-based proteomic analysis

For the in-solution digest of GST-III₉₋₁₀ fragment, the fragment was first glycated for 7 days in 0.1 mM MGO, 500 mM glucose, or EPPS buffer as described above. 5 µg (25 µl) of GST-III₉₋₁₀ fragment was added to 25 µl of 2x digestion buffer (0.2 M ammonium bicarbonate pH 8.0, 0.2% RapiGest, 0.01 M TCEP, 0.02 M chloroacetamide) and incubated for 45 minutes at 37 °C. 1 µl of 0.5 M cysteine was then added and the solution was incubated for another 15 minutes at 37 °C. Chymotrypsin (Pierce Chemical Co., Rockford, IL, USA) was added to a final concentration of 100 ng/µl to each sample and incubated for 2 hours at 37 °C. The same amount of chymotrypsin was spiked into samples and the incubation continued overnight at 37 °C. The following morning, the digests were acidified to 1% trifluoroacetic acid (TFA) and incubated at 37 °C for 20 minutes. The samples were centrifuged at 16,000 x g for 10 min to pellet insoluble material. The peptides in the supernatant were collected and desalted over SDB-RPS StageTips [52], concentrated to near dryness in autosampler vials, and resuspended in 20 µl of 1% formic acid/4% acetonitrile.

For the in-gel digestion of FN from decellularized matrices, glycated matrix was first prepared by incubating 10 cm dishes of glycated matrix for 7 days in 0.6 mM MGO, 500 mM glucose, or PBS alone at 4 °C. Decellularized matrices were washed once with PBS for 15 minutes, and then 400 µl of 4% SDS lysis buffer (4% SDS, 20 mM Tris-HCl pH 8.8, 2 mM EDTA, 2 mM PMSF) with protease inhibitor was added and remained on the plates for 10 minutes at room temperature. Aliquots (40 µl) of solubilized ECM were reduced and alkylated with 10 mM TCEP and 20 mM chloroacetamide, respectively, then were separated by SDS-PAGE on a 4 – 12% Bis-Tris NuPAGE gel and stained with Coomassie SimplyBlue (Life Technologies, Grand Island, NY, USA). The band corresponding to full-length fibronectin was excised, and subjected to in-gel enzymatic digestion, as previously described [64] with some modifications as detailed below. Briefly, gel pieces were destained, and then washed with 2 rounds of dehydration and rehydration in acetonitrile and 0.05 M ammonium bicarbonate, respectively. Gel pieces were dehydrated a final time and then incubated for 90 min on ice with 20 µl of 0.1 M ammonium bicarbonate containing 40 ng/µl chymotrypsin.

The chymotrypsin solution that was not absorbed by the gel pieces was removed, the gel pieces were covered with fresh 0.1 M ammonium bicarbonate and the incubation was continued overnight at 37 °C. The next day, the supernatant was retained and the gel pieces were extracted twice with 50 µl of 5% formic acid:acetonitrile (1:2) at 37 °C for 15 min with gentle agitation. The two extracts were pooled with the supernatant, evaporated by vacuum centrifugation (< 20 µl), and adjusted to 50 µl with TFA (1% final). Peptides were desalted, concentrated, and resuspended as described above.

Peptides (4 µl) were analyzed by nanoliquid chromatography-tandem mass spectrometry, using a Dionex Ultimate 3000 nRSLC coupled online to an LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Inc), as described [65]. Peptides were loaded directly on-column and resolved by reverse phase chromatography using a linear 90 min gradient of 4–35% buffer B (B: 97% ACN in 0.1% formic acid, A: 0.1% formic acid in H₂O). Eluted peptides were analyzed by data-dependent Top 15 selection, which entails acquisition of a single MS scan in the orbitrap (resolution = 30,000, lockmass enabled) followed by MS/MS fragmentation in the ion trap by collision-induced dissociation (min. signal = 1000, normalized collisional energy = 30) of the top 15 most intense precursor ions. This cycle was repeated across the LC gradient using the following instrument parameters: FTMS and ITMS target values, 1E6 and 1E4, respectively; FTMS and ITMS max ion times, 500 and 100 ms, respectively; dynamic exclusion enabled with a repeat count of 1.

MS/MS spectra were extracted from the instrument RAW files using Proteome Discoverer (ver 1.4) and submitted to the Byonic search engine (ver 2.3.8) for database searching against the UniProt-SwissProt mouse protein database (release 2013-08). The default Byonic settings were used, with the following exceptions: precursor and fragment ion tolerances, 5 ppm and 0.4 Da, respectively; semi-specific cleavage at FYWL (C-terminus); maximum missed cleavage, 3; and mzIdentML export, True. Amino acid modifications (max total common and rare modifications, 3 and 1, respectively) were specified as fixed modification of cysteine carbamidomethylation and common variable modifications of methionine oxidation and asparagine deamidation, which were allowed for all protein sequences. To increase the specificity and sensitivity for detection of modifications on FN, the following variable modifications were allowed, but only for the human (GST-III₉₋₁₀ fragment; UniProt accession P02751-15) and mouse (full-length, UniProt accession P11276) FN database sequences: common variable modifications of dioxidation on tryptophan; Amadori (+162), carboxymethyl (+58), and carboxyethyl (+72) on lysine (CML and CEL); glyoxal-derived hydroimidazolone/G-H (+40), glyoxal-derived dihydroxyimidazolidine/G-DH (+58), methyl glyoxal-derived hydroimidazolone/MG-H (+54), methyl glyoxal-derived dihydroxyimidazolidine/MG-DH (+72), argpyrimidine (+80), dihydropyrimidine (+126), tetrahydropyrimidine (THP, +144), and Amadori (+162) on arginine; and rare modification of pyro-Glu at peptide N-termini. Byonic search results (3 biological replicates x 3 treatment conditions) were exported in the mzIdentML format and analyzed by Scaffold (ver 4.4.1, Proteome Software, Inc) to calculate peptide/protein probabilities using the built-in LFDR algorithm. The peptide probability threshold was selected to maintain a global PSM FDR of < 1%, based on reverse database assignments. Datasets were exported to perSPECTives (ver 2.0.5, Proteome Software, Inc) and AGE-modified spectra were manually inspected to verify

the high confidence (< 1% peptide FDR) automated sequence assignments and the residue of AGE modification (see Supplemental Figure 1 for representative MS/MS spectra).

4.5 Cell binding to GST-III₉₋₁₀ and decellularized matrix

A 96-cell non-tissue culture-treated plate was coated with 0.5 µg/ml of GST-III₉₋₁₀ overnight at 4°C and then blocked with 1% BSA in PBS for 30 minutes at room temperature. Cells conditioned in 30 mM glucose as described above were seeded at 1×10^5 cells/well in serum-free medium with 30 mM glucose and allowed 1 hour to adhere. For attachment on glycated matrix, matrices were described as above in 6-well plates. Cells conditioned in 30 mM glucose as described above were seeded at 3.6×10^5 cells/well in serum-free medium with 30 mM glucose and allowed 2 or 6 hours to adhere. Unattached cells were washed away once with medium. Phase-contrast images from 10 randomly selected fields were captured using a Nikon inverted TS100 microscope equipped with a Cooke Sensicam QE cooled CCD camera for each well per condition per experiment and cells were counted.

4.6 Cell lysis and immunoblotting

Cell lysates were separated into DOC-soluble and insoluble fractions as previously described [42]. Total protein concentrations were measured in the DOC-soluble fraction using a BCA protein assay (Pierce Chemical Co., Rockford, IL, USA) and equal amounts of DOC-soluble protein or proportional volumes of DOC-insoluble samples were separated by SDS-PAGE on 5% polyacrylamide gel alongside Precision Plus Protein Standard (Bio-Rad, Hercules, CA, USA). Protein was then transferred to nitrocellulose membrane. Antibody incubations were performed in buffer A (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). Total FN was detected with R457 rabbit anti-FN antiserum diluted 1:5000 and rat FN was detected with IC3 monoclonal antibody against rat FN (ascites fluid diluted 1:1000). DOC-soluble samples were probed with rabbit anti-GAPDH antibody (1:2000, Cell Signaling, Danvers, MA, USA) to confirm equal loading. Horseradish-peroxidase-conjugated goat anti-rabbit-IgG and goat-anti-mouse-IgG antibody (Pierce Chemical Co., Rockford, IL, USA) secondary antibodies were diluted 1:10,000 in buffer A. Blots were developed using SuperSignal West Pico ECL reagents (Pierce Chemical Co., Rockford, IL, USA). Densitometry was performed on scanned films using Quantity One(R) software (Bio-Rad, Hercules, CA, USA) and exposures yielding signals within the linear range were quantified. FN levels were normalized to GAPDH.

4.7 Immunofluorescence and microscopy

Cells were fixed for 15 minutes at room temperature in 3.7% (w/v) formalin (Sigma, St. Louis, MO, USA) in PBS supplemented with 0.5 mM MgCl₂, and then washed with PBS. Alexa-Fluor-488-conjugated goat anti-mouse-IgG and Alexa-Fluor-568-conjugated goat anti-rabbit-IgG secondary antibodies (Invitrogen, Eugene, OR, USA) were diluted 1:600 in 2% ovalbumin serum in PBS along with DAPI at 1:1000. Coverslips were then mounted using ProLong Gold antifade reagent (Life Technologies, Grand Island, NY, USA). All images were captured of fields selected at random using Nikon Eclipse Ti microscope equipped with a Hamamatsu C10600 ORCA-R2 digital camera. Total mean fluorescence

measurements were performed using ImageJ on 10 randomly selected fields per condition and fluorescence fold-change was calculated.

4.8 Quantitative RT-PCR

RNA was extracted according to the manufacturer's instructions (Qiagen, Hilden, Germany). 1 µg of total RNA was reversed transcribed using random hexamer primers and Superscript II reverse transcriptase (Life Technologies, Grand Island, NY, USA). Primers for real-time PCR were designed using Mac Vector and reactions performed in a mix containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 nM of each primer on the Mx3000P QPCR System (Agilent Technologies, Waldbronn, Germany). PCR reaction conditions were: 10 min at 95°, followed by 40 cycles of 15 s at 95°, 30 s at 55°, and 30 s at 72°. Data analysis was performed using MxPro™ QPCR software (Agilent Technologies, Waldbronn, Germany). All data values were normalized to those for GAPDH. Primers used in this study were: fibronectin forward, 5'-AAGGCTGGATGATGGTGGACTG-3', and reverse 5'-TGAAGCAGGTTTCCTCGGTTG-3'; GAPDH forward 5'-AATGGTGAAGGTCGGTGTGAA-3', and reverse 5'-CCGTGAGTGGAGTCATACTGG-3'.

4.9 Statistical analysis

Results are reported as the mean ± standard error for a minimum of three independent samples and mean ± range for two independent samples. Computed means were compared using Student's t-test, with $p < 0.05$ considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ECM	extracellular matrix
FN	fibronectin
DOC	deoxycholate
AGE	advanced glycation endproduct
RAGE	receptor for advanced glycation endproducts
MGO	methylglyoxal
BSA	bovine serum albumin

References

1. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetologia*. 2001; 44:129–146. [PubMed: 11270668]
2. Ahmed N. Advanced glycation endproducts--role in pathology of diabetic complications. *Diabetes Res Clin Pract*. 2005; 67:3–21. [PubMed: 15620429]
3. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*. 1995; 46:223–234. [PubMed: 7598459]
4. Frye EB, Degenhardt TP, Thorpe SR, Baynes JW. Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins. *J Biol Chem*. 1998; 273:18714–18719. [PubMed: 9668043]
5. Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT. RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. *J Transl Med*. 2009; 7:17. [PubMed: 19292913]
6. Pamplona R, Portero-Otin M, Requena J, Gredilla R, Barja G. Oxidative, glycoxidative and lipoxidative damage to rat heart mitochondrial proteins is lower after 4 months of caloric restriction than in age-matched controls. *Mech Ageing Dev*. 2002; 123:1437–1446. [PubMed: 12425950]
7. Pamplona R, Naudi A, Gavin R, Pastrana MA, Sajnani G, Ilieva EV, Del Rio JA, Portero-Otin M, Ferrer I, Requena JR. Increased oxidation, glycoxidation, and lipoxidation of brain proteins in prion disease. *Free Radic Biol Med*. 2008; 45:1159–1166. [PubMed: 18703134]
8. Yang CW, Vlassara H, Peten EP, He CJ, Striker GE, Striker LJ. Advanced glycation end products up-regulate gene expression found in diabetic glomerular disease. *Proc Natl Acad Sci U S A*. 1994; 91:9436–9440. [PubMed: 7937785]
9. Dixon AJ, Burns J, Dunnill MS, McGee JO. Distribution of fibronectin in normal and diseased human kidneys. *J Clin Pathol*. 1980; 33:1021–1028. [PubMed: 7002956]
10. Ayo SH, Radnik RA, Garoni JA, Glass WF 2nd, Kreisberg JJ. High glucose causes an increase in extracellular matrix proteins in cultured mesangial cells. *Am J Pathol*. 1990; 136:1339–1348. [PubMed: 2356864]
11. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation*. 2006; 114:597–605. [PubMed: 16894049]
12. Rashid ST, Humphries JD, Byron A, Dhar A, Askari JA, Selley JN, Knight D, Goldin RD, Thursz M, Humphries MJ. Proteomic analysis of extracellular matrix from the hepatic stellate cell line LX-2 identifies CYR61 and Wnt-5a as novel constituents of fibrotic liver. *J Proteome Res*. 2012; 11:4052–4064. [PubMed: 22694338]
13. Sottile J, Hocking DC. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol Biol Cell*. 2002; 13:3546–3559. [PubMed: 12388756]
14. Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *J Biol Chem*. 2002; 277:37377–37381. [PubMed: 12145303]
15. Miller CG, Pozzi A, Zent R, Schwarzbauer JE. Effects of high glucose on integrin activity and fibronectin matrix assembly by mesangial cells. *Mol Biol Cell*. 2014; 25:2342–2350. [PubMed: 24943838]
16. Dallas SL, Sivakumar P, Jones CJ, Chen Q, Peters DM, Mosher DF, Humphries MJ, Kielty CM. Fibronectin regulates latent transforming growth factor-beta (TGF beta) by controlling matrix assembly of latent TGF beta-binding protein-1. *J Biol Chem*. 2005; 280:18871–18880. [PubMed: 15677465]
17. Voziyan P, Brown KL, Chetyrkin S, Hudson B. Site-specific AGE modifications in the extracellular matrix: a role for glyoxal in protein damage in diabetes. *Clin Chem Lab Med*. 2014; 52:39–45. [PubMed: 23492568]
18. Pozzi A, Zent R, Chetyrkin S, Borza C, Bulus N, Chuang P, Chen D, Hudson B, Voziyan P. Modification of collagen IV by glucose or methylglyoxal alters distinct mesangial cell functions. *J Am Soc Nephrol*. 2009; 20:2119–2125. [PubMed: 19608705]

19. Reigle KL, Di Lullo G, Turner KR, Last JA, Chervoneva I, Birk DE, Funderburgh JL, Elrod E, Germann MW, Surber C, Sanderson RD, San Antonio JD. Non-enzymatic glycation of type I collagen diminishes collagen-proteoglycan binding and weakens cell adhesion. *J Cell Biochem*. 2008; 104:1684–1698. [PubMed: 18348167]
20. Bartling B, Desole M, Rohrbach S, Silber RE, Simm A. Age-associated changes of extracellular matrix collagen impair lung cancer cell migration. *FASEB J*. 2009; 23:1510–1520. [PubMed: 19109409]
21. Tarsio JF, Reger LA, Furcht LT. Decreased interaction of fibronectin, type IV collagen, and heparin due to nonenzymatic glycation. Implications for diabetes mellitus. *Biochemistry*. 1987; 26:1014–1020. [PubMed: 3567152]
22. Tarsio JF, Wigness B, Rhode TD, Rupp WM, Buchwald H, Furcht LT. Nonenzymatic glycation of fibronectin and alterations in the molecular association of cell matrix and basement membrane components in diabetes mellitus. *Diabetes*. 1985; 34:477–484. [PubMed: 3987974]
23. Lapolla A, Fedele D, Reitano R, Arico NC, Seraglia R, Traldi P, Marotta E, Tonani R. Enzymatic digestion and mass spectrometry in the study of advanced glycation end products/peptides. *J Am Soc Mass Spectrom*. 2004; 15:496–509. [PubMed: 15047055]
24. Brock JW, Cotham WE, Thorpe SR, Baynes JW, Ames JM. Detection and identification of arginine modifications on methylglyoxal-modified ribonuclease by mass spectrometric analysis. *J Mass Spectrom*. 2007; 42:89–100. [PubMed: 17143934]
25. Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J*. 2003; 375:581–592. [PubMed: 12885296]
26. Xie J, Mendez JD, Mendez-Valenzuela V, Aguilar-Hernandez MM. Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cell Signal*. 2013; 25:2185–2197. [PubMed: 23838007]
27. Kolset SO, Reinholt FP, Jenssen T. Diabetic nephropathy and extracellular matrix. *J Histochem Cytochem*. 2012; 60:976–986. [PubMed: 23103723]
28. Forbes JM, Cooper ME, Oldfield MD, Thomas MC. Role of advanced glycation end products in diabetic nephropathy. *J Am Soc Nephrol*. 2003; 14:S254–S258. [PubMed: 12874442]
29. Stitt AW, He C, Friedman S, Scher L, Rossi P, Ong L, Founds H, Li YM, Bucala R, Vlassara H. Elevated AGE-modified ApoB in sera of euglycemic, normolipidemic patients with atherosclerosis: relationship to tissue AGEs. *Mol Med*. 1997; 3:617–627. [PubMed: 9323713]
30. Palinski W, Koschinsky T, Butler SW, Miller E, Vlassara H, Cerami A, Witztum JL. Immunological evidence for the presence of advanced glycosylation end products in atherosclerotic lesions of euglycemic rabbits. *Arterioscler Thromb Vasc Biol*. 1995; 15:571–582. [PubMed: 7749871]
31. Kume S, Takeya M, Mori T, Araki N, Suzuki H, Horiuchi S, Kodama T, Miyauchi Y, Takahashi K. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Am J Pathol*. 1995; 147:654–667. [PubMed: 7545874]
32. Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R. Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol*. 1993; 143:1649–1656. [PubMed: 8256853]
33. Stenman S, von Smitten K, Vaheri A. Fibronectin and atherosclerosis. *Acta Med Scand Suppl*. 1980; 642:165–170. [PubMed: 6935942]
34. Rohwedder I, Montanez E, Beckmann K, Bengtsson E, Duner P, Nilsson J, Soehnlein O, Fassler R. Plasma fibronectin deficiency impedes atherosclerosis progression and fibrous cap formation. *EMBO Mol Med*. 2012; 4:564–576. [PubMed: 22514136]
35. Bunn HF, Higgins PJ. Reaction of monosaccharides with proteins: possible evolutionary significance. *Science*. 1981; 213:222–224. [PubMed: 12192669]
36. Vander Jagt DL, Hunsaker LA. Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase-I, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. *Chem Biol Interact*. 2003; 143–144:341–351.

37. Turk Z, Nemet I, Varga-Defteardarovic L, Car N. Elevated level of methylglyoxal during diabetic ketoacidosis and its recovery phase. *Diabetes Metab.* 2006; 32:176–180. [PubMed: 16735968]
38. Bourajjaj M, Stehouwer CD, van Hinsbergh VW, Schalkwijk CG. Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus. *Biochem Soc Trans.* 2003; 31:1400–1402. [PubMed: 14641073]
39. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. *Annu Rev Cell Dev Biol.* 2010; 26:397–419. [PubMed: 20690820]
40. Leahy DJ, Aukhil I, Erickson HP. 2.0 Å crystal structure of a four-domain segment of human fibronectin encompassing the RGD loop and synergy region. *Cell.* 1996; 84:155–164. [PubMed: 8548820]
41. Mao Y, Schwarzbauer JE. Stimulatory effects of a three-dimensional microenvironment on cell-mediated fibronectin fibrillogenesis. *J Cell Sci.* 2005; 118:4427–4436. [PubMed: 16159961]
42. Wierzbicka-Patynowski I, Mao Y, Schwarzbauer JE. Analysis of fibronectin matrix assembly. *Curr Protoc Cell Biol Chapter.* 2004; 10 Unit 10 12.
43. Boel E, Selmer J, Flodgaard HJ, Jensen T. Diabetic late complications: will aldose reductase inhibitors or inhibitors of advanced glycosylation endproduct formation hold promise? *J Diabetes Complications.* 1995; 9:104–129. [PubMed: 7599349]
44. Mason RM, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol.* 2003; 14:1358–1373. [PubMed: 12707406]
45. Ng R, Argirova OK, Ahmeda N, Weigleb B, Thornalley PJ. Human serum albumin minimally modified by methylglyoxal binds to human mononuclear leukocytes via the RAGE receptor and is displaced by Nε-carboxymethyl-lysine and hydroimidazolone AGE epitopes. *International Congress Series.* 2002; 1245:77–81.
46. Huang KP, Chen C, Hao J, Huang JY, Liu PQ, Huang HQ. AGEs-RAGE system down-regulates Sirt1 through the ubiquitin-proteasome pathway to promote FN and TGF-β1 expression in male rat glomerular mesangial cells. *Endocrinology.* 2015; 156:268–279. [PubMed: 25375034]
47. Shimizu F, Sano Y, Tominaga O, Maeda T, Abe MA, Kanda T. Advanced glycation end-products disrupt the blood-brain barrier by stimulating the release of transforming growth factor-β by pericytes and vascular endothelial growth factor and matrix metalloproteinase-2 by endothelial cells in vitro. *Neurobiol Aging.* 2013; 34:1902–1912. [PubMed: 23428182]
48. Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A. Role of advanced glycation end products in cellular signaling. *Redox Biol.* 2014; 2:411–429. [PubMed: 24624331]
49. Brenner KA, Corbett SA, Schwarzbauer JE. Regulation of fibronectin matrix assembly by activated Ras in transformed cells. *Oncogene.* 2000; 19:3156–3163. [PubMed: 10918570]
50. Ahmed N, Thornalley PJ, Dawczynski J, Franke S, Strobel J, Stein G, Haik GM. Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins. *Invest Ophthalmol Vis Sci.* 2003; 44:5287–5292. [PubMed: 14638728]
51. Busby TF, Argraves WS, Brew SA, Pechik I, Gilliland GL, Ingham KC. Heparin binding by fibronectin module III-13 involves six discontinuous basic residues brought together to form a cationic cradle. *J Biol Chem.* 1995; 270:18558–18562. [PubMed: 7629186]
52. Zhong C, Chrzanowska-Wodnicka M, Brown J, Shaub A, Belkin AM, Burridge K. Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J Cell Biol.* 1998; 141:539–551. [PubMed: 9548730]
53. Verzijl N, DeGroot J, Ben ZC, Brau-Benjamin O, Maroudas A, Bank RA, Mizrahi J, Schalkwijk CG, Thorpe SR, Baynes JW, Bijlsma JW, Lafeber FP, TeKoppele JM. Crosslinking by advanced glycation end products increases the stiffness of the collagen network in human articular cartilage: a possible mechanism through which age is a risk factor for osteoarthritis. *Arthritis Rheum.* 2002; 46:114–123. [PubMed: 11822407]
54. Engler AJ, Chan M, Boettiger D, Schwarzbauer JE. A novel mode of cell detachment from fibrillar fibronectin matrix under shear. *J Cell Sci.* 2009; 122:1647–1653. [PubMed: 19401337]
55. Carraher CL, Schwarzbauer JE. Regulation of matrix assembly through rigidity-dependent fibronectin conformational changes. *J Biol Chem.* 2013; 288:14805–14814. [PubMed: 23589296]
56. Berrou J, Tostivint I, Verrecchia F, Berthier C, Boulanger E, Mauviel A, Marti HP, Wautier MP, Wautier JL, Rondeau E, Hertig A. Advanced glycation end products regulate extracellular matrix

- protein and protease expression by human glomerular mesangial cells. *Int J Mol Med*. 2009; 23:513–520. [PubMed: 19288028]
57. Serban AI, Stanca L, Geicu OI, Munteanu MC, Dinischiotu A. RAGE and TGF-beta1 Cross-Talk Regulate Extracellular Matrix Turnover and Cytokine Synthesis in AGEs Exposed Fibroblast Cells. *PLoS One*. 2016; 11:e0152376. [PubMed: 27015414]
58. Watanabe N, Kato T, Fujita A, Ishizaki T, Narumiya S. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat Cell Biol*. 1999; 1:136–143. [PubMed: 10559899]
59. Meng W, Numazaki M, Takeuchi K, Uchibori Y, Ando-Akatsuka Y, Tominaga M, Tominaga T. DIP (mDia interacting protein) is a key molecule regulating Rho and Rac in a Src-dependent manner. *EMBO J*. 2004; 23:760–771. [PubMed: 14765113]
60. Satoh S, Tominaga T. mDia-interacting protein acts downstream of Rho-mDia and modifies Src activation and stress fiber formation. *J Biol Chem*. 2001; 276:39290–39294. [PubMed: 11509578]
61. Engvall E, Ruoslahti E, Miller EJ. Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J Exp Med*. 1978; 147:1584–1595. [PubMed: 567240]
62. Aguirre KM, McCormick RJ, Schwarzbauer JE. Fibronectin self-association is mediated by complementary sites within the amino-terminal one-third of the molecule. *J Biol Chem*. 1994; 269:27863–27868. [PubMed: 7961716]
63. Chen X, Abair TD, Ibanez MR, Su Y, Frey MR, Dise RS, Polk DB, Singh AB, Harris RC, Zent R, Pozzi A. Integrin alpha1beta1 controls reactive oxygen species synthesis by negatively regulating epidermal growth factor receptor-mediated Rac activation. *Mol Cell Biol*. 2007; 27:3313–3326. [PubMed: 17339338]
64. Hopf M, Gohring W, Mann K, Timpl R. Mapping of binding sites for nidogens, fibulin-2, fibronectin and heparin to different IG modules of perlecan. *J Mol Biol*. 2001; 311:529–541. [PubMed: 11493006]
65. Diner BA, Li T, Greco TM, Crow MS, Fuesler JA, Wang J, Cristea IM. The functional interactome of PYHIN immune regulators reveals IFIX is a sensor of viral DNA. *Mol Syst Biol*. 2015; 11:787. [PubMed: 25665578]

Highlights

- Sites of non-enzymatic glycation on fibronectin within a matrix were identified.
- Our mass spectrometry-proteomics approach detected nine different species of AGEs.
- Matrix assembly was stimulated by cell interactions with an AGE-modified matrix.
- The mechanism of increased matrix assembly involves the AGE receptor (RAGE).

Figure 1. Identification of AGE modifications on the III₉₋₁₀ cell-binding domain
 (A) Fold change in fluorimetry readings (excitation/emission ratio of 335/385 nm). The GST-III₉₋₁₀ fusion protein was treated with MGO or buffer and fluorescence was measured at the indicated times. Bars are the average of two independent experiments; error bars indicate the range. (B) Diagram of the domains and modular organization of a FN subunit [39]. The rectangles, triangles, and ovals represent type I, II, and III repeats, respectively. The rectangular green/yellow box is the variable region. Binding domains for collagen (white), FN (dark orange), heparin (light orange), and integrins (green) are shown. The

III₉₋₁₀ integrin-binding domain is boxed. AGE-modified sites along the length of III₉₋₁₀ amino acid sequence are highlighted in green. The synergy site (PHSRN) and the integrin-binding RGD site are bolded. (C) Comparison of AGE modifications on individual sites within the III₉₋₁₀ modules. Nine types of AGE modification were identified and are listed across the top. The number of spectra detected for each treatment at each residue is listed in Supplemental Table 1. The heat map indicates the relative abundance of modifications for each amino acid residue. For each residue, the number of spectra for each type of modification was summed across n = 3 biological replicates. The modification with the highest number of spectra was set at 1 (dark red). The number of spectra for each of the other modifications at the site was divided by the highest number of spectra and that fraction is represented by the appropriate gradation of red.

Figure 2. Identification of AGE modification sites on FN from a decellularized matrix
 (A) Location of AGE-modified sites. FN was isolated from a decellularized matrix after treatment with MGO and AGE modifications were identified by mass spectrometry. Black lines with numbered amino acids indicate the positions of AGE modifications along the diagram of a FN subunit. See Figure 1 legend for description of FN modules and binding domains. R1614 in mouse FN (UniProt accession P11276) differs by one position from human FN (R1615; UniProt accession P02751-15). To illustrate the high quality of the spectra used to identify AGE modifications, representative spectra for each identified AGE

modification are shown in Supplemental Figure 1. (B) Protein sequence coverage of FN using tandem mass spectrometry. (Top) Coverage map consists of a mark for each identified peptide from MGO-treated FN. Identified peptides covered 86% (2138/2477 amino acids) of total FN sequence. Regions of the protein sequence not detected are indicated in white. (Bottom) Relative detectability of FN sequences expressed as a function of identified, summed spectra that overlap sequential windows of 20 amino acid residues. (C) Comparison of AGE modifications identified on FN isolated from a glycated decellularized matrix. Relative number of spectra at each site are presented as described in Figure 1C. Heat map is based on data in Supplemental Table 2.

Figure 3. Cell attachment on AGE-modified FN

(A) Mesangial cells were allowed to attach for 1 hr on a surface coated with 0.5 $\mu\text{g/ml}$ GST-III₉₋₁₀ that had been previously incubated for 7 days in MGO or control buffer. Error bars indicate the range ($n = 3$). $p < 0.05$. (B, C) Mesangial cells were allowed to attach to MGO or control-treated decellularized matrices for 2 hr (B) or 6 hr (C). Representative phase images are shown for each time and condition. Graphs show the mean of cell counts per field $\pm$ standard error (B; $n = 3$, $p < 0.001$) or $\pm$ range (C; $n = 2$). Scale bar is 200 μm .

Figure 4. Stimulation of matrix assembly by AGE-modified ECM

Mesangial cells were grown on an NIH 3T3 cell-derived decellularized matrix that had been incubated in buffer (control) or MGO for 7 days. (A) Left, mouse FN (no cells): Decellularized matrices were stained with R457 polyclonal anti-FN antiserum in the absence of cells. Middle, phase: Phase images of cells attached to decellularized matrices were captured at 24 hr. Right, rat FN: Cells were fixed and stained after 24 hr with IC3 anti-rat FN monoclonal antibody. Total mean fluorescence intensity was measured from 10 randomly selected fields per condition. Values were used to calculate the average FN staining intensity, which was increased by 1.25 fold $\pm$ standard error ($n = 3$, $p < 0.01$) in MGO compared to control-treated matrix. Scale bar is 200 μ m. (B) Samples were solubilized in DOC buffer at 24 hr. The DOC-insoluble fraction was separated by SDS-PAGE and immunoblotted with IC3 anti-rat FN monoclonal antibody. Relative densitometry values (below the lanes) are the mean of six experiments $\pm$ standard error normalized to GAPDH from the DOC soluble

fraction. Samples from the same blot and exposure time are shown and are representative of six independent experiments.

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Figure 5. Stimulation of FN matrix assembly by AGE-modified BSA

Mesangial cells were grown for 2 days on tissue culture plastic in the presence of AGE-BSA or BSA (20 μ g/mL) which were replenished after 1 day. 10 μ g/mL rat plasma FN was added for the final 24 hours. (A) Left, phase: Phase images of cells were captured after 2 days of culture. Right, rat FN: Immunofluorescence images of cells stained with IC3 anti-rat FN antibody. Relative average fluorescence intensities are indicated on the images ($\pm$ standard error, $n = 3$, $p < 0.05$). Scale bar is 200 μ m. (B) The DOC-insoluble fraction was separated by SDS-PAGE and immunoblotted with IC3 anti-rat FN monoclonal. Relative densitometry

values are the mean of three experiments $\pm$ standard error normalized to GAPDH from the DOC soluble fraction. Blot is representative of three independent experiments.

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Figure 6. RAGE function-blocking antibody reduces matrix assembly

Mesangial cells were grown for 2 days on tissue culture plastic + AGE-BSA or BSA (20 $\mu\text{g}/\text{mL}$) as in Figure 5 legend. 10 $\mu\text{g}/\text{mL}$ rat plasma FN and 15 $\mu\text{g}/\text{mL}$ RAGE function-blocking antibody were added for the final 24 hours. (A) Phase and fluorescence images were captured as described in Figure 5 legend. Average FN fluorescence intensity was decreased 1.16-fold to $0.86 \pm \text{range}$ ($n = 2$) in the presence of RAGE function-blocking antibody with AGE-BSA compared to AGE-BSA treatment alone. Scale bar is 200 μm . (B) The DOC-insoluble fraction was separated by SDS-PAGE and immunoblotted with IC3 anti-

rat FN monoclonal. Relative densitometry values are the mean of two experiments $\pm$ range normalized to GAPDH from the DOC soluble fraction. Blot is representative of two independent experiments.

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