Advanced Glycation Endproducts in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes

Running title: AGEs and ECM proteins in diabetic neuropathy

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Objective. To characterize glycation adducts formed both in vivo in extracellular matrix (ECM) proteins of endoneurium from streptozotocin (STZ)-induced diabetic rats, and in vitro by glycation of laminin and fibronectin with methylglyoxal (MG) and glucose. To investigate the impact of advanced glycation endproduct (AGE) residue content of ECM on neurite outgrowth from sensory neurons.

Research Design and Methods. Glycation, oxidation and nitration adducts of ECM proteins extracted from the endoneurium of control and STZ-diabetic rat sciatic nerve (3-24 weeks post-STZ), and of laminin and fibronectin that had been glycated using glucose or MG, were examined by liquid chromatography with tandem mass spectrometry. MG-glycated or unmodified ECM proteins were used as substrata for dissociated rat sensory neurons as in vitro models of regeneration.

Results. STZ-induced diabetes produced a significant increase in early glycation Nε-fructosyl-lysine (FL) and AGE residue contents of endoneurial ECM. Glycation of laminin and fibronectin by MG and glucose increased glycation adduct residue contents with MG-derived hydroimidazolone (MG-H1) and FL, respectively, of greatest quantitative importance. Glycation of laminin caused a significant decrease in both neurotrophin-stimulated and preconditioned sensory neurite-outgrowth. This decrease was prevented by aminoguanidine. Glycation of fibronectin also decreased preconditioned neurite-outgrowth, which was prevented by aminoguanidine and NGF.

Conclusions. Early glycation and AGE residue content of endoneurial ECM proteins increase markedly in STZ-induced diabetes. Glycation of laminin and fibronectin causes a reduction in neurotrophin-stimulated neurite outgrowth and preconditioned neurite-outgrowth. This may provide a mechanism for the failure of collateral sprouting and axonal regeneration in diabetic neuropathy.
The extracellular matrix (ECM) provides physical support for cells and tissue and also has a crucial role in regulating cell behaviour, mediating survival, proliferation, differentiation and migration via interaction with specific cell adhesion receptors such as integrins (1). Sensory neurons contain at least five laminin-binding integrins and two fibronectin-binding integrins (2-5) and we, and others, have shown the β1-integrins to be crucial mediators of neuronal adhesion and nerve regeneration (3;6). Modification of ECM proteins by glycation in diabetes may, therefore, have a severe impact on cellular function.

Non-enzymatic glycation of proteins involves the covalent linkage of saccharides and saccharide derivatives to proteins. Glucose reacts with amino groups of lysine and N-terminal amino acid residues. Early stage reactions lead to the formation of fructosyl-lysine (FL) and related fructosamine residues, which degrade slowly to form AGEs. In addition to degradation of glycated proteins, glycolytic intermediates and lipid peroxidation leads to the formation of the reactive dicarbonyl metabolites, glyoxal, methylglyoxal (MG) and 3-deoxyglucosone (3-DG). Dicarbonyls form AGE residues in proteins, largely, but not exclusively, on arginine residues. At the quantitative level, the most abundant AGEs are the hydroimidazolones derived from glyoxal, MG and 3-DG (denoted by the acronyms G-H1, MG-H1 and 3DG-H), the lysine-derived AGEs (Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL)), imidazolium crosslinks derived from glyoxal, MG and 3-DG (GOLD, MOLD and DOLD), and the trace fluorescent crosslink pentosidine.

AGE residues accumulate in both intracellular and extracellular proteins, especially in poorly-controlled diabetes and in tissues with metabolic dysfunction associated with high cellular glucose concentration. Accumulation of AGE residues is a risk marker for the development of diabetic neuropathy (7-9). AGE formation can be decreased by scavenging dicarbonyl precursors with aminoguanidine (10), which suppresses neurovascular dysfunction in STZ-diabetic rats(11;12).

ECM proteins are particularly long-lived, and they are potential targets of glycation. Changes in the composition and structure of ECM of peripheral nerve are observed in diabetes, notably increased endoneurial collagen, reduplication of basement membranes around endoneurial capillaries, and a thickening of basal lamina in both clinical and experimental diabetes (13-16). Glycation of ECM proteins modifies functionally important arginine residues of RGD and GFOGER motifs causing loss of charge and structural distortion, which is associated with decreased binding affinity of integrins and cell detachment (22). It also produces intramolecular crosslinking, causing structural distortion, and may confer resistance to proteolysis, leading to thickening of the basement membrane (17).

We have previously shown that cytosolic protein extracts of peripheral nerve of STZ diabetic rats have increased FL and AGE residue content compared to controls (7). In this current study, we firstly characterised and quantified AGE residue content in ECM protein extracts from the endoneurium of STZ-diabetic rat sciatic nerve over a 24 week timecourse. Secondly, we glycated the ECM proteins, laminin and fibronectin, in vitro, using glucose and MG and characterised and quantified the AGE residue contents of these glycated and unmodified control proteins. To address the functional impact of increased AGE residue content in ECM proteins on axonal outgrowth, we utilised two in vitro models of sensory nerve regeneration to model both collateral sprouting and axonal regeneration processes.
AGEs and ECM proteins in diabetic neuropathy

RESEARCH DESIGN AND METHODS

Reagents. All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. High purity methylglyoxal (MG) was prepared by acid hydrolysis of freshly distilled methylglyoxal dimethylacetal and purified by fractional distillation under reduced pressure (18).

Animal studies. All studies and procedures were licensed under the UK Animals (Scientific Procedures) Act 1986. Diabetes was induced in adult female Wistar rats (200 – 250g, Charles River, UK) via intraperitoneal injection of freshly dissolved streptozotocin (STZ; 55mg/kg in sterile saline) as previously described (19). Rats were euthanized at various time points following induction of diabetes (3, 6, 9, 12, and 24 weeks post-STZ, n=6 at each timepoint) and the sciatic nerves were rapidly removed and frozen on liquid nitrogen. Age- and weight-matched rats were used as non-diabetic controls (Time 0 and 24 weeks, n=4 at each timepoint).

A separate group of adult male Wistar rats (250–300 g, n=12) were anaesthetized with isoflurane (2% in oxygen), and, under sterile conditions, the left sciatic nerve was exposed at mid-thigh level. The sciatic nerve was crushed with the tips of watchmakers forceps (2 × 15 s), the wound was closed and animals recovered under observation. Seven days following nerve crush, rats were killed by concussion followed by decapitation. Ipsilateral and contralateral L4 and L5 dorsal root ganglia (DRG) were removed for cell culture experiments.

ECM protein extraction from sciatic endoneurium. Sciatic nerves were desheathed, homogenized in 0.25M sucrose on ice and centrifuged at 900g at 4°C. The ECM protein in the pellet was washed with PBS, then extracted with chloroform:methanol (2:1, v:v) and stirred continuously for 24 hours at 4°C and centrifuged at 900g at 4°C. The pellet was washed twice with PBS and frozen at -80°C until analysis.

Glycation of ECM proteins. Laminin and fibronectin modified by FL and AGEs (AGE-laminin and AGE-fibronectin, respectively) were prepared by incubating the proteins (6.6 mg/ml) with 50 mM glucose in 100 mM sodium phosphate buffer, pH 7.4, at 37°C, for 21 days. Laminin and fibronectin were also modified by incubating the proteins (6.6 mg/ml) with 500 µM methylglyoxal in 100 mM sodium phosphate buffer, pH 7.4, at 37°C, for 24 h. After the incubation, the glycated and control proteins were dialysed against 30 mM ammonium formate, pH 7.8 and 4°C, lyophilised to dryness and stored at –20°C.

Protein glycation, oxidation and nitration adduct determination by LC-MS/MS. Protein glycation, oxidation and nitration adducts were determined by stable isotopic dilution analysis liquid chromatography with tandem mass spectrophotometer detection (LC-MS/MS; 8). Glycation adducts determined were: FL and 10 AGEs (CML, CEL, G-H1, MG-H1, 3DG-H, GOLD, MOLD, DOLD, argpyrimidine and pentosidine). The protein oxidation adduct, dityrosine, the protein nitration adduct 3-NT, and amino acids lys, arg, met, tyr and trp were also determined. Pentosidine was determined by liquid chromatography with fluorimetric detection (20). Authentic standard analytes were prepared as described (7;21). Glycation, oxidation and nitration adduct residues of protein extracts were determined in exhaustive enzymatic digests (50 µg protein equivalent; 22). Samples were analysed using a module 2690 separation module with a Quattro Ultima triple-quadrupole mass spectrometric detector and 2475 fluorescence detector (Waters-Micromass, Manchester, UK) as previously described (20).

Sensory neuron culture. Control or preconditioned (7 days post-unilateral sciatic nerve crush injury) adult rat sensory neurons were mechanically and chemically dissociated, purified and resuspended in modified Bottenstein and Sato’s medium (BS) in Ham’s
F12 (containing 10mM D-glucose) as previously described (6;19;23).

Coating of slides with ECM. Labtek slides (VWR, UK) were either coated directly with 2µg/ml laminin or derivatised for 30 min with 1 mM m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co, USA), prior to coating for 1 h at room temperature with 10 µg/ml fibronectin (24). MG (500 µM), aminoguanidine (500 µM), MG with aminoguanidine (both 500 µM) or PBS was added to appropriate wells and slides incubated for 24 h at 37°C. Slides were washed three times, and neurons were seeded onto slides in BS and allowed to adhere to the substrate for 1 h prior to addition of NGF (10ng/ml) or GDNF (50ng/ml, Promega). Neurons were incubated for 18 h in 5% CO₂ at 37 °C and immunostained with anti-β(III)-tubulin antibody (1:1000, Sigma), a pan-neuronal marker labelling all cell bodies and neurites as previously described (23).

Neurite outgrowth analysis. For each experimental condition, images of neurons were acquired from 20 randomly selected fields of view per condition. Assessors were blinded to the experimental condition during analysis. The mean number of neurite-bearing cells was calculated (defined as those with neurites longer than 1.5 times the associated cell body diameter). The length of the longest neurite from each cell was calculated using SigmaScan software (SPSS, UK), as was a measure of total neurite outgrowth: a series of concentric circles was overlaid onto an image of each neuron, and the number of times neurites crossed each circle was calculated. This provided both a measure of total neurite outgrowth (total number of crosspoints) and neurite branching structure (crosspoints related to distance from the cell body). All these measurements were made from cells in the 20 randomly selected fields in each experiment and repeated in at least 4 independent cultures (23). Data are expressed as mean ± standard deviation. Statistical analysis was conducted using Graphpad Prizm software using ANOVA followed by Bonferroni post-hoc test.

RESULTS Glycation, oxidation and nitration adduct residue content of endoneurial ECM proteins in experimental diabetes. To determine whether diabetes altered the extent of adduct residue content on endoneurial ECM proteins, we assessed fourteen markers of glycation, oxidation and nitration by LC-MS/MS. Ten of the fourteen markers investigated were present in detectable amounts in endoneurial ECM proteins from control and STZ-diabetic rats. These were FL, CML, CEL, G-H1, MG-H1, 3DG-H, MOLD, pentosidine, MetSO and dityrosine. The protein damage marker residues below the limit of detection (LOD) were GOLD, DOLD, argpyrimidine and 3-NT. Estimates of pentosidine residues were <LOD in 33% of cases (STZ diabetic study groups) and were set to the LOD in such cases for statistical analysis. For indices of diabetes refer to table 1.

Following induction of diabetes, there were significant increases in the amount of FL, CML, G-H1 and MG-H1 adduct residue contents on endoneurial ECM proteins (Fig 1A-D). There were no significant changes in CEL (0.083 ± 0.025 mmol/mol lys), pentosidine (0.0016 ± 0.0009 mmol/mol lys), MOLD (0.004 ± 0.003 mmol/mol lys), MetSO (31.3 ± 8.7 mmol/mol met) and dityrosine (0.29 ± 0.13 mmol/mol tyr) residue contents and a one-fold increase of 3DG-H residues (1.26 ± 0.69 versus 0.67 ± 0.13 mmol/mol arg; P<0.05) after 6 weeks of diabetes. For MG-H1, there was a relative decline at weeks 9 and 12 of diabetes after reaching a maximum content at 6 weeks of diabetes and rebound to higher contents after 24 weeks. There were no age-related changes in adduct residue contents over the 24 week time course studied (Fig 1 A-D: no significant differences in AGE content between endoneurial ECM protein samples at
time 0 or 24 weeks post-STZ; p>0.05). These results indicate that the increase in FL, CML, G-H1 and MG-H1 residue contents of ECM proteins of the sciatic nerve endoneurium correlates with the development of STZ-induced diabetes.

**Glycation of ECM proteins by methylglyoxal and glucose in vitro.** To test the hypothesis that ECM proteins are susceptible to glycation under physiological conditions, we incubated laminin and fibronectin with MG or glucose. Note that similar conditions employed with human serum albumin (HSA) produced HSA modified minimally with glycation adducts (1 – 2 molar equivalents (25)). Control fibronectin and laminin had minor but significant contents of glycation adducts FL, CML, MG-H1 and 3DG-H, ranging from 0.2 – 2.3 mol/mol protein, and some protein oxidation (1 – 6 equivalents of MetSO residues), but no detectable dityrosine or 3-NT (Tables 2 & 3).

Glycation of fibronectin by MG produced ~28 equivalents of MG-H1. This was the major MG-derived AGE formed (97%), the others being CEL (0.06 mol; 0.2%), argpyrimidine (0.79 mol; 3%) and MOLD (0.002 mol; 0.01%); the total MG-derived adduct increase was ~29.2 molar equivalents, representing 88% of the added MG (Table 2). Similarly, glycation of laminin by MG produced ~56 equivalents of MG-H1 (88%), together with: CEL (0.53 mol; 0.8%), argpyrimidine (6.8 mol; 11%) and MOLD (0.04 mol; 0.07%). The total adduct MG-derived adduct increase was ~ 63.1 molar equivalents, representing 93% of the added MG (Table 3).

Glycation of fibronectin by glucose gave a relatively modest increase in glycation adduct residues: FL (0.52 mol; 61% detected adduct residue increase), 3DG-H (0.32 mol; 36%) and trace amounts of MG-H1 and pentosidine (Table 2). Glycation of laminin by glucose gave much higher increases of glycation adduct residues: FL (21 mol; 81%), 3DG-H (3.7 mol; 14%), CML (1.1 mol; 4%) G-H1 (0.2 mol; 0.8%) and trace amounts of CEL, MOLD and pentosidine. As expected, pentosidine residue content was very low in all proteins analysed; <0.01 mol percent (Table 3).

**Sensory nerve regeneration is impaired on glycated ECM.** Since, experiments utilising function-blocking antibodies have highlighted the essential role that laminin and fibronectin play in promoting axonal outgrowth, we hypothesised that glycation of these proteins may contribute directly to failure of axonal regeneration (26;27).

To test this hypothesis, we examined the ability of dissociated adult rat sensory neurons to form neurites in culture. The addition of neurotrophic factors during the first 18 h promotes neurite outgrowth in defined populations of sensory neurons, which thus enabled us to compare regeneration of different populations of neurons on glycated versus unmodified laminin and fibronectin. Sensory neurons in culture exhibit two distinct forms of neurite outgrowth: an initial, short arborizing form of neurite outgrowth, which occurs in the absence of neurotrophic factors (but is enhanced by addition of nerve growth factor (NGF), neurotrophin-3 (NT-3) or glial cell-line derived neurotrophic factor (GDNF)); followed by a transcription-dependent switch to axon elongation. The arborizing growth is analogous to the collateral sprouting of terminal fields seen in vivo, whilst elongation is analogous to axon regeneration in vivo (28).

Survival of sensory neurons was not altered on MG-glycated laminin in comparison to untreated laminin (assessed using trypan blue 18 h following plating, data not shown), nor was the percentage of neurite-bearing cells (Fig 2D).

Neurons plated on laminin extended highly-arborised neurites in the presence of NGF (Fig 2A). In contrast, neurite outgrowth was dramatically lower on MG-glycated laminin (Fig 2B). Quantification of NGF-stimulated neurite outgrowth showed no
significant decrease in the length of longest neurite (Fig 2E), but significant reduction in total neurite density and branching, as measured by crosspoint analysis (Total neurite density: laminin 160 ± 28 crosspoints vs MG-glycated laminin 90 ± 18 crosspoints, p<0.05. At 100µm from cell body: laminin 32 ± 3 crosspoints vs MG-glycated laminin 17.4 ± 3 crosspoints, p<0.001 Figs 2 F, G). Typically, the neurons which extended neurites in response to NGF on glycated laminin had fewer and less branched neurites than those plated on control unmodified laminin. To confirm this decrease was associated with glycation, MG-glycation was conducted in the presence of the glycation scavenger aminoguanidine (AG), which prevented the deficits in neurite outgrowth (Fig 2C,F,G).

The GDNF-responsive subpopulation of neurons was similarly disadvantaged when plated on glycated laminin (Fig 3). There were significant glycation-associated decreases in the length of longest neurite (Fig 3B,E), total neurite outgrowth (Total neurite density: 64.7 ± 28.6 crosspoints on laminin vs 21.2 ± 18.2 crosspoints on MG-glycated laminin, p<0.05 Figs 3B, F) and a reduction in branching structure (Fig 3B,G). These deficits were also prevented by inclusion of AG.

Diabetes is associated with the initial presence of regenerative axon profiles alongside degenerative structures in peripheral nerve(29). To model this regenerative phenotype we utilised a preconditioning nerve crush injury model which can potentiate the capacity of sensory neurons to mount a regenerative response following a subsequent injury to their axons. Control sensory neurons extend neurites very poorly on fibronectin compared to growth on laminin, however, neurite outgrowth is enhanced by a preconditioning crush to the sciatic nerve in vivo seven days prior to culture (23). We therefore utilised this model to investigate the impact of glycation of laminin and fibronectin on sensory nerve regeneration.

Preconditioned neurons plated on laminin extended some neurites even in the absence of exogenous neurotrophins (Fig 4A), but growth was strongly enhanced by addition of NGF (Fig 4D) or GDNF (Fig 4G). Glycation of laminin significantly reduced preconditioned neurite-outgrowth in all treatment conditions (Fig 4B,E,H). This reduction was prevented by inclusion of AG (Fig 4C,F,I). Quantification of preconditioned neurite outgrowth showed significant glycation-associated decreases in the length of longest neurite and total neurite density (Fig 4J,K).

Preconditioned neurite outgrowth was also observed on fibronectin in the absence of neurotrophic support (Fig 5A), which was strongly enhanced by GDNF, but not NGF (in agreement with our previous study (23)). In neurons plated on glycated FN, neurite outgrowth was significantly impaired, both in the absence of neurotrophins and in the presence of GDNF (Fig 5 B, D,J,K). As before, inclusion of AG prevented the glycation-mediated inhibition. Interestingly, the addition of NGF to preconditioned neurons also prevented glycation mediated inhibition of neurite-outgrowth (Fig 5, J,K).

These results show that ECM glycation has a dramatic inhibitory effect on the ability of sensory neurons to extend neurites in respond to neurotrophic factors as well as to regenerate following a preconditioning injury.

**DISCUSSION**

The accumulation of proteins damaged by formation of AGE residues in the peripheral nerve in diabetes has been linked to changes in nerve structure and neuronal function and development of diabetic neuropathy (32). We have now conducted experiments which suggest that the glycation of two central ECM proteins associated with neuronal regeneration, laminin and fibronectin, may be linked directly to the failure of axonal regeneration in diabetic neuropathy.

The accumulation of proteins damaged by formation of AGE residues in the peripheral nerve in diabetes has been linked to changes in nerve structure and neuronal function and development of diabetic neuropathy (32). We have now conducted experiments which suggest that the glycation of two central ECM proteins associated with neuronal regeneration, laminin and fibronectin, may be linked directly to the failure of axonal regeneration in diabetic neuropathy.
neuropathy. We have previously shown that cytosolic protein extracts of peripheral nerve of STZ diabetic rats have increased FL and AGE residue content (7). Here, we extend those findings by demonstrating that ECM proteins are also glycated in the endoneurium of rat sciatic nerves. Moreover, glycation of ECM proteins with MG increases AGE formation and decreases the ability of adult rat sensory neurons to extend neurites.

**Endoneurial ECM proteins are glycated in vivo.** The epidermis is innervated by the free axon terminals of small diameter unmyelinated peptidergic and nonpeptidergic sensory neurons (33). These subsets of sensory neurons differ in their responsiveness to specific neurotrophins. The peptidergic population expresses trkA and responds to NGF, whilst the non-peptidergic population expresses receptors for GDNF: ret and GFR (34;35). Since it is the peripheral processes of these small-diameter neurons which degenerate in clinical (36) and experimental diabetic neuropathy (37-39) we focused our attention on the response of these populations of small-diameter neurons to ECM glycation.

Experimental diabetes in rats led to a profound increase in early glycation adduct and AGE residues in endoneurial ECM proteins of the sciatic nerve. The most abundant AGE residue was MG-H1, which increased ~5-fold after 6 weeks of diabetes. Similarly, FL residue content increased from 6-fold after 3 weeks of diabetes to 13-fold after 12 weeks. This is a higher increase than that seen for plasma glucose concentration, but nevertheless is in keeping with the high (14-fold) increase of glucose concentration reported in sciatic nerve in STZ diabetic rats (40). The 5-fold increased content of MG-H1 residues is higher than the 2-fold increase in plasma concentration of MG in STZ diabetic rats and may reflect down regulation of glyoxalase 1, which provides the major defense against MG glycation, in diabetes (41;42). The accumulation of MG-H1 residues may be particularly damaging as it targets functional domains of ECM proteins – particularly arginine residues of RGD and GFOGER motifs, which are the major integrin-binding motifs (43;44).

**Glycation of laminin and fibronectin in vitro.** Further evidence for the reactivity of ECM proteins towards glycation was obtained by glycation of fibronectin and laminin in vitro. We used conditions for glycation by MG where similar preparation of HSA produced a low or minimal extent of modification (2.49 equivalents of MG-H1 per mol, (45)). Fibronectin and laminin were modified by MG to produce 28 and 56 MG-H1 residues per mol protein, respectively. The arginine residue equivalents in these incubations of HSA, fibronectin and laminin were: 2.40 mM, 1.86 mM and 2.50 mM and the concentration of MG-H1 residues formed in MG modified HSA, fibronectin and laminin was 2.5 mM, 4.2 mM and 4.1 mM. Assuming pseudo-first order reaction kinetics for the reaction of MG with arginine residues in these proteins, fitting of these data to a first order integrated rate equation indicates under the conditions studied the mean reactivity of arginine residues in fibronectin and laminin is ca. 3 – 4 fold higher than in HSA. As the RGD residues are preferentially modified, it is likely that the reactivities of arginine residues in RGD motifs of laminin and fibronectin are much higher than this. These deductions provide support for proposing ECM proteins as targets for hotspot dicarbonyl glycation in diabetes.

Similar considerations for the formation of FL residues in the glycation of HSA (22) fibronectin and laminin by glucose show that the final increased concentration of FL residues in the incubation were 114 µM, 8 µM and 152 µM. As neither lysine residues nor glucose were markedly depleted in these reactions, an appropriate kinetic comparison may be made by considering these data as a measure of initial rates of glycation. The lysine residue concentration in the incubations of 6.6
mg/ml HSA, fibronectin and laminin is ca. 5.9 mM, 1.2 mM and 2.3 mM. The reactivity of lysine residues towards formation of FL residues in HSA, fibronectin and laminin is in the ratio 1: 0.35: 3.5 (not accounting for FL residue degradation). Glycation of ECM proteins by MG in vivo is likely to originate from MG formed from cellular metabolism. High dose thiamine therapy decreased plasma MG concentration, and glycation of aortal collagen by MG, by correction of dysfunctional cell metabolism downstream of glucose, without change in FL residue content (46).

Together, our data show that laminin is particularly susceptible to glycation by glucose.

**ECM glycation dramatically impairs neuronal outgrowth and regeneration.** Glycation of laminin with glyceraldehyde (47) or glucose (48) has previously been shown to reduce attachment and neuritogenesis in neonatal sensory neurons (47) and neurite extension from mouse DRG explants (48). In this study, we use 2 models of regeneration to demonstrate that MG-glycation of laminin and fibronectin causes a reduction in neurite outgrowth from specific populations of sensory neurons following stimulation with neurotrophins and also a reduction in preconditioned neurite-outgrowth. Glycation of fibronectin with MG impairs adhesion of smooth muscle cells (49), retinal pericytes (50), and endothelial progenitor cells (51).

Similarly, MG glycation of the RGD domain of collagen IV, inhibits αvβ3 integrin binding and endothelial cell adhesion (43;44). We previously implicated α5β1 integrin binding to the RGD domain of fibronectin as being of key importance in the neurite outgrowth of preconditioned neurons (23) and therefore suggest that glycation of the RGD domain is also responsible for the impaired neurite outgrowth on glycated fibronectin. ECM-integrin binding not only provides a physical anchor point between the intracellular actin cytoskeleton and the outside environment, enabling traction to mechanically drive axonal outgrowth, but also directly regulates important intracellular signalling cascades and subsequent gene transcription at the site of the focal adhesion (52). Indeed, using Affymetrix gene microarray analysis we have identified over 500 genes whose expression is significantly altered in NGF-treated neurons plated on MG-glycated laminin compared to unmodified laminin (data not shown). Replacement therapy with exogenous NGF in diabetic rats normalizes key molecular and functional aspects of neuropathy in vivo (53;54). Whilst, the mechanism of the observed NGF-induced rescue of axonal regeneration on glycated fibronectin, remains to be elucidated our initial experiments suggest it is not mediated via RAGE (Receptor for AGEs), since function-blocking experiments using anti-RAGE did not affect neurite outgrowth on glycated fibronectin.

Failure of axonal regeneration is a key feature of both clinical and experimental diabetic neuropathy (55). In uninjured peripheral nerve samples from patients with diabetic neuropathy clusters of regenerating axons can initially be observed alongside degenerating axons, however, as the disease progresses there is a decline in the number of regenerating axons (29). The regenerative response of the peripheral nerve following injury is also blunted in diabetes - little axonal regeneration is observed following sural nerve biopsy (56) or epidermal nerve fibre injury (57). We suggest that AGE accumulation in the endoneurial ECM may contribute to this progressive failure of axonal regeneration in diabetic neuropathy.

In conclusion, we have shown that ECM proteins are glycated in the endoneurium of rat sciatic nerves in STZ-induced diabetes. Furthermore, glycation of laminin and fibronectin with MG caused a reduction in both neurotrophin-stimulated and preconditioned neurite-outgrowth from sensory
neurons. This may provide a potential mechanism for the failure of collateral sprouting and axonal regeneration observed in diabetic neuropathy and may represent an important, if challenging, target for therapeutic intervention.

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Figure Legends

Fig 1. Glycation adduct content of endoneurial ECM proteins of STZ-diabetic rat sciatic nerve. A, \( \text{N}_\varepsilon \text{-fructosyl-lysine FL} \); B, \( \text{N}_\varepsilon \text{-carboxymethyl-lysine CML} \); C. Glyoxal-derived hydroimidazolone G-H1, and D. MG-derived hydroimidazolone MG-H1. Data are given for non-diabetic control rats at 0 and 24 weeks of the experiment and for STZ diabetic rats with duration of diabetes (weeks post-STZ injection) of 3-24 weeks. Data are mean ± SEM (n=6 – 8 for STZ diabetic groups; n = 4 for non-diabetic controls). Significance: * p<0.05; ** p<0.01 compared to non-diabetic controls (Kruskal Wallis test).

Fig 2. Sensory neurons plated on glycated laminin and treated with NGF extend fewer, less branched neurites than those plated on control unmodified laminin. Representative photomicrographs of sensory neurons plated on laminin (A), laminin glycated with methylglyoxal (B) or laminin treated with methylglyoxal in the presence of aminoguanidine (C). NGF treatment (10ng/ml; 18 h) stimulated neurons plated on laminin to extend elaborate highly-branched neurites (A). In contrast, NGF-stimulated neurite outgrowth on MG-glycated laminin was much less extensive (B), this was prevented by inclusion of aminoguanidine (AG; C). Quantification of NGF-stimulated neurite outgrowth showed no significant reduction in length of longest neurite (E) but a significant reduction in total neurite density and branching structure, compared to control, as measured by crosspoint analysis (F, G), which was prevented by inclusion of the glycation scavenger aminoguanidine (C, F, G). Data are expressed as mean ± S.D. n=4 independent cultures; * p<0.05 ANOVA and Bonferroni’s Multiple Comparison post-hoc test). Aminoguanidine treatment alone had no significant effect on any of the indices examined (D-G; p>0.05). Scale bar = 100 µm.

Fig 3. GDNF-induced neurite outgrowth is reduced from sensory neurons plated on glycated laminin. Representative photomicrographs of sensory neurons plated on laminin (A), laminin glycated with methylglyoxal (B) or laminin treated with methylglyoxal in the presence of aminoguanidine (C). GDNF treatment (50ng/ml; 18 h) stimulated neurons plated on laminin to extend elaborate highly-branched neurites (A). In contrast, GDNF-stimulated neurite outgrowth on MG-glycated laminin was much less extensive (B), this was prevented by inclusion of aminoguanidine (AG; C). Quantification of GDNF-stimulated neurite outgrowth showed a significant reduction in length of longest neurite (D), total neurite density and branching structure compared to control, as measured by crosspoint analysis (F, G), which was prevented by inclusion of the glycation scavenger aminoguanidine (C, F, G). Data are expressed as mean ± S.D. n=4 independent cultures; * p<0.05 ANOVA and Bonferroni’s Multiple Comparison post-hoc test). Aminoguanidine treatment alone had no significant effect on any of the indices examined (D-G; p>0.05). Scale bar = 100 µm.
Fig 4. Enhanced neurite-outgrowth in response to a preconditioning sciatic nerve crush is reduced in neurons plated on glycated laminin. Representative photomicrographs of neurons from L4 and L5 DRG ipsilateral to a crush injury to the sciatic nerve (7 days prior to culture), which were dissociated and plated on laminin (A,D,G), laminin glycated with methylglyoxal (B,E,H) or laminin treated with methylglyoxal in the presence of aminoguanidine (C,F,I) for 18hrs with (D-I) or without (A-C) neurotrophic support (A-C), this was enhanced by neurotrophin treatment (D-I). Two parameters of neurite extension were quantified: total neurite outgrowth (J, crosspoints) and length of longest neurite (K). Neurons plated on glycated laminin showed a significant reduction in length of longest neurite and total neurite density compared to control, which was prevented by inclusion of the glycation scavenger aminoguanidine (C,F,I-K). All data are expressed as mean ± s.d, n=4 independent experiments, *p<0.05, **p<0.01; ANOVA with Bonferroni’s posthoc test). Scale bar = 100 µm.

Fig 5. Enhanced neurite-outgrowth in response to a preconditioning sciatic nerve crush is reduced in neurons plated on glycated fibronectin. Representative photomicrographs of neurons from L4 and L5 DRG ipsilateral to a crush injury to the sciatic nerve (7 days prior to culture), which were dissociated and plated on fibronectin (A,D,G), fibronectin glycated with methylglyoxal (B,E,H) or fibronectin treated with methylglyoxal in the presence of aminoguanidine (C,F,I) for 18hrs with (D-I) or without (A-C) neurotrophic support (A-C), this was enhanced by neurotrophin treatment (D-I). Two parameters of neurite extension were quantified: total neurite outgrowth (J, crosspoints) and length of longest neurite (K). Neurons plated on glycated fibronectin showed a significant reduction in length of longest neurite and total neurite density compared to control, which was prevented by inclusion of the glycation scavenger aminoguanidine (C,F,I-K) and also by NGF (H,J,K). All data are expressed as mean ± s.d, n=4 independent experiments, *p<0.05, ANOVA with Bonferroni’s posthoc test). Scale bar = 100 µm.
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AGEs and ECM proteins in diabetic neuropathy


Table 1. Indices of STZ-diabetes over 24 week timecourse.

<table>
<thead>
<tr>
<th>Experimental Group (n)</th>
<th>Body weight (g)</th>
<th>Terminal Blood Glucose levels (mmol/l)</th>
<th>Motor Nerve Conduction Velocity (m/s)</th>
<th>Sensory Nerve Conduction Velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 24 wk</td>
<td>239 ± 5</td>
<td>361 ± 53</td>
<td>n.t.</td>
<td>58.7 ± 9.2</td>
</tr>
<tr>
<td>STZ 3 wk</td>
<td>233 ± 7</td>
<td>215 ± 21</td>
<td>26 ± 3.7</td>
<td>n.t.</td>
</tr>
<tr>
<td>STZ 6 wk</td>
<td>242 ± 10</td>
<td>257 ± 15</td>
<td>26.9 ± 1.4</td>
<td>n.t.</td>
</tr>
<tr>
<td>STZ 9 wk</td>
<td>242 ± 17</td>
<td>271 ± 27</td>
<td>25.4 ± 2.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>STZ 12 wk</td>
<td>233 ± 13</td>
<td>264 ± 41</td>
<td>21.5 ± 7.6</td>
<td>n.t.</td>
</tr>
<tr>
<td>STZ 24 wk</td>
<td>233 ± 16</td>
<td>319 ± 55</td>
<td>23.3 ± 7.9</td>
<td>43.2 ± 5.4***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation. *** p<0.005: t-test, control vs Diabetic NCV at 24 week. n.t. = not tested.

Table 2. Glycation adduct residue contents of human fibronectin glycated by methylglyoxal (MG) and glucose (AGE).

<table>
<thead>
<tr>
<th>Glycation adduct</th>
<th>MG-fibronectin control-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MG-fibronectin AGE-fibronectin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AGE-fibronectin control-2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AGE-fibronectin AGE-fibronectin control-1&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>0.213 ± 0.034</td>
<td>0.215 ± 0.088</td>
<td>0.094 ± 0.022</td>
<td>0.088***</td>
</tr>
<tr>
<td>CML</td>
<td>0.119 ± 0.025</td>
<td>0.202 ± 0.041*</td>
<td>0.079 ± 0.021</td>
<td>0.092 ± 0.020</td>
</tr>
<tr>
<td>CEL</td>
<td>0.008 ± 0.001</td>
<td>0.067 ± 0.016*</td>
<td>0.007 ± 0.001</td>
<td>0.014 ± 0.005</td>
</tr>
<tr>
<td>G-H1</td>
<td>0.016 ± 0.004</td>
<td>0.046 ± 0.007**</td>
<td>0.005 ± 0.002</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>MG-H1</td>
<td>0.239 ± 0.013</td>
<td>28.42 ± 0.97***</td>
<td>0.176 ± 0.006</td>
<td>0.198 ± 0.006*</td>
</tr>
<tr>
<td>3DG-H</td>
<td>0.217 ± 0.068</td>
<td>0.819 ± 0.215</td>
<td>0.420 ± 0.066</td>
<td>0.727 ± 0.044**</td>
</tr>
<tr>
<td>Argpyrimidine</td>
<td>&lt;LOD</td>
<td>0.793 ± 0.060***</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>MOLD</td>
<td>0.0021 ± 0.0001</td>
<td>0.0039 ± 0.001</td>
<td>0.0015 ± 0.001</td>
<td>0.0030 ± 0.001</td>
</tr>
<tr>
<td>Pentosidine</td>
<td>0.000015 ± 0.00001</td>
<td>0.000129 ± 0.00001</td>
<td>0.000007 ± 0.00001</td>
<td>0.000012 ± 0.00001</td>
</tr>
<tr>
<td>MetSO</td>
<td>1.05 ± 0.09</td>
<td>3.15 ± 0.16***</td>
<td>0.40 ± 0.04</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

The data expressed as mol/mol fibronectin; mean ± S.D. (n = 3). Significance: * p<0.05; ** p<0.01; ***p<0.001; t-test). Key: a. Fibronectin incubated under the conditions to control for glycation by MG; b. Fibronectin incubated under the conditions to control for glycation by glucose. <LOD indicates argpyrimidine residue content is less than the limit of detection (<0.001 mol/mol fibronectin). Other adducts not detected were (mol/mol): GOLD and dityrosine <0.0006, DOLD <0.0002 and 3-NT <0.0001.
TABLE 3 Glycation adduct contents of laminin minimally modified by methylglyoxal (MG) and glucose (AGE).

<table>
<thead>
<tr>
<th>Glycation adduct</th>
<th>MG- laminin control-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MG- laminin</th>
<th>AGE- laminin control-2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AGE- laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>0.777 ± 0.100</td>
<td>0.698 ± 0.047</td>
<td>1.11 ± 0.35</td>
<td>21.84 ± 0.58***</td>
</tr>
<tr>
<td>CML</td>
<td>0.303 ± 0.052</td>
<td>0.332 ± 0.070</td>
<td>0.353 ± 0.027</td>
<td>1.420 ± 0.094***</td>
</tr>
<tr>
<td>CEL</td>
<td>0.035 ± 0.009</td>
<td>0.562 ± 0.047***</td>
<td>0.029 ± 0.004</td>
<td>0.055 ± 0.010*</td>
</tr>
<tr>
<td>G-H1</td>
<td>0.022 ± 0.009</td>
<td>0.021± 0.011</td>
<td>0.031 ± 0.001</td>
<td>0.229 ± 0.060**</td>
</tr>
<tr>
<td>MG-H1</td>
<td>0.87 ± 0.04</td>
<td>56.57 ± 1.78***</td>
<td>1.44 ± 0.10</td>
<td>1.66 ± 0.16</td>
</tr>
<tr>
<td>3DG-H</td>
<td>2.31 ± 0.29</td>
<td>1.34 ± 0.36*</td>
<td>2.26 ± 0.16</td>
<td>5.97 ± 0.57**</td>
</tr>
<tr>
<td>Argpyrimidine</td>
<td>&lt;LOD</td>
<td>6.82 ± 0.56</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>MOLD</td>
<td>0.0030 ± 0.0011</td>
<td>0.0448 ± 0.0081&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0018 ±0.0006</td>
<td>0.0074 ± 0.0012**</td>
</tr>
<tr>
<td>Pentosidine</td>
<td>0.0000022</td>
<td>0.000001*</td>
<td>0.000002</td>
<td>0.0000004***</td>
</tr>
<tr>
<td>MetSO</td>
<td>6.01 ± 0.23</td>
<td>5.91 ± 1.56</td>
<td>4.81 ± 0.88</td>
<td>3.89 ±1.32</td>
</tr>
</tbody>
</table>

The data are expressed as mol/mol laminin; mean ± S.D (n = 3). Significance: * p<0.05; ** p<0.01; ***p<0.001; t-test). Key: <sup>a</sup>Laminin incubated under the conditions to control for glycation by MG; <sup>b</sup>Laminin incubated under the conditions to control for glycation by glucose. <LOD indicates argpyrimidine residue content is less than the limit of detection (<0.002 mol/mol laminin). Other adducts not detected were (mol/mol): GOLD and dityrosine <0.0012, DOLD <0.0004 and 3-NT <0.0002.

![Figure 1](image-url)
Figure 2

A. LM  B. LM + MG  C. LM + MG + AG

D. % neurite-bearing cells

E. Length of longest neurite (μm)

F. Total crosspoints

G. Crosspoints vs. Distance from cell body (μm)
Figure 3

AGEs and ECM proteins in diabetic neuropathy
Figure 4

AGEs and ECM proteins in diabetic neuropathy

No NTs

GDNF

NGF

J. Total crosspoints

K. Length of longest neurite (µm)

LM

LM + MG

LM + MG + AG

A. B. C.

D. E. F.

G. H. I.

Bar scale: 100 µm