Nonenzymatic Glycosylation of Laminin and the Laminin Peptide CIKVAVS Inhibits Neurite Outgrowth

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Nerve regeneration in diabetic animals is delayed and qualitatively impaired, but the mechanisms responsible for these defects have not been elucidated. The extracellular matrix protein laminin promotes the extension of neuronal processes, and recent studies have localized neurite-promoting activity to a lysine-containing sequence (IKVAV) within the laminin molecule. Because long-lived molecules such as laminin are likely to accumulate excessive amounts of nonenzymatic glycosylation products in diabetic subjects, we have investigated whether such adduct formation on laminin or the IKVAV peptide affects their neurite-promoting properties. These studies used the murine neuroblastoma cell line NB2a, which extends neurites on laminin when differentiated by cAMP. Neurite outgrowth in NB2a cells plated on glycosylated laminin was significantly decreased from that occurring on unmodified laminin. Similarly, neurite outgrowth in NB2a cells plated on glycosylated IKVAV peptide was inhibited compared with that observed on native IKVAV. These data suggest that nonenzymatic glycosylation of a biologically active domain within laminin may contribute to impaired nerve regeneration in diabetes. *Diabetes* 42:509–13, 1993

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CIKVAVS, IKVAV peptide; cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HS, horse serum; PBS, phosphate-buffered saline; NGF, nerve growth factor.

Peripheral nerve regeneration is impaired in experimental diabetic animals (1,2), but the mechanisms have not yet been elucidated. In normal animals, the basal lamina of the peripheral nerve sheath is thought to provide a permissive substrate that promotes the sprouting of regenerating nerve fibers (3,4). Perturbations of this basal lamina or alterations of its molecular constituents such as laminin could thus impair its growth-promoting properties. Laminin is a major component of the basal lamina, and in culture systems has been shown to promote specific cellular responses, such as adherence (5,6), migration (6,7), and neurite outgrowth (5,6,8,9). Recent studies have defined domains within the laminin molecule that are specifically associated with particular cellular responses. Laminin is a 900,000-M_r glycoprotein composed of three nonidentical polypeptide chains (A,B1,B2), which adopt a cruciform configuration. One laminin domain from the A-chain containing the sequence IKVAV has been shown to promote neurite outgrowth (6).

Because nonenzymatic glycosylation of laminin causes decreased self-assembly, decreased binding to type IV collagen, and decreased binding of heparan sulfate proteoglycan (10), we hypothesized that excessive nonenzymatic glycosylation of endoneurial sheath laminin associated with uncontrolled diabetes also might contribute to impaired neurite outgrowth from damaged peripheral nerve tissue.

This study examines the effect of nonenzymatic glycosylation on the ability of laminin molecules and an IKVAV-containing peptide to promote neurite outgrowth. We demonstrate that glycosylation product formation impairs the ability of both laminin and the IKVAV peptide to promote neuritogenesis from cultured neuroblastoma cells.

RESEARCH DESIGN AND METHODS

Laminin and synthetic peptide. Mouse laminin was purchased from Collaborative Research (Waltham, MA). The synthetic peptide CIKVAVS (IKVAV peptide) was synthetized in the amide form, and its composition was verified by amino acid analysis.

Cell culture, neurite extension, and cell adhesion assays. Murine neuroblastoma cell line NB2a (25) was obtained from Dr. Thomas Shea and cultured in low glucose DMEM (5.5 mM) supplemented with 10% FCS and 5% heat inactivated HS in a humidified atmosphere containing 5% CO_2 at 37°C. Laminin dissolved in PBS (10 mM Na phosphate, 145 mM NaCl, pH 7.4) or IKVAV peptide in 20% (v/v) acetic acid was applied at different concentrations to sterile borosilicate coverslips and allowed to adhere for 4 h, after which time excess solution was removed. Dissociated NB2a cells suspended in growth media containing 1 mM dibutryl cAMP (Sigma, St. Louis, MO) were applied (200 μl of cell suspension) to the laminin-coated coverslips (in wells of a 24-well tissue culture dish) and then placed in the incubator. After 1–2 h, nonadherent cells were removed, and attached cells were fed with fresh media containing 1 mM cAMP. After 4–5 h in the incubator, the percentage of cells bearing neurites longer than one cell diameter was calculated. A minimum of 120 cells was counted in each assay.

For all glycosylation experiments and their controls, saturating concentrations of laminin (16 μg/ml; 11 nM) and IKVAV peptide (500 μg/ml; 575 mM) were used, because neurite extension was maximal at these concentrations of each of the substrates. Nonenzymatic glycosylation studies were performed with glycolaldehyde because the kinetics of adduct formation are more rapid than with glucose. Glycolaldehyde reacts with free amino groups, predominantly the epsilon amino groups of lysine residues (11,12). It undergoes the Amadori rearrangement to form fluorescent products in a manner identical to glucose (12). Glass coverslips were coated with either laminin or IKVAV peptide as above, after which they were treated with either 50 mM glycolaldehyde in PBS or PBS as a control. The coverslips were reacted overnight in a humidified incubator. Coverslips were then treated with 1 M glycine ethyl ester in PBS (pH 7.4) for 1 h to quench the glycosylation reaction. After removal of this solution and washing twice with PBS, NB2a cells were plated for a neurite assay as described above.

NB2a cell adhesion was measured by plating cells in culture media without cAMP on laminin-coated coverslips that had been glycosylated as described above or left untreated as a control. After 5 h, coverslips were gently washed with fresh media, and the number of adherent cells was determined then and again at 24 h by counting ≥20 low-power fields.

Quantitation of binding of laminin and peptide. To assure that similar amounts of native and glycosylated laminin were adhering to glass coverslips, [^{125}I]laminin was enzymatically prepared according to published methods (13). Coverslips ($n = 20$) were coated with labeled laminin (16 μg/ml of specific activity = 2.4×10^5 dpm/μg). Half of these coverslips were treated with glycoaldehyde (as they were for glycosylation assays) the other half, treated with PBS, were for controls. All coverslips were treated with 1 M glycine ethyl ester as described above. After rinsing with PBS, the amount of laminin bound to each coverslip was measured by γ-scintillation counting. In these studies, 12-mm coverslips coated with 16 μg/ml laminin (18 nM) had a mean ± SD of 0.133 ± 0.03 μg (0.148 pmol) bound. Peptide binding was measured by incubation of coverslips with 500 μg/ml (575 μM) peptide as described above. Determination of bound peptide was made after acid hydrolysis by the ninhydrin reaction using a leucine standard (14). In these studies, 12-mm coverslips coated with 500 μg/ml (575 μM) peptide had a mean ± SD of 7 ± 2.8 μg/coverslip (9.2 nmol) bound.

Statistical analysis. Statistical significance was assessed with the Z test for the difference between two proportions for analysis of neurite outgrowth data. A Student's t test was used for analysis of cell adhesion. $P < 0.05$ was considered statistically significant.

RESULTS

Neurite outgrowth on laminin and synthetic peptide. Previously, laminin had been demonstrated to promote the growth of neuronal processes in neural cell lines (6) and primary neurons (15,16). The neurite-promoting activity of laminin was verified in NB2a cells. The extent of neurite elaboration in NB2a cells was found to be proportional to laminin concentration, saturating at a concentration of 5.5 nM (Fig. 1*A*). At saturating laminin concentrations, ~40% of NB2a cells were neurite positive. The synthetic IKVAV peptide also promoted neurite outgrowth in a concentration-dependent manner (Fig. 1*B*), although a higher concentration (575 μM) was required to achieve maximum neurite outgrowth (57%) (Fig. 1*B*).

Neurite outgrowth on glycosylated substrates. We tested whether nonenzymatic glycosylation would affect the ability of laminin and synthetic peptide to promote neurite outgrowth. In these studies, the substrate was modified after application to a glass coverslip. Control coverslips were processed identically except that the glycosylating agent, glycolaldehyde, was omitted. Control experiments with [^{125}I]laminin demonstrated that treatment with glycolaldehyde did not remove preadsorbed laminin from the coverslips (data not shown). Neurite outgrowth on glycosylated laminin (Table 1) was measured and found to be significantly ($P < 0.0001$) impaired: 30.6% of NB2a cells plated on unmodified laminin had neurites, whereas only 8.9% of cells plated on glycosylated laminin extended neurites. Similar results were observed with glycosylated peptide: 42.8% of NB2a cells plated on unmodified peptide were neurite containing compared with 22.7% of NB2a cells plated on glycosylated peptide ($P < 0.0001$). These data indicated that nonenzymatic glycosylation of laminin and the IKVAV peptide inhibited neurite outgrowth.

Cell adhesion on glycosylated laminin. Because differences in neuritogenesis could arise by altering NB2a cell adhesiveness, we evaluated this possibility. An equal number of cells were applied to coverslips coated with

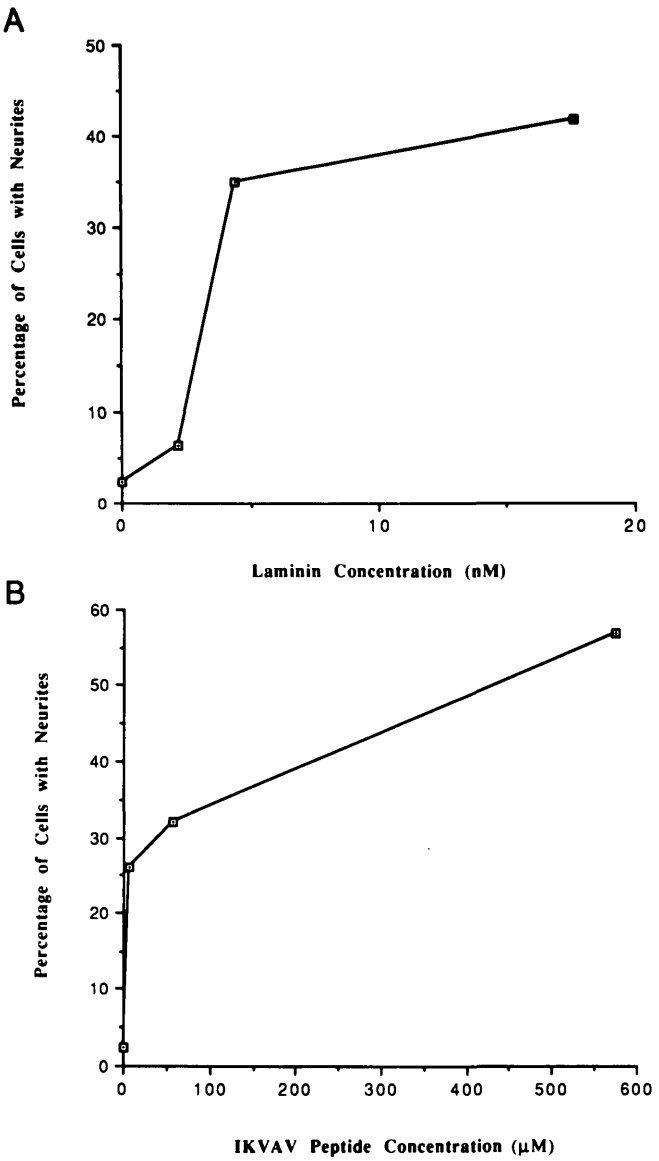

FIG. 1. Neurite outgrowth on laminin and IKVAV peptide. NB2a cells were plated on coverslips coated with laminin (*A*) or IKVAV peptide (*B*) at the concentrations shown and then stimulated with cAMP. The number of neurite-bearing cells on duplicate coverslips was determined 4–5 h later. Data from a single experiment are expressed as the percentage of cells with neurites. The number of neurite-bearing cells over the number counted were as follows: No laminin, 12 of 575 cells; laminin 2.2 nM, 13 of 199 cells; laminin 4.4 nM, 87 of 245 cells; laminin 17 nM, 79 of 187 cells. No IKVAV, 6 of 225 cells; IKVAV 5.7 μM, 58 of 226 cells; IKVAV 57 μM, 54 of 157 cells; IKVAV 575 μM, 140 of 245 cells.

either unmodified or glycosylated laminin. After 5 h, coverslips were washed and the number of adherent cells determined. The mean ± SD number of plated cells per 20 low-power fields that were adherent (Table 1)—508 ± 116 on unmodified laminin versus 521 ± 128 on glycosylated laminin—were not statistically different. These results indicated that cell adhesiveness was not affected by glycosylation of laminin.

TABLE 1
Properties of NB2a cells on unmodified and glycosylated substrates

	Unmodified substrate	Glycosylated substrate	Independent experiments (*n*)
Property			
Neurite extension on laminin (%)*	30.6	8.9†	4
Neurite extension on IKVAV (%)*	42.8	22.7†	2
Cell adhesion‡	508 ± 116	521 ± 128	2

*Neurite extension was quantified by counting the number of cells bearing a neurite greater than a cell body diameter. In 4 experiments with laminin consisting of 2 coverslips each, the number of neurite-bearing cells over the number counted was as follows: experiment 1 unmodified laminin 101/344 (29%), modified laminin 5/150 (3%); experiment 2 unmodified laminin 170/656 (26%), modified laminin 26/633 (4%); experiment 3 unmodified laminin 105/284 (37%), modified laminin 53/302 (18%); experiment 4 unmodified laminin 103/280 (37%), modified laminin 30/197 (15%). $Z = 14.18$, $P < 0.0001$. In two experiments with IKVAV peptide consisting of two coverslips each, the number of neurite-bearing cells over the number counted was as follows: experiment 1 unmodified peptide 117/254 (43%), modified peptide 55/252 (22%); experiment 2 unmodified peptide 118/294 (40%), modified peptide 35/144 (24%). $Z = 6.46$, $P < 0.0001$. The percentages shown in the table were calculated from cumulative data: unmodified laminin 479/1564 (30.6%), modified laminin 114/1282 (8.9%), unmodified peptide 235/548 (42.8%), and modified peptide 90/396 (22.7%).
†$P < 0.0001$ by Z test for difference between two proportions (see METHODS).
‡Cell adhesion was measured in 2 experiments consisting of 2 coverslips each by counting 20 low-power fields. The numbers of cells adherent to unmodified laminin and glycosylated laminin are means ± SD.

DISCUSSION

Diabetic neuropathy affects many patients with long-standing disease. The pathogenetic mechanisms that contribute to the alteration in peripheral neuron function and, in some cases, to peripheral neuron death, are incompletely understood. Because the severity of peripheral neuropathy generally parallels the duration of the disease, it is likely that chronic hyperglycemia and its metabolic sequelae produce neuron injury. One postulate is that early in the course of diabetes, hyperglycemic injury elicits a repair or regenerative response, similar to that observed in nondiabetic animals, although with progressive diabetes the capacity of peripheral nerve to repair itself becomes limited (1,2), producing clinically apparent neuropathy. Normal nerve regeneration involves a dynamic interplay between neurons and matrix-producing nonneuronal fibroblasts and Schwann cells. Schwann cells ensheath peripheral nerve fibers, and their synthesis of laminin contributes to the basal lamina of the endoneurium. One mechanism by which dysfunctional nerve regeneration may occur in diabetes is by depletion of neuronal levels of neurotrophic factors. In experimental diabetes, the transport of one neurotrophic factor, NGF, is diminished (17), leading to a depletion in the NGF content in peripheral ganglia (18).

A second mechanism is that hypergylcemia could affect the matrix elaborated by either Schwann cells or fibroblasts and consequently impede functional nerve regeneration. Studies on laminin function in vitro indicate

that it promotes the growth of neurites from cultures of primary neurons (15,16) and neural cell lines (6,9,15). Laminin and other components of the basal lamina are long-lived proteins and are therefore subject to accumulation of nonenzymatic glycosylation products in individuals with sustained hyperglycemia. Characterization of the laminin molecule, a trimer composed of nonidentical chains A, B1, and B2, has revealed numerous domains that appear to subserve different functions, including cell attachment, migration, and neurite outgrowth. The laminin A-chain contains 3084 amino acids forming 8 structural domains. Sequence data has permitted the exact identification of the amino acids that specify a polypeptide fragment, termed E8, shown to promote neurite outgrowth (16). Recent work with synthetic peptides covering the carboxyl end of the E8 region has demonstrated that the lysine-containing amino acid sequence IKVAV is a critical element within this region responsible for neurite outgrowth (6).

In this report, we describe studies that examined the effect of nonenzymatic glycosylation of laminin and synthetic IKVAV peptide on their neurite-promoting activity. We used the murine neuroblastoma line, NB2a, to study neurite elaboration. We first demonstrated that NB2a cells plated on unmodified laminin or synthetic IKVAV peptide and then differentiated with cAMP undergo robust neuritogenesis. Quantitative neurite outgrowth studies indicated that the substrate effect was concentration dependent and saturable. The data presented herein clearly show that glycosylation of either laminin or the synthetic IKVAV peptide significantly diminish their capacity to promote neurite outgrowth. This effect of glycosylation on neurite outgrowth was specific because NB2a cell adhesiveness was not affected. Our data directly implicate the epsilon amino group of the lysine residue within the IKVAV sequence as important for neurite outgrowth. This finding is in agreement with studies on succinic anhydride modification of lysine residues in laminin, which also block neuritogenesis (5). On a molar basis, much larger amounts of peptide than laminin were required to achieve neurite outgrowth. This has been noted in numerous studies that have used active fragments and peptides of laminin (6,19). Although it is not certain why this difference in molar amounts is observed, we speculate that some peptide-coverslip interactions alter the configuration or bioactivity of the peptide's active site.

Diabetic peripheral nerve endoneurium, its basal lamina and constitutent laminin may be implicated in contributing to the development of neuropathy. The data presented in this report suggest that hyperglycemic injury to nonneuronal elements, mediated by nonenzymatic glycosylation of laminin, alters the capacity of nondiabetic neural cells to elaborate neurites. In these experiments, incubation conditions were selected to accelerate the formation of nonenzymatic glycosylation products over a short period. The same degree of modification would require a much longer period of time to occur in vivo (20). Although the effect of intact basement membrane glycosylation on laminin/neurite interactions is not known, glycosylation of intact basement membrane does affect cell/matrix interactions significantly in other systems (21). These data complement other observations (1,2) and suggest that both hyperglycemic-induced injury and hyperglycemic-induced defects in repair and regeneration contribute to the pathogenesis of diabetic neuropathy.

We speculate that the development and progression of diabetic neuropathy involve both alterations in the function of peripheral neurons and alterations in their interactions with adajcent nonneuronal cells and extracellular matrix. Hyperglycemia damages neurons by a variety of mechanisms, and alters axonal transport such that neurotrophic factor delivery is reduced. Hyperglycemic injury stimulates ongoing diabetic peripheral nerve repair, but cumulative injury and sustained neurotrophic factor depletion limit this process. Concurrent nonenzymatic glycosylation of extracellular matrix proteins such as laminin occurs, ultimately producing an endoneurial substrate that is inadequate to support effective nerve repair. This model would predict that both treatments aimed at improving peripheral neuron function, such as neurotrophic factor administration, and those targeted at reducing the amount of glycosylation products, e.g., aminoguanidine, would likely slow the development of peripheral neuropathy produced by diabetes mellitus. Recent studies in our laboratory have demonstrated the efficacy of systemic NGF therapy for diabetic sensory neuropathy (22), and two recent reports have described the amelioration of diabetic peripheral neuropathy by aminoguanidine treatment (23,24).

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