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Hyperglycemic Glucose Concentrations Up-Regulate the Expression of Type VI Collagen *in Vitro*

Relevance to Alterations of Peripheral Nerves in Diabetes Mellitus

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Electron microscopy of peripheral nerves obtained from two diabetic patients revealed large deposits of microfibrils and the presence of Luse bodies in the vicinity of perineurial cells. Microfibrils were found to accumulate also in the sciatic nerves of diabetic BB rats; these microfibrillar deposits were shown to contain type VI collagen by immunoelectron microscopy. Connective tissue cells cultured from rat sciatic nerves were exposed to high glucose concentrations. High glucose concentrations up-regulated the mRNA steady-state levels of α_1(VI), α_2(VI), and α_3(VI) chains of type VI collagen and caused accumulation of type VI collagen-containing fibrils in the cultures. Immunostaining and in situ hybridizations demonstrated that perineurial cells, Schwann cells, and fibroblasts expressed type VI collagen at the mRNA and protein levels. The results suggest that the turnover and supramolecular assembly of type VI collagen are perturbed in diabetic nerves and that glucose per se increases the expression of type VI collagen in vitro. (Am J Pathol 1993, 142:1586–1597)

Diabetes mellitus is associated with alterations in connective tissue metabolism; these changes may relate to the development of diabetic functional complications, such as neuropathy, nephropathy, and retinopathy (for a review see Ref. 1). Connective tissue alterations observed in the peripheral nerves of diabetic humans and diabetic experimental animals include thickening of the basement membranes of perineurial cells, endothelial cells, and Schwann cells[2–4] and an increased diameter of endoneurial collagen fibrils.[5]

In diabetes mellitus, accumulation of microfibrils has been observed in a number of tissues, such as kidney,[6–8] pancreas, and arteriolar walls.[6] However, the biochemical composition of the microfibrils has remained obscure.

The microfibrillar component of the extracellular matrix has been shown to be heterogeneous, and proteins shown to participate in the formation of microfibrils include type VI collagen,[9] fibronectin,[10] and fibrillin,[11] as well as several minor proteins of smaller molecular weight.[12–14] Type VI collagen has been shown to be present in the 2–3-nm beaded filaments as well as aggregated forms (100-nm periodic fibrils, also referred to as Luse bodies).[9,15–17] Type VI collagen is a structural glycoprotein which is composed of three polypeptide chains, α_1(VI), α_2(VI), and (α_3VI).[18–20] It has been shown to be abundant in a variety of tissues,[21,22] including the peripheral nerve.[23] Type VI collagen has been suggested to play

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a role in determining the organization and architecture of the extracellular matrix.[16]

In this study, we investigated the ultrastructure of the connective tissue matrix of sciatic nerve samples obtained from human diabetic subjects and from spontaneously diabetic BB rats, with special emphasis on the microfibrillar component. Immunoelectron microscopy was utilized to examine the localization of type VI collagen in the sciatic nerves of BB rats. The effects of elevated glucose concentrations on the expression of type VI collagen *in vitro* were studied in primary cell cultures initiated from normal rat sciatic nerves at both the protein and mRNA levels, utilizing indirect immunofluorescence as well as Northern and *in situ* hybridizations.

Materials and Methods

Human Nerve Samples

Samples from the peripheral nerves of two male diabetic patients, 24 and 31 years of age, were obtained at autopsy. Both patients had insulin-dependent diabetes mellitus (duration of 22 and 15 years, respectively) with clinical manifestations of neuro-, retino-, and nephropathy. Neither one of the patients had other diseases affecting the peripheral nerves. The causes of death in these two cases were myocardial infarct and diabetic ketoacidosis, respectively. For control purposes, nerve samples were obtained also from 16- and 23-year-old apparently healthy male persons who had died in accidents. Samples of sciatic, sural, and tibial nerves were fixed in 5% glutaraldehyde in 0.16 mol/L s-collidin-HCl buffer (pH 7.4).

BB Rat Nerve Samples

Diabetic BB Wistar rats were kindly provided by Dr. Helle Markholst (Hagedorn Research Laboratory, Gentofte, Denmark). Sciatic nerve samples of four diabetic rats were studied by electron microscopy. Age- and sex-matched non-diabetes-prone BB rats served as controls. The age of the rats at the time of death varied from 4.5 to 9 months, and the duration of manifest diabetes from 2.5 to 6 months (Table 1). The rats were kept glucosuric but nonketotic by adjusting the insulin dose (a single daily dose of Insulin Ultralente, Novo, Gentofte, Denmark) according to daily determinations of urine glucose (Glukotest, Boehringer-Mannheim, Indianapolis, IN) and ketone bodies (Ketostix, Miles Laboratories, Elkhart, IN). The animals were killed by cervical dislocation under ether anesthesia, and samples of the sciatic nerves were fixed in 5% glutaraldehyde in 0.16 mol/L s-collidin-HCl buffer (pH 7.4) for conventional electron microscopy or snap-frozen in freon cooled in liquid nitrogen for immunoelectron microscopy.

Electron Microscopy

Nerve samples fixed in 5% glutaraldehyde were postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. After dehydration in a graded series of ethanol and infiltration of Epon 812 in hydroxypropyl methacrylate (Fluka Chemie AG, Buchs, Switzerland), the samples were embedded in Epon and polymerized at 60 C for 36 hours. For light microscopic observation, 1-μm sections were stained with toluidine blue. Thin sections were cut perpendicularly to the axis of the nerve, stained with uranyl acetate, lead citrate and phosphotungstic acid, and examined in a JEOL JEM-100C electron microscope.

Immunoelectron Microscopy

Cryosections, 15 μm thick, were cut perpendicularly to the axis of the nerve onto glass slides. The sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and pretreated with glycine (2 mg/ml in PBS), Triton X-100 (0.5% in PBS), and bovine serum albumin (1% in PBS) (BSA/PBS). Some sections were additionally treated with pepsin (0.4% in 0.9% NaCl, 0.1 N HCl) at 37 C for 15 minutes. Primary antibody incubations (rabbit

Table 1. *Diabetic BB Rats*

Rat	Age (months)	Sex	Duration of diabetes (months)	Insulin Dose* (IU/kg)	Weight* (g)	Weight of the control rat* (g)
1	4.5	F	2.5	16.3	270	260
2	9	F	4	8.6	290	310
3	7.5	M	5	6.7	430	490
4	8	M	6	7.0	400	470

* At the time of death.

antiserum to human type VI collagen and rabbit IgG to murine type IV collagen; see below) were carried out in appropriate dilutions in BSA/PBS overnight at 4 C, followed by thorough washes in PBS. In controls, the primary antibodies were replaced with serum from nonimmunized rabbits. The samples were subsequently incubated with gold-conjugated goat immunoglobulins to rabbit immunoglobulins (Sigma Chemical Company, St. Louis, MO; in a 1:3 dilution in a buffer containing 0.5 mol/L NaCl, 0.02 mol/L Tris-HCl, 0.1% BSA, 0.05% Tween-20, and 5% fetal calf serum) for 3 hours at room temperature. After washes in PBS, the sections were fixed in 5% glutaraldehyde and processed for electron microscopy essentially as described above. Some thin sections were examined without staining with uranyl acetate and lead citrate to enhance the detection of gold particles.

Indirect Immunofluorescence Staining

Cell cultures grown on glass slides were fixed in cold absolute ethanol, preincubated with BSA/PBS, and incubated with rabbit antiserum to human type VI collagen (see below; dilution 1:200 in BSA/PBS) overnight at 4 C. After washes with five changes of PBS, the samples were incubated with tetramethylrhodamine isothiocyanate-conjugated swine immunoglobulins to rabbit immunoglobulins (DAKO, Glostrup, Denmark; 1:100 dilution in BSA/PBS) for 1 hour at room temperature and washed with five changes of PBS. The samples were mounted with Glycergel (DAKO) and examined with a Leitz Aristoplan microscope equipped with an epiilluminator and a filter for tetramethyl-rhodamine isothiocyanate fluorescence. In control reactions, the primary antibody was replaced with BSA/PBS or with serum from nonimmunized rabbits.

Antibodies

Commercial rabbit antibodies to type IV collagen extracted from murine EHS tumor were purchased from the Pasteur Institute (Lyon, France). The antiserum (In7) to pepsin-solubilized human placental type VI collagen was raised in rabbits as described in detail previously.[24,25]

Cell Cultures

Primary nerve connective tissue cell cultures were established from the sciatic nerves of normal, 3–5-week-old male Sprague-Dawley rats, as described in detail elsewhere.[26–29] The identity of the different cell types present in these cultures, *i.e.*, perineurial cells, Schwann cells, and neural fibroblasts, has previously been confirmed by their characteristic morphology, as well as by the differential profiles of expression of matrix genes and S-100 protein.[26–29] The cells were grown in plastic tissue culture flasks (Primaria, Becton Dickinson Labware, Lincoln Park, NJ) for RNA isolations, on acetylated glass slides for *in situ* hybridizations, and on untreated glass slides for immunostainings. The cultures were routinely maintained in Dulbecco's modified Eagle's medium containing 5.5 mmol/L D-glucose and supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. To study the effects of elevated glucose concentrations on the steady-state levels of mRNA transcripts for the α-chains of type VI collagen and on the deposition of immunodetectable type VI collagen, confluent cultures were placed in the same medium as above, except that they contained 5.5, 15, or 25 mmol/L D-glucose. When the deposition of type VI collagen was studied, the culture medium was supplemented with 50 μg/ml ascorbic acid. Parallel sets of cultures were incubated in these glucose concentrations for 3, 7, or 14 days when mRNA steady-state levels were studied and for 7 or 14 days when deposition of type VI collagen was studied. When the effect of glucose incubations on the mRNA steady-state levels was studied, each set of cell cultures consisted of nine 25-cm^2 cultures (three cultures for each glucose concentration). During these incubations the media were changed daily to keep the glucose concentrations as steady as possible.

RNA Isolation and Northern Analysis

Cultured cells were lysed in 4 mol/L guanidine thiocyanate buffer, and total RNA was extracted by a single-step method.[30] Briefly, Tris-saturated phenol, 2 mol/L sodium acetate (pH 4.1), and chloroform: isoamylalcohol (49:1) were added to the guanidine thiocyanate solution, and the samples were vortexed and centrifuged at 10,000 × *g* for 20 minutes. RNA was precipitated from the water phase with two volumes of absolute ethanol. Purity of the RNA preparations was assessed by UV absorbance ratios A260/A280. RNA species were separated on 0.9% agarose gels under denaturing conditions, transferred to nylon membranes (Biodyne, Pall Biosupport, East Hills, NY), and immobilized by heating at 80 C for 2 hours. Prior to transfer, the integrity of

the RNA and the even loading of the gels were verified by visualizing ribosomal RNA subunits with ethidium bromide staining. The filters were prehybridized and hybridized with complementary DNAs (cDNAs) in a solution containing 50% formamide, 50 mmol/L sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), 5× standard saline citrate (SSC) (1× SSC = 0.15 mol/L sodium chloride and 15 mmol/L sodium citrate), 5× Denhardt's solution, and 250 μg/ml calf thymus DNA. The cDNAs used in this study were a 1.6-kb cDNA for mouse α_1(VI) collagen chain, a 1.5-kb cDNA for mouse α_2(VI) collagen chain, a 1.3-kb cDNA for mouse α_3(VI) collagen chain (Rui-Zhu Zhang et al., manuscript in preparation), a 1.3-kb cDNA for rat glyceraldehyde phosphate dehydrogenase (GAPDH),[31] and a 0.7-kb cDNA for human β-actin (pHFβA3′ut).[32] The cDNA probes were labeled radioactively with [α-^{32}P]dCTP by random priming to a specific activity of at least 5 × 10^8 cpm/μg.

Following hybridizations for 18–24 hours at 42 C, the filters were washed three times for 5 minutes each at room temperature in 2× SSC/0.1% SDS and then two times for 15 minutes at 60 C in 0.1× SSC/0.1% SDS. After washings, the filters were kept moist in sealed plastic bags and exposed to X-ray film (Kodak X-Omat, Eastman Kodak, Rochester, NY) at −70 C. For rehybridizations, the Northern filters were washed in water containing 0.1% SDS at 90 C for 10 minutes. Autoradiograms were quantitated by scanning densitometry at 633 nm with a HeNe laser densitometer (LKB, Bromma, Sweden). The data were evaluated for statistical significance by Wilcoxon's signed-rank test.

In Situ *Hybridization*

In situ hybridization was performed as described in detail earlier.[33,34] In brief, cells grown on acetylated slides were rinsed briefly in PBS, fixed in cold absolute ethanol, and postfixed in 4% paraformaldehyde in PBS. The cells were pretreated with glycine (2 mg/ml in PBS) and acetic anhydride (0.25% in 0.1 mol/L triethanolamine, pH 8.0), dehydrated in increasing series of ethanol, and heated at 90 C for 5 minutes. Hybridizations with [α-^{32}P]dCTP-labeled cDNA for α_2(VI) were carried out under conditions described previously[33,34] for 18 hours at 42 C. The samples were subsequently washed at 42 C to a final stringency of 0.2× SSC, followed by dehydration in a graded series of ethanol. The [^{32}P]cDNA-mRNA hybrids were detected by dipping the samples into NTB-3 emulsion (Eastman Kodak) and exposing them for 7–14 days at 4 C. The samples were developed using D-19 developer (Eastman Kodak), stained with hematoxylin, dehydrated in increasing series of ethanol, cleared in xylene, mounted with Permount (Fisher Scientific, Fair Lawn, NJ), and examined with a Leitz Aristoplan microscope.

Results

Ultrastructure of Connective Tissue Component of Diabetic Nerves

Human Tissues

Sciatic nerve samples obtained from a 16-year-old person and a 23-year-old person, were examined by electron microscopy as controls. The findings on these normal sciatic nerves were in good agreement with those described earlier.[35] In particular, large clusters of microfibrils or any Luse bodies were not encountered. The ultrastructure of nerve samples obtained from two diabetic patients (24 and 31 years of age) was examined by electron microscopy. Both patients had suffered from advanced complications of diabetes, including polyneuropathy. In the diabetic nerve samples, cross-banded collagenous structures with a periodicity of ~110 nm (Luse bodies) were occasionally found immediately adjacent to the basement membranes of perineurial cells (Figure 1). Large deposits of microfibrils were found between the perineurial cells, as well as subperineurially in the diabetic human sciatic, sural, and tibial nerves (Figure 1). The microfibrils were rather homogeneous in size, with a

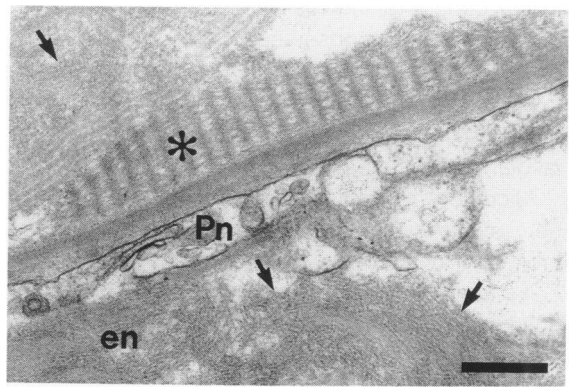

Figure 1. *Electron micrograph of a sural nerve sample obtained from a 24-year-old diabetic man. Luse body (*) with a periodicity of ~110 nm is seen immediately adjacent to the thickened basement membrane of a perineurial cell (Pn). Large deposits of microfibrillar material (arrows) are observed between the perineurial cell layers and subperineurially. Bar = 500 nm. en, endoneurium.*

diameter of ~10 nm. Also, a network of beaded filaments, 2–3 nm in diameter, which often was continuous with the microfibrillar clusters, was prominent in the human diabetic nerve samples (not shown). Perineurial basement membranes were markedly thickened in the human diabetic nerve samples (Figure 1); the thickness of the diabetic basement membrane varied from 135 to 405 nm. In contrast, the thickness of the perineurial basement membrane in the control nerves varied from 65 to 85 nm. The basement membrane material often filled the whole space between two perineurial cell layers in the diabetic nerves, and collagen fibrils were occasionally embedded within the basement membranes.

Rat Tissues

In further studies, the sciatic nerves of four diabetic BB rats and four age- and sex-matched control BB rats were examined by electron microscopy. The animal tissues were examined to complement the findings on humans for two principal reasons. First, in the case of human tissue samples, there is usually a time lapse of several days after the death before the tissue specimens can be obtained at autopsy. Secondly, age- and sex-matched normal controls for the experimental animals are easily obtained.

The diabetic syndrome of spontaneously diabetic BB rats has been reported to closely mimic the insulin-dependent diabetes mellitus of humans.[36] In all diabetic BB rat nerves studied, large deposits of microfibrils with a diameter of ~10 nm were found between the perineurial cell layers and subperineurially (Figure 2A). Morphologically, the microfibrils closely resembled those of human diabetic nerves. In the control rat nerves only small clusters of microfibrils could be observed (Figure 2B). In the sciatic nerve samples obtained from diabetic rats, the microfibrillar deposits occasionally caused marked widening of the spaces between adjacent perineurial cell layers (Figure 2A).

Microfibrillar deposits of the diabetic BB rat nerves were further studied by immunoelectron microscopy for type VI collagen. Antibody to type IV collagen served as a positive control, since type IV collagen is characteristically localized to basement membranes.[37] As expected, the gold particles decorated the basement membranes of perineurial cells and Schwann cells when an antibody against type IV collagen was used (Figure 3). Type VI collagen epitopes localized to the extracellular matrix

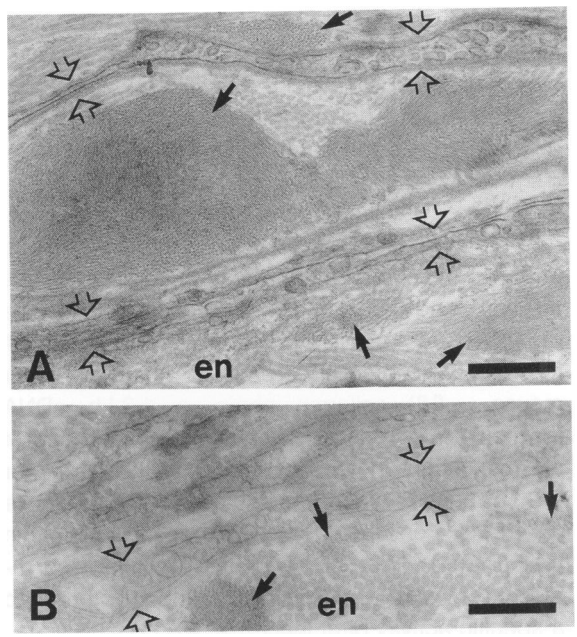

Figure 2. **A**, *electron micrograph of a sciatic nerve sample of a diabetic BB rat. Massive deposits of microfibrils (small arrows) are seen between layers of perineurial cells (open arrows).* **B**, *electron micrograph of a sciatic nerve sample of a non-diabetes-prone BB rat. Only small clusters of microfibrils, predominantly in the subperineurial space can be detected (small arrows). Open arrows point to a perineurial cell layer. Bar = 500 nm. en, endoneurium.*

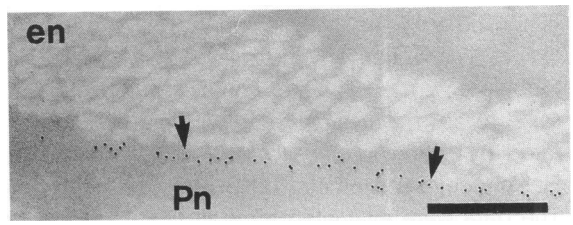

Figure 3. *Immunoelectron microscopy for type IV collagen in a sciatic nerve sample obtained from a diabetic BB rat. Gold particles decorate the basement membrane (arrows) of a perineurial cell (Pn). The section was not stained with uranyl acetate or lead citrate, in order to enhance the detection of gold particles. Bar = 500 nm. en, endoneurium.*

in close proximity to the basement membranes of perineurial cells and Schwann cells (Figure 4A), as well as between bundles of thick collagen fibrils (Figure 4B). However, the 10-nm microfibrils were only rarely labeled with gold particles (Figure 4A). Since type VI collagen antiserum was raised against pepsin-solubilized type VI collagen, some sections were treated with pepsin prior to staining with the antibodies in order to expose antigenic sites which may have been masked in the native samples. After the samples were treated with 0.4% pepsin for 15 minutes, the microfibrillar deposits stained positively, demonstrating the presence of type VI collagen in the deposits (Figure 4C). In the

control sections, in which the primary antibody was replaced with serum from nonimmunized rabbits, only a few scattered gold particles were observed.

Modulation of Type VI Collagen Gene Expression by Glucose in Cultured Connective Tissue Cells of Peripheral Nerve

In order to determine whether elevated concentrations of D-glucose alter the steady-state levels of mRNAs for the α_1(VI), α_2(VI), and α_3(VI) chains of type VI collagen, connective tissue cell cultures were initiated from normal rat sciatic nerves. These cultures consisted of a mixture of perineurial cells, Schwann cells, and neural fibroblasts, as described in detail previously.[26–29]

Type VI collagen gene expression in confluent cell cultures exposed to different concentrations of D-glucose (5.5, 15, and 25 mmol/L) for 3, 7, or 14 days was analyzed by Northern hybridization. Hybridization with a cDNA for α_1(VI) collagen chain sequences revealed a mRNA transcript of ~4.2 kb in size; a cDNA corresponding to α_2(VI) collagen chain hybridized to a ~3.7-kb mRNA transcript; and a cDNA corresponding to α_3(VI) collagen chain hybridized to a 8.0–10.0-kb mRNA transcript (Figure 5A). Hybridization with a cDNA for GAPDH revealed a characteristic 1.4-kb mRNA transcript (Figure 5A), and hybridization with a cDNA for β-actin revealed a transcript of 2.5 kb.

First, total RNA extracted from cells incubated in different glucose concentrations for 3, 7, or 14 days (total, 17 sets of cell cultures) was subjected to hybridization with cDNA for the α_2(VI) chain of type VI collagen. Visual inspection and quantitation of the mRNAs by scanning densitometry of the autoradiographic films indicated that incubation of the cell cultures in high glucose concentrations elevated the mRNA steady-state levels of α_2(VI) collagen chain, whereas the steady-state levels of GAPDH and β-actin were not altered. In cultures incubated in medium containing 15 mmol/L glucose, the mRNA level for α_2(VI) collagen chain was 179 ± 27% (mean ± SEM; n = 16, P = 0.002) of control (*i.e.*, cultures incubated in low-glucose medium), when corrected for the GAPDH or β-actin mRNA levels in the same RNA specimens. In cultures incubated in medium containing 25 mmol/L glucose the corresponding value was 200 ± 24% (n = 17, $P <$ 0.001) of controls (Figure 5B). The effect of glucose on the mRNA steady-state levels was essentially the same in cultures incubated for 7 or 14 days. The means were 180% (n = 5) and 180% (n = 8) of controls in cultures incubated in 15 mmol/L glucose for 7 and 14 days, respectively, and 185% (n = 6) and 211% (n = 8) of controls in cultures incubated in 25 mmol/L glucose for 7 and 14 days, respec-

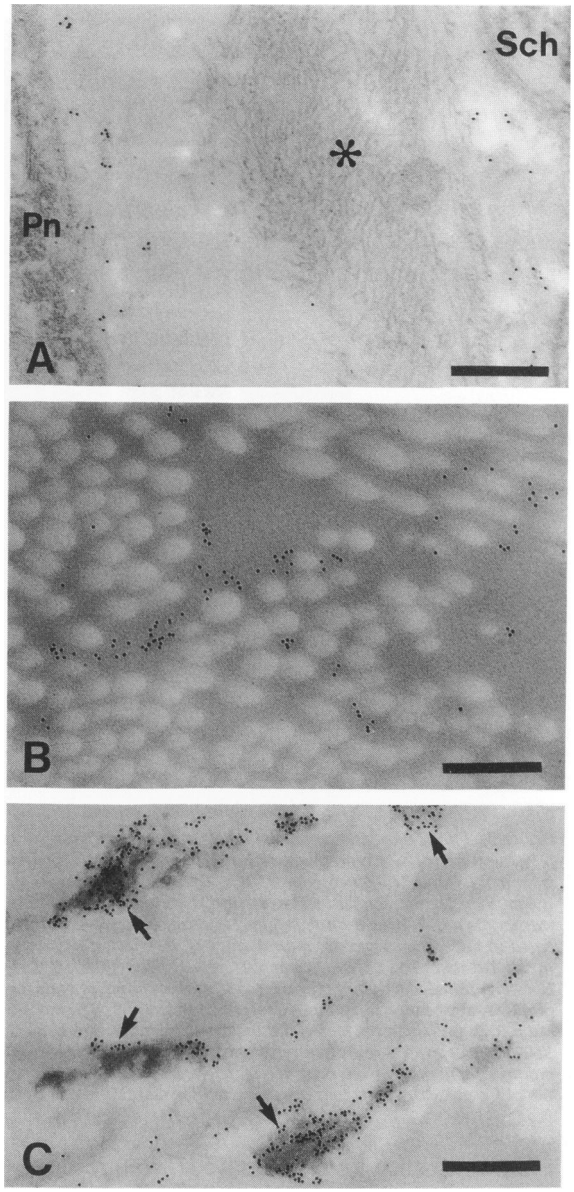

Figure 4. *Immunoelectron microscopy for type VI collagen in sciatic nerve samples obtained from a diabetic BB rat.* **A**, *gold particles in the proximity, but not within, the basement membranes of a perineurial cell (Pn) and a Schwann cell (Sch). A deposit of microfibrils (*) in the subperineurial space is almost completely devoid of gold particles.* **B**, *detection of gold particles in spaces separating bundles of thick collagen fibrils in the epineurium of a diabetic BB rat.* **C**, *immunoelectron microscopy of the sciatic nerve sample of a diabetic BB rat after treatment with 0.4% pepsin for 15 minutes prior to immunostainings. Microfibrillar deposits (arrows) between the perineurial cell layers are prominently labeled with gold particles. Bars = 500 nm.*

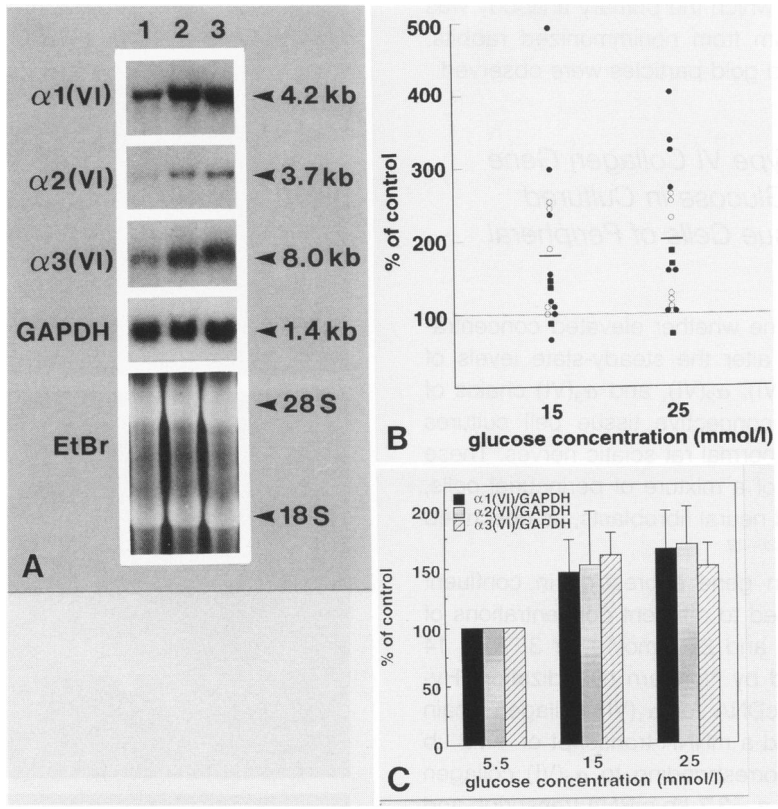

Figure 5. *Effects of D-glucose on the mRNA steady-state levels for α-chains of type VI collagen in connective tissue cell cultures derived from rat peripheral nerve.* **A**, *a representative Northern blot analysis. Confluent cell cultures were incubated in the presence of 5.5 (lane 1), 15 (lane 2), or 25 mmol/L (lane 3) D-glucose for 7 days. Total RNA was extracted, and 15 μg/lane were separated on an agarose gel followed by transfer onto a nylon filter. The same filter was successively hybridized with cDNAs for α1(VI), α2(VI), and α3(VI) collagen chains and GAPDH. Sizes of the mRNA transcripts are indicated on the right. Ethidium bromide staining of the gel is shown, and migration positions of the ribosomal subunits are indicated in the bottom panel.* **B**, *quantitation of the effect of D-glucose on the steady-state level of α2(VI) collagen chain mRNA. The cell cultures were incubated with 5.5, 15, or 25 mmol/L D-glucose for 3 (■), 7 (○), or 14 (●) days. Quantitation was achieved by scanning densitometry of the Northern films, and values represent mRNA abundance in cultures incubated with 15 (n = 16) or 25 (n = 17) mmol/L D-glucose, as a percentage of mRNA abundance in control cultures incubated with 5.5 mmol/L D-glucose. The values were corrected for GAPDH or β-actin abundance in the same RNA preparations. Means are indicated by transverse bars.* **C**: *After hybridization with cDNA for α2(VI) collagen chain, a filter containing RNA samples from 5 sets of cultures was rehybridized with α1(VI) and α3(VI) collagen chain cDNAs. The values represent mRNA abundance in cultures incubated in the presence of 15 or 25 mmol/L D-glucose as a percentage of mRNA abundance in control cultures incubated with 5.5 mmol/L D-glucose. The values are corrected for GAPDH mRNA abundance in the same RNA preparations. Columns, means; error bars, SEM.*

tively. Incubation for only 3 days (n = 3) resulted in a smaller effect (means, 128% of control in 15 mmol/L and 143% of control in 25 mmol/L) (see also Figure 5B).

A filter containing RNA samples isolated from five sets of cultures incubated in different glucose concentrations for 7 days was subsequently rehybridized with α_1(VI) and α_3(VI) collagen cDNAs. The results also indicated that the steady-state levels of α_1(VI) and α_3(VI) mRNAs were elevated to approximately the same extent as the steady-state level of α_2(VI) mRNA. The results obtained from the quantitation of the mRNA steady-state levels in these five sets of cultures are summarized in Figure 5C.

Interestingly, α_3(VI) collagen chain mRNA isolated from the cells that had been incubated in elevated glucose concentrations was slightly larger than α_3(VI) chain mRNA isolated from control cells. The presence of α_3(VI) mRNAs with slightly different sizes has been observed earlier, eg, in cultures initiated from neurofibromas.[28] Incubation of the cells in high concentrations of glucose did not have an effect on the sizes of mRNAs for α_1(VI) and α_2(VI) chains of type VI collagen (Figure 5A).

Deposition of type VI collagen in the cell cultures was visualized by immunostaining with antibodies to type VI collagen after incubation of the confluent cells in either 5.5 or 25 mmol/L D-glucose for 7 or 14 days in the presence of ascorbic acid. Type VI collagen epitopes were detected in fibrillar networks that covered the cell layers (Figure 6). The fibrils were irregularly distributed, the areas of high cell density showing a denser network. When areas with apparently similar cell densities were compared, incubation in high glucose concentration was observed to cause increased deposition of

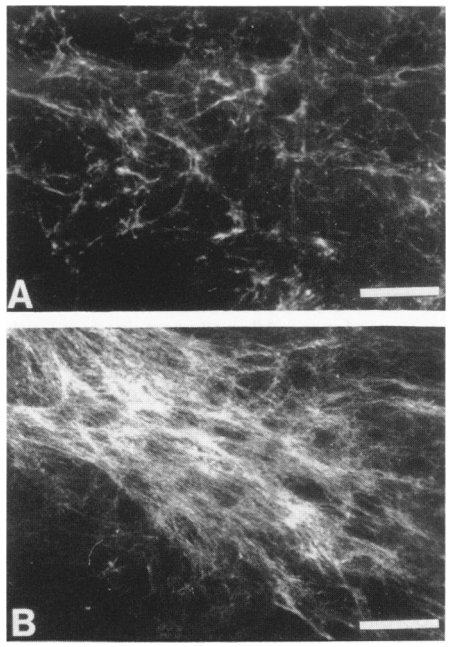

Figure 6. *Indirect immunofluorescence staining of rat neural connective tissue cell cultures for type VI collagen. Cell cultures were incubated in medium containing 5.5* **(A)** *or 25* **(B)** *mmol/L D-glucose in the presence of ascorbic acid (50 μg/ml) for 7 days. Immunofluorescence staining, photographing, and processing of the prints were performed in parallel under identical conditions to enable comparison between the staining intensities. Fibrillar deposits positive for type VI collagen cover larger areas of the cell layer in cultures incubated in the higher glucose concentration* **(B)** *when areas with apparently similar cell densities are compared. Bars = 50 μm.*

type VI collagen, as evaluated by indirect immunocytochemistry (Figure 6).

The cell types responsible for the production of type VI collagen in these cultures were characterized further by a high magnification of the immunostaining and by *in situ* hybridization. A perinuclear, apparently intracellular staining for type VI collagen, as well as autoradiographic grains representing α_2-(VI) collagen chain mRNA-cDNA hybrids, were detected in the three cell types identified in these cultures, ie, the perineurial cells (Figure 7), Schwann cells (Figure 7), and fibroblasts, indicating that all of these cell types express type VI collagen at both the mRNA and protein levels.

Discussion

Type VI collagen is an abundant structural protein of the extracellular matrix in a variety of tissues, eg, in normal peripheral nerves and in nerve-derived tumors, such as cutaneous neurofibromas.[23,38] In diabetes mellitus, marked changes in the synthesis and structure of the extracellular matrix have been observed. However, only one report describing altered turnover of type VI collagen in diabetes is available to date: Mohan et al[39] demonstrated an increased amount of type VI collagen in human diabetic kidneys utilizing solid-phase radioimmunoassays. They suggested that accumulation of type VI collagen may account for the mesangial expansion of diabetic kidneys that is known to correlate well with the manifestation of clinical nephropathy.[40]

In this study, we detected the so-called Luse bodies in the peripheral nerves of human diabetic subjects. Luse bodies, which have been shown to represent a form of aggregated type VI collagen,[9] have been reported in neural tissue only under pathological conditions, such as in neurofibromas,[41] schwannomas,[15,42] and compressed nerves.[43] It is unclear why type VI collagen monomers interact with each other in this characteristic way only under certain conditions. Specifically, it is not known whether an altered rate of synthesis of type VI collagen explains this phenomenon, or whether the structure of the protein is somehow altered. Furthermore, brief treatment of tissue samples with bacterial collagenase has been shown to enhance the formation of the 100-nm periodic fibrils which contain type VI collagen, suggesting that proteolytic enzymes present in the extracellular space under pathological conditions may contribute to the aggregation of type VI collagen.[17]

Deposition of extracellular microfibrils in the kidneys, arteriolar walls, and pancreas of diabetic patients has been described previously.[6–8] The biochemical composition of these microfibrils has remained obscure, however. It has been suggested that the fibrils may represent fibronectin[8] or elastin-associated microfibrils,[7] but direct evidence is lacking. Sohar et al[6] suggested the term "diabetic fibrillosis" to describe the generalized accumulation of microfibrils in diabetic tissues. Accumulation of microfibrils around the vasa nervorum of diabetic human femoral nerves has previously been described.[6] In this study, microfibrils were found to accumulate between the perineurial cell layers and in the subperineurial space of peripheral nerves of both human diabetic patients and spontaneously diabetic BB rats. The BB rats were diabetic for 2.5–6 months before obtaining the samples; this time period was not long enough to cause a notable thickening of the neural basement membranes, but deposits of microfibrillar material could readily be observed. Thus, it appears that the accumulation of microfibrils is an early sign of altered connective tissue metabolism in diabetic nerves, and it precedes the thickening of basement membranes. Our immunoelectron microscopic studies suggest that type VI collagen is a component of the microfibrillar depos-

its accumulating in the diabetic nerves. The antigenic sites of type VI collagen were exposed only after a limited pepsin digestion. It should be noted that fibrillin in a previous study was detectable by immunoelectron microscopy in certain extracellular microfibrils without enzymatic pretreatment.[11] Also, our immunoelectron microscopic studies on the distribution of fibronectin within peripheral nerve revealed a clearly different labeling pattern compared to type VI collagen (Muona et al, unpublished data). Thus, it is not likely that our antibody to type VI collagen would have cross-reactions with either fibronectin or fibrillin, two other components of extracellular microfibrils.

The pathophysiological significance of accumulation of microfibrillar material in diabetic tissues (eg, relevance to diabetic functional complications) is not known. Nevertheless, it may be regarded as an early indication of perturbed regulation of connective tissue metabolism in diabetes mellitus. It is also possible that the microfibrils accumulating in the perineurium of diabetic nerves may contribute to the compression of transperineurial blood vessels and disturb the blood flow within the nerve.[44]

In an attempt to study the mechanisms that possibly underlie the accumulation of type VI collagen in diabetic nerves, we investigated the effects of elevated concentrations of D-glucose on the expression of type VI collagen by nerve-derived connective tissue cells *in vitro*. Under cell culture conditions, it is possible to study the effects of various factors operative in diabetic tissues *in vivo*, dissociated from the multiple other factors present in diabetic tissues. Ambient glucose concentration is capable of regulating the expression of genes coding for matrix proteins in various cell types *in vitro*, including peripheral nerve-derived connective tissue cells.[29,45–47] In the present study we demonstrate that a high concentration of D-glucose *in vitro* increases the steady-state levels of mRNAs for all three type VI collagen chains. It has been suggested that the synthesis of α_3(VI) chain is rate limiting for the synthesis of type VI collagen molecules. For example, γ-interferon has been shown to down-regulate the synthesis of type VI collagen molecules by selectively decreasing the production of α_3(VI) chain.[48] However, cell density has been shown to coordinately regulate the synthesis of all three types of α-chains of type VI collagen in cultured fibroblasts: confluent cells express all α-chains of type VI collagen at a higher level than cells in preconfluent cultures.[49] Yet maintenance of the cells in a postconfluent state further increases the synthesis of α_1(VI) and α_2(VI) collagen chains, while the expression of α_3(VI) chain is not altered, thus limiting the increase in the formation of heterotrimeric type VI collagen molecules.[50] In the present study, glucose coordinately up-regulated the mRNA steady-state levels of all α-chains of type VI collagen *in vitro*. The glucose-induced increase in the synthesis of type VI collagen was observed also at the protein level, as indicated by the increased deposition of fibrils staining positively for type VI collagen epitopes in cell cultures incubated in high glucose concentrations. Thus, increased glucose concentration in tissues may contribute to the enhanced production of type VI collagen in diabetes. The exact mechanism of how glucose excerts its effects on the expression of genes encoding α-chains of type VI collagen remains to be elucidated. However, glucose does not cause a generalized up-regulation of steady-state levels of all mRNA species: elevated glucose concentrations did not alter the steady-state levels of β-actin mRNA in the same samples which displayed increased type VI collagen mRNA steady-state levels. Interestingly, the mRNA steady-

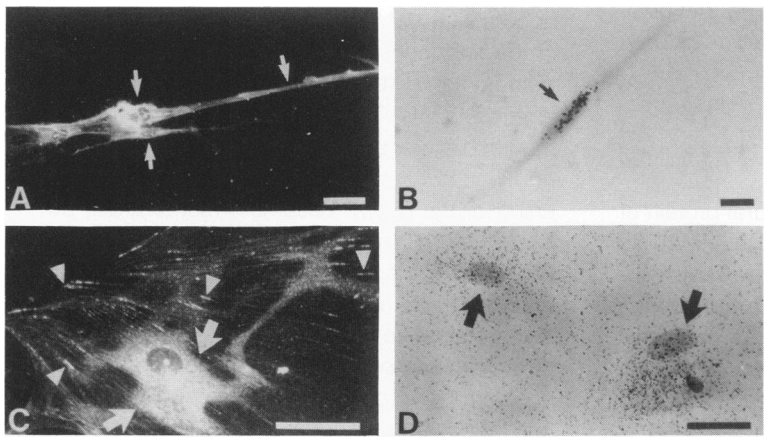

Figure 7. *Expression of type VI collagen by Schwann cells and perineurial cells in culture.* **A** *and* **C**, *indirect immunofluorescence stainings for type VI collagen. An array of Schwann cells* (**A**, *arrows*) *shows bright fluorescence. In a large perineurial cell* (**C**), *type VI collagen epitopes are observed both perinuclearly (arrows), probably representing intracellular, newly synthesized type VI collagen, and as short stripes (arrowheads), probably representing type VI collagen deposited on the cell surface.* **B** *and* **D**, in situ *hybridizations with a cDNA for* α_2*(VI) chain of type VI collagen. Autoradiographic grains representing* α_2*(VI) collagen mRNA (arrow) are seen perinuclearly in a Schwann cell* (**B**). *The nuclei of two perineurial cells* (**D**) *are indicated with arrows. Autoradiographic grains are seen predominantly in the perinuclear region within the perineurial cells. Bars:* **A**, **C**, **D**, *50 μm;* **B**, *10 μm.*

state levels of GAPDH, an enzyme of the glycolytic pathway, also remained unchanged. Furthermore, using in part the same RNA samples as utilized in the present study, we have demonstrated that incubation of neural connective tissue cells in high concentration of D-glucose resulted in a 33% decrease in the mRNA level of glucose transporter GLUT1.[51] Also, our earlier studies have not revealed differences in the mRNA steady-state level of laminin B2 chain in response to incubation in high glucose concentrations.[29] In addition to type VI collagen, high concentrations of D-glucose increase the expression of type IV collagen and fibronectin in various cell types.[29,45–47]

It is interesting that the cells incubated in high glucose concentrations express α_3(VI) mRNA transcript of apparently slightly larger size than the cells incubated under control conditions. It has been shown that alternative splicing of the primary transcript for the human α_3(VI) chain gene generates mRNA transcripts of different sizes.[52] Previously, different sizes of α_3(VI) mRNA have been described in different passages of neurofibroma-derived cells: in RNA preparations extracted from primary cultures with relative predominance of Schwann cells the smaller α_3(VI) mRNA transcript was detected, whereas cultures of later passages with enriched populations of perineurial cells predominantly expressed the larger α_3(VI) mRNA transcript.[28] Since incubation in high glucose concentrations does not seem to influence the ratios of different cell types in the nerve-derived mixed connective tissue cell cultures, it is tempting to speculate that the perineurial cell would be especially sensitive to elevated glucose concentrations with respect to an increase in the expression of type VI collagen genes.

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