Talin immunogold density increases in sciatric nerve of diabetic rats after nerve growth factor treatment

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**Keywords:** talin, sciatric nerve, diabetic neuropathy, Schwann cells, endoneurium, perineurium.

**Summary.** Background. Diabetic neuropathy is a debilitating disorder whose causation is poorly understood. Recent studies have shown significant reduction in the activity of nerve growth factor (NGF) and in the amount of talin cytoskeleton protein immunoreactivity in the perineurium in patients with diabetic neuropathy.

Objective. Since talin is involved in transmembrane connections between extracellular matrix and cytoskeleton, this study investigates the subcellular pattern of talin immunoreactivity and the effect of NGF treatment of diabetic rats on the distribution of talin in the sciatric nerve.

Materials and methods. Post-embedding immunogold electron microscopy using monoclonal antibody against talin in combination with quantitative procedures was employed to localize talin-like immunoreactivity in the sciatric nerve of normal, diabetic and NGF treated diabetic rats.

Results. We found the highest densities of gold particles in the Schwann cells (139.6±5.6 particles/µm²) and in the fibroblasts (127.4±4.1 particles/µm²). A moderate amount of immunoreactivity was also present in the endothelial cells of vasa nervosa (32.3±9.1 particles/µm²). The myelinated and unmyelinated nerve fibers and the extracellular matrix profiles were not labeled (8.7±2.1 particles/µm², 4.2±2.2 particles/µm², 6.1±3.2 particles/µm², 9.5±5.3 particles/µm², respectively). The immunogold localization of talin in diabetic rats was significantly (p<0.001) reduced in Schwann cells (66.3±6.5 particles/µm²) and perineurial and epineurial fibroblasts (56.8±3.9 particles/µm²). Diabetic rats treated with NGF for 12 weeks showed significant (p<0.005) increase in talin-like immunogold density in Schwann cells and fibroblasts. Talin immunogold density in Schwann cells and fibroblasts increased approximately 68% and 58%, respectively, after NGF treatment. The endothelial cells of endoneurial and epineurial vessel walls showed no significant change in the talin-like immunogold particle density among control, diabetic and NGF treated diabetic animals.

Conclusions. These results have shown that the administration of exogenous NGF may be essential for inducing functionally significant regenerative mechanisms in diabetic neuropathy through maintaining the permeability of the barrier properties of the peripheral nerve.

**Introduction**

Nerve growth factors (NGF) are required during development for the survival and growth of peripheral sensory dorsal root ganglia (DRG) and sympathetic neurons (1). The exact function of NGF in adult sensory DRG neurons is unknown. Several studies (2–4) indicated that NGF loss in adult sensory DRG neurons is responsible for the production of at least three components of the axon reaction: 1) somatofugal axonal atrophy, which is the reduction in axonal caliber beginning in the proximal axon resulting from a decrease in NGF receptor (pNF) synthesis; 2) nuclear eccentricity; and 3) aberrant expression of pNF epitopes in neuronal soma. Together, these observations suggest that NGF influences axonal size while serving in the broader capacity of a more generalized “trophic” factor which regulated the functional status of mature sensory neurons. It is unclear how NGF increases pNF synthesis in mature DRG neurons. NGF is also produced by Schwann cells in the distal stump following axotomy (5) which primarily appears to act locally to promote axonal regeneration. The function of NGF synthesized by Schwann cells in the distal stump is unknown. Schwann cells in the axotomized distal stump increase both NGF synthesis and the number of their low-affinity NGF receptors (5). It is known

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that NGF specifically supports small-fiber sensory neurons and sympathetic neurons. These neurons degenerate early and prominently in the course of diabetic polyneuropathy and their degeneration is responsible for most of the symptoms and signs that are particularly bothersome to the diabetic patient. Administration of NGF prevented the reduction of substance P and calcitonin gene-related peptide (CGRP) levels in the sciatic nerve in streptozotocin (STZ)-treated rats (6). However, NGF administration did significantly improve diabetic nerve conduction abnormality, but this was not unexpected because nerve conduction measures are dependent primarily on large fiber function (7).

Talin, a high molecular weight protein of 225–235 kDa, is found in a variety of tissues and cell types (8). Talin immunoreactivity is normally found at endoneurial and epineurial vessel walls, perineurial and epineurial fibroblasts in all the human sural nerves except diabetic nerves. In vitro studies have proposed that talin can interact with at least two other proteins that are localized at adhesion plaques, vinculin and integrin, and that they are involved in transmembrane connections between the extracellular matrix and the cytoskeleton (9). A. Mazzeo and her co-workers showed a decrease in 235 and 190 kDa bands of talin in the muscle homogenate of diabetic neuropathy patients by Western immunoblotting. A striking feature is the reduced amount of talin immunoreactivity at inner and outer lamellae of the perineurium only in patients with diabetic neuropathy (10). Since talin is involved in transmembrane connections between extracellular matrix and cytoskeletal (8) we propose to expand this work by assessing the pattern of talin immunoreactivity in diabetic rats treated with NGF.

The possibility that abnormal availability of neuromorphometric factors, in particular NGF, contributes to the pathogenesis of diabetic neuropathy has been extensively investigated in recent years. Several studies suggest that retrograde transport of endogenous NGF is impaired in diabetic animals (7, 11, 12). It was shown that rats’ NGF levels were increased in target tissues and decreased in sciatic nerve and sympathetic ganglia in diabetic rats. These data suggested that retrograde axonal transport of NGF was impaired (13). Other studies have shown that NGF expression itself is reduced in experimental and clinical diabetes (14). However, a direct connection between abnormal availability on NGF and the pathogenesis of peripheral neuropathy has not been demonstrated yet. Likewise, the relation between the NGF and the cytoskeleton proteins such as talin in diabetic neuropathy has not been fully investigated. In order to understand the mechanism of NGF action in diabetes and its relation with talin, the changes in the immunoactivity and localization of talin protein were assessed in sciatic nerves of diabetic rats after treatment with NGF.

Materials and methods

Animals. All animals were maintained and cared for as outlined in the guide for the care and use of laboratory animals (Kuwait University, Faculty of Health Publication). Animals were housed in area which is well-ventilated, air-conditioned and provided with independently adjustable light-dark cycle system and temperature regulation system. The rooms and animal cages were cleaned daily and the animals were provided with fresh food and water on a daily basis. In addition, all animals were inspected daily for any possible signs of inflammation, respiratory or gastrointestinal disease. If such signs were present, the individual animal was not used in this protocol.

Experimental protocols and treatments. Adult male Sprague-Dawley rats (Kuwait Animal Laboratory Center colony) were group-housed (4–5 animals per cage) in standard plastic cages with wood chip bedding. Bedding was changed daily for all animals to maintain sanitary conditions. In individual experiments, rats (180–220 g) were injected intraperitoneally (ip) in groups of 5–10 animals with 75 mg/kg (15) streptozotocin (STZ) (Sigma, St. Louis, MO) (40–50 mg/kg (16)) dissolved in 0.9% saline. Control animals were injected with an equal volume of vehicle (0.9% saline). Non-diabetic rats were studied at this age as onset control, to provide a starting value against which to judge any diabetes induced neuropathic deterioration. STZ solutions were freshly prepared due to the limited stability of the compound (17). Blood glucose levels were determined in all animals using an Encore Glucometer (Bayar, Elkhart, IN) from blood samples obtained by tail vein bleeds. Since elevated blood glucose levels appear to stabilize approximately 3 weeks post STZ treatment (18), blood glucose levels were assessed 3–4 weeks following STZ treatment. Rats with blood glucose levels ≥250 mg/dl (>14 mM) were considered diabetic and used for further studies.

Diabetic rats were verified 24 hours by the presence of hyperglycemia and glucosuria using Visidex II and Diastix kits (Ames Slough, UK). After final experiments, plasma glucose was estimated (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) in samples taken from the tail vein. While few detailed investigations of the time-course for the expression of STZ-induced alterations in nociceptive

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responses have been reported, other data also suggest that there is some variability between studies in the temporal expression of altered nociception following STZ-treatment. For example, the onset of mechanical hyperalgesia (decreased withdrawal latency to paw pressure) has been reported to range from one (19) to 3–4 weeks (18). C. Courteix and his co-workers (18) reported that STZ-induced thermal allodynic responses were present as early as 2 weeks post STZ treatment in a subpopulation of diabetic rats, and that this subpopulation increased in number with time following STZ treatment. Similarly, STZ induced increases in nociception in the formalin test were found to develop over time requiring at least 4 weeks to fully develop (18, 20). After 2 weeks of uncontrolled diabetes, blood glucose was assessed and the animals were divided into diabetic control group and experimental group for treatment with nerve growth factor (NGF) for 12 weeks.

*NGF experimental protocol.* Human recombinant NGF (hrNGF: Boehringer Mannheim GmbH, Biotechnology Center Pensaerg, FRG) was administered at a dosage of 0.5 mg/kg according the procedure described by J. W. Unger et al. (21). Briefly, NGF was stored at −80°C and diluted in phosphate buffered saline, pH 7.4, containing cytochrome c (Boehringer Mannheim GmbH) at a concentration of 0.1 mg/ml as a carrier protein to minimize loss of NGF due to adsorption to plastic ware. Dilutions were prepared fresh each day. A volume of 1 ml/kg body wt was injected subcutaneously in the neck region on a three times weekly dosing schedule for 12 weeks using syringes with Ultra-Fine gauge needles (Beckton Dickinson, Heidelberg, FRG).

*Specimens collection and electron microscopy.* Animals were deeply anesthetized (Pentobarbitone, 50 mg/kg ip) and perfused transcardially with 200 ml of phosphate buffer saline (PBS) followed by 500 ml of 4% paraformaldehyde / 0.2% glutaraldehyde. The sciatic nerves were dissected out and post-fixed for 48 hours. Then the tissues were processed for EM as described previously by M. W. Renno et al. (22). Briefly the tissue sections were rinsed in phosphate buffer and post-fixed in osmium tetroxide and dehydrated in a series of graded ethanol. After infiltration with propylene oxide and Araldite for 4 h and then with 100% Araldite overnight, the tissues were embedded in fresh Araldite resin. Semi-thin sections were assessed first for proper tissue orientation. Ultrathin sections were cut and mounted on nickel grids. Finally, the tissue sections were counterstained with uranyl acetate and lead citrate and viewed in the transmission electron microscope (EM unit at the Health Science Center, Faculty of Medicine).

For immunocytochemical post-embedding labeling, the protocol used has been described previously (22). Briefly, the sections were washed in Tris buffer saline (TBS) pH 7.6 buffer (0.05 M Tris pH 7.6 with 0.9% NaCl and 0.1% Triton X-100) and drained by touching the edge of grids to Kimwipe, followed by incubation in anti-talin primary monoclonal antibody (Sigma T3287; clone 8d4 ascites fluid; Sigma-Aldrich, USA) diluted 1:20 in TBS pH 7.6 overnight in a moist chamber at room temperature. The next day, the sections were washed twice in TBST pH 7.6 buffer 5 min each followed by a third wash for 30 min. Then the sections were conditioned in TBS pH 8.2 buffer (0.05 M Tris pH 8.2 with 0.9% NaCl and 0.1% Triton X-100) for 5 min. Grids were then incubated with anti-mouse IgG (Sigma) conjugated to 10 nm gold particles (diluted 1:20 in TBS pH 8.2 buffer) for 1 h, washed twice in TBS pH 7.6 for 5 min each, and rinsed in double distilled water (ddH2O). Finally, the sections were counterstained with uranyl acetate and lead citrate. Since myelin is a phospholipid, its staining often appears weak, whenever low percentage of glutaraldehyde is used. In our study, we had to use a low percentage of glutaraldehyde since higher percentage would mask the talin antigen. Also, we used 0.1% Triton X-100 as an etching agent to remove the osmium and Araldite plastic in order to improve antigenic immunoreactivity by exposing antigenic sites and restoring antigenic affinity to the antibody. This in turn may affect the ultrastructural preservation of myelin (22–24). However, the usage of low percentage of glutaraldehyde and etching agent does not affect our interpretation of the results since we are comparing and contrasting the specific staining for talin, not myelin.

For morphometric analysis, three sections from each block (two blocks from each rat) were subjected to quantitative analysis according to procedures described earlier by many investigators (21–23, 25). Sections from the five rats from each group were quantitated for sciatic nerve profiles. Random electron micrographs were taken from the sciatic nerve to assess the immunogold labeling densities in the different profiles examined in this study (Schwann cells, endoneurial and perineurial fibroblast, myelinated and unmyelinated nerve fibers, extracellular matrix and endothelium of vasa nervosa).

*Quantitation of post-embedding immunostaining.* Ultrathin sections were examined under a JEOL 1200EX II transmission microscope at 80 kV. Sections
were photographed randomly at a plate magnification of \( \times 15,000 \) and printed at a final magnification of \( \times 18,000 \). All gold particles were counted over each profile. Aggregates of two or more gold particles were counted as one. Aggregates could be recognized from the uniform small distance among the particles. Occasionally, close by, independently attached particles may have been counted as one. This would occur mainly in densely labeled structures resulting in the underestimation of particle density. Since, at the most, only a few percent of the particles were aggregates, this underestimation would not affect the differences among cell profiles in the present study. For quantitative analysis, the number of gold particles per square micrometer for each structure was calculated according to the morphometric technique of M. Ben-dayan et al. (24) as previously described (26). Briefly, the surface area (Sa) of individual histological structures was first measured using a digitizing tablet (Sigma Scan V3.90; Jandel Scientific, Corte Madera, CA) and associated microcomputer (computer model software WSYE pc+). An electron micrograph was positioned on the tablet, and the perimeter of the profile to be measured was traced. The number of gold particles (Ni) present over individual Schwann cells, endoneurial and perineurial fibroblast, myelin sheath, myelinated and unmyelinated nerve fibers, extracellular matrix and endothelium of vasa nervosa was then counted and the density of labeling (Ns) calculated as Ns=Ni/Sa. A background gold particle density per square micrometer was calculated for each animal from immunohistochemically-processed sections in which the talin primary antibody was replaced with normal rabbit serum. This background density of gold particles was then subtracted from the calculated talin gold particles densities to obtain a final density per square micrometer. A one-way analysis of variance (ANOVA) was applied to assess significant differences between the mean values of gold particles among the different neuronal structures quantified in control, diabetic and NGF treated diabetic rat groups. The Scheffe F-test was used to determine individual probability values for multiple comparisons. In addition, the surface density of gold particles was quantitated over individual neuronal and non-neuronal elements identified on the basis of their fine structural features and their location in the sciatic nerve in order to determine whether the observed differences in talin immunolabeling were statistically significant. All gold particles were counted over each profile (Table), and the results are summarized in Fig. 1 and described in the text.

**Table. Number of each type of profile measured in this study**

<table>
<thead>
<tr>
<th>Cellular profiles</th>
<th>Number measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schwann cells</td>
<td>88</td>
</tr>
<tr>
<td>Myelinated nerve fibers</td>
<td>110</td>
</tr>
<tr>
<td>Myelin sheath</td>
<td>110</td>
</tr>
<tr>
<td>Unmyelinated nerve fibers</td>
<td>102</td>
</tr>
<tr>
<td>Perineural and epineurial fibroblasts</td>
<td>69</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>78</td>
</tr>
<tr>
<td>Endothelium of vasa nervosa</td>
<td>44</td>
</tr>
</tbody>
</table>

**Results**

**Specific control.** Routine control experiments (blocking with the talin antigen, omission of primary antiserum, or substitution of primary monoclonal antibody with preimmune serum) confirmed the specificity of the immunolabeling. The talin monoclonal antibody had been shown to possess considerable specificity for talin (27). The specificity of the staining observed at the electron microscopic levels is further demonstrated by the following. First, there was no staining when the talin antibody was absorbed by preincubation with 100 μM talin. Figure 2A is an example of preabsorption of talin antibody with synthetic talin that significantly reduced the gold labeling density of talin to background levels. Second only background level was observed in the sections following the deletion of anti-talin monoclonal antibody (Fig. 2B). Finally, the labeling was not observed when normal mouse serum was substituted for talin monoclonal antibody during staining procedure (Fig. 2C). These control experiments support the conclusion that the anti-talin monoclonal antibody used in this study recognizes talin. At the ultrastructural level, only background labeling (5.7±4.3 particles/μm²) was observed in sections treated with normal mouse serum. This immunogold background labeling was subtracted from the values presented in Fig. 1 to provide an index of specific labeling in the sciatic nerve profiles.

**Talin immunolabeling.** A low background density of gold particles was distributed over the entire tissue section. Within labeled profiles, however, the gold particles were found at much higher density; thus immunolabeled profiles were readily distinguished from the backgrounds. Morphological analysis of the data (Fig. 1) shows two distinctive populations: “Labeled” or “immunoreactive” profiles contain more that 20 particles/μm² and “unlabeled” profiles contain less
Fig. 1. Quantitative analysis of the distribution of talin-like gold particle densities in different cellular profiles of the sciatic nerve of control, diabetic and NGF treated diabetic rat groups

Values represent number of particles/μm² ± SEM after the background density of gold particles was subtracted from the calculated densities of talin gold particles to obtain a final density per μm². Twelve weeks following the induction of diabetes, talin-like immunogold density in STZ-induced rats was significantly decreased compared to non-diabetic controls and diabetic rats treated with NGF (ANOVA: *p<0.001). Administration of NGF, subcutaneously three times a week over the same time course, prevented the decrease in talin-like immunogold staining (***p<0.005). The gold particle density staining over the endothelial cells of capillaries was significantly (+p<0.02) different from the background in the control and diabetic rats. Administration of NGF, however, did not change the gold particle density of talin-like immunoreactivity in the endothelial cells of nerve capillaries. A Scheffe F-test of multiple comparisons revealed that no significant difference existed between the gold particle density overlying the myelinated nerve fibers, unmyelinated nerve fibers, extracellular matrix and that of background.

than 11 particles/μm² and are not statistically significant from gold density labeling of extracellular matrix (control group 9.5±5.3 particles/μm²; diabetic group 10.9±4.1 particles/μm²; NGF treated diabetic group 8.5±3.9 particles/μm²). Using these criteria, 88 Schwann cells, 69 fibroblasts, 110 myelinated nerve fibers, 102 unmyelinated nerve fibers, 69 extracellular areas, and 44 endothelial cells of vas nervosa were classified as talin immunolabeled in the sciatic nerve (Table).

Electron microscopic examination of sections through the sciatic nerve revealed intense immunogold labeling over the Schwann cells (Fig. 3A) and fibroblasts (Fig. 4A). Analysis of the cellular distribution of talin immunoreactivity in the sciatic nerve showed that particle density for talin was greatest over labeled profiles (Fig. 1). These labeled Schwann cells and fibroblasts contained approximately 139.6±5.6 particles/μm² and 127.4±4.1 particles/μm², respectively, in sciatic nerve of control group. The immunogold localization of talin in diabetic rats was significantly (p<0.001) reduced in Schwann cells (66.3±6.5 particles/μm²) (Fig. 3B) and perineurial and epineurial fibroblasts (56.8±3.9 particles/μm²) (Fig. 4B). Talin immunogold labeling decrease in Schwann cells and fibroblasts of diabetic group was approximately 47% and 45%, respectively. Diabetic rats treated with NGF for 12 weeks showed significant (p<0.005) increase (Fig. 1) in talin-like immunogold density in Schwann cells (Fig. 3B) and fibroblasts (Fig. 4C). Talin immunogold density in Schwann cells and fibroblasts increased approximately 68% and 58%, respectively, after NGF treatment when compared to diabetic rats.

In the five control animals studied, talin-like immunogold labeling over the endothelial cells of capillaries was significantly (p<0.02) different from the background levels (Fig. 1). The density of talin gold particles over the endothelial cells of vas nervosa was approximately 77% and 75% less than gold density over the Schwann cells and fibroblasts of the
Fig. 2. Examples of electron micrographs of control sections from the sciatic nerve of the control and diabetic rat groups
A – effect of talin pre-absorption on immunolabeling. Note that pre-absorption of talin primary antibody abolished immunolabeling (arrows) significantly (Ax, axon; asterisks, myelin sheath; U, unmyelinated nerve fiber), ×20,000; B – control section in which the first antibody (anti-talin antibody) was deleted during the immunogold labeling. Note the insignificant amount of gold particles was seen on the different profiles examined (Sc, Schwann cells), ×20,000; C and D – control sections in which the talin antiserum was replaced with normal mouse serum followed by secondary anti IgG conjugated to 10 nm gold particles. Note that only a small number of background gold particles (arrows) were seen on different profiles examined in this study (E, endothelium), ×18,000 and ×18,000.

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Fig. 3. Electron micrographs of talin-like immunoreactive Schwann cells (Sc) of the rat sciatic nerve
A – electron micrograph depicting talin-like immunoreactivity within Schwann cells of the rat sciatic nerve of control group. Gold particles representing talin immunolabeling are indicated by arrows, ×20,000; B – example of talin immunolabeling of Schwann cells of diabetic STZ induced rat group. Note the significant decrease in gold labeling in Schwann cells in these diabetic animals, ×20,000; C – electron micrograph of talin-immunoreactive gold-labeled Schwann cells in NGF treated diabetic rat group. Notice the significant gold particles (arrows) increase in NGF treated diabetic rat group compared to diabetic one, ×20,000 (asterisks, myelin sheath; Ax, axons).
**Fig. 4.** Electron micrographs depicting immunogold talin-like immunoreactive perineurial fibroblasts (Fb) of the rat sciatic nerve

A – micrograph depicting talin-like immunoreactivity (arrows) within the fibroblasts of the rat sciatic nerve of control group, ×18,000; B – example of talin immunolabeling of fibroblast cells of diabetic rat group. Note the significant decrease in gold labeling (arrows) in fibroblast in these diabetic animals, ×18,000; C – electron micrograph of talin-immunoreactive gold-labeled fibroblast in NGF treated diabetic rat group. Note the significant gold particle (arrows) increase in the fibroblasts of the NGF treated diabetic rat group compared to diabetic group, ×18,000 (C, collagen fibers).

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Fig. 5. Electron micrographs of talin-like immunoreactive endothelium cells of vasa nervosa (perineurial and epineurial vessels) of the rat sciatic nerve

A – micrograph showing talin immunogold labeling of endothelium cell of a capillary in the sciatic nerve of rat control group, ×20,000; B – electron micrograph of epineurial vessel from a section of sciatic nerve from diabetic rat group, ×20,000; C – micrograph showing talin-like immunoreactivity (arrows) of endothelium cells of epineurial vessel in the sciatic nerve of NGF treated diabetic group. Note that the endothelial cells of vas nervosa are significantly labeled with talin antibody compared to background gold density level; however, there is no significant difference of gold particle density in these cells among control and treated groups, ×20,000 (E, endothelium; Fb, fibroblast; L, lumen; C, collagen).
control rats, respectively. The endothelial cells of endoneurial and epineurial vessel walls showed no significant change (Fig. 1) in talin-like immunogold particle density among control (32.3±9.1 particles/μm²) (Fig. 5A), diabetic (21.9±7.6 particles/μm²) (Fig. 5B) and NGF treated diabetic (26.5±8.1 particles/μm²) (Fig. 5C) animals.

The mean gold particle densities of talin-like immunoreactivity over the myelinated (nerve fibers (8.7±2.1 particles/μm²) and myelin sheaths (4.2±2.2 particles/μm²)) (Fig. 6) and the unmyelinated nerve fibers (6.1±3.2 particles/μm²) (Fig. 7) and the extracellular matrix (9.5±5.3 particles/μm²) (Fig. 8) profiles were insignificant from background levels. Talin-like immunoreactivity in these profiles did not show any significant changes in diabetic or NGF treated animals (Fig. 1).

Discussion
In order to understand the talin distribution in the normal sciatic nerve and the effect of NGF on the diabetic rats, we utilized immunogold post-embedding technique at the electron microscopic level to examine the relationship between talin distribution and NGF treatment in diabetic animals. Our morphometric analysis was performed on the sciatic nerve because the common type of peripheral neuropathy associated with diabetes in humans is the loss of the distal region of long and large-diameter axons with survival of most neuronal somata. These pathological changes are characteristically associated with a stocking-and-glove pattern of sensory loss, which is later accompanied by a similar distribution of motor weakness. Such changes are found in a number of vitamin-deficiency states, metabolic disorders such as diabetes mellitus and advanced age. These conditions are supplemented by a large number of localized mechanical nerve injuries that lead directly to distal axonal degeneration. The presence of axonal loss, together with the dependency of the axon of the neuronal soma, suggests that defects or interruptions in the delivery of materials via axonal transport are the key to the pathogenesis of axonal degeneration. In the course of this disease, many diabetic patients end up suffering from a variety of neuropathies, which may include acute painful diabetic neuropathy, ataxic and acerdyostrophic neuropathy (ataxia, foot ulceration, and neuropathic arthropathy). Neuropathies are undoubtedly the commonest type of neurological dysfunction found in diabetes, although the exact prevalence varies according to the diagnostic criteria used (28–30).

In the normal sural nerve, the endoneurial and epineurial vessels are intensely positive for vinculin and talin. There is spindle-shaped immunoreactivity for talin which correspond to fibroblasts. Strong and concentric lines of immunofluorescence for vinculin and talin are usually seen in the perineurium. Gold immunoelectron microscopy showed both proteins labeling the membrane of the perineurial fibroblasts (31, 32). A variety of osmiophilic inclusion bodies (dense osmiophilic staining and paracrystalline laminated appearance) are often associated with disrupted myelin sheath lamellae, lysosomal bodies and periaxonal expansions of Schwann cell cytoplasm in diabetic animals. An unusual Schwann cell immunoreaction is usually evident in nerves of more severely diabetic rats, involving lysosomal digestion of large, pinched-off portions of myelinated nerve fibers. Unmyelinated nerve fibers and associated Schwann cells of both diabetic and control rats rarely contained inclusion bodies and secondary lysosomes (25). Experimental (streptozotocin-induced) diabetic neuropathy is also associated with a reduced amount of fast anterogradely transported proteins but no overall change in transport range (13). A defect in fast anterograde transport of specific receptors and neurotransmitter enzymes as measured by their accumulation proximal to nerve ligation has been demonstrated in STZ-induced diabetic neuropathy (33, 34). Decreased retrograde axonal transport of glycoproteins, measured by accumulation distal to a nerve ligation (13), has also been found in experimental STZ-induced diabetic neuropathy. These data might suggest that an early defect in retrograde axonal transport underlie the development of axonal degeneration in these various neuropathies. However, recent evidence indicates that a defect in retrograde axonal transport leads to a loss of target tissue-derived “trophic” support. This hypothesis appears to be consistent with the decrease in retrograde transport of NGF that has been observed in diabetic neuropathy (12).

In the present study, we found that intense talin-like immunogold labeling over the Schwann cells and fibroblasts of normal sciatic nerves where as the endothelial cells of capillaries were moderately labeled. These findings are in agreement with the results reported by A. Mazzeo and her co-workers (10). In addition, the immunogold localization of talin in diabetic rats was significantly reduced in Schwann cells and perineurial and epineurial fibroblasts. Talin immunogold labeling decrease in Schwann cells and fibroblasts of diabetic group was approximately 47% and 45%, respectively. Previous investigation (10) reported that talin immunoreactivity was found at endoneurial and epineurial vessel walls, perineurial and
Fig. 6. Electron micrographs of talin-like immunoreactive myelinated nerve fibers of the rat sciatic nerve
A – micrograph showing talin immunogold-labeled myelinated nerve fibers in the sciatic nerve of the control group, \( \times 20,000 \); B – micrograph depicting talin immunogold labeling in myelinated nerve fibers of the sciatic nerve in diabetic rat group, \( \times 20,000 \); C – example of a micrograph showing talin-immunogold labeling in the myelinated nerve fibers of the sciatic nerve taken from diabetic rats treated with NGF for 12 weeks after the induction of diabetes, \( \times 20,000 \). Note that only a small number of background gold particles (arrows) are evident in these micrographs (asterisks, myelin sheath; Ax, axon).

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**Fig. 7.** Electron micrographs of unmyelinated nerve fibers of rat sciatic nerve sections stained with talin monoclonal antibody

A – micrograph from control group showing a number of unmyelinated nerve fibers (U) enclosed with significantly talin-like immunoreactive Schwann cell (Sc). Note that the unmyelinated nerve fibers show no immunogold labeling, ×20,000; B – electron micrograph of a group of unmyelinated nerve fibers of diabetic rat group showing only background immunolabeling (arrows) of talin-like immunoreactivity, ×20,000; C – Micrograph showing sections of sciatic nerve fibers from NGF treated diabetic rat group. The unmyelinated nerve fibers show insignificant immunogold labeling for talin monoclonal antibody. Note the significant labeling of Schwann cell which is enclosing the unmyelinated nerve fibers, ×20,000 (asterisks, myelin sheath; Ax, axon).
**Fig. 8.** Electron micrographs of talin-like immunogold staining of the extracellular matrix of the rat sciatic nerve

A – electron micrograph depicting talin-like immunoreactivity in the extracellular matrix of the rat sciatic nerve of control group, ×20,000; B – example of talin immunolabeling of extracellular matrix of STZ-induced diabetic rat group, ×20,000; C – micrograph of talin immunoreactive gold-labeled extracellular matrix in NGF treated diabetic rat group. Note that only a small number of background gold particles (arrows) are evident in control and treated animals, ×20,000 (asterisks, myelin sheath; Ax, axon; Fb, fibroblast; C, collagen).

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epineurial fibroblasts in normal human sural nerves; in addition, talin binding was markedly reduced in the perineurium of the sural nerves from patients with diabetic neuropathy. This total talin decrease in patients with diabetic neuropathy was confirmed by Western immunoblotting. However, neither immunofluorescence light microscopic study nor Western immunoblotting techniques (10) could show the specific decrease in Schwann cells or the changes in different profiles. Using immunogold localization at electron microscopic level in conjunction with quantitative analysis, we were able to precisely determine the talin-immunoreactive cellular profiles in the sciatic nerve and determine the approximate changes in the talin immunogold labeling, quantitatively.

Our results demonstrate that diabetic rats treated with NGF for 12 weeks showed significant increase in talin-like immunogold density in Schwann cells and fibroblasts of the diabetic sciatic nerves. Talin immunogold density in Schwann cells and fibroblasts increased approximately 68% and 58%, respectively, after NGF treatment when compared to diabetic rats. These data support the therapeutic role of the NGF in restoring the normal functions of the peripheral nerves in diabetic neuropathy as indicated by many recent studies (6, 7, 35). In addition, our study suggests that the NGF mechanism of action in diabetic neuropathy involves the modulation of talin protein in Schwann cells and fibroblasts in peripheral neuropathic nerve.

The physiologic source of NGF for DRG cells is provided by local synthesis in Schwann cells or by expression in the peripheral innervation area. NGF reaches the soma by retrograde transport after intracellular uptake (36). However, NGF receptors are not restricted to DRG neurons, but are also present in the projection area within the spinal cord (lamina 1 and 2), suggesting a modulatory role of NGF in the sensory pathways also at the level of the central nervous system (37). In addition, neurotrophins exert their influence not only along the central direction of the neuronal pathway of the sensory system, but also within the receptor field of the primary sensory neuron, i.e. receptors in the skin, A delta- for mechano-nociception or C-fibers for thermo-nociception. A previous study has demonstrated that collateral sprouting of A delta- and C-fibers in the partially denervated skin is supported by endogenous NGF and can be blocked by the application of NGF antibodies, whereas regrowth of crushed nociceptive axons occurred surprisingly independent from NGF (38). In light of these findings, J. Diamond and his co-workers (38) suggested that the cells possess additional properties for the induction of regenerative mechanisms.

These previous investigations have shown potent effects of NGF on the structure and function of the somato- and viscerosensory system. In addition, recent studies, using animal models of type I diabetes mellitus, have pointed toward a neurotrophic deficit accompanying the metabolic disease which may play a critical role in the development of neuropathy, i.e., reduced axonal NGF transport of the DRG in sciatic nerves of diabetic rats with no change in NGF binding sites (11). Furthermore, NGF protein and NGF mRNA were found to be reduced in skin and muscle of untreated diabetic rats four weeks after induction of diabetes. NGF treatment for 8 weeks was able to correct these defects (6). Based on the data summarized above, a “neurotrophic hypothesis” of diabetic neuropathy was developed stating that insulin deficit and/or hyperglycemia causes reduced growth factor synthesis in target areas and locally in Schwann cells and disturbance of retrograde axonal transport to the perikaryon. These changes subsequently lead to reduced gene expression of neurotrophin-dependent substances, i.e. transmitters, structural proteins, and neurofilaments. Thus, lack of neurotrophic support may be partially responsible for degenerative changes in the neuronal and axonal structure that may contribute to the impairment in nerve function such as reduced conductivity due to axoglial disjunction with shifting of voltage-dependent sodium-ion channel (14).

The blood-nerve barrier of the perineurial and endoneurial blood vessels maintains the normal homeostasis of the nerve. Perineurium protects the endoneurium from substances that might leak from permeable epineurial vessels and it also controls transporting selectivity materials and fluid through the endoneurial spaces (39). At the level of the tight junctions, the extracellular space between perineurial fibroblasts is obliterated and this cell-to-cell communication is most likely related to the barrier function of the perineurium. The localization of talin at Schwann and perineurial fibroblasts in normal nerve suggests that this protein may be implicated in the permeability barrier property of the perineurium. Previous studies (10, 31) also support this role. In diabetic nerves, thickening of the basal laminae of the perineurial fibroblasts (40) and perineurial calcifications which are mainly localized to the outer lamellae (41) have been documented. In addition, disorganized perineurial tight junctions in diabetic polyneuropathy (42) and endoneurial edema in experimental galactose neuropathy which is associated with altered tight junc-
tions of the perineurial fibroblasts (43) suggest that similar mechanisms could result in the alterations of the perineurial tight junctions in diabetic neuropathy and account for its impaired permeability properties. Our study supports the above investigations and conclusions; in addition, our data implicate a strong role for NGF to restore the normal functions of the tight junctions by restoring the talin, cytoskeleton protein, in diabetic sciatic nerve.

Although talin-like immunogold labeling over the endothelial cells of capillaries was significantly different from the background levels, these cells of endoneurial and epineurial vessel walls showed no significant change in talin-like immunogold particle density among control, diabetic and NGF treated diabetic animals. The density of talin gold particles over the endothelial cells of vas nervosa was approximately 77% and 75% less than gold density over the Schwann cells and fibroblasts of the control rats, respectively. Our data indicate that talin may play a minor role in the vascular abnormalities of the peripheral nerves of diabetic animals. Furthermore, this study does not support any role for the NGF on talin distribution in the endothelial cells of nerve capillaries. In spite of the fact that the barrier properties in the peripheral nerve are attributed to the presence of extensive tight junction complexes in the perineurium and the cells of endoneurial vasculature, A. A. Sima and his co-workers (44) concluded from their study that the endothelial tight junctions abnormalities found in diabetic neuropathy are unlikely to have any pathogenetic significance. Our study, indeed, supports such a conclusion for the tight junctions in endothelial cells of vasa nervosa of diabetic sciatic nerve.

In conclusion, these data suggest that in diabetic neuropathy the role of NGF is mainly confined to Schwann cells and fibroblasts of peripheral nerve. The talin, cytoskeleton protein, in these two profiles seems to play a major role in maintaining normal function in the nerve fibers and its decrease in diabetic animals suggests a possible role in the pathogenesis of diabetic neuropathy seen in peripheral nerves. In light of the fact that talin is a major component of the tight junctions in perineurial and Schwann cells, the alteration of talin in diabetes could lead to abnormal function of blood-nerve barrier which in turn may expose the axonal fibers to harmful components. In addition, this study shows that neurotrophic (NGF) support may be essential for maintaining talin protein which in turn preserves functional significant tight junctions in the peripheral nerves of diabetic neuropathy.

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Talino, žymėto imunoaukso dalelėmis, tankumo padidėjimas žiurkėms, kurioms sukeltas cukrinis diabetas, sėdmens nervo gydymo augimo faktoriumi

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Raktažodžiai: talinas, sėdmens nervas, diabetinė neuropatija, Švano laštelės, endoneuriumas, perineuriumas.


Darbo tikslas. Taliną dalyvauja tarpmembraninėje sąveikoje tarp tarplastelinio užpildo ir laštelės griauciai, todėl šio tyrimo tikslas – ištirti talino imunoreaktyvumo pobūdį ir nervų augimo faktoriaus poveikį talino pasiskirstymui žiurkėms, kurioms sukeltas cukrinis diabetas, sėdmens nervo.

Medžiagos ir metodai. Talino imunoreaktyvumas sėdių žiurkėms, žiurkėms, kurioms sukeltas cukrinis diabetas, ir nervų augimo faktoriaus gydytą žiurkėms, kurioms sukeltas cukrinis diabetas, sėdmens nervo stebėtas naudojant elektroninę mikroskopiją, talinui susijungus su monokloniniu antikūnu, žymėtui aukso dalelėms.

Rezultatai. Didžiuosius aukso dalelių tankumas nustatyti Švano laštelėse (139,6±5,6 dalelės/µm²) ir fibroblastuose (127,4±4,1 dalelės/µm²). Vidutinis imuninis reaktyvumas užfiksuotas nervų kraujagyslių endotelio laštelėse (32,3±9,1 dalelės/µm²). Mielinės ir nemielinės nervinės skaidulos bei tarplastelinio užpildo struktūros nebuvo žymėtos (atitinkamai – 8,7±2,1 dalelės/µm², 4,2±2,2 dalelės/µm², 6,1±3,2 dalelės/µm²).

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9.5±5.3 dalelės/μm²). Talino, žymėto imunoaukso dalelėmis, tankumas statistiškai reikšmingai (p<0.001) sumažėjo žiurkių, kurioms buvo sukelta cukrinis diabetas, Švano laštelese (66.3±6.5 dalelės/μm²) bei perineuriumo ir epineuriumo fibroblastuose (56.8±3.9 dalelės/μm²). Žiurkių, kurioms buvo sukelta cukrinis diabetas ir 12 sąvaitių duodama nervų augimo faktoriaus, Švano laštelese bei fibroblastuose nustatytas statistiškai reikšmingas (p<0.005) talino, žymėto imunoaukso dalelėmis, tankumo padidėjimas. Žiurkėms davus nervų augimo faktoriaus, talino imunoaukso dalelės tankumas Švano laštelese bei fibroblastuose padidėjo atitinkamai – 68 ir 58 proc. Endoneuriumo bei epineuriumo kraujagyslių sienelių endotelio laštelėse statistiškai reikšmingo talino, žymėto imunoaukso dalelėmis, tankumo pokyčio tarp kontrolinių, žiurkių, kurioms sukelta cukrinis diabetas, bei nervų augimo faktorių gydytojų gyvūnų nuostata. 

Išvados. Tyrimo duomenimis, egzogeninio nervų augimo faktoriaus vartojimas, sergent diabetine neuropatija, palaikant periferinio nervo pralaidumo barjera, gali būti svarbus sukeliant funkciškai svarbus regeneracinus procesus.

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