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Advanced glycation endproducts in peripheral nerve in type 2 diabetes with neuropathy

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Abstract Advanced glycation endproducts (AGE) accumulate over proteins as a consequence of diabetic hyperglycemia, and thus contribute to the pathogenesis of diabetic complications. To improve the understanding of the pathology of diabetic neuropathy, AGE accumulation was analyzed in sural and/or femoral nerves obtained under spinal anesthesia from 8 type 2 diabetic patients with both distal symmetrical polyneuropathy and proximal neuropathy. Pronounced AGE immunoreactivity was detected on axons and myelin sheaths in 90% of diabetic peripheral nerves but not in the control specimen. The intensity of axonal AGE immunopositivity significantly correlated with the severity of morphological alterations ($p<0.005$). AGE localization, demonstrated by immunohistochemical methods, was also present in the endoneurium, perineurium and microvessels. Morphometric analysis of the diabetic peripheral nerve showed perineurial thickening (diabetic vs. control, 15.5±4.9 vs. 6.6±2.1 μm, $p<0.001$), narrowing of the microvessel lumina (66.6±50.5 vs. 579.5±38.4 x10^3 μm^2, $p<0.001$) and significant reduction in the number of preserved axons (3.6±3 vs. 8.9±2.3 per 10^5 μm^2 per area, $p<0.037$). The sera of diabetic patients contained epitope(s) of AGE structure and soluble immune complexes containing AGE moiety. In conclusion, to the best of our knowledge, this is the first study providing evidence for excessive AGE formation on peripheral nerve components, primarily axons, and a significantly higher level of circulating AGE-immune complexes in patients with both distal diabetic polyneuropathy and proximal neuropathy. Humoral immune mechanisms, including the production of anti-AGE autoantibody, may potentially be involved in the development of structural abnormalities described in this report.

Key words Diabetic polyneuropathy • Advanced glycation endproducts • Peripheral nerve • Immunohistochemistry

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Introduction

Diabetic neuropathy is one of the most common long-term complications of diabetes, affecting as many as 60% of the diabetic population. In spite of ample research, the pathogenesis of diabetic neuropathy has not yet been fully understood. The proposed mechanisms include cellular damage in the peripheral nervous system, altered neuronal metabolism, microvascular abnormalities, slowing of axonal transport mechanisms, deficiency of neurotrophic

substances, and impaired repair capabilities [1, 2]. Neuropathy has been described in patients with type 1 and type 2 diabetes. The key role of hyperglycemia has received support from large prospective studies such as DCCT and UKPDS [3–5]. The major metabolic changes caused by hyperglycemia include an increased polyol pathway flux, elevated oxygen free radical formation, and advanced glycation process [1, 6]. Although a number of biochemical changes have been described in diabetic nerve, the exact sequence of events leading from insulin deficiency and accompanying hyperglycemia to the functional and structural manifestations characterizing clinical neuropathy has not yet been fully elucidated.

Nonenzymatic glycation leading to advanced glycation endproduct (AGE) formation is one of the important biochemical pathways involved in the development of long-term complications of diabetes [7], with a potential role also in diabetic neuropathy [8–10]. Products of advanced glycation are generated from series of reactions involving the attachment of reducing sugars or oxoaldehydes to protein [11]. The final reaction step, which is irreversible, gives rise to AGE products. AGEs are a heterogeneous group of molecules that accumulate in plasma and tissues. These toxic macromolecules interact with specific receptors and elicit pleiotropic response. There are several possible pathways by which AGE formation could be directly pathogenic. Extracellular AGEs induce cross-linking of proteins, modification of matrix components, and may interfere with cellular adhesion and interaction. Intracellular AGE may induce alteration of DNA and nuclear proteins, and may alter protein transport and function.

Extensive investigations of the advanced glycation process in human diabetic neuropathy are generally limited by analitical tissue unavailability. To improve the understanding of the pathophysiology of diabetic neuropathy, the human diabetic peripheral nerve was investigated by examining AGE modifications in situ. Biopsy specimens of the sural and/or femoral nerve from type 2 diabetic patients with distal symmetrical polyneuropathy and proximal neuropathy were examined. This report provides detailed immunohistologic description, however, with a limited body of relevant clinical data.

Patients and methods

The study included eight type 2 diabetic patients with both distal polyneuropathy and proximal neuropathy, mean age 62.1±6 years, diabetes duration of 1–15 years, HbA_{1c} 7.9±1.2%, distal polyneuropathy duration of 2.5 years, and proximal neuropathy duration of 4.2±1.8 months, pelvifemoral weakness MRC grade 15.9±3.5/30, pain – visual analogue scale 7.8±1.6/10 above the knee. Clinical data on the study patients are presented in Table 1. The presence of microangiopathy was defined as a positive history of retinopathy (n=4), nephropathy (n=3) or neuropathy (n=8). Simple or proliferative diabetic retinopathy was diagnosed ophthalmoscopically by an ophthalmologist, and graded according to the European protocol. The diagnosis of diabetic neuropathy was made or ruled out by neurologic examination (subjective discomfort, clinical signs of disease, eletromyography [EMG]), according to international classification. Renal impairment was assessed on the basis of complete clinical examination. Normal renal function was defined as serum creatinine concentration <120 μmol/l; urinary albumin excretion <30 mg/24 h, and creatinine clearance >0.90 ml/s. Microalbuminuria was considered at values of ≥30 mg/24 h and ≤200 mg/g creatinine; macroalbuminuria was diagnosed at >200 mg/g creatinines, and overt proteinuria (>0.5 g/24 h) was defined as elevated serum creatinine (>130 μmol/l) and creatinine clearance <0.80 ml/s. Macrovascular disease (n=4) was considered to be present if there was a history of myocardial infarction, coronary artery disease, stroke or peripheral arterial disease.

Specimens of sural and femoral nerve were obtained by biopsy in spinal anaesthesia. Longitudinal and transverse 2- to 5- mm nerve specimens were embedded in tissue cryopreservative (Cryomatrix) and frozen in liquid nitrogen. Thus prepared, the specimens were stored at -75° C until analysis. The Vuk Vrhovac University Clinic Ethics Committee approved the study protocol, and informed consent was obtained from each study patient before entering the study.

Neurophysiology

Prior to biopsy, electrophysiological assessment of the lower limb was performed with a Dantec Counterpoint EMG system using surface electrodes. Nerve conduction velocity was measured proximally on the femoral nerve and distally on the sural nerve. Skin temperature was maintained over 32° C using a surface heater.

Tissue sampling and histologic examination

Ten peripheral nerve cylindrical specimens, 3–5 mm in length and <1 mm in diameter, were analyzed. Ten peripheral nerve specimens were obtained by biopsy of the sural and/or femoral nerves of type 2 diabetic patients. Control specimens were obtained from a healthy, morphologically intact sural nerve from a healthy individual, used as allograft to bypass a peripheral nerve defect caused by gunshot wound; a part of the allograft was referred for histologic analysis to assess the viability of its structures. Frozen transverse never sections previously made by a cryomicrotome were fixed in 10% formalin, then dehydrated in a series of increasing ethanol concentrations (50%–100%), xylene, and embedded in paraffin blocks. The paraffin-embedded nerves were cut transversally, perpendicularly to longitudinal axis, into 5-μm sections. One section was stained with hematoxylin-eosin (HE), and the other was immunohistochemically stained by use of PAP technique. Immunohistochemical staining of paraffin sections with rabbit antibody against AGEs was done for more precise morphological analysis and positivity distribution in particular parts of the nerve; precise analysis cannot be performed on

frozen sections by the immunofluorescence method. Prior to applying it onto the tissue, primary rabbit polyclonal antibody was diluted in phosphate-buffered saline (PBS) at a 1:30 ratio, and incubated for 45 min. Secondary porcine IgG to rabbit IgG, PAP and diethylaminobenzidine (DAB; Dako, Denmark) were used according to the manufacturer's instructions.

Immunohistochemistry

Nerve specimens were cut to 4–8 μm sections on a cryostar (Leica, Germany) at a temperature of -25° C to -30° C. These sections were applied on microplates previously overlayered with a fixation membrane (PAP pen). Before testing, the sections were allowed to dry in cold air stream and rehydrated in PBS (pH 7.4) for 10 min. The sections were preincubated with proteinase K (0.5 mg/ml) at room temperature to allow for primary AGE antibody binding. After washing with PBS for 30 min (in three fresh portions), the sections were incubated for 30 min with normal rabbit serum (1:10) to inhibit nonspecific binding. After careful washing with PBS at least 3 times for 10 min, the sections were incubated with primary antibody (polyclonal rabbit antibody against AGEs) for 2 h at room temperature. Primary antibody was obtained by rabbit immunisation and prepared for testing by serial dilution at 1:5 to 1:80 with PBS, pH 7.4. After washing with PBS, secondary antibody was added for 30 min at room temperature. Tetramethylrhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG antibody was used as secondary antibody. The labelled antibody was used at a 1:80 dilution for rhodamine or at 1:100 and 1:150 dilutions for fluorescein. After washing, the sections were observed under fluorescence microscope (Opton-Zeiss) with appropriate filters for rhodamine (IF 545) and fluorescein (IF 490).

Analysis of advanced glycation products

The antibodies referred to in our previous reports [12, 13] describing the preparation of AGE antigen, production of antibodies against AGE, and specificity assessment of anti-AGE antibody against a particular antigen were used in the present study. The competitive-type enzyme-linked immunosorbent assay with polyclonal anti-AGE antibodies was used for detection of AGE content in serum samples. The assay utilizes AGE-human serum albumin (AGE-HSA) as a standard. The immunoplate was coated with AGE-HSA antigen. The serum containing an unknown quantity of antigens was incubated together with a constant, known quantity of polyclonal anti-AGE antibody. Solid-phase antigen-antibody complexes were generated based upon the competition between serum AGEs and immobilized AGE antigen. AGE antigen-antibody complexes were detected using (secondary antibody) alkaline phosphatase-conjugated anti-rabbit IgG. A colorimetric signal, which was inversely proportional to the amount of AGEs in serum, is obtained following the addition of chromogenic substrate. Competitive immunoreactivity of the samples was read from the calibration curve and expressed relative to AGE-albumin standard in μgEq/ml. The precision of the method was determined in serum pools from diabetic patients and from normoglycemic control individuals. The within-run coefficients of variability of AGE-ELISA were 7.90% and 7.11%, respectively. Anti-AGE antibodies in human serum were determined by use of blocking ELISA [13]. The presence of immune complexes containing AGE moiety was determined by two independent criteria: (a) serum AGE immune complexes (AGE-IC) were detected by ELISA using an immunochemical bridge; and (b) soluble AGE-IC were precipitated from serum by polyethylene glycol and analysed as previously reported [13]. The within-run C.V. was 8.80% and the between-run was 10.72%. The products of advanced glycation were measured in the serum of diabetic patients (n=8) as well as in the serum of non-diabetic healthy subjects (n=20, control group).

HbA_{1c} measurement

Samples were prepared according to Jeppsson et al. [14]: heparinized blood (600 μl) was incubated for 4 h at 37° C with saline and centrifuged. The supernatant was discarded and the erythrocyte pellet was hemolysed by the addition of H_2O and CCl_4. Separation of cell ghosts was accomplished by centrifugation; the clear supernatant was diluted with malonic buffer and analysed. HbA_{1c} was analyzed using Fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden), on a Mono S HR 5/5 column at pH 5.7, and expressed as percentage of the total amount of hemoglobin with normal range of 3.5% to 5.7%.

Statistical analysis

All morphometric analyses and evaluation of intensity of AGE immunoreactivity were performed in a blinded manner. The analysis of AGE-immunopositivity was semiquantitatively evaluated by the following symbols (-no AGE positivity; ±, borderline AGE positivity; +, weak AGE-positivity; ++, strong AGE positivity), as shown in Table 3. The intensity of immunostaining signals was also numerically scored (-as 0; ±as 1; + as 2 and ++ as 3). Morphological alterations were classified as mild (*=1), moderate (**=2) and severe (***=3). The scores of total morphological changes correlated with the graded AGE-immunostaining intensity. Quantitative analysis of peripheral nerves was performed on transverse sections (Mallory three-chrome staining) with ISSA and SFORM programs (VAMS, Zagreb, Croatia). The statistical methods employed included Mann-Whitney test for comparison of two groups of data, and Spearman's rank correlation coefficients for bivariate analysis. A value of $p<0.05$ was considered to be statistically significant.

Results

We evaluated the presence of advanced glycation endproducts (AGEs) in 8 patients with type 2 diabetes and diabetic neuropathy (Table 1). The patients had a mean age of 62 years (SD=6 years) and mean HbA_{1c} concentration of 7.9% (SD=1.2%). Mean pelvifemoral weakness score was 15.9 (SD=3.5) and mean pain score (VAS) was 7.8 (SD=1.6). The patients had distal symmetrical diabetic

Table 1 Clinical characteristics of 8 patients with type 2 diabetes and diabetic neuropathy

Case	Age, years	Sex	DM duration, years	Treatment	HbA_{1c}, %	Neuropathy	Nephropathy	Retinopathy	Macrovascular disease[a]
1	65	M	11	OHA	8.36	+	–	+	–
2	65	M	8	OHA	7.18	+	+	–	+
3	66	M	15	Insulin	8.57	+	–	+	+
4	61	M	1	OHA	7.60	+	–	–	–
5	57	M	13	Insulin	6.91	+	+	+	–
6	64	F	10	Insulin	10.10	+	–	–	+
7	50	F	3	Insulin	7.27	+	–	+	–
8	69	F	11	Insulin	6.15	+	+	–	+

DM, type 2 diabetes mellitus; *HbA_{1c}*, hemoglobin A1c; *OHA*, oral hypoglycemic agents
[a] History of myocardial infarction, coronary artery disease or peripheral arterial disease

Table 2 Neurophysiological parameters of 8 patients with type 2 diabetes and diabetic neuropathy

Case	PDN duration, months	DSDP		Nerve	NCV, m/s
		Duration, months	Pain		
1	6	48	No	Sural	0.0
2	3	24	No	Sural	35.8
				Femoral	0.0
3	3	60	Yes	Sural	0.0
				Femoral	43.1
4	5	10	No	Sural	40.2
5	5	36	Yes	Sural	0.0
6	5	12	No	Femoral	41.7
7	3	4	No	Femoral	44.5
8	4	24	Yes	Sural	0.0

PDN, proximal diabetic neuropathy; *DSDP*, distal symmetrical diabetic polyneuropathy; *NCV*, nerve conduction velocity (motor NCV in femoral nerve, and sensory NCV in sural nerve)

Table 3 Semiquantitative analysis of advanced glycation endproduct (AGE) immunopositivity and morphological alteration in biopsied diabetic peripheral nerves and control sural nerve

		AGE immunopositivity[a]					
Case	Nerve	Perineurium	Endoneurium	Myelin sheath	Axons	Blood vessels	Morphological alteration
1	Sural	–	±	+	+	–	Moderate
2	Femoral	–	–	±	–	–	Mild
	Sural	++	++	++	++	+	Severe
3	Sural	–	–	–	±	+	Mild
	Femoral	+	+	+	++	+	Moderate
4	Sural	–	+	+	+	+	Severe
5	Sural	±	±	+	++	+	Severe
6	Femoral	+	±	+	–	+	Mild
7	Femoral	–	–	+	±	–	Mild
8	Sural	–	±	++	++	+	Moderate
Control	Sural	–	–	–	–	–	None

[a] AGE immunopositivity scores: - none; ± borderline; + weak; ++ strong

polyneuropathy for a mean of 2.5 years and proximal diabetic neuropathy for 3–6 months. Clinical picture was predominated by pronounced pain with hyperesthesia and dysesthesia in upper legs, atrophy of upper leg musculature, loss of pelvifemoral muscle strength, and weight loss.

Neurophysiological parameters are presented in Table 2.

Nerve pathohistology

Transverse sections of ten peripheral nerves (6 sural and 4 femoral) from 8 type 2 diabetic patients showed similar morphological changes of varying severity (mild, moderate or severe; Table 3). Some 4–6 fascicles were observed in all the nerves examined. In all diabetic patients, the perineurium and vascular walls were markedly thickened and hyalinated, with occasional endothelial proliferation and microvascular thrombosis. Proliferation of the endoneurial connective tissue and a reduced number of nerve fibers were observed. Degeneration in the myelin sheath region and absence of axons varied from mild through moderate and severe.

Mild pathological alterations were observed in 4 of 10 peripheral nerves from diabetic patients (Table 3). These showed focal reduction of nerve fibers, myelin degeneration in one or two fascicles, thickening of the perineurium and endoneurium, and thickening and hyalinization of blood vessels. Immunhistochemical staining for AGE in these samples yielded weak or borderline myelin sheath positivity in 3 of 4 cases. In 2 cases, weak AGE positivity was also present in the axonal region and blood vessels. Borderline or weak positivity in the endoneurium and perineurium was only present in one case with mild pathologic alterations (Fig. 1).

Moderate pathologic alterations were detected in 3 of 10 peripheral nerves from diabetic patients. These were characterized by a reduced number of nerve fibers and manifest myelin degeneration in more than half of the fascicles of each nerve sample, thickening of the perineurium and endoneurium, and thickening and hyalinization of the blood vessels. Either weak or strong AGE positivity was present in the myelin sheath and axonal region of all 3 nerve samples (Table 3). AGE immunopositivity of borderline or weak intensity was also present in the endoneurium. A weak vascular immunopositivity was observed in 2 samples, and in only one perineurium of these 3 nerves (Table 3).

In the remaining 3 peripheral nerve samples, all fascicles on transverse nerve sections were diffuse, with a severe loss of nerve fibers and myelin degeneration. The perineurium and endoneurium as well as vascular walls were markedly thickened and frequently hyalinated. AGE immunopositivity was present in the myelin sheath, axonal region, endoneurium and blood vessels of all samples (Table 3). The intensity of AGE positivity in the axons and myelin was strong or weak, while microvascular immunopositivity was weak. In the endoneurium, AGE immunopositivity ranged from borderline to strong, while in the perineurium it was absent, bordeline or strong (Table 3, Fig. 2).

In 9 of 10 peripheral nerves from diabetic subjects, AGE immunopositivity was present in the myelin sheath and axons. The intensity of axonal positivity significantly

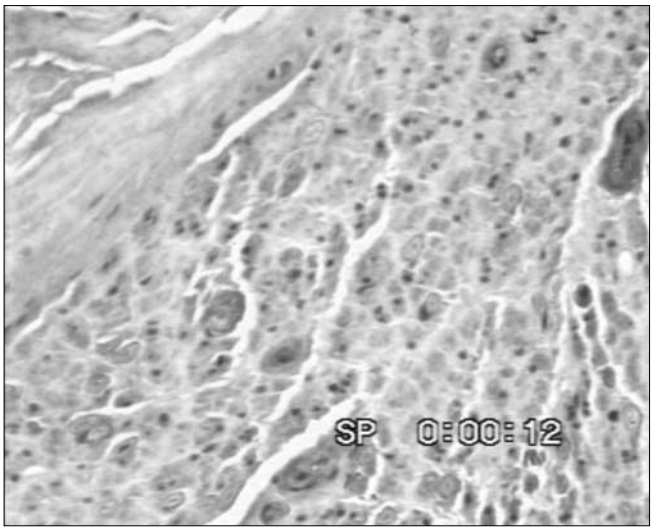

Fig. 1 Transverse section of diabetic sural nerve (case 5) shows markedly thickened and hyalinated perineurium with no AGE immunopositivity. Vascular walls as well as some axons and myelin sheath are thickened and hyalinated with strong AGE positivity (PAP 200x)

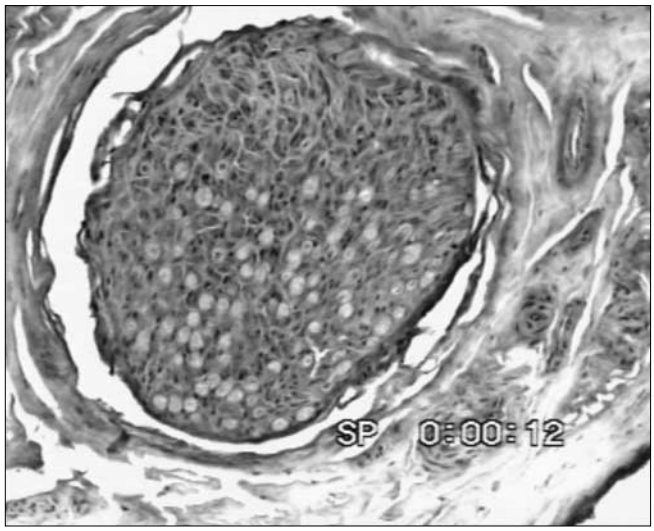

Fig. 2 Transverse sections of sural nerve (case 2) shows thickened and hyalinated perineurium with strong AGE-immunopositivity. Vascular walls and endoneurium are thickened and hyalinated with strong AGE positivity. In almost all preserved axons, AGE positivity is strong, while in myelin sheath it is weaker (PAP 100x)

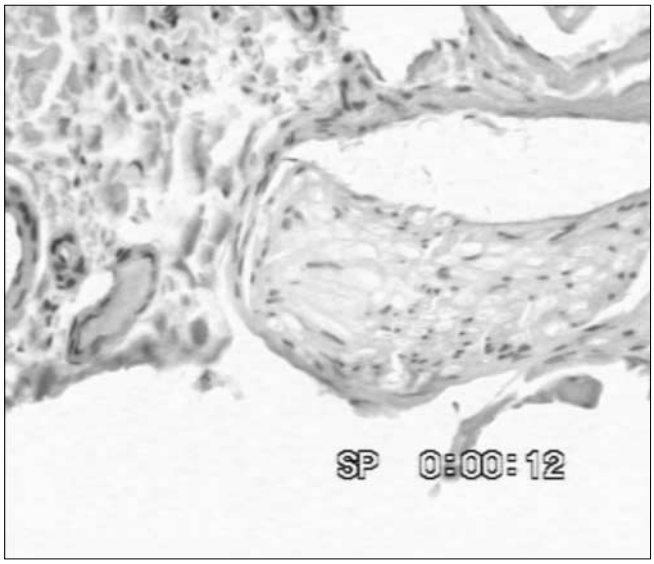

Fig. 3 Transverse section of peripheral nerve shows regular morphology with no AGE immunopositivity (PAP 200x)

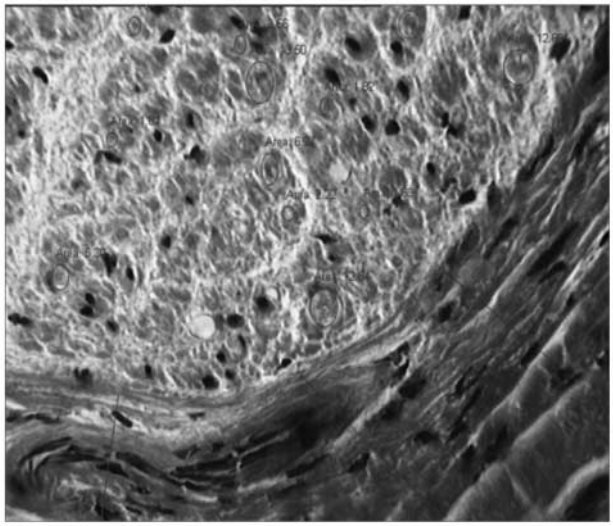

Fig. 4 Quantitative analysis of axon surface in transverse sections of peripheral nerve of diabetic patients (400x, Mallory three-chrome staining, ISSA and SFORM programs)

correlated with the severity of morphological alteration in the nerves ($p<0.005$). The same correlation was observed in the endoneurium of 7 of 10 nerve samples with lower intensity of AGE immunopositivity ($p<0.008$). Microvascular immunopositivity of a weak intensity, which yielded no correlation with the severity of morphological alteration in the nerves of diabetic patients, was found in 7 of 10 nerve samples. Perineurial AGE immunopositivity was detected in only 4 nerve samples, and the intensity of reaction did not correlate with the severity of morphological alteration. The control sural nerve was free from pathologic changes, and AGE immunopositivity was absent (Fig. 3).

Morphometric analysis of the peripheral nerves was performed on transverse sections (Mallory three-chrome staining) (Fig. 4). Comparison of diabetic and control nerves showed perineurial thickening (15.5±4.9 vs. 6.6±2.1 μm, $p<0.001$), narrowing of microvascular lumen (66.6±50.5 vs. 579.5±38.4 x10^3 μm^2, $p<0.001$), and significant reduction in the number of intact axons in diabetes (3.6±3 vs. 8.9±2.3 per 10^5 μm^2 area, $p<0.037$). Femoral nerve samples contained a slightly greater number of preserved axons than sural nerve samples; however, the difference was not statistically significant.

Immunofluorescence analysis

Indirect immunofluorescence with the polyclonal AGE antibody revealed formation of AGE products in tissue sections of sural and femoral nerves from diabetic patients. Most specimens showed positive reaction to AGE, recorded in the endoneurium, perineurium and periaxonal area. The intensity of fluorescence, indicating the severity of AGE lesions, differed among the specimens tested; thus they were difficult to compare. Analysis of each specimen showed the intensity of fluorescence to vary among different cellular structures. Almost all specimens of the sural and femoral nerves expressed AGE positivity, which was recorded in at least one of the above mentioned cell segments. Out of 10 specimens analyzed, the immunofluorescence was obscure in only one specimen of the sural nerve (specimen no. 4). The specimen was found to have poorly preserved tissue structure, which may have been the reason for unclear or negative result. AGE accumulation in sural nerve samples was observed to yield a more diverse pattern (Fig. 5a). In most of these specimens, positive findings were recorded in the endoneurium, perineurium and around axons, however, differing according to the intensity of fluorescence from specimen to specimen. In femoral nerve specimens, the distribution of AGE immunopositivity was more uniform and was mostly recorded in the periaxonal area (Fig. 5b).

Plasma AGE immunoreactivity

The total level of circulating AGEs determined by competitive ELISA was significantly higher in serum of 8 patients with diabetic neuropathy than of 20 nondiabetic healthy subjects (38.6±6.9 vs. 25.1±7.2 μEq/ml, $p<0.0009$). The presence of antibodies against AGE epitope(s) was detected in all serum samples examined, with a higher AGE antibody titer recorded in the group of diabetics (58.7%±20.2% vs. 17.4%±15.4% inhibition, $p<0.0001$). Higher serum levels of circulating AGE-IC were demonstrated in diabetic patients as compared with control subjects (5.9±2.4 vs. 3.39±1.1

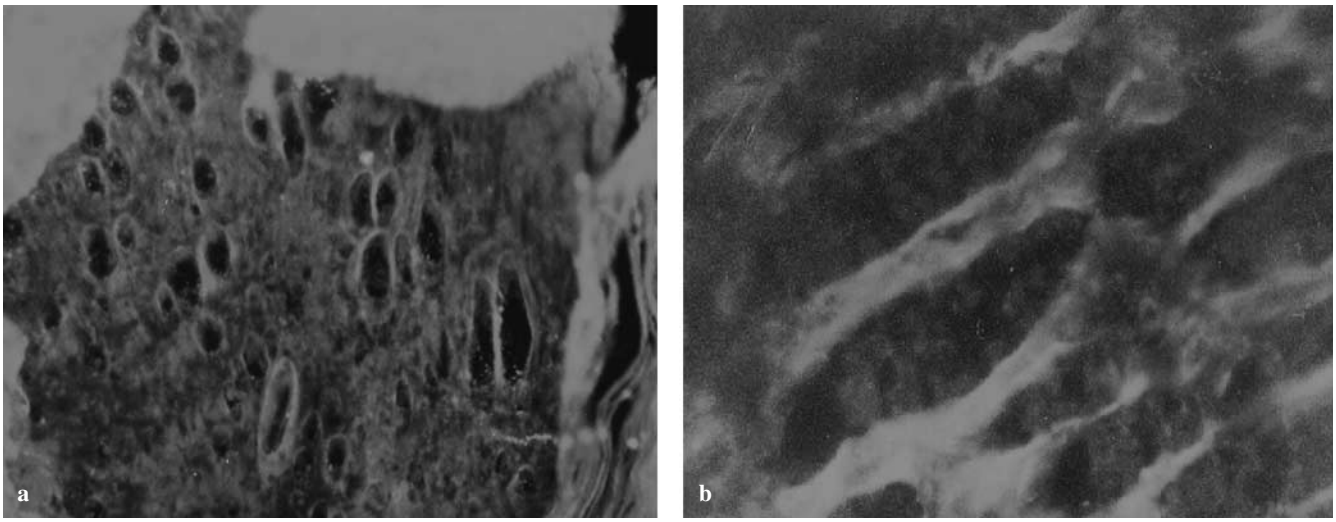

Fig. 5a, b *Immunofluorescence analysis of AGE immunopositivity with rabbit polyclonal primary antibody and fluorescein-labelled secondary antibody.* **a** AGE deposits on the transverse section of sural nerve (case 1). Positive AGE reaction was recorded all over the section surface, but was unevenly distributed in different section segments. High AGE positivity was found in the perineurium and in the area of myelin sheaths. A reaction of lower and uneven intensity was recorded in the endoneurium (x160). **b** Femoral nerve section (case 7) revealed clearly positive AGE reaction in the area of myelin sheaths. AGE positivity was absent in the preserved axonal cylinders (x400)

μEq/ml, $p<0.0004$). There was no significant correlation between serum AGE content and AGE-IC with either actual HbA_{1c} values or those assayed over the previous year.

Discussion

The patients with type 2 diabetes included in the study exhibited clinical signs and symptoms of distal symmetrical diabetic polyneuropathy of a mean duration of 2.5 years. This complication of diabetes develops in some 50% of patients in the first ten years of disease. It is of a gradually progressive course that correlates with diabetes duration and glycemia control. In the study patients, distal symmetrical diabetic polyneuropathy was superimposed by proximal diabetic neuropathy, a rare but extremely troublesome syndrome distinct from the former [15]. As differentiated from it, proximal diabetic neuropathy has an acute onset, frequently of a fulminant course, thus leading abruptly to a significant neurologic disability.

Upon neurological examination, the patients underwent femoral and sural nerve electroneurography. Biopsy specimens of these nerves were obtained under spinal anesthesia. The nerve samples available for analysis were small, thus they were frozen as a whole in a cutting medium. Because of prefreezing, the tissue was not suitable for ultrastructural analysis. Morphometric analysis of the diabetic peripheral nerve showed thickening of the perineurium and narrowing the microvessel lumen with change in the axon density. We demonstrated localization of AGE immunoreactivity in the peripheral nerve of diabetic patients but not in the control specimen.

The overall intensity of AGE immunopositivity as a total sum of the scores of tissue components showed that the most intensive positive reaction was detected in the area of axons and myelin sheaths, whereas lower scores of AGE immunoreactivity were recorded in the endoneurium, perineurium, and microvessel walls. The intensity of total morphological changes correlated significantly with the intensity of AGE immunoreactivity in axonal components, suggesting that structural changes of the axon may be related to the reduction of axonal conductivity and impulse conduction impairments, thus contributing to functional events. The neurophysiological findings in all study patients indicated significant retardation of the motor and sensory conductivity of the nerves analyzed, with very low amplitudes of the muscular and neural responses. Excessive accumulation of AGE content in the endoneurium could be explained by the fact that endoneurium is rich in collagenous connective tissue. Collagens are abundant in lysine and hydroxylysine NH_2 residues, and therefore collagen is highly susceptible to glycation [16, 17]. Abnormally glycated collagen in the endoneurium of the nerve trunks might act as a physical barrier to elongation of the axonal sprouts. Additionally, it is difficult to separate the effects of glycation on the axonal cytoskeleton from glycation of the extracellular matrix because they are interrelated. If connection between the axon and its end tissues were damaged by glycation, this could alter transport functions.

There have been reports on glycation and AGE accumulation in the myelin fraction of peripheral nerve [8, 18, 19]. In the present study, we observed AGE immunoreactivity in myelin sheaths. However, we found no correlation

between the index of total morphological changes and index of myelin AGE immunoreactivity. This observation is open to speculation. Namely, the myelin AGE products can act as ligands for the uptake and degradation by macrophages via scavenger or AGE receptors [20]. The interaction between peripheral nerve AGE myelin and AGE cell surface receptor might contribute to the segmental demyelinization by a variety of mechanisms. However, the presence of AGE-modified myelin in experimental as well as in human diabetes demonstrated in our study, appears to be less relevant for distal diabetic neuropathy than the glycation of axonal cytoskeletal proteins. Diabetic polyneuropathy is a distal, length-related axonal neuropathy where axonal damage probably plays a major role. AGE formation on axonal proteins may influence their turnover, alter phosphorylation and dephosphorylation, and affect molecular architecture and transport mechanisms, which may contribute to axonal atrophy and degeneration.

In the present study, the serum of both diabetic patients and nondiabetic individuals contained autoantibodies to epitope(s) of AGE structure. However, a higher AGE-antibody titer was observed in diabetic subjects. Additionally, we demonstrated the presence of circulating immune complexes containing AGE moiety, predominantly in diabetic serum. Immune complexes may form consequentially to the association of AGE-modified proteins and antibodies against AGE epitope(s). As many body proteins are AGE modified, they may become a substrate for the formation of insoluble immune complexes formed at the tissue level. Therefore, we speculate that excessive glycated components of diabetic peripheral nerve are a substrate for the association with anti-AGE antibodies in situ [13, 21, 22]. All patients included in the present study had proximal diabetic neuropathy. There is a growing body of evidence suggesting the potential involvement of humoral immune mechanisms in the generation of neural structural abnormalities, or of some of them in this particular type of neuropathy.

In conclusion, the results presented provide evidence that nonenzymatic glycation leading to AGE formation primarily involves axonal proteins and endoneurium. We are inclined to believe that the lack of correlation between the index of total morphological alterations and index of AGE immunoreactivity on the myelin protein may have resulted from the fact that AGE myelin probably was partially internalized via AGE receptor. Also, humoral immune mechanisms, including the production of anti-AGE autoantibody, may potentially be involved in the development of structural abnormalities described in this report. However, because of the small number of specimens and diversity of lesions, no regularity related to morphological changes and AGE immunopositivity could be definitely established. It can only be emphasized that we confirmed the peripheral nerve structure involvement by AGE modification. Additional studies are necessary to elucidate the mechanisms by which the process of advanced glycation could be involved in the pathogenesis of diabetic neuropathy.

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