Relationship Between Electroneurographic Changes and Serum Ubiquitin Levels in Patients With Type 2 Diabetes

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OBJECTIVE — The aim of the present study was to investigate any relationship between serum ubiquitin levels and electroneurographic changes in peripheral nerves for patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — The study involved 34 patients (19 men, 15 women; mean age 46 ± 13 years) with type 2 diabetes. Serum ubiquitin values were measured by sandwich enzyme-linked immunosorbent assay. Measurement of nerve conduction velocity (NCV) was performed on three motor (median, tibial, and peroneal) and three sensory (median, ulnar, and sural) nerves. The value of motor compound muscle action potential (CMAP) was obtained from the sum of median, tibial, and peroneal motor nerve amplitudes, and sensory compound nerve action potential (CNAP) was computed as the sum of median and ulnar sensory nerve amplitudes.

RESULTS — Patients with diabetes were divided into three groups: group 1 (n = 8) had normal electroneurography results, group 2 (n = 8) had slowed NCV, and group 3 (n = 18) had low values of motor CMAP and/or sensory CNAP as well as slowed NCV. Mean ubiquitin level in group 3 (20.4 ± 2.9 ng/dl) was significantly higher than that in group 1 (11.2 ± 1.1 ng/dl, t = 11.5, $P < 0.0001$) and group 2 (13.2 ± 2.7 ng/dl, t = 5.9, $P < 0.0001$). Serum ubiquitin levels were inversely correlated with motor CMAP ($r = -0.68$) and sensory CNAP ($r = -0.61$) values.

CONCLUSIONS — The results of this study indicate that there could be a relationship between the diminished amplitudes of axons of the peripheral nerve and the increase in serum ubiquitin levels in patients with type 2 diabetes. Further studies are required to confirm this relationship.

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Ubiquitin is composed of 76 amino acids and is present in the cytoplasm and nucleus of eukaryotic cells. The protein can be covalently conjugated to cellular proteins by the enzymes of the ubiquitin conjugating system, which plays a role in selective protein degradation. Ubiquitin also plays a variety of regulatory roles in cellular processes, including stress response, cell cycle, gene expression, and apoptosis. The presence of ubiquitin within inclusion bodies was noted in neurofibrillary tangles and dystrophic neuritis associated with a variety of neurodegenerative and muscular diseases (1,2). These inclusion bodies were also detected in motor neuron disease, giant axonal neuropathy (3), brain ischemia (4,5), and cancers (6). Additionally, increased ubiquitin concentration in body plasma was detected in patients with chronic renal failure, acute viral hepatitis, and amyloidosis (1,7).

Diabetes is one of the diseases that activates the ubiquitin proteasome pathway, and it has been proposed that activation of this pathway is responsible for the wasting of muscle because of insulinopenia. That the ubiquitin proteasome pathway is especially activated in some neuronal diseases and during insulinopenia suggests that ubiquitin may play a role in diabetic neuropathy (8). In this study, the relationship between serum ubiquitin levels and motor and sensory amplitudes and nerve conduction velocity (NCV) was examined in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

— The study involved 34 patients (19 men, 15 women) with type 2 diabetes. Physical examination assessed peripheral sensation (light, touch, position, temperature, and pin-prick) and deep-tendon reflexes. In the absence of other known causes of neuropathy, abnormal findings in two of these three categories (neuropathic symptoms, sensory deficit, or impaired reflexes attributable to a distal symmetric polyneuropathy) constituted a definite abnormal neurologic examination indicating clinical neuropathy (9). All subjects were taking oral hypoglycemic agents. None of the patients took insulin. Duration of diabetes was between 5 and 25 years. Subjects who had macroalbuminuria (albumin excretion rate >200 μg/min or >300 mg/day) (10) and higher levels of serum creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) than normal were

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Abbreviations: ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; CMAP, compound muscle action potential; CNAP, compound nerve action potential; MCV, motor nerve conduction velocity; MMCV, median MCV; MSCV, median SCV; NCV, nerve conduction velocity; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; PGP, protein gene product; PMCV, peroneal MCV; SCV, sensory nerve conduction velocity; SSCV, sural SCV; TMCV, tibial MCV; USCV, ulnar SCV.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

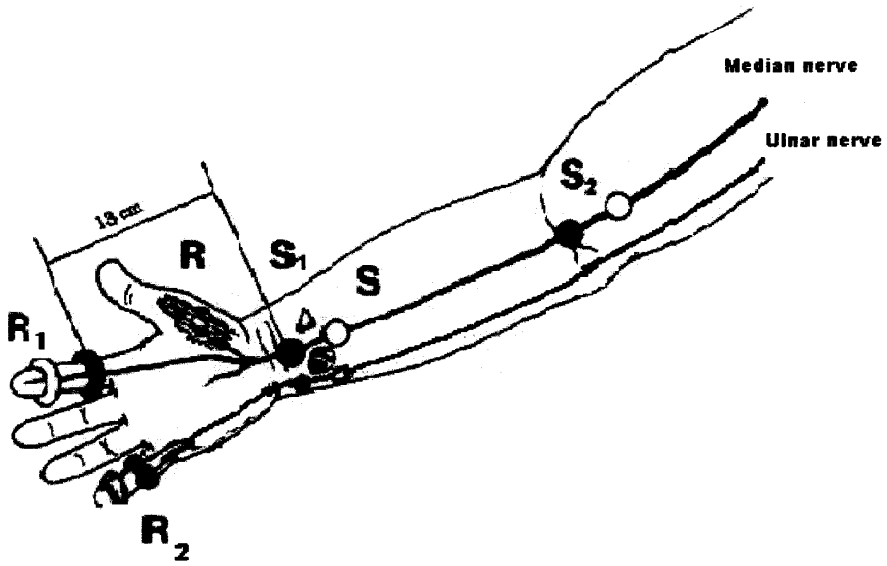

Figure 1—*Motor and sensory nerve conduction of median and ulnar nerves. Median and ulnar sensory nerve condition (antidromic method): for recording, active ring electrodes are placed on the midportion of the second (R_1) and fifth fingers (R_2) and reference electrodes on the midportion of the middle phalanx of the same fingers (2.5 or 3.0 cm from the active electrodes). For stimulation (S), the median and ulnar nerves are stimulated with an active surface electrode at the wrist (S_1 and S_2, respectively). For measurement, distal latencies are measured from the stimulus onset to the peak of the negative deflection of sensory CNAP. The amplitudes of sensory CNAP are measured from peak to baseline. Median motor nerve conduction: for recording, surface electrodes are placed on the thenar muscle. For measurement, latency measurement is conventional. The amplitude motor CMAP is measured from peak to peak.*

excluded from the study (the upper limits of normal were 1.2 mg/dl for creatinine and 40 U/l for AST and ALT). Fundoscopic examination was evaluated according to the following classification (11): *1*) no signs of diabetic retinopathy, 2) nonproliferative diabetic retinopathy (NPDR), or 3) proliferative diabetic retinopathy (PDR). Fundoscopic examination was normal in 5 subjects, 10 subjects displayed NPDR, and 11 subjects exhibited PDR.

To compare ubiquitin levels, 25 healthy control subjects (10 men, 15 women) were included in the study. HbA_{1c} was measured using a routine biochemical test. Ubiquitin levels were measured in both the diabetic and control groups. Electroneurographic measurements were performed only in diabetic subjects.

Ubiquitin assay procedure

Serum ubiquitin values were measured by sandwich enzyme-linked immunosorbent assay. For the ubiquitin levels of patients, 3 ml blood was taken, and serums were separated without hemolysis. Commercially available anti-ubiquitin antibody, purified ubiquitin protein, and peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins were purchased from Sigma (U5379, U6253, and A9044, respectively; Sigma, St. Louis, MO). Monoclonal anti-ubiquitin antibody was provided by R. Layfield (Nottingham University, Queens Medical Center, U.K.).

Maxisorp plates (catalog number 469949; Nunc, Roskilde, Denmark) were passively coated at 37°C for 24 h with polyclonal anti-ubiquitin antibody (200 µl/well). After washing with washing buffer (distilled water containing 0.5 ml/l Tween 20), 100 µl of different purified ubiquitin protein concentrations (concentration range 1–30 ng/ml) and 100 µl of serum samples from the control and diabetic patients were added to the wells. Later, 100 µl monoclonal anti-ubiquitin antibody (1:750 dilution) was added to each well. We then added to each well 100 µl of peroxidase-labeled anti-mouse immunoglobulin antibody (1:500 dilution). After 4 h incubation and washing 10 times with 300 µl of washing buffer, we added to each well 200 µl of substrate solution (0.1% wt/wt ABTS [2,2 azino-bis, 3-ethylbenzthiazoline-6-sulfonic acid] and 0.003% vol/vol H_2O_2 [100 volumes] in citrate/phosphate buffer, pH 4). After incubation for 15 min, the absorbance of the plates was read at 405 nm with a spectrophotometer. The color change was proportional to the amount of antigen in the test solution.

Electroneurography

Figures 1, 2, and 3 indicate the information concerning electroneurographic assessment methods carried out in this study. Measurement of motor nerve conduction velocity (MCV) was performed on median (MMCV), tibial (TMCV), and peroneal (PMCV) nerves. Measurement of sensory nerve conduction velocity (SCV) was done on median (MSCV), ulnar (USCV), and sural (SSCV) nerves. The value of motor compound muscle action potential (CMAP) was obtained from the sum of median, tibial, and peroneal motor nerve amplitudes, and sensory compound nerve action potential (CNAP) was computed as the sum of median and ulnar sensory nerve amplitudes (12). The measurements were performed by a device for electroneurography (Medelec Premier Plus; TECA, New York).

In our electrophysiology laboratory, cutoff values for motor CMAP and sensory CNAP were considered to be 6 mV and 10 µV, respectively. Cutoff values for MMCV and MSCV were set as 50 and 40 m/s, respectively, for other sensory and motor nerves studied.

Unpaired Student's *t* test, analysis of variance (ANOVA), Fisher's exact test, Pearson's χ^2 test, and Pearson's correlation tests were used for statistical analyses (SPSS 6.1). A *P* value of <0.05 was the criterion for significance.

RESULTS — Sex distributions (male/female) were similar for diabetic patients (19/15) and control subjects (15/10) (Pearson χ^2 test, $\chi^2 = 0.1$, $P > 0.05$). Mean serum ubiquitin levels were 16.5 ± 4.9 ng/ml for the diabetic group and 9.2 ± 2.2 ng/ml for the control group (unpaired Student's *t* test, $t = 9.8$, $P < 0.0001$).

Patients with diabetes were divided into three groups: patients in group 1 (n = 8) had normal electroneurography results, those in group 2 (n = 8) had slowed NCV, and those in group 3 (n = 18) had low values of motor CMAP and/or sensory CNAP as well as slowed NCV. SSCV could be obtained for patients in group 1, but not for those in groups 2 and 3 (Table 1).

Male-to-female ratios by group were 4/4 in group 1, 4/4 in group 2, and 10/8 in group 3 (Fisher's exact test, $P > 0.05$). The mean age was 55.6 ± 9.8 years in group 1, 55.3 ± 3.2 years in group 2, and 52.8 ± 3.5

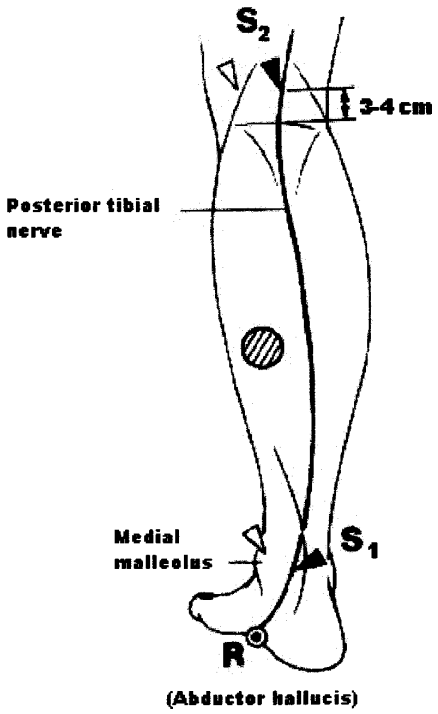

Figure 2—*Motor nerve conduction of the tibial nerve. For recording (R), a surface electrode is placed on the abductor hallucis muscle. For stimulation, the tibial nerve is stimulated distally at the ankle above the flexor retinaculum (S_1) and proximally at the popliteal fossa (S_2). For measurement, latency measurement is conventional. Amplitude is measured from peak to peak.*

years in group 3, and the differences among the groups were not statistically significant (ANOVA, $F = 0.4$, $P > 0.05$). HbA_{1c} values were 9.3 ± 2.5% in group 1, 10.0 ± 1.5% in group 2, and 10.7 ± 2.6% in group 3, and there was no significant difference among these groups (ANOVA, $F = 1.0$, $P > 0.05$).

We detected a statistically significant difference among the groups for serum ubiquitin concentration (11.2 ± 1.1, 13.2 ± 2.7, and 20.4 ± 2.9 ng/dl for groups 1, 2, and 3, respectively; ANOVA, $F = 43.7$, $P < 0.0001$).

Ubiquitin levels in group 3 were significantly higher than those in group 1 (unpaired Student's t test, $t = 11.5$, $P < 0.0001$) and group 2 ($t = 5.9$, $P < 0.0001$). There was no significant difference between groups 1 and 2 in terms of serum ubiquitin levels ($t = 1.9$, $P > 0.05$).

There was also a statistically significant difference among group 1 (7.6 ± 2.3 years), group 2 (13.9 ± 3.3 years), and group 3 (11.6 ± 4.6 years) in terms of mean diabetes duration (ANOVA, $F = 5.2$, $P < 0.05$). Duration of diabetes in both groups 2 and 3 was longer than that in group 1 (unpaired Student t test, $t = 4.4$, $P < 0.01$, and $t = 2.3$, $P < 0.05$, respectively). No significant difference was detected between groups 2 and 3 in terms of diabetes duration.

Serum ubiquitin levels were inversely correlated with motor CMAP and sensory CNAP values ($r = -0.68$, $P < 0.0001$, and $r = -0.61$, $P < 0.001$, respectively). Neither sensory CNAP nor motor CMAP values were correlated with the factors that can affect the measurements of NCV, such as age ($r = -0.14$ and -0.07), HbA_{1c} ($r = -0.06$ and -0.08), and diabetes duration ($r = -0.08$ and -0.03).

CONCLUSIONS — At present, little is known about the mechanisms underlying abnormalities in diabetic neuropathy. However, available clues indicate that both metabolic and ischemic damages to nerves are possible factors, including increased protein glycation, accumulation of polyols, altered lipid metabolism, decreased *myo*-inositol content, abnormal Schwann cell function, microangiopathy resulting in ischemia, and lack of neurotrophic factors (13,14). Plasma cell adhesion molecules (15) and deficiency of some neurotropins (16) could play a role in the progression and development of diabetic polyneuropathy, and additionally, autoimmune destruction may contribute to this process (17).

In our study, serum ubiquitin levels were higher in diabetic patients with low motor and sensory amplitudes and slow conduction velocities compared with patients with normal conduction measurements. This inverse relationship was significant ($r = -0.61$, $P < 0.01$, for sensory amplitudes, and $r = -0.68$, $P < 0.0001$, for motor amplitudes). On the other hand, the significant decrease in protein gene product (PGP) 9.5, an enzyme involved in the deconjugation of ubiquitinated proteins and one of the ubiquitin COOH-terminal hydrolases, was immunohistochemically detected in skin biopsies before any change in conventional neurophysiological tests in patients with diabetes (18,19). We might speculate that there may be inadequacies in the deconjugation of ubiquitinated nerve fibers and deterioration in the functions of ubiquitinated nerve fibrils with the decrease of PGP 9.5 activity in diabetic neuropathy.

As known, ubiquitination of proteins in neurons is a process that protects those neurons from chronic attacks of damaged neural proteins and organelles. Pathologically activated protein ubiquitination in the late stage of cell damage can cause neuronal death as a part of the irreversible catabolic process (1,3). Lanteri et al. (20) reported increased content of ubiquitin in motor neurons of rats in which axonal damage had been produced. Savedia and Kierna (21) also suggested that synthesis of ubiquitin mRNA in motor neurons increased after axotomy. Taking into account that diabetic neuropathy is mainly axonal, the explanation for neuropathy may be a catabolic process in the peripheral nerve, when the early protective effect of ubiquitination has vanished (22). In this context, elevated serum ubiquitin levels in patients who have diminished amplitudes of axons might be related to increased neuronal catabolism rather than axonal regeneration.

In our study, there was no significant difference in HbA_{1c} among the three groups of patients ($P > 0.05$). On the other hand, duration of diabetes for patients in groups 2 and 3 was significantly longer than duration in group 1 ($P < 0.05$). But neither HbA_{1c} nor duration of diabetes exhibited a correlation with motor and sensory amplitudes. Haimanot and Abdulkadir (23) also sug-

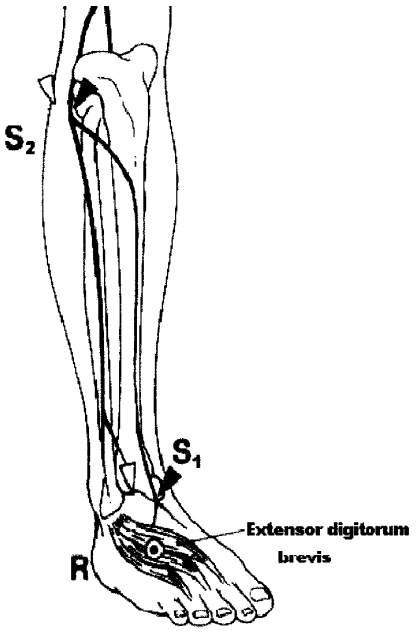

Figure 3—*Motor nerve conduction of the peroneal nerve. For recording (R), a surface electrode is placed on the extensor digitorum brevis muscle. For stimulation, the peroneal nerve is stimulated proximally at the fibular head (S_1) and distally over the anterior ankle (S_2). For measurement, latency measurement is conventional. Amplitude is measured from peak to peak.*

Table 1—*Clinical and electroneurographic findings from diabetic patients*

	Group 1	Group 2	Group 3
n	8	8	18
MMCV (m/s)	53.5 (51.0–56.0)	40.5 (36.0–46.1)	39.3 (32.3–47.0)
MSCV (m/s)	52.5 (51.0–55.0)	43.0 (37.0–46.1)	40.6 (35.0–48.0)
USCV (m/s)	42.0 (41.0–44.0)	34.3 (32.0–38.0)	35.5 (28.6–39.0)
TMCV (m/s)	43.0 (41.0–46.0)	36.5 (34.2–38.0)	36.0 (33.0–39.0)
PMCV (m/s)	43.0 (42.0–44.0)	36.5 (34.3–38.0)	35.5 (32.0–38.0)
SSCV (m/s)	42.0 (41.0–44.0)	—	—
MotorAMP (mV)	6.8 (6.2–6.9)	6.9 (6.4–7.3)	5.4 (4.6–5.8)
SensoryAMP (μV)	10.6 (10.1–11.1)	10.7 (9.3–11.2)	9.4 (8.3–9.8)
Clinical neuropathy	—	6 (75)	18 (100)

Data are median (range) or *n* (%). Clinical neuropathy was defined by at least two of the following: symptoms consistent with peripheral neuropathy, abnormal sensory examination findings, or absent or decreased deep-tendon reflexes (9).

gested a relation between diabetic neuropathy and duration of diabetes, but no relation between neuropathy and the age of patients. In another study, Tkac and Bril (24) reported the relationship of electrophysiologic findings in diabetic sensorimotor neuropathy with glycated hemoglobin. Likewise, it is known that long duration of diabetes is associated with the appearance of neuropathy (25), but some claim that there is no correlation between diabetes duration and functional deterioration (26). In the present study, the relationships between electroneurographic findings and some parameters such as age, diabetes duration, and glycated hemoglobin are comparable with those mentioned above. However, investigation of more specific parameters for diagnosing diabetic neuropathy that is not able to be established by conventional electrophysiologic studies may help to explain the relation of neuropathy with both glycemic control and duration of diabetes (27).

Consequently, high serum ubiquitin levels in type 2 diabetic patients who had diminished amplitudes of axons of peripheral nerves and inverse correlation between the levels of serum ubiquitin and amplitudes of axons may indicate that ubiquitin may play a role in abnormalities of peripheral nerve function in type 2 diabetes. More detailed studies are needed to establish the relationship between diabetic neuropathy and ubiquitin.

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