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Original Article

Serum levels of TGF-β1 in patients of diabetic peripheral neuropathy and its correlation with nerve conduction velocity in type 2 diabetes mellitus

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ABSTARCT

Aims: To correlate serum levels of TGF-β1 with motor and sensory nerve conduction velocities in patients of type 2 diabetes mellitus

Materials and methods: The study was conducted in diagnosed type 2 diabetes mellitus patients which were divided in patients with clinically detectable peripheral neuropathy of shorter duration ($n = 37$) and longer duration ($n = 27$). They were compared with patients without clinical neuropathy ($n = 22$). Clinical diagnosis was based on neuropathy symptom score (NSS) and Neuropathy disability score (NDS) for signs. Blood samples were collected for baseline investigations and estimation of serum TGF-β1. Nerve conduction velocity was measured in both upper and lower limbs. Median, Ulnar, Common Peroneal and Posterior Tibial nerves were selected for motor nerve conduction study and Median and Sural nerves were selected for sensory nerve conduction study

Results: In patients of type 2 diabetes mellitus with clinically detectable and serum TGF-β1 showed positive correlation with nerve conduction velocities

Conclusion: High level of TGF-β1 in serum of T2DM patients with neuropathy show possible contribution in development of neuropathy. Due to its independent association this cytokine might be used as biomarker for diabetic peripheral neuropathy

1. Introduction

Neuropathy is the most common complication and greatest source of morbidity and mortality in diabetes patients [1]. Diabetic peripheral neuropathy (DPN) is the most commonly reported long term complication of type 2 diabetic patients (T2DM) [2]. Despite the significant pathology associated with nerve degeneration, the etiology of neuropathy is still unclear. Several pathways have been suggested to be associated with glucose neurotoxicity and diabetic neuropathy [3]. The typical DPN is a chronic symmetrical, length-dependent sensory motor polyneuropathy and is thought to be the most common variety [4]. It is predominantly sensory neuropathy, with autonomic involvement which is usually subclinical. A number of neuropoietic cytokines exhibit pleiotrophic effects on glia cells and neurons important for the homeostasis of the peripheral, central and autonomic nervous systems. These cytokines including interleukin -1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1) leukaemia inhibitory factors (LIF), ciliary neurotrophic factors (CNTF) are produced locally by residual and infiltrating macrophages, lymphocytes, mast cells, schwann cells, fibroblasts and sensory neurons [5]. There are multiple metabolic pathways which are dysregulated in T2DM [6,7] and this dysregulation occurs after diagnosis of diabetes or may begin years or even decades before diagnosis of disease [8,9]. It develops on (or with) a background of long standing hyperglycemia, associated metabolic derangements (increased polyol flux, accumulation of advanced glycation end products, oxidative stress and lipid alterations among other metabolic abnormalities and cardiovascular risk factors [10,11]. The transforming growth factor (TGF)-β family members are stable multifunctional polypeptide growth factors that fulfill key functions during development and

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maintain adult tissue homeostasis. The TGF-β family of cytokines is involved in multiple pathways regulating SC and neuronal development, SC proliferation, production of neurotrophic factors, ECM deposition, and expression of CAMs [12]. The cytokine TGF beta1 is also involved in Schwann cells apoptosis [13]. TGF-βs may promote cell survival or induce apoptosis, stimulate cell proliferation or induce differentiation, and their immune functions are mostly anti-inflammatory [14]. TGF-β is reported to be increased by elevated glucose and is a known powerful stimulus of extra cellular matrix production [15,16]. Thus, we hypothesized that TGFβ1 might serve as a novel biomarker for human DPN and/or it could be implicated in its pathogenesis.

2. Materials and methods

2.1. Study design

Design of the study was cross sectional. The study was conducted in Rajiv Gandhi Centre for Diabetes and Endocrinology (RGCDE) and Department of Physiology on patients of Type 2 Diabetes Mellitus attending Diabetes clinic during year 2011–2012 after approval from the ethical committee of Jawaharlal Nehru Medical College Hospital (JNMCH). Aligarh Muslim University, Aligarh, India. Only T2DM patients aged 30–69 years were included in study and assessed for diabetic peripheral neuropathy. The diagnosis of diabetes was made on the basis of revised American Diabetes Association Criteria i.e. fasting plasma glucose ≥126 mg/dl (≥6.1 mmol/1) and 2 h postprandial plasma glucose ≥200 mg/dl (≥11.1 mmol/1). Informed Consent for nerve conduction study and sampling was taken. Patients with previous history of any systemic condition related to peripheral neuropathy (malnutrition, alcoholic neuropathy, renal failure), any neuromuscular diagnosis, such as myopathy, familial polyneuropathy or chronic polyneuropathy or GB syndrome, neuropathies associated with exogenous toxins, metals, or drugs, trauma, Skin lesions or swelling that would interfere with nerve conduction, were excluded from study. All neuropathy patients were divided in two groups based on duration of T2DM, Group I ($n = 37$) with less than 8 years and Group II ($n = 27$) with equal to or more than 8 years. The findings were also compared with group of 22 age, sex and BMI matched T2DM patients without clinical neuropathy.

2.2. Baseline characteristics and clinical examination

Patients general information as gender, age, diabetes duration, height, weight, waist circumference, hip circumference were obtained through proper history as per a pre-designed proforma. Body mass index (BMI)[weight (kg)/height $(m)^2$]and waist hip ratio[WC (cm)/HC (cm)] were calculated. Patients with symptoms of Paraesthesia/Burning/numbnesss/tingling/cramping/aching were assessed on basis of Neuropathy symptom score (NSS) and Clinical examination for Neuropathy disability score (NDS). Neuropathy symptom score consist of score 2 for Burning/numbness/tingling or 1 for Fatigue/cramping/aching. Further score for localization was added as 2 for feet/1 for leg/0 for elsewhere. Then score for exacerbation or symptom improvement was added. Maximum possible score was 9 and the patients having score ≥3 were regarded as clinical Peripheral neuropathy

Neuropathy Disability Score (NDS) was based on four tests: vibration perception test, temperature perception on dorsum of foot, pin-prick and Achilles reflex. Assessment of vibration sensation was done using 128-Hz tuning fork tested over the tip of the great toe bilaterally. The response was considered abnormal when the patient loses vibratory sensation while the examiner still perceived it. Temperature perception was tested by cold sponge to see the cold temperature perception. Inability to perceive pinprick just proximal to the toe nail on the dorsal surface of the hallux, would be regarded as an abnormal test result. Ankle reflex was assessed with a tendon hammer and was recorded as either present or absent. Total absence of ankle reflex either at rest or upon reinforcement was regarded as an abnormal result. The patients having score ≥3 in were regarded as clinical Peripheral neuropathy.

2.3. Sample collection and biochemical analysis

Selected patients were asked to report endocrinology laboratory after an overnight fasting of 10–12 h in fasting state for all baseline investigations. Blood samples were collected in EDTA-Na vials for estimation of glycosylated hemoglobin HbA_{1C}, Fluorode vials for plasma glucose, in plain vials for serum lipids and lipoproteins, serum creatinine. Spot Blood samples for fasting glucose and postprandial glucose were collected on same day. Blood was collected aseptically by venepuncture, for estimation of serum TGF-β1, allowed to clot, and the serum was separated from the clot as soon as possible. No additives or preservatives were used to maintain integrity of the sample. Samples having particulate matter, turbidity, lipaemia or erythrocyte debris required clarification by filtration or centrifugation before testing. The samples were stored frozen at −20 °C or below in vials for storage.

Plasma Glucose was measured by Glucose oxidase peroxidase enzymatic method. Estimation of Glycosylated Hemoglobin (HbA_{1C}) was done by cation exchange resin method, reagent supplied by Pointe Scientific Inc. Michigan, USA and Serum Creatinine by Jaffe Manners Method. Serum triglycerides, HDL cholesterol, Total cholesterol were measured by enzokits, Ranbaxy diagnostics and cholesterol reagent set supplied by Pointe Scientific Inc. Michigan, USA. Serum LDL cholesterol concentration was calculated indirectly by using Friedwalds equation:

$$\text{LDL} = \text{Total cholesterol} - \text{HDL conc} - \text{Triglycerides}/5$$

An enzyme immunoassay ELISA kit provided by DRG international, Inc USA was used for the quantitative *in vitro diagnostic* measurement of TGF-β1 in serum. Prior to testing the standards and patient samples were diluted in assay buffer, acidified with HCl and then neutralized with NaOH. Afterwards, the neutralized standards and samples were added to the antibody coated (polyclonal) microtiter wells. After the first incubation the unbound sample material was removed by washing. Then a monoclonal mouse anti TGF-β1 antibody, a biotinilated anti mouse IgG antibody and the Streptavidin-HRP Enzyme complex were incubated in succession. An immune enzyme sandwich complex was formed. After incubation the unbound conjugate was washed off. Having added the substrate solution, the intensity of colour developed was proportional to the concentration of TGF-β1 in the patient sample. The absorbance (OD) was read of each well at 450 nm with a microtiter plate reader. It was recommended that the wells be read within 10 minutes after adding the Stop Solution. The concentration of the samples was read directly from standard curve. The results were multiplied by the initial dilution factor.

2.4. Nerve conduction velocity

Nerve conduction velocity measurement was performed using Neuroperfect software on windows based Computerized EMG/NCV/EP system supplied by Medicaid System, Chandigarh, India. Nerve conduction velocities were measured with standard surface stimulating and recording techniques. Electrodes were coated with electro-conductive gel and held in place with adhesive tape. Nerve conduction velocity (m/s) was measured in both upper and lower