ABSTRACT

Objective: The mechanism/s leading to diabetic neuropathy is/are complex. In our study we evaluated the probable links between peripheral diabetic neuropathy with transforming growth factor beta 1 (TGF-β1), IL6 raised glycosylated hemoglobin (HbA1c), and c-reactive protein (CRP) in patients with type 2 diabetes mellitus and also the potential relationship between these biomarkers.

Patients: Sixty patients with type 2 diabetes mellitus were categorized into two groups according to the clinical and electrophysiological evaluation, 30 patients with neuropathy, and 30 without neuropathy. The two groups of our patients were subjected to HbA1c, CRP, IL6, and TGF-β1 and the results were analyzed by statistical assessment. Results: There were statistically significant raised serum levels of TGF-β1, IL-6, CRP and HbA1c in our patients with neuropathy if compared to non-neuropathic group. There were statistically significant positive correlations between these biomarkers.

Conclusions: Peripheral neuropathy in type 2 DM is associated with raised plasma levels of glycated hemoglobin, C-reactive protein, IL6, and Transforming growth factor β1. There is also a positive correlation among these biochemical markers.

Keywords: Diabetic neuropathy, TGF-β1, IL6, HbA1c, CRP.

INTRODUCTION

Diabetes mellitus is linked to multi-organ complications of which the commonest is neuropathy that take place in about 60% of diabetic patients (1). In spite of the crucial pathology accompanied with degeneration of peripheral nerves, the cause of peripheral neuritis is still uncertain. Metabolic pathway derangements so often observed in animal models have only partially been confirmed in humans. Clinical experiments of therapeutic agents outlined to counter these derangements have been assumed untimely (2). Epidemiological data lend support to the conflict that the duration and seriousness of exposure to hyperglycemia is linked to increased risk of neuropathy in humans (3). However, Hyperglycemia may not necessarily be fundamental to the development of human diabetic neuropathy but may play an important role in determining advancement and hence increased risk of nerve damage (2). Hyperglycemia results in the formation of advanced glycation end products (AGE's), which in turn act on specific receptors (RAGE) to activate monocytes and endothelial cells increasing the production of cytokines, adhesion molecules and tissue growth factor (4). Glycation has also recently been shown to have an effect on matrix metalloproteinase's (MMPs) and transforming growth factor-beta (TGF-β) (5).

Inflammation, is considered to be an important etiologic agent in the occurrence of type 2 diabetes mellitus, has also been suggested to play an important role in the progression and occurrence of diabetic peripheral neuropathy in animal models (6,7). In addition, numerous growth factors, such as vascular endothelial growth factor (VEGF), nerve growth factor, and TGF-β, have also been concerned in the pathophysiological alterations of diabetic neuropathy and have been aimed as a likely new therapeutic modalities (8, 9). Regulation of these growth factors and their intermediate signaling pathways has been suggested to be associated with glucose neurotoxicity & diabetic neuropathy (10, 11).

Three isoforms of TGF-β are reported in animal studies (TGF-β1, β2, & -β3). TGF-β composed of a superfamiliy of immunocytokine growth factors that have a diversity of biological processes including control of cell proliferation, differentiation, apoptosis, development and extracellular matrix formation (12, 13). It has been generally recognized that the functions of TGF-β superfamiliy components may differ as regard to the cellular status and cell types. TGF-β isoforms have been implicated in a wide range of biological events, including cell maturation, apoptosis, cell differentiation, inflammatory processes, and immunological reactions, by modifying the presentation of specific groups of target genes (14, 15). TGF-β has been served to be both pro- and anti-apoptotic, affected by both context and situation. Variability in the production of TGF-β has been associated with various disease states, such as atherosclerotic and fibrotic diseases of the kidney, liver and lung. TGF-β is reported to be increased by...
elevated glucose and is a known powerful stimulus of extra cellular matrix production (16,17). High levels of glucose significantly increase levels of TGF-β1 and lead to fibrosis of these organs (18, 19) and induction of diabetic nephropathy (20).

In neuronal tissues, it is clear that TGF-βs play a neurotrophic role in some situations (21, 22, 23, 24), while they elicit cell-death-inducing effects in other situations (13, 25). However, the precise role of different TGF-β isoforms and its purposes are not yet understood in the human peripheral nervous system or in diabetic neuropathy (9).

**PATIENTS AND METHODS**

60 patients (type II diabetes) were included in cross sectional cohort, Case-Control, comparative study. They divided into two cross matched groups for age, sex, race, height, age of disease onset and disease duration. The first group including, 30 diabetic patients with clinical and neurophysiological evidence of distal symmetrical polyneuropathy. The second group (control group) including, 30 diabetic patients without clinical or neurophysiological evidence of distal symmetrical polyneuropathy. Laboratory investigations including (HBA1C, CRP, IL6 and Transforming growth factor β1), were done for both groups and the results were recorded. The clinical assessment of distal symmetrical polyneuropathy was done by using diabetic neuropathy examination scoring system (see Appendix I) (26). Also, the Diabetic Neuropathy Examination (DNE) score was used to quantify the neuropathy and a score > 3 was considered significant for presence of neuropathy (see Appendix II) (27). Any patients with other confounding factors precipitating neuropathic changes were excluded from the study (e.g. metabolic, nutritional, toxic, alcohol abuse, drug induced, inflammatory, dysimmune or paraneoplastic factors). Patients on anti-inflammatory or cholesterol-lowering drugs within the previous 30 days were excluded. Also hypertensive patients, dyslipidemic, heavy smokers and patients with diabetic nephropathy or chronic renal failure were excluded.

CRP and Hemoglobin A1c were measured by immunoturbidimetric assay (Using COBAS INTEGRA 400 machine; Roche Diagnostics, Indianapolis, Ind USA).

C - reactive protein: Was determined by particle enhanced immunoturbidimetric assay in which human CRP agglutinates with latex particles covered with monoclonal anti- CRP antibodies. The precipitate is determined turbidimetrically at 552 nm (28).

Hemoglobin A1C:Total hemoglobin and HbA1c concentrations were determined after hemolysis of the heparinized whole blood specimen. Total Hb is measured colorimetrically. HbA1c is determined immunoturbidimetrically. The final result was expressed as percent HbA1c and is calculated from the HbA1c/Hb ratio, including a conversion equation to match a HPLC reference method. HbA1c (%) = HbA1c/Hb) x 175.8 + 1.73 (29).

For IL-6 and TGF β 1 blood samples were collected in sterile tubes without additives. The sera were stored at −70 C until thawed for the assays.

Human IL-6 Immunoassay: The plasma concentration of IL-6 was measured by a commercially available enzyme-linked immunosorbent assay Quantikine HS (R&D Systems,) (30).

Human TGF-β1 Immunoassay: Was determined using Quantikine Human TGF-β1 Immunoassay from R&D Systems (31). The activation procedure of sera was followed according to the Quantikine TGF-β1 immunoassay instructions to activate latent TGF-β 1 to immunoreactive TGF- β 1 to be detectable. The readings were measured on an ELISA reader (HumaReader HS) at 490 and 450nm for IL-6 and TGF-β1 respectively. The method sensitivity for TGF-β1 was 7.0 Pg /ml.

**Statistical analysis:** data were collected, revised, verified then edited on personal computer and then analyzed using SPSS version 15. Qualitative variables were given in percentages and number of cases. Quantitative variables were expressed as mean, range and standard deviation using Levene's test for equality of Variances and t-test for equality of means. The relationship between DPN and other variables was evaluated by chi square (χ2). Significance of correlations was determined using Spearman’s partial correlation coefficient. All P values <0.05 were considered statistically significant.

**RESULTS**

Baseline characteristics of the patients are depicted in table 1. Mean values of HbA1c were (8.1 ± 1.101) and (6.465± 0.689) in diabetic patients with and without polyneuropathy respectively (figure 1). TGF- β 1 levels in patients with diabetic polyneuropathy was 56.850±11.773 pg/ml and in participant without diabetic polyneuropathy it was 42.7± 9.315 pg/ml.
In our study population, patients with diabetic polyneuropathy had higher levels of C-reactive protein (CRP), HBA1c, TGF-β1 and interleukin (IL)-6 compared with those in patients without diabetic polyneuropathy (P<0.001) (Table 1).

### Table (1): Baseline characteristics of the HbA1C, CRP, IL6 and TGF-β1 level in patients with diabetic neuropathy and without diabetic neuropathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DPN</th>
<th>NDPN</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>8.1 ± 1.101(6.0 - 9.8)</td>
<td>6.465 ± 0.689(5.5 - 7.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP (mg / l)</td>
<td>4.7 ± 2.308(0.4 - 10.6)</td>
<td>1.7 ± 0.897 (0.2 - 3.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6 (pg / ml)</td>
<td>6.2 ± 1.760(2.5 - 8.7)</td>
<td>3.007 ± 0.869(1.3 - 4.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>TGF-B1(pg / ml)</td>
<td>56.850 ± 11.773(28.6 - 72.1)</td>
<td>42.7 ± 9.315(17.4 - 60.1)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are given in Mean ± SD, range and p value. DPN: patients with diabetic neuropathy. NDPN: patients without diabetic neuropathy

### Table (2): Spearman correlation coefficient between serum TGF-β1 levels and laboratory hyperglycemic and inflammatory markers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TGF-B1</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1C</td>
<td>0.473</td>
<td>0.000</td>
</tr>
<tr>
<td>CRP</td>
<td>0.362</td>
<td>0.004</td>
</tr>
<tr>
<td>IL6</td>
<td>0.536</td>
<td>0.000</td>
</tr>
</tbody>
</table>

### Table (3): Spearman correlation coefficient between serum HbA1C levels and TGF-β1 and inflammatory markers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HbA1C</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>0.473</td>
<td>0.000</td>
</tr>
<tr>
<td>CRP</td>
<td>0.777</td>
<td>0.000</td>
</tr>
<tr>
<td>IL6</td>
<td>0.710</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Alternative markers of diabetic neuropathy are being actively searched to promote the diagnosis, assess the progression, and to evaluate the advantages of therapeutic
modality in patients with diabetic neuropathy (32).

In our study, there were elevated concentrations of serum HbA1c, CRP, IL-6 and TGF-β in neuropathic diabetic group in comparison to the non-neuropathic group. Also, there were significantly positive correlations among these biomarkers. In spite of conflicting evidence has previously been published regarding the relationship of diabetic neuropathy to the quality of diabetic control, this finding is going with the most recent studies where the association between poorly controlled diabetes and higher risk of polyneuropathy is well established (33).

The American Diabetes association recommends that glycosylated hemoglobin (HbA1c) should be less than 7 % and several studies used higher HbA1c cut points mostly above 7.5%, indicating that those were mostly poorly controlled patients and focused on neurologically symptomatic patients , provided evidence of this association and decided that glycemic control, as estimated by HbA1c , was identified as the best correlated modifiable risk factor for severity of DSP by univariate and multivariate analyses(3,33, 34, 35, and 36).

In addition some investigators suggest that the level of hyperglycemia is important determinant of neuropathy, however neuropathy can develop despite intensive control of the glucose level. Thus risk factors in addition to hyperglycemia are probably involved in the evolution of neuropathy, identifying them, particularly if they are changeable, might lead to new risk–reduction strategies (37, 38).

On the other hand, very few studies did not show significant association between glycemic control and polyneuropathy indicating that factors rather than the degree of control of diabetes are important in the pathophysiologic alterations of diabetic neuropathy. They decided that poor control of blood glucose was not the sole pathogenic factor that plays a role in the pathogenesis of diabetic polyneuropathy. The occurrence of neuropathy during glycemic control and its occasional occurrence following the initiation by diet control, insulin or oral hypoglycemic agents has been noticed and this weakens the hypothesis that neuropathy may be the result of poor control. One of the main troubles in alleviating this problem has been in defining the category of diabetic control. This has, in the past, relied upon estimation of blood and urine glucose which are prone to large variation during the day. Recent investigations have indicated that estimations of the concentration of HbA1c gives a more reliable indication of the degree of diabetic control, as it reflects the degree of glucose elevation over a period of several weeks duration (39 , 40 , and 41).

The association between subclinical inflammation and diabetic polyneuropathy has only been investigated in small studies without definitive results (42, 43). Therefore, the aim of our study was to investigate systematically whether patients with type2 diabetes with or without diabetic polyneuropathy exhibit a different immune profile.

CRP is an acute-phase protein that is produced in the liver. It is a marker of systemic inflammation and its main inducer is IL-6. Associations of CRP and IL-6 with cardiac autonomic neuropathy have been previously reported in small studies in patients with type 1 and 2 diabetes mellitus (44, 45, 46, and 47).

Our results were in agreement with recent study of Herder et al. (48), where they demonstrate that CRP and IL-6 were most consistently associated with diabetic polyneuropathy and some neuropathic deficits. It is important that these links remained statistically significant after correction of multiple confounded factors, including diabetic duration and HbA1c, so that immune activation in diabetic polyneuropathy cannot be explained solely as a consequence of hyperglycemia or other metabolic disturbances (48). Recently, Herder et al reported that systemic inflammation anticipated both the onset and progression of diabetic polyneuropathy over 6.5 years for the large population of elderly adults in the Cooperative Health Research in the Region of Augsburg F4/FF4 cohort prospective study. Elevated plasma levels of high-sensitivity C-reactive protein, IL-6, TNF-α, IL-1RA and soluble intracellular adhesion molecule-1 (ICAM-1), and decreased adiponectin concentrations were accompanied with incident DPN. After adjustment for known DPN risk factors, increased IL-6 and TNF-α remained associated with incident DPN. Elevated plasma soluble ICAM-1 and IL-1RA were accompanied with advancement of DPN (49).

Additionally, the main findings of the present study were in agreement with Doupis et al., where they concluded that diabetic patients with peripheral neuropathy had elevated serum levels of inflammatory cytokines and changes in the levels of various growth factors. It should be emphasized that the observed measurements in their study were correlative and do not necessarily indicate causality (50).
Pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, monocyte chemoattractant protein-1 and C-reactive protein, are primarily composed in adipocytes, activated immune cells and resident macrophages. In addition to the previously mentioned inflammatory molecules, circulating and locally produced ICAM-1, vascular cell adhesion molecule-1, E-selectin and chemokines have raised their expression in diabetes and DPN, and have been shown to be associated with the occurrence and progression of DPN only by cross sectional trials. Although, in the prospective study done by Janahi, who described that elevated values of IL-6 and TNF-α increased the proportion of DPN development, and these elevations in levels of these systemic cytokines are not specific to DPN, but is determined in obesity, cardiovascular disease, and other diabetic complications, such as diabetic nephropathy. Therefore, we should confirm the difference in systemic cytokine change between DPN and other low-grade inflammatory disease (51). These observed findings were consistent with some studies that illustrate the pathogenic role of inflammation, especially the cytokine and chemokine production in diabetic neuropathy. For example, Dandona et al, reported increased serum levels of inflammatory cytokines, including TNF-α and CRP, and markers of endothelial dysfunction in subjects with DN (52). Another two studies by Wu et al and King et al., (53, 54); they demonstrated a higher HbA1c is significantly associated with a greater likelihood of elevated CRP among adults with diabetes. One of the several mechanisms proposed is oxidative stress on the endothelium, which potentiates inflammation and is enhanced by hyperglycemia (55, 56). Such evidence is in accordance with our findings in this study, which further confirms the relationship between hyperglycemia and the inflammatory process in patients with diabetes.

On the other hand, there are other evidences indicating that the observed changes are mostly associated to the presence of neuropathy rather than diabetes itself. Firstly, there are significant changes were detected between non-neuropathic and neuropathic diabetic patients. Second, the noticed results were not related to the drugs that were used for treatment of hyperglycemia, hypertension, and hyperlipidemia. Third, most of prior studies found that there is no advancement of the severity of diabetic neuropathy on follow up and there were also no alterations in the cytokine and growth factor levels. Lastly, it is of interest that in addition to classic proinflammatory cytokines that are known to be related to the diabetic state, such as TNF and CRP, peripheral neuropathy was also linked to extra inflammatory cytokines that are the results of numerous cells that have not heretofore been involved in the occurrence of this condition. However, longitudinal studies that will let the development of alterations in the nerve function and assessment of the role of inflammatory cytokines and growth factors will be needed to reach vigorous conclusions (50, 57).

Few data are available regarding the association between DPN and TGFβ1 besides a recent report of TGFβ1 inducing cellular injury in investigational diabetic neuropathy (9). Thus, we hypothesized that TGFβ1 might serve as a novel biomarker for human DPN and/or it could be implicated in its pathogenesis (57).

A number of inflammatory cytokines mediate atherosclerosis. Transforming growth factor beta 1 (TGFβ1) regulates important incidents like macrophage and fibroblast chemotaxis, inhibitions of lymphocyte function, collagen formation, and initiation of extracellular matrix synthesis (57, 58).

In our study, there was a statistically significant increase of TGFβ1 in our patients with neuropathic in comparison to non-neuropathic group; this finding is in agreement with Hussain et al (57). One likely clarification for this correlation might be related to the complex and multifactorial etiology of peripheral diabetic neuropathy: hyperglycemia and hyperlipidemia give rise to oxidative stress and formation of advanced glycation and lipoxidation end products. The inflammatory mediators (i.e., TGFβ1) lead to nuclear factor kappa B (NF-kappa B) activation (58, 59, and 60). Moreover, Expressed genes down-regulated in response to TGFβ1 are mainly those associated with oxidative stress (i.e., NF-kappa B) and several other genes implicated in glutathione production and maintenance (60, 61).

There are some limitations in our study, first one that this is a cross-sectional study, so one cannot distinguish between true risk factors and the possible links that could be due to inverse etiology or simply by chance. Second, the study lacked a non diabetic control group, which would have been important to determine unambiguously that the diabetic study candidates had elevated levels of proinflammatory markers compared with those in healthy individuals without type 2 diabetes. Third, this study relied on assessment of immune mediators at a single time point only.
and not at various stages of disease. Prospective studies will be needed to evaluate the time course and etiological relevancy of subclinical inflammatory process in the occurrence of diabetic polyneuropathy in order to study whether immune modulation could become a therapeutic choice for patients with diabetic polyneuropathy.

In conclusion, the present study has shown that peripheral diabetic neuropathy is linked to elevated active biological markers of inflammation and endothelial dysfunction. These results indicate that inflammatory cascade and endothelial dysfunction may be an important contributing factors in the development of peripheral neuropathy in diabetic patients. Simple effective methods are required to recognize the diabetic population at high risk of DPN and progression of the disease should be halted by controlling the modifiable risk factors. In our series, DPN's categorical diagnosis can be significantly and independently predicted by TGFβ1, HbA1c, CRP and IL6. Accordingly, these biomarkers in particular the vaso-active peptide TGFβ1 (as a key cytokine effectors), could be used as a novel biomarker molecule for DPN diagnosis. Further prospective studies are warranted.

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