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For further information about this article or if you need reprints, please contact:

Rania Shafik Salah
3 Bostan el Gaish Street,
Abbasia, Cairo, Egypt

Tel: (+202) 0101074984
Fax: (+202) 24051107

Study of Some Inflammatory Factors in Type 2 Diabetic Patients with Nephropathy

¹H.O. El-Mesallamy, ¹R.S. Salah and ^{1,2}M.Z. Gad

In this study we explored the relationship between the serum levels of the inflammatory factors tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and endothelin-1 (ET-1) and the severity of diabetic nephropathy (DN). The study included 50 type 2 diabetics with various degrees of nephropathy (20 patients with normoalbuminuria, 15 with microalbuminuria and 15 with macroalbuminuria) compared to 17 healthy volunteers. ELISA technique was used to measure the serum levels of these inflammatory factors. Significant increase in the serum levels of TNF- α and IL-6 were observed in diabetic patients compared to control subjects and this increase was in concert with the progression of renal disease as indicated by urinary albumin excretion (UAE). Regarding ET-1, serum levels were higher in micro- and macroalbuminuric patients as compared to normoalbuminuric patients and control subjects. ET-1 was significantly positively correlated with both TNF- α ($r = 0.595, p < 0.01$) and IL-6 ($r = 0.739, p < 0.01$). These results suggest that the inflammatory factors; TNF- α , IL-6 and ET-1 are associated with DN and may have some etiopathogenic roles in disease development.

Key words: Diabetic nephropathy, inflammation, TNF- α , IL-6, ET-1

INTRODUCTION

Diabetic nephropathy, a devastating complication of type 2 DM, is now considered to be a medical catastrophe of world wide dimensions (Mora and Navarro, 2006). At present, 20-30% of type 2 diabetic patients develop evidence of nephropathy (Sasso *et al.*, 2006). DN is characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hypertrophy together with increased urinary albumin excretion (UAE). Advanced DN is characterized by proteinuria, decline in renal function, glomerulosclerosis and interstitial fibrosis (Schrijvers *et al.*, 2004).

The pathogenesis of DN is still a matter of debate and the true pathogenic mechanism is not known, but there may be a common denominator of micro- and macrovascular diabetic complications. Such a factor may be low grade inflammation and endothelial dysfunction (Festa *et al.*, 2000; Stehouwer *et al.*, 2002). The extent to which inflammation is a primary abnormality involved in the pathogenesis of type 2 DM and associated complications such as atherosclerosis or whether inflammation is developed secondary to hyperglycemia, obesity and dyslipidemia, or other common features of the disease needs to be clarified (Pickup, 2004).

Originally thought of as selective anti-tumor agent, TNF- α is now grouped among the major inflammatory cytokines. In addition, it exerts a myriad of biological actions in different tissues, many of these can perturb the normal regulation of energy and metabolism (Sethi and Hotamisligil, 1999). Several studies have shown that the level of expression of TNF- α correlated with obesity and hyperinsulinaemia suggesting that it may be the link between obesity and insulin resistance which are of the key features of type 2 DM (Kern *et al.*, 2001). TNF- α had been suggested as a critical factor contributing to renal alterations that occur during the initial stage of DN taking in consideration that this cytokine is cytotoxic to glomerular, mesangial and renal epithelial cells and may induce significant renal damage (Navarro and Mora-Fernandez, 2005). IL-6 is another inflammatory cytokine that also seems to be associated with visceral obesity, insulin resistance and type 2 DM (Kern *et al.*, 2001). Expression studies had shown increased IL-6 mRNA in the mesangium of renal specimen from diabetic patients (Saraheimo *et al.*, 2003). In addition an association was observed between glomerular basement membrane thickness, a crucial lesion of DN and the acute phase markers: fibrinogen and IL-6 (Dalla Vestra *et al.*, 2005).

Endothelial dysfunction (ED) as an early sign of diabetic vascular disease is related to the presence of a

vascular low-grade inflammation (Stam *et al.*, 2003). ED can be a cause and/or a consequence of inflammation and the two are tightly linked (Stehouwer, 2004). ET-1 a vasoactive peptide of the vascular endothelium that is widely known for its vasoconstrictor and mitogenic actions (Chang *et al.*, 2007). Recent evidence suggests that ET-1 may be involved in the induction of inflammatory mechanisms in the diabetic kidney, including the production of cytokines and growth factors as well as chemoattraction (Touyz *et al.*, 2000) and infiltration (Tostes *et al.*, 2002) of macrophages, this may be supported by the observation that ET receptor blockade may reduce diabetic renal injury via an anti-inflammatory mechanism (Sasser *et al.*, 2007).

In Type 2 DM elevated serum levels of TNF- α , IL-6 and ET-1 have been observed: a finding suggestive of the association of these inflammatory factors and the pathogenesis of DM. The present study was designed to explore whether these inflammatory factors are further associated with the renal complications of diabetes as well as to define the link between these the inflammatory factors and the progression of nephropathy in type 2 diabetic patients.

MATERIALS AND METHODS

Subjects: This study was conducted on 67 subjects comprised of 40 females and 27 males with age ranging from 45-60 years, of which 17 served as healthy volunteers and considered as control group (group I). The other 50 subjects were type 2 diabetics with a duration of diabetes 5-15 years (group II). The diabetic patients were selected from patients attending the outpatients' diabetes and endocrinology clinic, Ain Shams University Hospital (ASUH), Cairo, Egypt, in the period from February to July 2006. All 50 patients suffering from type 2 diabetes had been diagnosed according to the criteria established by the American Diabetes Association (1997) and were treated with oral hypoglycemic drugs. The diabetic group was further divided according to their UAE into 3 subgroups: the first subgroup was diabetic patients with normoalbuminuria (group IIa) defined as UAE <25 mg L⁻¹, which consisted of 20 diabetic patients (11 females + 9 males). The second subgroup was diabetic patients with microalbuminuria (group IIb) defined as UAE 25-250 mg L⁻¹, which consisted of 15 patients (8 females + 7 males). The third subgroup was diabetic patients with macroalbuminuria (group IIc) defined as UAE >250 mg L⁻¹, which consisted of 15 patients (9 females + 6 males). A detailed medical history and drug treatment(s) were collected for all subjects.

The following were the exclusion criteria: Smoking, recent use of anti-inflammatory drugs, clinical evidence of cardiovascular/hepatic, acute or chronic inflammatory disease as well as clinical evidence of non diabetic renal disease, urinary tract infection or stones.

Study protocol and sampling: The day before the test, subjects were instructed to follow an isocaloric diet. On the test day, blood pressure was measured, weight and height were recorded and body mass index (BMI) was calculated as an index of the weight (kg)/height² (m²) (overweight was defined as BMI = 25-30 while subjects with BMI ranging 30-40 are obese) (Kuczmarski *et al.*, 1997). Patients were instructed to collect first morning urine samples for urinary albumin determination.

Blood samples were collected from subjects by vein puncture of the antecubital vein after an overnight fasting period. Samples were divided into two aliquots; one containing Na₂-EDTA (final concentration 1 mg mL⁻¹) for glycosylated hemoglobin (HbA_{1c}) determination in whole blood. The other aliquot was added to vacutainer clotted tubes and was used for preparation of sera. Sera were obtained by centrifugation of blood after complete clotting at 4000 rpm for 15 min, at 4°C. Sera were separated and aliquoted first for the measurement of fasting blood glucose (FBG), serum creatinine (Cr), triacylglycerol (TAG), total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C) by using standard enzymatic techniques. The other Serum aliquots were kept frozen at -70°C until used for TNF-α, IL-6 and ET-1 ELISA determinations.

Analytical procedures: Urinary albumin was assayed using microalbuminuria ELISA kit supplied from Orgentic, USA and was used in the classification of diabetic patients into groups as mentioned earlier. FBG level was determined using glucose oxidase method according to Trinder (1969). HbA_{1c} was measured by ion exchange method according to Geiger and Binder (1986). Creatinine was assayed according to the method of Henry (1974). Glomerular filtration rate (GFR) was calculated according to Cockcroft and Gault (1976) equation:

GFR = (140-age)*Wt/(S Cr*72) multiplied by 0.85 for females. TAG was measured by glycerol oxidase method (Wahlefeld, 1974). TC by cholesterol oxidize method (Richmond, 1973) as well as HDL-C after precipitation of apolipoprotein B-containing lipoproteins. LDL-C was calculated according to Friedewald *et al.* (1972) formula: LDL-C = TC-(HDL-C+TAG/5) (mg%). All spectrophotometric measurements were done by UV/Visible spectrophotometer Shimadzu, 1650, USA.

Determination of serum TNF-α, IL-6 and ET-1: TNF-α and IL-6 were assayed using Quantikine® kits, provided by R and D systems, USA. ET-1 was assayed using Titerzyme® kit, provided by Assay Designs, USA. All ELISA procedures were done by Hyprep® automated ELISA system, USA according to the instructions of the manufacturer.

Statistical analysis: All statistical calculations were done using SPSS version 10. The results were expressed as mean±standard deviation (Mean±SD). The data followed parametric distribution. To determine differences between groups, analysis of variance (ANOVA) followed by Bonferoni's post-hoc analysis were used for multiple comparisons between different groups. The mean difference is considered significant at p<0.05. Pearson's correlation coefficient was used to determine correlation between different parameters.

RESULTS

All subjects enrolled in this study were not different with respect to age (p = 0.39) and sex distribution (p = 0.822). As for BMI, all subjects were overweight or slightly obese. Group IIc showed longer duration of diabetes than either subjects of group IIa or IIb (p = 0.04). Systolic and diastolic blood pressures were significantly higher in all DM patients when compared to the control group with statistically significant increases in systolic and diastolic pressure with advanced renal disease (p<0.05) (Table 1).

Table 1: Demographic and clinical data of control subjects (group I), diabetic patients with normoalbuminuria (group IIa), diabetic patients with microalbuminuria (group IIb) and diabetic patients with macroalbuminuria (group II c)

Variables	Group I (n = 17)	Group II (n = 20)	Group II b (n = 15)	Group II c (n = 15)
N (f/m)	17 (11/6)	20 (11/9)	15 (8/7)	15 (10/5)
Age (years)	51.6±4.4	52.1±4.5	53.1±4.3	54.1±4.9
Duration (years)	-	7.4±2.5	8.8±2.6	10.5±4.0 ^b
BMI (kg m ⁻²)	26.8±2.3	28.3±4.6	30.3±4	30.3±3.7
SBP (mm Hg)	114.7±12.1	122.5±19 ^a	132.7±12.8 ^a	144.7±16.9 ^{a,b,c}
DBP (mm Hg)	74.7±5.1	81.8±7.3 ^a	85.7±8.0 ^a	89.6±9 ^{a,b,c}

Values are expressed as Mean±SD, BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure. Comparisons were done using Bonferoni's post-hoc analysis. ^a: Significantly different from control group at p<0.05, ^b: Significantly different from diabetic patients with normoalbuminuria at p<0.05, ^c: Significantly different from diabetic patients with microalbuminuria at p<0.05

Table 2: Biochemical parameters of control subjects (group I), diabetic patients with normoalbuminuria (group II a), diabetic patients with microalbuminuria (group II b) and diabetic patients with macroalbuminuria (group II c)

Variables	Group I (n = 17)	Group II a (n = 20)	Group II b (n = 15)	Group II c (n = 15)
UAE (mg L ⁻¹)	12.00±8.2	19.10±6.3 ^a	119.6±66.9 ^{ab}	512.7±89.2 ^{abc}
FBG (mg%)	88.00±8.7	181.20±62.3	274.7±87.8 ^{ab}	263.5±72.4 ^{ab}
HbA _{1c} (%)	5.12±1.0	6.50±1.2 ^a	8.3±2.2 ^{ab}	9.2±1.9 ^{ab}
Serum Cr (mg%)	0.80±0.2	0.86±0.2	1.0±0.2	1.3±0.3 ^{abc}
GFR (mL min ⁻¹)	104.70±8.9	100.30±10.9	96.7±11	68.6±7.3 ^{abc}
TAG (mg%)	125.50±20.9	197.90±28.2 ^a	214.0±22.1 ^{ab}	238.7±17.3 ^{abc}
TC (mg%)	160.30±18.5	242.70±49.3 ^a	256.2±54.1 ^{ab}	296.4±66.3 ^{ab}
HDL-C (mg%)	50.10±8.0	37.30±11.4 ^a	33.1±10.7 ^a	31.5±11.1 ^a
LDL-C (mg%)	85.75±24.0	165.80±52.6 ^a	180.3±59 ^a	217.2±71.0 ^{ab}

Values are expressed as Mean±SD, FBG: Fasting blood glucose; HbA_{1c}: Glycosylated hemoglobin; Cr: Creatinine; GFR: Glomerular filtration rate. TAG: Triacylglycerol; TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol. Comparisons were done using Bonferoni's post-hoc analysis, ^a: Significantly different from control group at p<0.05, ^b: Significantly different from diabetic patients with normoalbuminuria at p<0.05, ^c: Significantly different from diabetic patients with microalbuminuria at p<0.05

Table 3: Serum levels of TNF-α, IL-6 and ET-1 in control subjects (group I), diabetic patients with normoalbuminuria (group II a), diabetic patients with microalbuminuria (group II b) and diabetic patients with macroalbuminuria (group II c)

Variables	Group I (n = 17)	Group II (n = 20)	Group II b (n = 15)	Group II c (n = 15)
TNF-α (pg mL ⁻¹)	37.9±7.8	59.4±16.1 ^a	96.8±12.9 ^{ab}	144.7±21.0 ^{abc}
IL-6 (pg mL ⁻¹)	5.0±2.1	13.3±2.5 ^a	23.0±2.8 ^{ab}	26.6±5.3 ^{abc}
ET-1 (pg mL ⁻¹)	5.5±1.4	12.8±3.0 ^a	23.6±6.5 ^{ab}	22.4±4.7 ^{ab}

Values are expressed as Mean±SD, TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin-6; ET-1: Endothelin-1, ^a: Significantly different from control group at p<0.05, ^b: Significantly different from diabetic patients with normoalbuminuria at p<0.05, ^c: Significantly different from diabetic patients with microalbuminuria at p<0.05

Regarding glycemic status; serum glucose and HbA_{1c} were higher in diabetic patients as compared with control subjects. Furthermore, Groups IIb and IIc showed a statistically significant rise in the serum levels of both glucose and HbA_{1c}% as compared to group IIa (Table 2). However no statistically significant difference was observed between groups IIb and IIc regarding the glycemic status (p =1.0 for glucose and 0.552 for HbA_{1c}). While for kidney function, Serum Cr levels were markedly higher in group IIc associated with significant reduction in GFR. It is noteworthy to mention that, there was no statistically significant difference in those two parameters between both control and other diabetic groups (p>0.05). As for the lipid profile of the studied groups, TAG, TC as well as LDL-C were significantly higher in the three diabetic groups as compared with the control group (p<0.05). On the other hand HDL-C values were decreased.

Concerning TNF-α serum levels, a statistically significant increase in group II a, group II b and group II c as compared to group I was revealed (p<0. 5) (Table 3). In addition a statistically significant difference was observed among the three diabetic groups with those of group II c showing the highest values. There was a strong positive correlation between serum TNF-α and UAE (r = 0.863, p<0.01). In addition TNF-α was also positively correlated with FBG, TAG, TC (r = 0.522, 0.68 and 0.412, respectively at p<0.01) (data not shown).

When comparing serum levels of IL-6 among different groups a similar pattern to TNF-α was revealed. The serum level of group I was (5.0±2.1 pg mL⁻¹), which

was further significantly increased in accordance with the increase in UAE in groups (II a, II b and II c), respectively. IL-6 was positively correlated with UAE (r = 0.762, p<0.01). Furthermore a significant positive correlation was observed between IL-6 and HbA_{1c} (r = 0.573 and TAG (r = 0.563, p<0.01) (data not shown).

Table 3 shows also the serum levels of ET-1 in the studied groups. Group II a level was significantly higher than that of group I. When comparing groups II b and II c both were significantly elevated in comparison with groups (I and II a), However, no statistically significant difference was observed between both groups as regarding serum ET-1 level (p>0.05). ET-1 showed a positive correlation with UAE (r = 0.488, p<0.01), Moreover it was positively correlated TC (r = 0.322, p<0.05) and negatively correlated with HDL-C (r = -0.322, p<0.01) (data not shown).

In addition ET-1 was also positively correlated with both TNF-α and IL-6 serum levels as shown in Fig. 1.

DISCUSSION

Multiple factors contribute to the pathogenesis of DN such as metabolic abnormalities, hemodynamic alterations and various genetic and growth factors (Schijvers *et al.*, 2004). However, the intimate mechanisms progressing from chronic hyperglycemia to the development of renal injury are complex and need to be raveled (Mora and Navarro, 2005). Prospective studies do support the notion that chronic low grade inflammation plays a contributory role in the genesis of type 2 DM

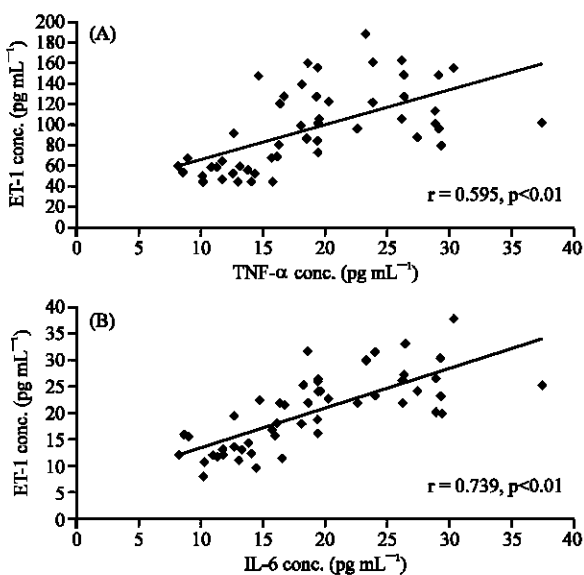


Fig. 1: Correlation of ET-1 (pg mL^{-1}) with TNF- α (pg mL^{-1}) (Panel A), ET-1 (pg mL^{-1}) with IL-6 (pg mL^{-1}) (Panel B) in diabetic patients ($n = 50$). Each individual value is represented by a symbol (\blacklozenge), $r =$ Pearson correlation coefficients

(Williams and Nadler, 2007). In addition, abnormal activation of inflammatory processes is gaining recognition as a possible unifying explanation for the micro-vascular and macro-vascular injury that occurs in the setting of DM (Mora and Navarro, 2006). In this study we report the elevated serum levels of the inflammatory factors TNF- α , IL-6 and ET-1 in type 2 diabetic patients and the association between these inflammatory factors with the renal complications of diabetes as indicated by UAE. Urinary levels of these inflammatory factors should have been also determined but the obstacle was the difficulty in recruiting suitable number of in-patients for accurate collection of 24 h urine samples.

Age, sex and BMI were not statistically different among different study groups. Only macroalbuminuric patients showed statistically significant rise in serum creatinine associated with decrease in GFR. This agrees with Mogensen (2000) who stated that kidney function remains apparently normal in normo- and microalbuminuria until the stage of overt nephropathy (macroalbuminuria) where GFR begins to decline. This is accompanied by a modest rise in serum Cr.

Results revealed that glucose and HbA_{1c} were significantly higher in diabetic groups as compared with the control group with those diabetics with incipient or overt nephropathy showing the highest values. These findings confirm that hyperglycemia is toxic to renal cells in support to Wendt *et al.* (2003). Moreover, Lappin *et al.*

(2002) considered hyperglycemia as one of the major driving forces of renal injury in DN. According to lipid profile, all diabetics showed significant dyslipidemia compared with the controls. Lipid profile parameters, except for HDL-C were positively associated with UAE (data not shown). Diabetic dyslipidemia could be partially attributed to hyperglycemia (Veiraiah, 2005) and dyslipidemia plays in turn its role in exacerbation of renal lesions in DN.

Concerning serum levels of TNF- α and IL-6, several previous studies have reported their elevated levels in type 2 diabetic patients (Mavridis *et al.*, 2007; Pedersen *et al.*, 2003). Our study support this increase that was even observed in female diabetic patients in whom TNF- α was not found to be elevated (Pfeiffer *et al.*, 1997). The mechanism of the elevation of these cytokines in the setting of DM may be contributed to hyperglycemia, insulin resistance, hyperlipidemia as well as hemodynamic alterations. Hyperglycemia induced oxidative stress, along with the formation of soluble oxidative glycation end products possibly serve as activators of transcription factors, leading to induction of gene expression of proinflammatory cytokines including TNF- α and IL-6. This suggests a casual role for hyperglycemia in the immune activation of diabetes (Esposito *et al.*, 2002; Wright *et al.*, 2006). The elevated levels of TNF- α and IL-6 in type 2 DM may in turn interfere with insulin action, suppressing the insulin transduction mechanisms and therefore exacerbating insulin resistance. In addition, this might interferes with the anti-inflammatory effect of insulin, which in turn might promote further inflammation (Dandona *et al.*, 2004). The direct effects of TNF- α on the functions of adipose tissue including induction of lipolysis, alteration of the adipocytes gene expression as well as their pertinence to insulin sensitivity of adipocytes (Ruan and Lodish, 2003; Shoelson *et al.*, 2006). The effect of TNF- α in increasing lipolysis agrees with the positive correlation observed in our study between TNF- α and serum lipids. IL-6 was also found to induce insulin resistance and enhanced lipolysis in adipocytes by a mechanism similar to that of TNF- α (Rotter *et al.*, 2003).

Microalbuminuria as a marker for DN is associated with markers of inflammation in patients with diabetes (Lane, 2004). Present study revealed that serum TNF- α and IL-6 levels increased with the progression of DN with the highest levels for both observed in those patients with macroalbuminuria. Shikano *et al.* (2000) had previously reported an increase in serum and urinary levels of IL-6 in type 2 diabetic patients with DN. IL-6 might be involved in the pathogenesis of renal lesions in DN by stimulating mesangial cells proliferation (Dalla Vestra *et al.*, 2005). Navarro *et al.* (2003) had found

a significant and positive association between serum TNF- α and urinary protein excretion in diabetic subjects with normal renal functions and macroalbuminuria as well as in subjects with overt nephropathy and renal insufficiency. Kalantarina *et al.* (2003) found an early rise in urinary TNF- α levels, which precedes the rise in UAE by about 2 weeks suggesting a possible contribution in the pathogenic process. TNF- α may be important in the pathogenesis of microalbuminuria possibly through damage to the glomerular basement membrane through disruption of sulphated glycosaminoglycans (Navarro and Mora-Fernandez, 2006).

To gain more insight into the inflammatory mechanisms underlying the renal complications of type 2 DM, serum levels of ET-1 were also measured. ET-1 level has been previously reported to be elevated in DM and is thought to be an inflammatory factor that relates ED with inflammation induced cardiovascular and renal injury in DM (Schneider *et al.*, 2002; Khan and Charabati, 2003). Our study supports this speculation. The activity of ET-1 in diabetic patients has received special interest and several explanations were proposed for the activation of ET-1 in the setting of DM. Most of these proposals are initiated/ activated secondary to hyperglycemia. Glucose mediated ED as a result of increased free radical generation and subsequent oxidative stress may lead to ET-1 upregulation (Sethi *et al.*, 2006). ET-1 is also incriminated in the pathogenesis of many chronic kidney diseases especially proteinuric nephropathies (Stehouwer, 2004).

Present results revealed the elevated serum level of ET-1 in patients with DN suggesting an etiopathogenic role in the development of the disease. However, in contrast to TNF- α and IL-6, this elevated level was not indicative for the progression of nephropathy because no significant difference in serum ET-1 was observed between the micro- and macroalbuminuric patients. Renal actions of ET-1 relevant to the pathogenesis of DN include remodelling, vasoconstriction, vascular smooth muscle cell proliferation and glomerulosclerosis (Dhaun *et al.*, 2006).

ET-1 was reported to be involved in the induction of inflammatory mechanisms in the kidney of diabetic rats. ET-1 receptor activation mediates renal inflammation and TGF- β production in diabetes (Tostes *et al.*, 2002) Furthermore, ET receptor blockade may reduce diabetic renal injury via an anti-inflammatory mechanism (Sasser *et al.*, 2007). ET-1 had been reported to induce gene expression of several growth factors including IL-6 in human mesangial cells (Mishra *et al.*, 2003) and this may be an explanation for the positive correlation observed in our study between ET-1 and IL-6. Moreover, the diabetes-induced increased expression of glomerular TNF- α was found to be completely blocked by treatment

with an ET-1 receptor antagonist (Nakamura *et al.*, 1995) giving an indication that ET-1 may be involved in induction of TNF- α production in the diabetic kidney. This is in harmony with the positive correlation observed between TNF- α and ET-1. On the other hand, Kohan (1992) had previously reported that TNF- α is a potent stimulator of mesangial cell ET-1 production and raise the possibility that ET-1 could mediate, at least in part, renal dysfunction associated with high glomerular TNF- α levels. Remains to be determined the cause-effect relationships between TNF- α , IL-6, ET-1 and DM or DN and whether the induction of synthesis of these cytokines occurs in the diabetic kidney or that the metabolic derangements in DN mainly hyperglycemia induces these three inflammatory factors independently needs to be subjected to further research.

In conclusion, the present study demonstrated elevated serum levels of the inflammatory factors TNF- α , IL-6 and ET-1 in type 2 DM patients especially those with nephropathy thus suggesting an important role for these factors in the development of diabetes induced renal injury. Moreover, in contrast to ET-1, TNF- α and IL-6 levels increased with the progression of nephropathy. The predictive value of these inflammatory factors needs to be assessed as well as to consider modulating these inflammatory factors activity as a therapeutic target in patients with DN.

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