

TNF- α and homocysteine levels in type 1 diabetes mellitus

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مستويات عامل النخر الورمي ألفا والهوموسيستين في النمط 1 من السكري

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الخلاصة: تمت دراسة دور الهوموسيستين كعامل اختطار مستقل من عوامل تلف البطانة الوعائية، والصلة المحتملة بين الهوموسيستين وعامل النخر الورمي ألفا، باعتبارهما عاملين متأثرين من عوامل اختطار الموت الخلوي المبرمج لخلايا بيتا في النمط 1 من السكري. وقد تبين ارتفاع مستويات هوموسيستين البلازما، بصورة ملموسة، في صفوف جميع السكريين أكثر مما لدى الشواهد، كما اتضح ارتفاع هذه المستويات بين السكريين المصابين بمضاعفات وعائية عنها في غيرهم. واتضح أيضا وجود علاقة إيجابية يُعتدُّ بها إحصائياً بين ارتفاع مستويات الهوموسيستين ودرجة بيلة المكروألبيومين. كما كانت مستويات عامل النخر الورمي ألفا مرتفعة في جميع السكريين بالمقارنة مع الشواهد. وقد تكون لهذه النتائج مُستَبَعَات علاجية.

ABSTRACT The role of homocysteine as an independent risk factor for vascular endothelial damage, and the possible link between homocysteine and tumour necrosis factor- α (TNF- α) as two synergistic risk factors for beta-cell apoptosis in type 1 diabetes mellitus was studied. Plasma homocysteine levels were significantly elevated in all diabetic patients compared with controls and diabetic patients with vascular complications showed higher elevations. Furthermore, homocysteine levels showed significant positive correlation with the degree of microalbuminuria. TNF- α levels were elevated in all diabetic patients compared with controls. These results may have therapeutic implications.

Taux de TNF- α et d'homocystine dans le diabète sucré de type 1

RESUME Le rôle de l'homocystine en tant que facteur de risque indépendant des lésions endothéliales vasculaires, et le lien éventuel entre l'homocystine et le facteur nécrosant des tumeurs alpha (TNF- α) en tant que deux facteurs de risque synergiques d'apoptose des cellules bêta dans le diabète sucré de type 1 ont été étudiés. Les taux d'homocystine plasmatique étaient considérablement élevés chez tous les patients diabétiques par rapport aux témoins et les patients diabétiques ayant des complications vasculaires affichaient des élévations plus importantes. Par ailleurs, les taux d'homocystine montraient une corrélation positive avec le degré de microalbuminurie. Les taux de TNF- α étaient élevés chez tous les patients diabétiques par rapport aux témoins. Ces résultats peuvent avoir des implications thérapeutiques.

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Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that accumulates in the genetic disorder homocysteinemia as a consequence of decreased activity of enzymes involved in Hcy metabolism [1]. Human plasma contains both reduced (homocysteine) and oxidized (homocystine) species of Hcy. Disulfide forms also exist with cysteine or proteins containing reactive cysteine residues (protein-bound Hcy). The latter oxidized forms are referred to as mixed disulfides. The oxidized forms of Hcy usually comprise 98%–99% of total Hcy in human plasma, 80%–90% of which is protein-bound [2]. Hcy concentration in the plasma of healthy adults ranges from 5 $\mu\text{mol/L}$ to 15 $\mu\text{mol/L}$ and a plasma Hcy concentration exceeding 15 $\mu\text{mol/L}$ is now termed hyperhomocysteinemia. Hyperhomocysteinemia has been identified as an independent risk factor for atherosclerosis, including coronary artery disease, cerebrovascular disease, peripheral vascular disease and thromboembolism [3].

Progressive vascular disease of the large and small vessels is characteristic of diabetes. Microvascular complications of diabetes have a complex pathogenesis involving dysfunction of and damage to vascular endothelial cells. Vascular endothelial cells are sensitive to stimulatory factors such as increased glucose concentrations, oxidative stress and advanced glycation endproducts [4].

Accumulating evidence suggests that around 35% of people with type 1 diabetes mellitus have hyperhomocysteinemia [5]. Hcy may produce vascular endothelial damage in vessels exposed to advanced glycation endproducts by several mechanisms, including induction of oxidative stress [6], impaired generation of nitric oxide [7,8] and decreased anticoagulant en-

dothelial properties with suppression of thrombomodulin expression in endothelial cells [9]. Thrombomodulin is predominantly expressed in vascular endothelial cells and is an important anticoagulant receptor for thrombin. A soluble form of thrombomodulin, most probably released after endothelial cell damage, is found in serum and plasma of diabetic patients and correlates with microvascular complications [10].

It is well known that type 1 diabetes mellitus is a T cell-dependent autoimmune disease resulting in selective destruction of beta cells of the islets of Langerhans with subsequent programmed cell death, i.e. apoptosis [11]. Tumour necrosis factor- α (TNF- α) is involved in the apoptotic pathways that are implicated in the beta-cell destruction. Ratter et al. proved that S-adenosyl-L-homocysteine potentiates TNF- α -mediated cytotoxicity *in vivo* and *in vitro*, via blockage of the methylation of prenylated proteins in the mitochondrial membranes [12].

The aim of this work was to study the relationship between TNF- α and Hcy in type 1 diabetic patients with and without microvascular or macrovascular complications.

Methods

Forty-five (45) type 1 diabetic patients selected from the diabetes mellitus outpatient clinic at Abo El-Reish were recruited for this study. Fifteen (15) healthy individuals were also recruited as controls. The participants were divided into four groups:

- Group I: 15 healthy control individuals
- Group II: 15 type 1 diabetic patients without microvascular or macrovascular diabetic complications
- Group III: 15 type 1 diabetic patients with microalbuminuria

- Group IV: 15 type 1 diabetic patients with other microvascular or macrovascular complications, such as retinopathy, neuropathy or cardiac autonomic neuropathy.

For each participant, a complete medical history was taken and clinical examination was carried out and the following parameters were evaluated: lipid profile including triglycerides (TG), cholesterol, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) [13–16]; glycosylated haemoglobin, HbA1c [17]; microalbuminuria [18]; plasma Hcy by enzyme-linked immunoassay [19]; and plasma TNF- α by solid phase, two-site chemiluminescent enzyme-linked immunoassay using an IMMULITE automated analyser [20].

Results

The results of the study are summarized in Table 1. There was a significant elevation of plasma Hcy levels in all the diabetic patients compared with the control group ($P < 0.0001$). Diabetic patients with vascular complications exhibited significantly greater elevation in Hcy levels than diabetic patients without vascular complications ($P < 0.0001$). TNF- α was also significantly raised in all diabetic patients compared with controls ($P < 0.002$). Diabetic patients with other vascular complications were significantly older ($P < 0.05$) and had a longer duration of diabetes ($P < 0.001$) than those without vascular complications.

Diabetic patients with microalbuminuria had a longer duration of diabetes ($P < 0.02$) than those without vascular complications. There was a significant elevation of HbA1c levels in all diabetic patients studied compared to controls ($P < 0.0001$). However, the difference in HbA1c levels in diabetic

patients with vascular complications, i.e. Groups III and IV, versus diabetic patients without vascular complications, was not significant. There was a significant positive correlation between the increase in Hcy levels and the degree of microalbuminuria ($r = 0.946$, $P < 0.001$).

There was a non-significant correlation between Hcy levels and the age of the patients, duration of diabetes, level of HbA1c, lipid profile and TNF- α levels. There was a significant positive correlation between the increase in TNF- α levels and the elevation in HbA1c ($r = 0.539$, $P < 0.001$), cholesterol levels ($r = 0.307$, $P < 0.05$) and LDL-C levels ($r = 0.312$, $P < 0.05$). In contrast, the correlations between TNF- α levels and age of the patients, duration of diabetes, Hcy, TG, HDL-C and the degree of microalbuminuria were not significant.

The percentage of diabetic patients with hyperhomocysteinaemia was 88.88% (40/45). Among the patients with microalbuminuria, the percentage with hyperhomocysteinaemia was 86.66% (13/15). The proportion with hyperhomocysteinaemia among patients with other vascular complications was 100% (15/15), and among patients without vascular complications 73.33% (11/15). Elevated TNF- α levels were found in 66.66% of all diabetic patients (30/45), in 73.33% (11/15) of those without microvascular complications, 66.66% (10/15) of those with other vascular complications and 66.66% (10/15) of those with microalbuminuria.

Discussion

Homocysteine is a thiol-containing amino acid, produced by intracellular demethylation of methionine. Remethylation of Hcy to methionine is catalysed by B₁₂-dependent methionine synthase in the presence of 5-

Table 1 Comparison of age, sex, duration of diabetes and biochemical parameters between type 1 diabetic patients with and without microvascular or macrovascular diabetic complications versus control subjects

Variable	Group I Control	Group II Type 1 diabetic subjects without vascular complications	Group III Type 1 diabetic subjects with microalbuminuria	Group IV Type 1 diabetic subjects with other vascular complications
No.	15	15	15	15
Sex (M/F)	8/7	8/7	8/7	7/8
Age (years)	13.21 ± 3.20	13.71 ± 4.26	15.67 ± 3.86	17.78 ± 5.13 ^{a,b}
Duration of diabetes (years)	—	5.48 ± 3.22	9.03 ± 4.43 ^b	12.21 ± 5.84 ^b
Homocysteine (µmol/L)	11.10 ± 2.56	20.10 ± 3.24 ^a	30.16 ± 8.05 ^{a,b}	27.37 ± 2.71 ^{a,b}
TNF-α (pg/mL)	6.61 ± 1.97	14.00 ± 10.67 ^a	17.92 ± 21.34 ^a	12.82 ± 7.48 ^a
HbA1c%	6.73 ± 0.96	9.53 ± 1.18 ^a	12.06 ± 7.77 ^a	12.23 ± 7.74 ^a
Triglycerides (mg/dL)	116.73 ± 23.53	129.06 ± 24.81	150.07 ± 14.82 ^{a,b}	127.06 ± 26.18
Cholesterol (mg/dL)	169.33 ± 32.17	176.00 ± 32.34	192.53 ± 27.70 ^a	161.85 ± 35.99
LDL-C (mg/dL)	106.93 ± 26.00	118.31 ± 33.78	122.98 ± 23.06	116.98 ± 11.41
HDL-C (mg/dL)	54.13 ± 14.63	37.28 ± 5.77 ^a	41.16 ± 3.07 ^{a,b}	37.10 ± 3.11 ^a
Microalbuminuria (µg/mg creatinine)		11.33 ± 4.29	52.23 ± 21.20 ^b	13.12 ± 4.19

^aSignificant at $P < 0.05$ comparing all diabetic groups versus control group.

^bSignificant at $P < 0.05$ comparing diabetic patients with microalbuminuria or other complications versus diabetic patients without vascular complications.

Results are expressed as mean ± standard deviation.

TNF-α = tumour necrosis factor-α.

LDL-C = low-density lipoprotein cholesterol.

HDL-C = high-density lipoprotein cholesterol.

methyl-tetrahydrofolate. The latter is the product of the reduction of 5-10-methylene-tetrahydrofolate by methylene tetrahydrofolate reductase. Homocysteine also enters the catabolic trans-sulfuration pathway, where it is converted to cysteine by two sequential enzymatic reactions catalysed by cystathionine-β-synthase and cystathionase [27].

Genetic defects in enzymes involved in the methylation or trans-sulfuration path-

ways lead to classic homocysteinuria. Individuals with this genetic disease have elevated plasma Hcy levels. Within the past few years several studies *in vivo* and *in vitro* have proved that hyperhomocysteinemia induces vascular endothelial damage with subsequent atherogenic and thrombotic effects [22,23]. More recently, Ratter et al. reported a novel apoptogenic effect of Hcy through potentiation of TNF-α-induced cytotoxicity [12].

The demographic data of our study show that diabetic patients of Group IV (with other vascular complications) were older ($P < 0.05$) and had a longer duration of diabetes ($P < 0.001$) than diabetic patients without vascular complications, i.e. Group II. On the other hand, diabetic patients with microalbuminuria (Group III) had a longer duration of diabetes than those without vascular complications ($P < 0.02$), a finding consistent with the fact that vascular complications of diabetes occur after a prolonged period without adequate glycaemic control. HbA1c levels were found to be significantly elevated in all diabetic groups studied compared to controls ($P < 0.0001$).

Plasma levels of Hcy were significantly elevated in most (88.88%) of the diabetic patients whether or not they had complications. A significant positive correlation was noticed between the elevation of plasma Hcy and the degree of microalbuminuria, whereas there was a non-significant correlation between plasma Hcy levels and glycated proteins, lipid profile, duration of diabetes, age of the patients and TNF- α levels. Hyperhomocysteinaemia was more frequently found in patients with microalbuminuria: 86.6% of microalbuminuric patients compared to 73.33% of normoalbuminuric patients. These observations are in agreement with data obtained by Hofmann et al. [5] and Jacobsen [21].

Several hypotheses have been proposed to explain the mechanisms of Hcy-induced vascular endothelial damage. Wang et al. reported that Hcy inhibited the growth of vascular endothelial cells by a mechanism involving decreased carboxymethylation of p21^{ras} [24]. Upchurch et al. stated that Hcy decreased the expression of glutathione peroxidase and nitric oxide synthase in bovine aortic endothelial cells, with impairment of endothelium-mediated vasodilation

[25]. Furthermore, Tsai et al. proved that Hcy was mitogenic for smooth muscle cells by a mechanism involving synergistic induction of cyclin mRNA expression by serum, with subsequent accumulation of matrix proteins [26]. Apolipoprotein(a) is increased in type 1 diabetic patients with nephropathy [27] and the affinity of lipoprotein(a) for fibrin is increased by the presence of Hcy, leading to lipoprotein (a)-mediated impairment of fibrinolysis and atherosclerosis [28]. Furthermore, Hcy promotes low-density lipoprotein oxidation [8].

Hcy has also been shown to stimulate collagen production in cultured aortic smooth muscle cells [29]. Jacobsen showed that Hcy upregulated the expression of the chemokine monocyte chemoattractant protein-1 in cultured human aortic endothelial cells [21]. On the other hand, Hcy oxidation products that include reactive oxygen species directly alter vascular cell functions [21].

All these effects induce atherogenesis. Hcy also has thrombogenic effects, decreasing anticoagulant endothelial properties with suppression of thrombomodulin expression in endothelial cells [9].

Colwell [4] and Hofmann et al. [5] reported a high prevalence of hyperhomocysteinaemia in patients with type 1 diabetes mellitus. Diabetic patients with hyperhomocysteinaemia also have a significantly higher incidence of macrovascular and microvascular complications than those with normal Hcy levels. These observations agree with our data of a high incidence of macrovascular or microvascular diabetic complications among hyperhomocysteinaemic patients (86.66% in those with microalbuminuria and 100% in those with other vascular complications), whereas only 6.66% of patients with normal lev-

els of homocysteine have microvascular diabetic complications.

TNF- α has been implicated in several apoptotic pathways that are involved in the destruction of islets of Langerhans beta-cells [11]. TNF- α -induced cytotoxicity originates in the TNF- α receptor-1 (55–60 kDa) signal transduction pathway, which in turn recruits caspase-8 to the receptor complex [30,31]. Caspase-8 is considered to play a role in the activation of other proteases that are responsible for cell death. More recently, TNF- α -mediated cell death has also been shown to occur independently of caspase [32]. TNF- α -induced cytotoxicity also involves the G-protein-coupled activation of phospholipase A2 [33], the generation of reactive oxygen intermediates [34] and DNA damage [35]. Moreover, it has been shown that TNF- α induces the opening of mitochondrial permeability transition pores resulting in dissipation of the mitochondrial transmembrane potential [36].

Several investigators have reported enhanced expression of cytokines, including TNF- α , in both patients and healthy first-degree relatives of patients with type 1 diabetes mellitus [20,37,38]. These findings agree with our results showing significant elevation of TNF- α in 66.66% of all diabetic patients. Altered levels of TNF- α expression in type 1 diabetic patients may contribute to the insulin resistance that has been described in diabetes mellitus and a number of other diseases, including cancer, sepsis, endotoxaemia and alcoholism, and in trauma [39]. One mechanism that has been suggested for TNF- α -induced insulin resistance is the inhibition of the insulin receptor's autophosphorylation signal and a loss of its ability to phosphorylate, on tyrosine residues, its major substrate insulin receptor substrate-1 [40]. More recently, TNF- α has been reported to induce serine

phosphorylation of insulin receptor substrate-1 which in turn prevents the insulin receptor from phosphorylating this substrate in adipocytes and hepatocytes [41].

Furthermore, Ahmad and Goldstein reported that TNF- α mediates insulin resistance through modulation of expression of specific protein tyrosine phosphatase that has been implicated in the regulation of insulin receptor signalling [42]. Increased abundance of protein tyrosine phosphatase modulates the action of TNF- α to inhibit signalling. The authors proved that TNF- α dose-dependently decreased ligand-stimulated autophosphorylation of insulin receptors and insulin-stimulated insulin receptor substrate-1 phosphorylation in cultured KRC-7 and hepatoma cells. Conversely, Stephens et al. reported that TNF- α -induced insulin resistance is accompanied by a reduction in the expression of insulin receptors (50%), its major substrate insulin receptor substrate-1 (80%), and the insulin responsive glucose transporter GLUT 4 (80%), without loss of insulin receptor-mediated signal transduction in 3T3 adipocytes [39]. The different biochemical effects of TNF- α in different cell lines are thought to result from altered gene expression that often follows the activation of the STATS (signal transducers and activators of transcription) family of transcription factors [39].

Feugeas et al. studied the influence of metabolic and genetic factors on TNF- α production in type 1 diabetic patients [43]. They reported that poor glycaemic control (HbA1c > 8%) is associated with enhanced TNF- α production by peripheral blood mononuclear cells. This finding concurs with our demonstration of a significant positive correlation between TNF- α levels and HbA1c levels ($P < 0.001$). Furthermore, Feugeas et al. found that the presence of the TNF- α 1 allele in the

microsatellite region in HLA-(DR3) subjects was associated with an increased risk of type 1 diabetes mellitus [43].

More recently, Ratter et al. showed a novel effect of the cellular methylation state on TNF- α -mediated cytotoxicity [12]. The authors proved that S-adenosyl-L-homocysteine accumulates in individuals with hyperhomocysteinemia and as a consequence decreases the cellular methylation state, i.e. the ratio of S-adenosyl-methionine to S-adenosyl-L-homocysteine. This decrease leads to inhibition of isoprenylcysteine-carboxyl-methyl-transferase that catalyses the carboxymethylation of C-terminal cysteine residues on isoprenylated proteins on mitochondrial membrane. This fact suggests that the methylation state of prenylated proteins is important in TNF- α -mediated cytotoxicity. Blockage of methylation reactions was associated with enhancement of the TNF- α -induced disruption of the mitochondrial membrane potential and increased cell death [44].

Proteins that have C-terminal modifications include members of the RAS superfamily, such as the λ subunit of the

heterotrimeric G-binding proteins. Many of these proteins are involved in signal transduction processes. For instance, the methylated forms of certain G-binding proteins effectively activate enzymes such as phosphoinositol-3-kinase and phospholipase C-B, while their unmethylated counterparts are virtually inactive [45,46]. Moreover, phosphoinositol-3-kinase and phospholipase C-B are implicated in a pathway that conveys survival signals from various cell surfaces to mitochondria [47].

We conclude that the hyperhomocysteinemia found in diabetes has two major consequences. First, it induces vascular endothelial damage that leads to microvascular and macrovascular diabetic complications. Second, hyperhomocysteinemia has a novel apoptogenic effect through potentiation of TNF- α -induced cytotoxicity. These results may therefore have therapeutic implications. We recommend dietary supplementation with folate, vitamin B₁₂, B₆, choline or betaine at the recommended dosage for all diabetics with elevated Hcy levels [48,49].

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