Clinical validation of a link between TNF- α and the glycosylation enzyme core 2 GlcNAc-T and the relationship of this link to diabetic retinopathy

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Abstract

Aims/hypothesis Increasing evidence suggests that chronic, subclinical inflammation plays an important role in the pathogenesis of diabetic retinopathy. We recently reported that a glycosylating enzyme, core 2 β -1,6-*N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), is implicated in increased leucocyte–endothelial cell adhesion in diabetic retinopathy via an upregulation mechanism controlled by TNF- α .

Subjects, materials and methods We examined the functional link between circulating TNF- α and the activity and phosphorylation of core 2 GlcNAc-T in polymorphonuclear leucocytes of patients with type 1 and type 2 diabetes.

Results Plasma levels of TNF- α , although similar in patients with type 1 and type 2 diabetes, were significantly higher than in age-matched healthy controls, and correlated well with the severity of retinopathy. Core 2 GlcNAc-T activity followed the same trend and was associated with phosphorylation of the enzyme. Finally, the observation that TNF- α levels are also linked to glycaemic values suggests that in patients, as well as in vitro, the glycosylation-mediated cell adhesion process that plays a role in diabetic retinopathy may involve glucose- and TNF- α -

induced protein kinase β 2 activation, and subsequently raise activity of core 2 GlcNAc-T through increased enzyme phosphorylation.

Conclusions/interpretation Our results reveal a novel rationale towards a specific treatment of diabetic retinopathy, based on the inhibition of core 2 GlcNAc-T activity and/or the blockage of cognate glycans.

Keywords β -1,6-*N*-acetylglucosaminyltransferase · C-reactive protein · Hyperglycaemia · Leucocyte · Non-proliferative diabetic retinopathy · Proliferative diabetic retinopathy · Subclinical inflammation · TNF- α · Type 1 diabetes · Type 2 diabetes

Abbreviations

Core 2	core 2 β -1,6- <i>N</i> -acetylglucosaminyltransferase
GlcNAc-T	
CRP	C-reactive protein
NPDR	non-proliferative diabetic retinopathy
PDR	proliferative diabetic retinopathy
PKC	protein kinase C
PMN	polymorphonuclear
PSGL-1	P-selectin glycoprotein ligand-1

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Introduction

Diabetic retinopathy is a leading cause of blindness for working-age people living in Western countries [1]. As shown by fluorescein angiography, an early clinical feature of this disease is the occlusion of capillaries [2, 3], although the pathogenic mechanism whereby this arises remains unclear. However, growing evidence suggests that retinopathy is a low-grade, chronic inflammatory condition associ-

ated with increased leucocyte entrapment in retinal capillaries and areas of capillary non-perfusion followed by endothelial cell damage [4–7].

Earlier studies have demonstrated that structural changes in *O*-linked carbohydrates expressed on the surface of leucocytes play a prominent role in controlling adhesion to endothelial cells [8, 9]. In this regard, our group has described, through use of an *in vitro* cell-based assay method, that plasma of diabetic patients affected by retinopathy causes a catalytic upregulation of a key glycosylating enzyme of the *O*-linked pathway, i.e. core 2 β -1,6-*N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), leading to a comparable increase of cell adhesion properties [10]. The activity of core 2 GlcNAc-T is significantly higher in type 1 and type 2 diabetic patients with retinopathy [11]. Furthermore, we have reported biochemical studies suggesting that TNF- α modulates core 2 GlcNAc-T activity via protein phosphorylation mediated by serine/threonine protein kinase C β 2 (PKC β 2) [10, 12].

The present study was designed to clinically validate the association between plasma levels of TNF- α , phosphorylation of leucocytic core 2 GlcNAc-T and corresponding enzyme activity at different stages of retinopathy in diabetic patients.

Subjects, materials and methods

Subjects

This study included a total of 25 type 1 and 41 type 2 diabetic patients recruited from the Diabetes Outpatient Clinic and the Eye Unit at St Thomas' Hospital, London, UK. Age-matched non-diabetic healthy controls ($n=15$) were found among the accompanying relatives of the patients or from hospital employees. The duration of type 1 and type 2 diabetes was 18.9 ± 2.2 and 9.2 ± 1.8 years, respectively. The levels of severity of retinopathy were determined according to the lesions as non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR). Informed, written consent was obtained from all participants in our investigation, which was approved by Guy's and St Thomas' Medical Ethics Committee and carried out in accordance with the Declaration of Helsinki as revised in 2000 (<http://www.wma.net/e/policy/b3.htm>).

Collection of blood and preparation of plasma

Whole blood was drawn and collected in EDTA tubes. Blood glucose was measured using a glucose analyser (Clandon Scientific; YSI, Yellow Springs, OH, USA). Plasma was isolated after density gradient centrifugation

over Histopaque 1070 (Sigma, Poole, UK). Briefly, 10 ml of whole blood were layered onto an equal volume of Histopaque and centrifuged (Heraeus, Brentwood, Essex, UK) at $400\times g$ for 30 min. Plasma above the polymorphonuclear (PMN) leucocyte-rich buffy coat was carefully removed under sterile conditions and stored at -20°C until used for experiments. The PMN leucocyte-rich buffy coat was carefully removed and resuspended in PBS. After centrifugation at $400\times g$ for 15 min, the pellet was stored at -20°C until used in the assay for core 2 GlcNAc-T activity.

Measurement of core 2 GlcNAc-T activity

To measure core 2 GlcNAc-T activity, leucocytes were washed in PBS, frozen and lysed in 0.9% NaCl, 0.4% Triton X-100 at 0°C . The activity of core 2 GlcNAc-T was then measured as described previously [8]. Endogenous activity of core 2 GlcNAc-T was measured in the absence of the added acceptor. The specific activity was expressed as picomoles per hour per milligram cell protein. In each case, the protein concentration was determined using a BCA Protein Assay Kit (Sigma, Poole, Dorset, UK).

Immunoassays

Plasma concentrations of TNF- α were measured in the diabetic patients and age-matched healthy controls using a commercially available ELISA kit (R&D Systems, Abingdon, Oxon, UK), which was standardised and implemented based on the manufacturer's instructions. Sensitivity of the kit for plasma concentrations of TNF- α , expressed as minimum detectable dose, was 0.12 pg/ml, while reproducibility of the assay method, evaluated as inter-assay variability, was quantified by a 10% CV.

C-reactive protein (CRP) was determined using a high-sensitivity anti-CRP monoclonal antibody kit with immunonephelometry (Dade Behring, Milton Keynes, Bucks, UK). This method had a lower detection limit of 0.2 mg/l and an inter-assay CV of 2.8%.

Immunoprecipitation of core 2 GlcNAc-T and Western blot analysis

For core 2 GlcNAc-T immunoprecipitation, as well as for Western blots, a polyclonal antibody against core 2 GlcNAc-T was used [12]. Cells were lysed on ice in lysis buffer (20 mmol/l Tris-HCl, pH 7.4, 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l sodium vanadate, 1 mmol/l phenylmethylsulphonyl fluoride, 1 $\mu\text{g/ml}$ aprotinin and 10 $\mu\text{g/ml}$ leupeptin). The lysate was incubated at 4°C for 20 min with constant agitation and insoluble material removed by centrifugation

Table 1 Clinical and metabolic characteristics of the study population

	Type 1 diabetes	Type 2 diabetes
<i>n</i>	25	41
Sex (F/M)	11/14	18/23
Age	55.1±2.7	59.8±2.1
Known duration of diabetes (years)	18.9±2.2	9.2±1.8
HbA _{1c} (%)	6.2±0.15	6.0±0.11
BMI (kg/m ²)	23.7±0.72	28.7±0.79
Diabetic retinopathy		
Absent	4	16
NPDR	12	16
PDR	9	9
Diabetic nephropathy		
Absent	25	41
Incipient	0	0
Diabetic neuropathy	6	4
Ischaemic heart disease	5	2
Peripheral vascular disease	3	3

Data are expressed as number, percentage and means±SEM

(14,000×g for 5 min at 4°C). The clarified lysate was incubated with Protein A-Sepharose Cl-4B-conjugated primary antibody for 2 h with constant agitation at 4°C. The immunoprecipitates were washed with Tris-buffered saline (10 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl) containing 0.5% Triton X-100, resuspended in 2×SDS-PAGE sample buffer and analysed by SDS-PAGE. After immunodetection of core 2 GlcNAc-T, the membranes were stripped in a buffer containing 50 mmol/l Tris-HCl, 2% SDS and 100 mmol/l mercaptoethanol at 55°C for 30 min,

washed and immunoblotted with anti-phosphoserine antibody (Chemicon, Chandlers Ford, Hants, UK). Immunoreactive bands were quantified by scanning densitometrically and calculating the density of individual bands using ImageQuant software (Molecular Dynamics, Kemsing, Kent, UK). The level of serine phosphorylation was expressed as a ratio of the intensity of the phosphorylation immunoreactive band to the intensity of core 2 GlcNAc-T immunoreactive bands.

Protein measurement

Total protein was measured using a BCA protein assay kit.

Statistical analysis

The statistical software Graph Pad Prism version 3.0 was used (GraphPad Software, San Diego, CA, USA). An unpaired two-tailed Student's *t*-test was used to test the significance of variables. Linear regression and correlation were used to evaluate the relationship between two variables. Data are expressed as means±SEM of measurements in the different experiments. Differences were considered statistically significant at *p*<0.05.

Results

Subjects

Table 1 shows the baseline clinical characteristics of patients with type 1 and type 2 diabetes recruited for this study.

Fig. 1 Activity of core 2 GlcNAc-T in PMN leucocytes is associated with severity of diabetic retinopathy. PMN cells were isolated from diabetic patients with no retinopathy (NR, *n*=20), non-proliferative diabetic retinopathy (NPDR, *n*=28) and proliferative diabetic retinopathy (PDR, *n*=18), and age-matched healthy controls (ND, *n*=15). The activity of core 2 GlcNAc-T was measured in lysates and expressed as

picomoles per hour per milligram protein. **a** The activity of core 2 GlcNAc-T in PMN cells from patients with type 1 and type 2 diabetes (*D*, *n*=66 and age-matched healthy controls (*ND*, *n*=15). Means±SEM; **p*<0.0001. **b** Relationship between the activity of core 2 GlcNAc-T in PMN cells and severity of diabetic retinopathy. Means±SEM: **p*=0.0007 vs ND; ***p*=0.0138 vs NR; ****p*<0.0001 vs NPDR

Activity of core 2 GlcNAc-T in leucocytes of diabetic patients

The activity of core 2 GlcNAc-T was several-fold higher in PMN leucocytes of patients with type 1 and type 2 diabetes than in those of healthy control subjects ($4,888 \pm 475$ vs 287 ± 147 pmol·h⁻¹·mg⁻¹ protein, $p < 0.0001$) (Fig. 1a). Furthermore, we observed a close relationship between enzyme activity in PMN leucocytes and severity of diabetic

retinopathy (Fig. 1b). PMN leucocytes of diabetic patients with PDR ($n=18$) exhibited core 2 GlcNAc-T activity that was approximately 2.6-fold higher than that from patients with NPDR ($n=23$) ($9,372 \pm 532$ vs $3,601 \pm 443$, $p < 0.0001$).

Plasma TNF- α in diabetic patients

Values of circulating TNF- α were significantly higher (Fig. 2a) in diabetic patients (5.89 ± 0.41 pg/ml, $n=51$) than in healthy subjects (1.78 ± 0.11 pg/ml; $p < 0.0001$, $n=15$). Conversely, our study revealed no evidence of differential TNF- α levels in plasma from patients with type 1 and type 2 diabetes (Fig. 2b). There was a positive correlation between plasma levels of CRP and TNF- α ($r=0.4126$, $p=0.0024$) (Fig. 2c). CRP is the main acute-phase protein and elevated CRP levels are an indicator of systemic inflammation.

Data analysis showed a significant, positive correlation between TNF- α and the severity of diabetic retinopathy (Fig. 3). In this regard, plasma levels of TNF- α in diabetic patients with PDR ($n=16$) were significantly higher than in subjects affected by NPDR ($n=23$) (8.24 ± 0.38 vs 5.73 ± 0.56 pg/ml).

Positive correlation between plasma TNF- α levels and core 2 GlcNAc-T activity

We have recently reported that in U937 cells, used as a model of circulating leucocytes, exogenous TNF- α causes a dose-dependent elevation of core 2 GlcNAc-T activity via PKC β 2-phosphorylation of the enzyme protein [12]. To confirm the implication of this pathway in diabetic patients affected by retinopathy, plasma levels of TNF- α were compared with the activity of core 2 GlcNAc-T measured in PMN cells. Figure 4 shows that the two values were closely related ($r=0.540$, $p < 0.0001$). Additionally, data

Fig. 2 Plasma levels of TNF- α are higher in diabetic patients than in age-matched healthy control subjects. EDTA-plasma samples were collected and stored at -80°C . **a** Plasma levels of TNF- α in diabetic patients (D , $n=66$) vs age-matched healthy controls (ND , $n=15$). Means $\pm$ SEM; $*p < 0.0001$. **b** Plasma levels of TNF- α in patients with type 1 ($n=25$) and type 2 ($n=41$) diabetes. Means $\pm$ SEM; difference non-significant. **c** Significant relationship ($r=0.4126$, $p=0.0024$) between plasma levels of TNF- α and plasma levels of CRP. Data points represent single plasma samples

Fig. 3 Plasma levels of TNF- α strongly correlate with the severity of diabetic retinopathy. Plasma and PMN leucocytes were isolated from total blood of diabetic patients with no retinopathy (NR , $n=20$), non-proliferative diabetic retinopathy ($NPDR$, $n=28$), proliferative diabetic retinopathy (PDR , $n=18$), and age-matched healthy controls (ND , $n=15$). Means $\pm$ SEM: $*p=0.0005$ vs ND ; $**p=0.018$ vs NR ; $***p=0.0018$ vs $NPDR$

Fig. 4 Plasma levels of TNF- α strongly correlate with the activity of core 2 GlcNAc-T in PMN leucocytes of diabetic patients. Plasma and PMN cells were isolated from total blood of patients with type 1 and type 2 diabetes ($n=66$). Each data point represents the TNF- α level of a single plasma sample. Activity of core 2 GlcNAc-T was measured in the PMN cell lysates and expressed as picomoles per hour per milligram protein ($r=0.540$, $p<0.0001$)

analysis indicated a close correlation between serine phosphorylation of the enzyme protein and both the level of TNF- α (Fig. 5b) and the activity of core 2 GlcNAc-T (Fig. 5c) ($r=0.434$, $p=0.00386$ and $r=0.412$, $p=0.017$, respectively), thereby substantiating the results previously obtained in vitro.

Correlation between glycaemia and core 2 GlcNAc-T activity and plasma TNF- α levels

Blood glucose showed a significant correlation with the activity of core 2 GlcNAc-T in PMN leucocytes ($r=0.572$, $p=0.0003$) (Fig. 6a), and also plasma TNF- α levels ($r=0.423$, $p=0.0248$) (Fig. 6b) in diabetic patients.

Discussion

The present study validates the pivotal role of circulating plasma TNF- α in the induction of a key glycosyltransferase of the *O*-linked glycosylation pathway, namely core 2 GlcNAc-T, which is associated with the biosynthesis of carbohydrate moieties affecting cell adhesion events. In this regard, the functional significance of higher core 2 GlcNAc-T activity, and subsequent appearance of cognate *O*-glycans, during the initial course of the inflammatory cascade has been well documented [13, 14].

The extent of the increase in core 2 GlcNAc-T activity in leucocytes of diabetic patients is similar to that observed in a number of physiological and pathological events, such as immune responses, diabetic cardiomyopathy and cancer, where a common element of these conditions is the altered dynamics of cell–cell or cell–matrix [15–18] interactions.

Fig. 5 Plasma levels of TNF- α are associated with the phosphorylation of core 2 GlcNAc-T in PMN leucocytes of diabetic patients. Plasma and PMN cells were isolated from total blood of patients with type 1 and type 2 diabetes ($n=22$). **a** Core 2 GlcNAc-T was immunoprecipitated from PMN cell lysates, followed by immunoblotting with anti-phosphoserine antibody. Blots were re-probed with anti-core 2 GlcNAc-T antibody. The blots were analysed densitometrically and level of phosphorylation expressed as a ratio (intensity of phosphorylation immunoreactive band : intensity of core 2 GlcNAc-T immunoreactive band). **b** Significant relationship between the levels of TNF- α in plasma and phosphorylation of core 2 GlcNAc-T in PMN cells of diabetic patients ($r=0.434$, $p=0.00386$). **c** Significant relationship between the activity and phosphorylation of core 2 GlcNAc-T in leucocytes of diabetic patients ($r=0.412$, $p=0.017$). Data points (**b**, **c**) represent the TNF- α level in single plasma samples

The central and causal role of leucocytes in vaso-occlusive processes and endothelial cell injury leading to the pathogenesis of diabetic retinopathy suggests a physiological significance of the enzyme in diabetes, probably through P-selectin glycoprotein ligand-1 (PSGL-1)-mediated adhesion events [11].

Using U937 cells as a model of circulating leucocytes, our group has previously shown that a human recombinant

Fig. 6 Activity of core 2 GlcNAc-T in PMN leucocytes and plasma levels of TNF- α strongly correlate with blood glucose in diabetic patients. Plasma and PMN cells were isolated from total blood of patients with type 1 and type 2 diabetes ($n=28$). Each data point represents the TNF- α level of a single plasma sample. **a** Significant relationship between blood glucose and the activity of core 2 GlcNAc-T in PMN cells ($r=0.572$, $p=0.0003$). **b** Significant relationship between blood glucose and plasma levels of TNF- α ($r=0.423$, $p=0.0248$)

version of TNF- α controls core 2 GlcNAc-T activity in a dose-dependent fashion via PKC β 2-phosphorylation of the enzyme protein [12]. This study confirms that, in diabetic patients, the functional link between plasma TNF- α and leucocytic core 2 GlcNAc-T through protein phosphorylation closely reflects the correlation observed in vitro. Consistent with previous reports [19–22], we noted significantly higher levels of TNF- α in the plasma of patients affected by either type 1 or type 2 diabetes vs healthy control subjects.

Our results indicate that this overzealous production of the cytokine, which may be released from various sources such as activated leucocytes, adipocytes and endothelial cells, is directly correlated with glucose values. This is in agreement with a previous report, which showed that chronic high glucose can dramatically increase levels of circulating TNF- α in human monocytic cells via oxidant stress-dependent and -independent pathways [23]. It is conceivable that higher levels of glucose and/or TNF- α may upregulate expression of adhesion molecules, such as

E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 [24–26], on the surface of endothelial cells and cause leucocyte adhesion to the diabetic retinal vasculature. In this regard, leucocytes of CD18 and ICAM-1 knock-out mice exhibited significantly decreased adhesion properties, which was associated with fewer damaged endothelial cells and less vascular leakage [27]. The contribution of TNF- α to the onset and progression of diabetic retinopathy is clearly supported by a number of previous studies [28–34], suggesting the therapeutic potential of anti-inflammatory treatments.

The demonstration that glucose levels, plasma TNF- α and core 2 GlcNAc-T activity are functionally linked with diabetic retinopathy confirms the physiological relevance of the cytokine, and the involvement of a downstream, glycosylation-mediated pathway in the onset and/or progression of the disease. Although the relative importance of this mechanism in diabetic retinopathy has yet to be elucidated, the validation of the in vitro system based on U937 cells allows further studies to be carried out in this regard. The use of core 2 GlcNAc-T gene-silencing strategy with RNA interference (RNAi) is a potential strategy, as well as the blockage of PSGL-1, the enzyme's predominant substrate expressed on the cell surface of leucocytes, which binds to P-selectin and primarily mediates the rolling phase of the adhesion cascade [35, 36]. These studies would provide insights regarding the possible use of small molecule inhibitors of core 2 GlcNAc-T as therapeutics.

Given the wide spectrum of biological activities shown by TNF- α [37] and, in particular, its important role in the general responses of the immune system, our results reveal a novel rationale for a specific treatment of diabetic retinopathy.

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