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Elevated plasma CD105 and vitreous VEGF levels in diabetic retinopathy

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Abstract

Diabetic retinopathy is the leading cause of blindness in the industrialized world. Hyperglycaemia induces retinal hypoxia that upregulates a range of vasoactive factors which may lead to macular oedema and/or angiogenesis and hence potentially sight threatening retinopathy. In this study, we have focused on the association of CD105 and vascular endothelial growth factor (VEGF) with the development and progression of diabetic retinopathy by means of quantifying their expression in the plasma and vitreous of diabetic patients. CD105 levels were quantified in the plasma of 38 type I diabetic patients at various stages of retinopathy and 15 non-diabetic controls. In an additional cohort of 11 patients with advanced proliferative retinopathy and 23 control subjects, CD105 and VEGF were measured in the vitreous. The values were expressed as median (range) and statistical analysis was carried out using the non-parametric Mann-Whitney U test. Plasma CD105 levels were significantly increased in diabetic patients [1.8 (1.1–2.4) ng/ml] compared with non-diabetic controls [0.7 (0.3–1.8) ng/ml] ($p<0.01$). Plasma CD105 levels were elevated in diabetic patients with all stages of retinopathy, the highest level was observed in background retinopathy [2.3 (2.1–2.5) ng/ml] followed by proliferative retinopathy [2.1 (0.9–2.8) ng/ml] and advanced proliferative retinopathy [1.4 (0.6–1.8) ng/ml]. Vitreous contents of CD105 did not differ between controls and patients with advanced proliferative retinopathy, but vitreous levels of VEGF were elevated by ~3-fold in patients with advanced proliferative retinopathy [7.2 (1.90–15.60) ng/ml] compared with the control subjects [1.80 (1.10–2.210)] ($p<0.01$). These observations indicate that plasma levels of CD105 and vitreous levels of VEGF are associated with diabetic retinopathy, suggesting that CD105 and the angiogenic factor VEGF may play a critical role in the development and progression of diabetic retinopathy. Further studies are required to determine whether circulating CD105 levels could serve as a surrogate marker for early stage retinopathy and for monitoring disease progression.

Keywords: diabetic retinopathy • angiogenesis • CD105 • VEGF

Introduction

Diabetic retinopathy is the major cause of premature blindness amongst adults in the western world [1]. Characterised by microvascular occlusion and leakage due to endothelial and pericyte cell damage and basement membrane thickening, macular oedema, neovascularisation and vitreous haemorrhage can eventually result in blindness [1]. Sustained hyperglycaemia results in retinal under perfusion, hypoxia and retinal ischaemia. These changes induce the production of

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Table 1 Clinical details of patients with various stages of diabetic retinopathy and controls

Group (number)	Age (years)	Duration of diabetes (years)	HbA1c (%)
Controls (15)	30 (21.5–33.5)	0	<7
No retinopathy (6)	25 (19.7–34.5)	4 (1.5–9.3)	8.7 (6.6–9.5)
Background retinopathy (10)	35 (26–57)	21 (15–24)	9.7 (8.3–10.7)
Proliferative retinopathy (6)	30 (26.3–40.3)	20 (16.3–25)	9.9 (9.3–11.5)
Advanced retinopathy (16)	30 (26–44)	22 (18–26)	10.1 (8.6–11.5)

vasoactive growth factors and their receptors that initiate retinal angiogenesis.

Many angiogenic factors have been incriminated in the development and progression of diabetic retinopathy. A previous report showed that VEGF was elevated in the vitreous and aqueous humor of diabetic patients with proliferative retinopathy [2]. In addition to VEGF, CD105 (endoglin) is also important in angiogenesis and its gene expression is upregulated by hypoxia [3, 4]. CD105 is a homodimeric membrane glycoprotein strongly expressed in angiogenic vascular endothelial cells and binds TGF-β1 and TGF-β3 [5, 6]. Experimental investigations have confirmed the role of CD105 in angiogenesis. Reduced CD105 gene expression in human vascular endothelial cells leads to inhibition of *in vitro* angiogenesis and CD105 null mice develop severely impaired vasculature in the yolk sac and heart defects [9–11]. A number of studies have demonstrated the augmented expression of CD105 in malignant tissues where it has been related to angiogenesis and inversely correlated with prognosis [5, 7, 8]. Circulating levels of CD105 have been shown to correlate with the presence of various types of cancers and their progression [12, 13]. In patients with early stage atherosclerosis, serum levels of CD105 are significantly elevated [14].

In view of the recognised function of CD105 and the pivotal role of angiogenesis in sight threatening diabetic retinopathy, we have examined the plasma and vitreous expression of CD105 and vitreous VEGF in a well characterised cohort of diabetic patients.

Patients and methods

Clinical details

Plasma samples were collected from 38 Type I diabetic patients. Their grade of retinopathy was characterised according to a modified Airlie house technique [15]: (a) no retinopathy (n=6), (b) background retinopathy (n=10), (c) proliferative retinopathy (n=6), and (d) advanced proliferative retinopathy requiring vitrectomy (n=16). Plasma samples from 15 non-diabetic age-matched control subjects were also collected. The duration of diabetes was significantly greater in patients with retinopathy compared to those without retinopathy ($p<0.01$) (Table 1). HbA1c did not differ significantly between diabetic patients (Table1). The clinical details of patients in the vitrectomy study are presented in Table 2. Vitreous samples were collected from 11 patients with advanced proliferative diabetic retinopathy prior to undergoing vitrectomy. Research Ethics Committee's approval and informed consent was obtained from all patients.

Plasma and vitreous samples

Venous blood samples were collected from the patients and control subjects. Plasma was harvested by centrifugation, aliquoted and stored at –70°C. Approximately 0.5–1ml of undiluted vitreous fluid was collected from the eye prior to irrigation of the vitreous, transported on dry ice and stored at –70°C.

Table 2 Clinical details of patients with various stages of diabetic retinopathy and controls used in vitrectomy study

Group (number)	Age (years)	Duration of diabetes (years)	HbA1c (%)
Controls (23)	67 (54–70)	0	<7
Advanced retinopathy (11)	60 (47–65)	22 (12.3–30.8)	8.9 (7.6–9.5)

Immunodetection of CD105, CD105/TGFβ1 and VEGF

Enzyme-linked immunosorbent assay (ELISA) for CD105

The sandwich ELISA for CD105 was developed by using optimised conditions [14]. Briefly, 96-well white micro-titre plates were coated with anti-CD105 Mab E9 (100µl/well) diluted 1/1000 in 0.1M PBS, and incubated in a humidified chamber overnight at 4°C. The coated plates were blocked using 1% BSA and 0.1% Tween 20 in 0.1M PBS (PBS-Tween) for 2h at room temperature. Test samples ½ diluted in PBS-Tween, were added to the plates in duplicate. Plasma with pre-determined CD105 (100 ng/ml) was titrated to make a standard curve in each plate. After overnight incubation at 4°C, 100µl/well of biotinylated Mab E9 (1/2000 dilution) was added to the plates and incubated at 4°C in a humidified chamber for 3h followed by addition of 100µl/well of HRP-conjugated avidin at 1/2000 dilution in PBS-Tween and 1% BSA, and incubated at room temperature for 30 min. Three washes with PBS-Tween were carried out between each of the procedures. Finally, 100µl/well of Amerlite signal reagent (Amersham UK) were added to each well and the light emission was measured immediately at 420nm in an Amerlite plate reader (Kodak Clinical Diagnostics, Aylesbury, UK).

Indirect immunoassay for VEGF

The procedure of immunoassay for measuring plasma VEGF has been published [16]. For this assay, 96-well white plates were coated with 100µl/well of goat anti-VEGF-165 antibody (R&D systems) diluted 1/1000 (1µg/ml) in 0.1M carbonate buffer (pH 9.6), and incubated in a humid box overnight at 4°C. The coated plate was blocked with 1% (w/v) bovine serum albumin (BSA), 0.01% (v/v) Tween 20 in 0.1M PBS (PBS-Tween) for 2h at room temperature. Serum samples in duplicate were added to the plates (100µl/well, diluted 1/2 in PBS-Tween). A standard curve was generated using recombinant human VEGF (R&D systems) at a range of 0.1–40ng/ml on each plate. After overnight incubation at 4°C, 100µl/well of rabbit anti-VEGF antibody (Santa Cruz Biotechnology) were added to the plate at 1/2000 dilution (1µg/ml) in PBS-Tween and incubated for 3h at 4°C. This was followed by the addition of HRP-conjugated goat anti-rabbit antibody (0.5µg/ml) (diluted 1/2000 with 1%BSA in PBS-Tween), which was incubated with shaking for 30 min at room temperature. Three washes with PBS-Tween were carried out between each of the steps. Finally, 100µl/well of Amerlite chemiluminescence signal reagent were added and the plate was read immediately in a plate reader. The measured values of light emission were converted into absolute concentration by reference to the VEGF standard curve.

Statistical analysis

All results are expressed as the median and range. Differences between groups were calculated employing the non-parametric Mann-Whitney U test. Correlation analysis was performed by calculating the Spearman's Rank correlation coefficient.

Results

Increased plasma CD105 levels in diabetic patients with or without retinopathy

Plasma CD105 levels in 38 diabetic patients were compared with 15 non-diabetic control subjects. The CD105 levels in the control subjects were 0.70 (0.30–1.80)ng/ml and were significantly increased

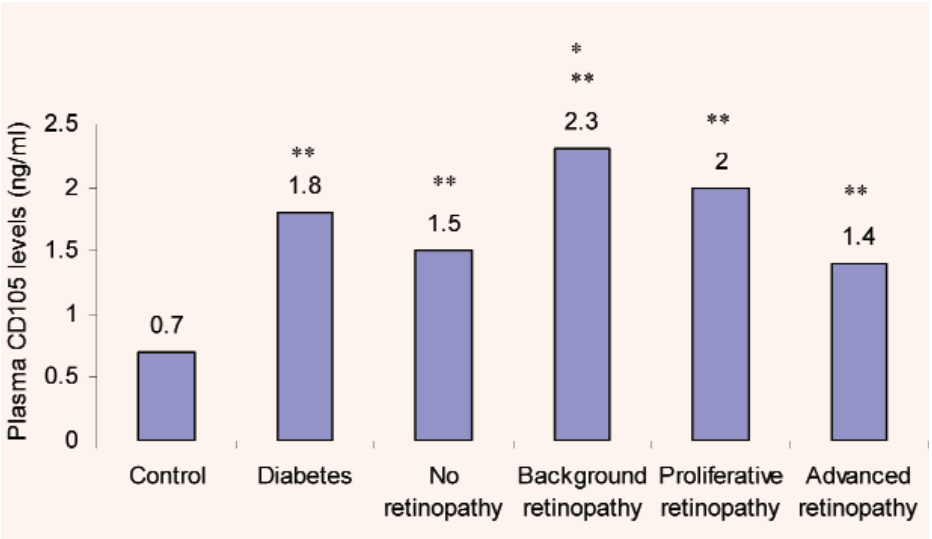

Fig. 1 Plasma levels of CD105 in non-diabetic controls and diabetic patients with various stages of retinopathy. The highest levels were observed in patients with background retinopathy (*p<0.05 compared to patients with no retinopathy or with advanced retinopathy. **p<0.01 compared to controls).

to 1.80 (1.10–2.80) ng/ml in the diabetic patients (p<0.001). CD105 levels, when assessed in accordance with the severity of diabetic retinopathy, were increased in those without retinopathy [1.50 (0.90–2.40) ng/ml], background retinopathy [2.30 (2.10–2.50) ng/ml], proliferative retinopathy [2.0 (0.90–2.80) ng/ml] and advanced proliferative retinopathy [1.40 (0.60–1.80) ng/ml]. Furthermore, CD105 levels were markedly higher in patients with background retinopathy compared to those with advanced proliferative retinopathy or without retinopathy (p<0.05) (Fig. 1). There was no significant correlation between CD105 levels and age (r = 0.13, p>0.05), duration of diabetes (r = 0.09, p>0.05), or HbA1c (r = 0.11, p>0.05).

Vitreous levels of CD105 in advanced proliferative retinopathy

Although CD105 in vitreous was detected, its levels in both patients with advanced proliferative retinopathy and controls were much lower than that in the plasma and did not differ between diabetic patients and controls (p>0.05) (Fig. 2). This led us to quantify the CD105/TGFβ1 complexes in the vitreous samples. The vitreous levels of the complexes were increased compared with the plasma levels, but there was no significant difference between patients and controls (data not shown).

Vitreous levels of VEGF in advanced proliferative retinopathy

Vitreous levels of VEGF were quantified in the same patients and controls. In agreement with a previous report [2], dramatically elevated vitreous VEGF levels were observed in patients with advanced proliferative retinopathy [7.20 (1.90–15.60) ng/ml] in comparison to 1.80 (1.10–2.21) ng/ml in the controls (p<0.001) (Fig. 2).

Discussion

The data presented in this report have shown significantly elevated plasma CD105 levels in diabetic patients, particularly in those with background and proliferative retinopathy. The relatively low levels of CD105 in the vitreous are likely to be the result of the formation of CD105/TGFβ complexes and/or sequestration by other unknown factors in the vitreous. VEGF levels in vitreous were markedly increased in advanced proliferative retinopathy compared with control subjects. These findings suggest that both CD105 and VEGF may contribute significantly to the development and progression of diabetic retinopathy.

This is the first study to show an association between circulating CD105 levels and the presence of diabetic retinopathy. It is intriguing that plasma from patients with early stage diabetic retinopathy possessed the greatest amount of CD105 in their

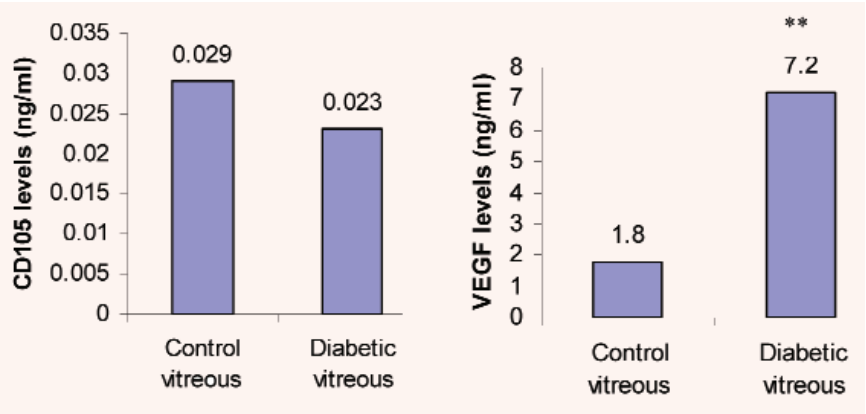

Fig. 2 Vitreous levels of CD105 and VEGF in controls and patients with advanced proliferative retinopathy. Vitreous levels of CD105 did not differ between controls and patients with diabetic retinopathy (barchart on the left), but vitreous levels of VEGF were increased 3-fold in patients with advanced retinopathy (barchart on the right, **p<0.01).

circulation, implying its potential as an early marker for this diabetic complication. In our previous investigations, CD105 was significantly elevated in early stage atherosclerosis and diminished in advanced atherosclerosis [14]. We proposed that this represented neovascularisation in the atherosclerotic plaque which contributes to plaque growth and lesion instability [14, 17]. The present study has shown that patients with early stage retinopathy (background retinopathy) had higher CD105 levels compared with those with late stage retinopathy (advanced proliferative retinopathy). Several factors may lead to such a pattern of circulating CD105 in patients with atherosclerosis and diabetic retinopathy. First, the gene expression of CD105 is regulated by vasoactive factors, such as TGFβ1 and TNFα, the former up-regulates and the latter down-regulates CD105 gene expression [18, 19]. The alteration of CD105 levels could reflect a dynamic change of these factors in the progression of both atherosclerosis and diabetic retinopathy. Second, CD105 is strongly expressed in proliferating angiogenic endothelial cells, the elevated CD105 levels in the early stage atherosclerosis may reflect occurrence of active angiogenesis at this stage. Third, CD105 binds TGFβ1 and TGFβ3, the formation of such complexes could lower the detectable free CD105.

VEGF is a well documented angiogenic factor which is thought to play a key role in the development of diabetic retinopathy [2, 20]. In agreement with a previous report [2], we have shown that VEGF levels were significantly increased in the vitreous of patients with retinopathy, supporting its role in promoting angiogenesis and vessel leakage leading to advanced proliferative retinopathy.

In conclusion, we have observed elevated plasma levels of CD105 in diabetic patients. Its levels are increased most significantly in patients with early stage retinopathy and decreased in advanced retinopathy, suggesting that it may be involved in the development and progression of diabetic retinopathy. The marked elevation of vitreous VEGF in patients with advanced suggests that VEGF is a key player in proliferative diabetic retinopathy. Thus there appears to be a complex interaction between CD105 and VEGF at different stages of diabetic retinopathy which requires further study. The correlation of CD105 levels with various stages of retinopathy suggests that circulating CD105 could be useful for detecting early stage diabetic retinopathy and for monitoring disease progression.

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