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Early changes in glomerular size selectivity in young adults with type 1 diabetes and retinopathy.

Results from the Diabetes Incidence Study in Sweden – DISS.

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Short title: Urinary markers in patients with diabetes and retinopathy.

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Key words : diabetic retinopathy, glomerular, glycosaminoglycans, immunoglobulin IgG2 and IgG4, size selectivity, Tamm-Horsfall protein, transforming growth factor (TGFβ1).

Abbrevations: albumin/creatinine ratio (ACR), diabetic nephropathy (DN), glycosaminoglycans (GAG), Tamm-Horsfall protein (THP), transforming growth factor (TGFβ1).

Abstract

Objective. To investigate the relationship between early-onset retinopathy and urinary markers of renal dysfunction.

Research Design and Methods. The Diabetes Incidence Study in Sweden (DISS) aims to register all new cases of diabetes in young adults (15-34 years). In 1987-1988, 806 patients were reported and later invited to participate in a follow-up study focusing on microvascular complications after ~ 10 years of diabetes. In the present study, 149 patients with type 1 diabetes, completed eye examination, and urine sampling were included.

Results. The patients with retinopathy (n = 58, 39%) had higher HbA_{1c} ($p<0.001$) and urinary IgG2/creatinine ($p<0.05$) and IgG2/IgG4 ratios ($p<0.05$). Patients with maculopathy had the highest levels. No significant differences in urinary albumin/creatinine, glycosaminoglycans (GAG)/creatinine, Tamm-Horsfall protein (THP)/creatinine and IgG4/creatinine were found. Women had higher urinary albumin/creatinine ($p<0.01$) and urinary IgG2/creatinine ratios ($p<0.01$) than men.

Conclusions. Young adults with type 1 diabetes and early-onset retinopathy had higher IgG2/creatinine and IgG2/IgG4 ratios than patients without retinopathy indicating that retinopathy is associated with a change in glomerular size selectivity. This was found in association with normal urinary albumin and THP excretion and may be suspected to reflect early general vascular changes.

Introduction

Retinopathy and nephropathy are microvascular complications in diabetes. Retinopathy is often connected with signs of nephropathy such as albuminuria and proximal tubular proteinuria. (Skrha et al. 1991) (Nielsen, Holm and Hemmingsen 1990). Albuminuria has been suggested as a risk marker for proliferative retinopathy in some (Klein, Moss and Klein 1993; Johansen et al. 1994; Rossing et al. 1998) but not all studies (Porta et al. 2001) (Agardh, Agardh and Torffvit 1996). Likewise, independent of glycemic control and diabetes duration, the development of nephropathy is associated with an increased risk of developing proliferative retinopathy in patients with type 1 diabetes (Gilbert et al. 1998). Hence, although there is an association between retinopathy and nephropathy, the link between is unclear.

A study of the transcapillary escape rate and the urinary excretion rate of albumin has suggested that macular edema reflects generalized vascular hyperpermeability at least in type 2 diabetic patients (Knudsen et al. 2002). Measurements of urinary IgG2 together with IgG2/IgG4 ratios are used to assess glomerular size selectivity whereas urinary IgG4 evaluates charge selectivity (Tencer et al. 1999). A decrease in the glomerular synthesis of glycosaminoglycans (GAGs) results in a loss of negative charge of the glomerular basement membrane and thereby increased passage of negatively charged molecules such as albumin and IgG4 in patients with type 1 diabetes (Torffvit and Rippe 1999). TGFβ1 increases the synthesis of matrix proteins (Mason and Wahab 2003; Pueyo et al. 2004). Inhibition of TGFβ1 reduces albuminuria, fibrosis, atrophy of the tubular system (Mifsud et al. 2003), mesangial expansion and sclerosis of the glomeruli (Cruzado et al. 2004). In agreement the synthesis of matrix proteins may be monitored by following TGFβ1 in urine. An increase in proximal tubular proteinuria has been found in patients with retinopathy (Nielsen, Holm and Hemmingsen 1990; Skrha et al. 1991). A distal tubular proteinuria has not been studied in

patients with retinopathy but is possible to disclose by measuring Tamm-Horsfall protein (THP) (Torffvit et al. 1992) .

The aim of this study was to investigate the relationship between early-onset retinopathy and urinary markers of renal dysfunction. To that end, we examined the size and charge selectivity of the glomerular basement membrane, distal tubular dysfunction, and the promotion of matrix proteins in a well characterized Swedish cohort of young adults with type 1 diabetes (Svensson et al. 2003).

Research design and Methods

Study cohort

Among 806 diabetic patients reported to the Diabetes Incidence Study in Sweden (DISS) 1987-88, 782 were invited and 579 (74%) accepted to participate in a follow-up study after ~ 10 years of diabetes. In the present analysis the following exclusion criteria were used: unable to collect urine (n = 399), missing data of retinopathy (n = 7) and type 2 diabetes (n = 24). Thus, 149 patients with type 1 diabetes with at least one timed overnight urine collection were included. The study protocol included a questionnaire for clinical data and information of all HbA_{1c} values collected since diagnosis of diabetes. Mean HbA_{1c} for each patient was calculated using the area under the curve of HbA_{1c} over time to compensate for the occasionally irregular intervals between the measurements (Matthews et al. 1990). In addition, patients were asked for a blood sample for measurement of C-peptide and HbA_{1c} and two consecutive over-night urine collections (Svensson et al. 2003). The Ethics Committee at the University of Lund approved the study.

Diabetes classification

The clinical classification of type 1 and type 2, was based on WHO criteria (World Health Organization 1985). However, as a clinical classification is uncertain (Arnqvist et al. 1993; Littorin et al. 1999), the results of islet antibody analysis, at diagnosis 1987-88, were used to

make a more accurate classification (Landin-Olsson et al. 1992; Schölin et al. 2004). Patients reported as having type 1 diabetes at follow-up and patients clinically considered as having type 2 or unclassifiable diabetes but displaying islet antibodies at diagnosis were all classified as having type 1 diabetes. Patients reported as type 2 or unclassifiable diabetes and lacking islet antibodies at diagnosis were all considered as having type 2 diabetes.

Retinopathy

Retinal status was evaluated by a central assessment of all retinal photographs taken at the patients local hospital. If no photograph had been taken, ophthalmoscopy or slit lamp biomicroscopy data from the patients records were used. Retinopathy was graded according to the method suggested by Klein et al (Klein et al. 1986). Two independent graders examined the photographs. If there was a disagreement, a third grader determined the retinopathy level (Henricsson et al. 2003). The patients were divided into four groups: no retinopathy, background (simplex), proliferative retinopathy, and macula oedema.

Nephropathy (DN)

The diagnosis clinical diabetic nephropathy (DN) was extracted from the medical and laboratory records and not from the urine collection conducted in the present study. The following criteria were used: at least two of three consecutive urine samples displaying an albumin excretion rate >20 µg/min (timed collections) or an albumin concentration >30 mg/L (spot samples). The criteria thus include both micro- and macroalbuminuria. If any other renal disease than diabetes was suspected, the patient was not considered to have DN (Svensson et al. 2003).

Blood and Urine chemistry

HbA_{1c} was measured using ion-chromatography at the local hospital laboratories (Jepsson et al. 1986) whereas urine analysis was done at a central laboratory, the Renal laboratory at the University Hospital in Lund. Urinary albumin (Torffvit and Wieslander 1986),

immunoglobulins G2 (IgG2) and G4 (IgG4) (Tencer et al. 1999)and Tamm-Horsfall protein (THP) (Torffvit et al. 1992) were measured using enzyme-linked immunosorbent assay techniques. Urinary glycosaminoglycans (GAG) were assessed by a colour reaction assay (Tencer et al. 1997). This method does not measure hyaluronate. Urinary transforming growth factor β1 (TGF-β1) was evaluated by a commercially available assay (Promega Corporation, WI, USA). The urinary data are given as the urine/creatinine ratio to correct for diuresis and failure in urine collection.

Statistical methods

When analyzing differences between groups, Mann-Whitney U nonparametric test was used. To analyse differences in frequencies between groups, we used Fisher´s exact test. Data are presented as mean $\pm$ SD or as indicated. Logistic regression analysis with all forward stepwise selection (Likelihood ratio) was used to assess the association between the dependent dichotomy variable retinopathy and the independent variables –urinary markers, age at follow-up, age at onset of diabetes, C-peptide levels, gender, smoking, blood pressure and HbA_{1c}. Urinary markers were log10 transformed due to skewed distribution. The association between the dependent continuous urine variables and independent variables retinopathy and the above mentioned confounder variables were tested with stepwise linear regression analysis.

A p-value of < 0.05 was considered statistically significant. SPSS 12.0.1 (Chicago, IL, USA) was used to analyse data.

Results

There were no significant differences in age at onset of diabetes, mean HbA_{1c}, smoking, systolic or diastolic blood pressure between men and women (data not shown). Urinary albumin/creatinine ratio (0.6 [0.04-5.9] vs. 0.4 [0.05-3.4] mg/mmol) (p=0.001) and IgG2/creatinine ratio (0.27 [0.02-5.4] vs. 0.14 [0.01-2.3] mg/mmol) (p=0.004), respectively,

were significantly higher in women whereas IgG4, IgG2/IgG4 ratio, GAG and THP creatinine ratios were similar to those in men (data not shown). Among patients with clinically diagnosed nephropathy, urinary albumin/creatinine (1.13 [0.5-5.8] vs. 0.44 [0.04-5.9] mg/mmol) ($p<0.001$) and TGFβ1/creatinine ratios (3.8 [0.01-10.1] vs. 0.6 [0.01-222] mg/mmol) ($p=0.002$), respectively, were significantly higher compared with those patients without nephropathy. HbA_{1c} and BP levels, respectively, were similar compared with patients without nephropathy (data not shown). No differences in urinary markers were found between smokers and non-smokers (data not shown).

Retinopathy and urinary markers

51 patients had developed background (simplex) retinopathy, one proliferative retinopathy and six maculopathy. Among the 91 patients without retinopathy, 33 were smokers and three had antihypertensive therapy. 23 were smokers and three had antihypertensive therapy among the patients with retinopathy. Among the patients with maculopathy, four were smokers and none were treated for hypertension. Patients with retinopathy had significantly higher HbA_{1c}, ($p<0.001$) and IgG2/creatinine ratio ($p=0.03$) compared with those without retinopathy. The highest levels of HbA_{1c} ($p=0.009$), IgG2/creatinine ($p=0.04$), and IgG2/IgG4 ratios ($p=0.04$) were found in patients with maculopathy (Table 1).

Multivariate analyses

Retinopathy was associated with 2.7 (1.3-5.7, 95% CI) times higher urinary IgG2/creatinine excretion ($p = 0.007$) than no retinopathy. The model explained the variation in the retinopathy by 20% (R-square). Conversely, urinary IgG2/creatinine retinopathy ($p= 0.01$) and IgG2/IgG4 ratios were associated with retinopathy ($p = 0.03$) but this model only explained 4% of the variation (R square) in the ratios.

Discussion

In the present study, despite similar urinary albumin/creatinine ratio type 1 diabetic patients with retinopathy had higher urinary IgG2/creatinine and IgG2/IgG4 ratios than patients without retinopathy. The highest ratios were found in patients with maculopathy. This finding indicates a change in glomerular size but not in charge selectivity or tubular function in patients with type 1 diabetes and retinopathy.

In patients with type 1 diabetes, a correlation between the degree of retinopathy and the severity of glomerulopathy has previously been found (Chavers et al. 1994). However, some patients with advanced retinopathy have normal urinary albumin excretion and glomerular morphology (Chavers et al. 1994). Furthermore, early retinal changes, diagnosed by retinal vascular leakage utilizing fluorescein angiography, do not always predict progression to renal insufficiency (Mosier et al. 1997). Our study cohort with young adults with type 1 diabetes and ~ 10 years of diabetes duration had near-to- normal albumin excretion in men and slightly above normal in women. Measurements of IgG2 together with IgG2/IgG4 ratios are often used to assess size selectivity and IgG4 charge selectivity (Tencer et al. 1999). The urinary IgG2 levels in this study cohort were above normal in most patients while IgG4 was normal indicating alterations in size selectivity. Our study infers that IgG2 may be a more sensitive than albumin for the detection of early diabetic microangiopathy.

The association between glycemic control and development or progression of retinopathy has been described in several clinical long-term studies in patients with type 1 diabetes (Nørgaard et al. 1989; Chase et al. 1990; Kullberg and Arnqvist 1993; Klein, Klein and Moss 1995; Rossing et al. 1998; Porta et al. 2001; Lövestam-Adrian et al. 2001; Voutilainen-Kaunisto et al. 2001). The results in this study confirm these observations; HbA_{1c} levels were higher in patients with retinopathy than in patients without retinopathy.

The presence of hyperglycemia activates a cascade of biological events. TGFβ1 increases the synthesis of matrix proteins (Mason and Wahab 2003; Pueyo et al. 2004). The levels of

TGFβ1 in this study are above those previously published by Chaturvedi (Chaturvedi et al. 2002). However, the urinary levels of TGFβ1/creatinine were not increased in patients with retinopathy although a clear tendency to higher levels in patients with maculopathy was seen. This may indicate that TGFβ1 is of minor importance in the development of retinopathy compared with diabetic nephropathy.

A decrease in the glomerular synthesis of glycosaminoglycans (GAGs) results in a loss of negative charge of the glomerular basement membrane and increased passage of negatively charged molecules such as albumin and IgG4 in patients with type 1 diabetes (Torffvit and Rippe 1999). In the current study, urinary GAG levels did not differ between patients with or without retinopathy. Hence, it is unlikely that GAG in urine is associated with retinal leakage (Witmer et al. 2001).

Only in long-standing changes of the retinal vessels, the permeability is sufficiently altered to allow passage of higher-molecular-weight proteins such as IgG (Rahu and Chignell 1975). Widening of endothelial intercellular clefts, tight junctions, rather than damage to the endothelial cell body may form the cellular basis for the microvascular leakage of albumin, fibrinogen and IgG (Schlingemann et al. 1999) (Vinores et al. 1999). The magnitude of retinal leakage of fluorescein correlates with the severity of retinopathy and visual acuity and is more important in macular oedema (Sander et al. 2002). In agreement, in our study, patients with maculopathy had the highest urinary levels of IgG2 and IgG2/IgG4 suggesting a widespread capillary damage with a general change in size selectivity of the basements membrane in the capillaries. We thus confirmed a previous study in type 2 diabetic patients with macular oedema who had a generalized vascular hyperpermeability with an increased transcapillary escape rate and urinary excretion rate of albumin (Knudsen et al. 2002). In a previous study of patients with type 1 diabetes, both an increased excretion of IgG2 and IgG4 was found in normoalbuminuric patients (Torffvit and Rippe 1999). Thus, a normal albumin excretion does

not exclude glomerular barrier damage. The observations mentioned above may be caused by a generalized damage of the capillary system in the diabetic patient. The defect may be size related and increase the number of large pores (90Å in radius) allowing IgG2 to pass. Whether this is the case or it is simply a widening of the tight junctions as mentioned above for the patients with macular oedema need to be further studied. The cause may be a high systemic blood pressure putting a high degree of shear stress to the capillaries. In such case it may be possible to prevent with ACE inhibitors or AII blockers.

As glucosuria exert stress on the tubular system the tubular reabsorption of IgG2 may be affected and an increase in proximal tubular proteinuria has been found in patients with retinopathy (Nielsen, Holm and Hemmingsen 1990; Skrha et al. 1991). In the present patient cohort, distal tubular function, as reflected in THP/creatinine was unaffected by retinopathy.

Conclusions

Despite similar levels of albumin/creatinine and THP/creatinine ratios, type 1 diabetic patients with early-onset of retinopathy have higher urinary levels of IgG2 and IgG2/IgG4 than patients without retinopathy. Thus, retinopathy was associated with a change in the size but not charge selectivity of the glomerular filter or distal tubular dysfunction, and this may apply to the microvasculature throughout the body.

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Table 1. Clinical and laboratory characteristics at follow-up in 149 patients without and with retinopathy (RP) but without maculopathy and patients with maculopathy, respectively.

	Without RP (n=91)	With RP (n=52)	Maculopathy (n=6)
Men/women (n)	51/40	34/18	6/0
Nephropathy (n)	9	3	1
Age at diagnosis (years)	25±6	25±6	23+5
Age at follow up (years)	33±6	34±5	31+5
Systolic BP (mmHg)	120±13	123±13	118+8
Diastolic BP (mmHg)	73±9	77±9	73+4
Mean HbA1c (%)	6.8±1.4	7.7±1.7***	8.7+1.6**
Urinary substrate/creatinine			
Normal levels in parenthesis			
Albumin (mg/mmol)	0.49 (0.05-5.9)	0.40 (0.04-5.8)	0.61(0.3-1.3)
IgG2 (mg/mmol)(0.07 (0.01-0.4))a	0.16 (0.01-2.2)	0.26 (0.01-5.4)*	0.75(0.03-1.6)*
IgG4 (mg/mmol) (0.03 (0.01-0.5))a	0.02 (0-1.0)	0.02 (0-4.1)	0.05(0-0.1)
GAG (mg/mmol)(2.9 (2-4.4))a	2.5 (0-8.4)	2.6 (0.4-6.0)	3.0(1.5-3.8)
IgG2/IgG4 (3.7 (0.6-17.4))a	9.0 (0.2-200)	13.8 (1.7-175)	21.3(10.5-37.5)*
THP (mg/mmol)(5.7 (1.2-16.2))b	1.3 (0.2-14.2)	1.3 (0.3-11.5)	2.8(0.9-20.32)
TGFβ1 (ng/mmol) (0.003 (0.002-0.003))c	0.7 (0.01-17.3)	0.4 (0.01-222)	2.3(0.01-6.2)

Urine parameters are given as median and range of ratios between substance and urine creatinine (mg/mmol). * P <0.05; ** P<0.01; ***P<0.001 vs. without RP. Normal value from a: (Torffvit and Rippe 1999), b: (Torffvit 1999), c: (Chaturvedi et al. 2002).