Indian Journal of Clinical Biochemistry, 2008 / 23 (3) 159-165

NON ENZYMATIC GLYCOSYLATION OF IgG AND THEIR URINARY EXCRETION IN PATIENTS WITH DIABETIC NEPHROPATHY

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ABSTRACT

Diabetic nephropathy is a major cause of end stage renal disease. Increased excretion of albumin has widely been recognized as an early manifestation of diabetic nephropathy particularly in subjects with diabetes mellitus. However, certain other proteins besides albumin may be excreted in high amount during early phase of diabetic nephropathy. The serum and urinary IgG, Glycosylated hemoglobin, fructosamine and glycosylated IgG were evaluated in the present study. Thirty-two patients of Type 2 Diabetes without any complications, thirty-one patients of Type 2 Diabetes with nephropathy, twenty-six patients of non-diabetic nephropathy and forty normal healthy individuals were enrolled in this study. Subjects were grouped based on their serum creatinine level. Serum IgG, glycosylation of IgG and urinary IgG excretion were increased significantly in diabetic patients compared to healthy controls, which were further increased significantly in chronic renal failure patients with respect to the clinical stage of nephropathy. A positive correlation was observed between glycosylation of IgG and IgG excretion (R^2=0.5995, 0.7114 respectively) in diabetic patients without any complications and diabetic nephropathy patients only, suggesting a significant role of IgG glycosylation in the vascular clearances of IgG during diabetic nephropathy.

KEY WORDS

Urinary and serum IgG, Glycosylation of IgG, Fructosamine, Glycosylated hemoglobin, Diabetic nephropathy.

INTRODUCTION

Nephropathy is one of the critical secondary complication of diabetes mellitus leading to progressive loss of kidney function, dialysis and generally to end stage renal disease (1). Diabetic nephropathy has emerged as the single largest cause of end stage renal failure and accounts for nearly 40% of the new patients who need dialysis each year. Diabetic patients poorly respond to dialysis due to high rates of infection, access failure and cardiovascular complications. Of all the long-term complications of diabetes, nephropathy imposes the highest cost, both in terms of money and human sufferings (2). Non enzymatic end products are heterogeneous cross-linked sugar-derived proteins which could accumulate in glomerular basement membrane, mesangial cells, endothelial cells, and podocytes in patients with diabetes and/or end-stage renal failure. Non enzymatic end products thought to be involved in the pathogenesis of diabetic nephropathy via multifactorial mechanisms such as oxidative stress generation and overproduction of various growth factors and cytokines (3). The progression of nephropathy can be monitored from its incipient stage by increased serum creatitine levels, defined by slight increase in the excretion rates of certain plasma proteins (called microproteinuria) to its overt stage, characterized by gross proteinuria and ultimately renal failure (4). As on today abnormal urinary albumin excretion is the hallmark of diabetic nephropathy, generally recognized to be sufficiently specific, particularly in subjects with diabetes mellitus, to predict the subsequent development of clinically overt diabetic nephropathy (5,6), however, certain other proteins besides albumin may be excreted in abnormal amounts during this early phase of diabetic nephropathy. The permselectivity of the kidneys vary with respect to size and charge of the filtered plasma proteins (4). Renal permselectivity

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is the result of glomerular filtration and tubular reabsorption of plasma proteins, and urinary albumin is a limited indicator of nephropathy, because it is insensitive to renal tubular damage. Proteinuria may play a central role in the induction of tubulointerstitial injury, which is more frequently seen with non-selective proteinuria suggesting that loss of high molecular weight proteins such as growth factors, or complement components may be involved in tubular cell damage. The excretion of IgG may then reflect urinary loss of these high molecular weight injurious factors. Urinary IgG excretion, which reflects the combination of selectivity and magnitude of proteinuria, thus measuring urinary IgG excretion, may be of help in guiding the treatment of these patients. Several studies have shown that increased urinary excretion of some kinds of plasma proteins with different molecular radii <55A and different isoelectric points such as IgG (4,7), transferrin (8), ceruloplasmin (7,9) and orosomucoid (10) may precede the development of microalbuminuria in diabetic patients.

The present study was undertaken to investigate the serum IgG concentration, their glycosylation and urinary clearance of IgG to predict the relationship of excessive glycosylation of IgG and their urinary clearance. The urinary IgG (molecular radius 55A isoelectric point 7.4) might be the better marker of renal injury as it combines information on the level of proteinuria and loss of size selectivity.

Table 1: Glycosylated hemoglobin and serum fructosamine levels in patients with Type-2 Diabetes without any complication, with non -diabetic nephropathy and with diabetic -nephropathy

Groups	*Glycosylated Hemoglobin (g/dl)*	*Serum Fructosamine (mmol/L)*
Group I (Control, n = 40)	4.28 ± 0.10	1.2 ± 0.02
Group II (Diabetes mellitus without any Complication, n = 32)	6.77 ±0.21 $p<0.001^a$	2.51 ±0.15 $p<0.001^a$
Group III (n = 26) Non-diabetic nephropathy CRF (creatinine<4)	5.89 ± 0.28 $p<0.001^a$	1.94 ± 0.12 $p<0.001^a$
Group IV (n = 31) Diabetic nephropathy CRF (creatinine <4)	8.58 ± 0.46 $p<0.001^a$ $p<0.01^b$ $p<0.001^c$	3.10 ± 0.26 $p<0.001^a$ $p<0.05^b$ $p<0.001^c$

Values are mean ± SE, Values in parenthesis represent sample size. Statistical significances between different groups were evaluated by student's test. p value < 0.05 were considered significant; a:-Compared with group I (Control); b:- Compared with group II (DM); c:- Compared with group III CRF(Creatinine <4)

MATERIALS AND METHODS

Male and female patients registered in Muljibhai Urological Hospital, Nadiad and P.S Medical College and Hospital, Karamsad were screened by the physician for diabetes, diabetic nephropathy, and non-diabetic nephropathy. The consent of patients and approval of the protocol was confirmed by the ethical committee of the hospitals.

Patients were subjected to detailed clinical evaluation regarding duration of diabetes, parameters of renal involvement (odema, proteinuria, hypertension, and renal failure), treatment modalities, other associated complication of diabetes such as cardiovascular, peripheral neuropathy, and retinopathy. The laboratory investigations included: urine analysis, 24 hr urinary protein, hemoglobin, total and differential leucocytes count, serum urea, serum creatinine, total serum protein, serumA: G ratio, serum electrolyte level such as serum sodium, potassium, phosphate, complete lipid profile, blood glucose (random, fasting and postprandial), glucose tolerance test, ultra sonography for kidneys and bladder and renal biopsy.

Based on the above clinical and laboratory investigations, patients were diagnosed by the physician and collectively grouped as diabetes mellitus patients without any complications and patients with diabetic-nephropathy and non-diabetic nephropathy of different stage. Thirty-two patients of Type 2 Diabetes without any complications, thirty-one patients of Type 2 Diabetes with nephropathy (CRF patients with creatinine <4 and were not on dialysis), twenty-six patients of non-diabetic nephropathy (CRF patients with creatinine <4 and were not on dialysis) and forty age and sex matched healthy individuals not having any disease and infection from last one year were enrolled in this study. Fasting blood samples and urine samples were collected from the patients of all the above-mentioned groups and from normal healthy individuals. The morning first voided urine sample was pooled with two subsequent samples after the interval of three hours as many of them were from out patient department.

The urine samples were centrifuged in refrigerated centrifuge at 1500 r.p.m. to remove cell debris and stored at- 20°C with sodium azide (0.2%) as preservative. Serum was separated and stored at 4°C till further use. The serum and urinary IgGs were measured by sandwich ELISA (Enzyme Linked Immunosorbent Assay) within one week of storage using reagents supplied by Bangalore Genei, India.

The 96 wells ELISA plate (Tarsons) were coated with 100µl of capture antibody (goat anti human IgG, 10µg/ml) in carbonate

Table 2: Serum IgG (mg/dl) and Glycosylation level of IgG (Carbohydrate µg/mg of purified IgG) in in patients with Type 2 Diabetes without any complication, with non -diabetic nephropathy and with diabetic -nephropathy

Groups	*Serum IgG (mg/dl)*	*Glycosylation level of IgGµg/mg of IgG*
Group I (Control, n = 40)	772.10 ± 8.0	3.67 ± 0.20
Group II (Diabetes mellitus without any Complication, n = 32)	1168.4 ±29.7 $p<0.001^a$	9.77 ± 0.322 $p<0.001^a$
Group III (n = 26) Nondiabetic nephropathy CRF (creatinine<4)	910.1 ± 27.5 $p<0.001^a$	3.86 ± 0.095 $N.S^a$
Group IV (n = 31) Diabetic nephropathy CRF (creatinine <4)	1041.2 ± 32.97 $p<0.001^a$ $p<0.01^b$ $p<0.001^c$	14.68 ± 4.0 $p<0.001^a$ $p<0.001^b$ $p<0.001^c$

Values are mean ± SE, Values in parenthesis represent sample size
Statistical significance between different groups was evaluated by student's 't' test. p value < 0.05 were considered significant
a :-Compared with group I (Control); b:- Compared with group II (DM)
c:- Compared with group III CRF(Creatinine <4)

buffer (50 mM pH 9.6). The plates were incubated at 4°C under humid conditions over night. The wells were aspirated out and washed thrice with PBST (Phosphate buffer saline tween - 20 - 0.1 M pH 7.2). The vacant sites were blocked with 100ml of blocking solution (3% BSA in PBST), incubated at room temperature for 1 hour and washed thrice with PBST. 100µl of samples and standards were added to wells in conjugate diluent (0.1% BSA in PBST). The plates were incubated at 37°C for 1 hour, and washed thrice with PBST. Goat anti human IgG HRP 1:1000 diluted in PBST was added 100µg/well and incubated at room temperature for 1 hour. The plates were washed four times with PBST, 100µl of substrate TMB/ H_2O_2 was added and the reaction was stopped with 50µl/well 1N sulphuric acids after 5 minutes and the O. D. was read at 450 nm in ELISA reader (Molecular device model spectra max 190).

Serum immunoglobulin (Ig) fraction was precipitated by 40% ammonium sulphate saturation and precipitated protein was suspended in distilled water and reprecipitated by 40% ammonium sulphate. This fraction was used to purify IgG, using DEAE-sephadex ion exchange chromatography (11) and purity was verified by SDS- PAGE. Purified IgG fractions were lyophilized and used for carbohydrate estimation by Orcinol method (12). Serum fructosamine and Glycosylated hemoglobin were measured by NBT (Nitro blue terazodium) method (13), and TBA (Thio barbituric acid) method (14) respectively.

Statistical significance of the data was evaluated by Student's 't' test and the p values less than 0.05 were considered significant. The correlation between various parameters was evaluated by simple linear regression curve and its significant level was calculated by Fisher Z test for R^2 >0.55 for different groups and p values less than 0.05 were considered significant.

RESULTS

Glycosylated hemoglobin is considered a gold standard for long–term glycemic control, was increased 1.5 times in diabetic patients without any signs of complications as compared to the normal individuals, whereas, fructosamine, a short term (1-4 weaks) glycemic control marker was increased twice (Table 1). However, in the patients with diabetic nephropathy a more pronounced increase in fructosamine and glycosylated levels was observed in diabetic patients with nephropathy as compared to the diabetic patients without any complications and with the corresponding group of non-diabetic nephropathy.

Sandwich ELISA quantified serum IgG and urinary IgG. Compared to normal individuals, serum IgG level showed a significant increase (52%) in diabetic patients without any complication while, an increase of 35% was observed in diabetic nephropathy patients (Table 2). In comparison to diabetic patients without any complications, diabetic nephropathy patients showed a significant decrease in serum IgG levels. The serum IgG was not significantly changed in non-diabetic nephropathy patients as compared to normal healthy individuals.

Glycosylation of IgG was quantified by estimation of total carbohydrate content in purified pooled IgG samples (Table 2). There was a significant increase (166%) in carbohydrate content in IgGs of diabetic patients without any complications as compared to the non- diabetic control while the increase was more prominent (300%) in patients with diabetic nephropathy without any change in glycosylation of IgGs in non-diabetic nephropathy patients.

As shown in Table 3 urinary IgG excretion was increased significantly in all the three groups of patients of different types compared to the normal healthy control group. A small amount of IgG excretion was observed in normal subjects, while, diabetic patients without any complications showed significant increase of 156% in urinary IgG compared to the normal individuals. Interestingly, the increase in IgG excretion was

Table 3: Urinary excretion of IgG in patients with Type 2 Diabetes without any complications, with non -diabetic nephropathy and with diabetic -nephropathy

	Group I Control	*Group II DM without any complication*	*Group III Non-diabetic nephropathy with CRF (Creatinine < 4)*	*Group IV Diabetic nephropathy with CRF (Creatinine < 4)*
Urinary IgG(mg/L)	2.5 ± 0.05	6.4 ± 0.31 $p<0.001^a$	189.4 ± 1.6 $p<0.001^a$ $p<0.001^b$	329.1 ± 23.0 $p<0.001^a$ $p<0.001^c$

Values are mean ± SE; Values in parenthesis represent sample size

Statistical significances between different groups were evaluated by student's t test. p value < 0.05 were considered significant; a :-Compared with group I (Control); b:- Compared with group II (DM); c:- Compared with group III CRF(Creatinine <4)

radically increased in diabetic patients with nephropathy compared to all other groups. The IgG excretion in diabetic nephropathy patients was 75% higher as compared in the non-diabetic nephropathy patients.

The linear regression analysis has shown the positive correlation of glycosylated IgG with urinary excretion of IgG in all diabetic patients. As shown in Figure 1. Diabetic patients without any complications and diabetic nephropathy patients has shown the positive correlation of glycosylated hemoglobin with urinary excretion of IgG (R^2=0.5995, 0.7114) respectively, whereas, there was no significant correlation observed in the patients with non-diabetic nephropathy and normal healthy individuals. Further the linear regression analysis between fructosamine and urinary excretion of IgG was not significantly correlated in any of the groups studied.

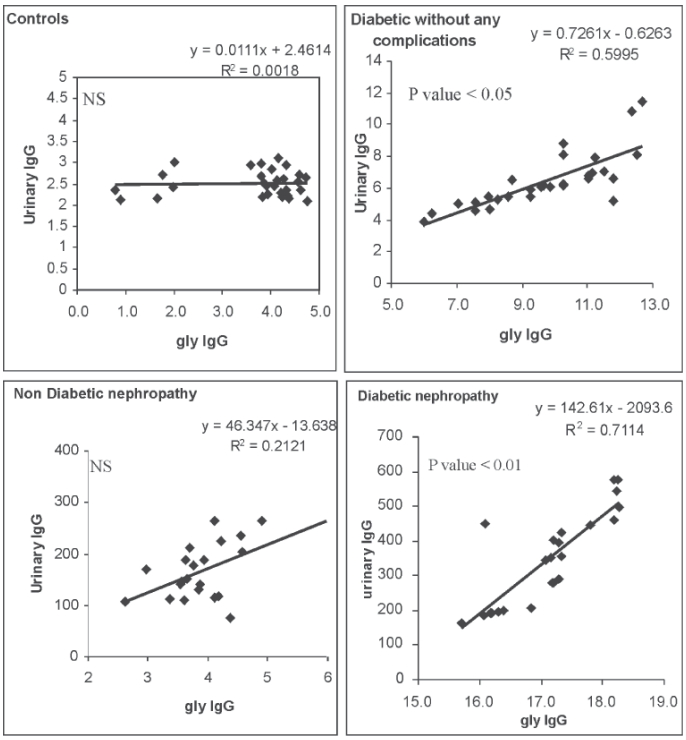

Figure 1: Relationship between glycosylation of IgG with urinary IgG in in patients with Type 2 Diabetes without any complications, with non -diabetic nephropathy and with diabetic -nephropathy.

DISCUSSION

Fructosamine and glycosylated hemoglobin concentration increased significantly in the all diabetic patients without any complication, non-diabetic nephropathy patients, diabetic nephropathy patients as compared to normal healthy individuals, however, it showed a significant elevation in the patients with diabetic nephropathy. The relationships between $GHbA_{IC}$, blood glucose concentration and late complications have been reported earlier (15-17). These reports support our present data where, patients suffering from diabetic nephropathy, showed a significantly increased concentration of glycosylated hemoglobin. Fructosamine also exhibited a marked increase in all groups compared to the control. The plasma fructosamine concentration has been measured in non-diabetic and diabetic nephropathy patients in past and higher plasma fructosamine values in non-diabetic patients than in the control population was reported, which are in accordance with our findings where, we found a significant increase in fructosamine level in non-diabetic nephropathy patients compared to controls. Fructosamine levels showed an increase in diabetic nephropathy patients compared to the diabetics without nephropathy (19, 20). Our findings suggest that there was a significant increase in the level of fructosamine, glycosylated hemoglobin in non-diabetic nephropathy patients. Selvaraj et al (21) suggested that an increased oxidative stress might be playing a critical role in promoting protein glycosylation in non-diabetic nephropathy patients.

It is generally assumed that serum IgG may play an important role in the host defense mechanism in diabetic patients (22). We measured the concentration of IgG in all the groups and found 52% increase in IgG levels in diabetics compared to

normal healthy individuals. Abnormal levels of immunoglobulins are very common in diabetic patients (23) and the probability that changes in immunolglobulin levels are implicated in the pathogenesis of infection requires further exploration. Compared to diabetic patients without any complications, patients with diabetic nephropathy showed a marked depletion of serum IgG level, which could be attributed to their increased vascular clearance and to the extent of nephropathy. There was no significant increase in IgG level observed in non-diabetic nephropathy. Thus, our results suggest that the increase in IgG level might be playing a role in diabetics as well as in pathogenesis of diabetic nephropathy (24, 25).

We also observed excessive glycosylation of IgGs in all diabetic patients without any complication and in diabetic nephropathy compared to the control. The levels of non-enzymatically glycosylated total plasma proteins, albumin and IgG have been measured in the diabetics and non-diabetics patients in past (26-29) and a significant increase in both glycosylated albumin and IgG, in diabetics were reported. These evidences also support our present findings. We observed no significant change in glycosylation of IgG in non-diabetic nephropathy (30) Non enzymatic modification of proteins may produce in changes charge, solubility, and conformation leading to molecular dysfunction as well as disrupting interactions with other proteins. Non enzymatic product also interact with specific receptors and binding proteins to influence the renal expression of growth factors and cytokines, implicated in the progression of diabetic renal disease (31,32,33). The glycosylation of the IgG significantly increases its vascular clearance rate have been reported in past (34) Thus it has been suggested that excessive glycosylation in vivo alters the Fc functions in the same way as the glycosylation of Fab region of IgG in vivo will alter its binding capacity to antigen. The significant increase in vascular clearance of glycosylated IgG may thus play a significant role in diabetic nephropathy.

The permeability and selectivity i.e. permselectivity of the kidney varies with respect to the size and charge of the filtered plasma protein (4). Renal permselectivity is the result of both glomerular filtration and tubular reabsorption of plasma proteins. Urinary albumin is a limited indicator of nephropathy because it is insensitive to renal tubular damage. In addition because of its acidic isoelectric point and its susceptibility to glycosylation, increases in urinary albumin are susceptible to matrix effects (4).

Our results have suggested that the urinary excretion of IgG was significantly increased in diabetic patients without any complication as compared with the healthy controls. Which was further increased in diabetic nephropathy (35, 36). They have shown a significant increase in IgG level with respect to the clinical stage of nephropathy and the progress of glomerular diffuse lesion and in the stage of normo albuminuria. The urinary excretion of IgG showed a significant increase in parallel with the progress of glomerular diffuse lesions whereas there was no relationship between the urinary excretion of albumin and progress of glomerular diffuse lesions while the excretion of IgG corrected with that of albumin and transferrin as per their study. In our study we observed the increased urinary excretion of IgG in both diabetic nephropathy and non-diabetic nephropathy which was much higher in patients with diabetic nephropathy as compared to non–diabetic nephropathy patients, indicative of the role in extent of renal damage. The urinary IgG excretion may be a good marker as it reflects urinary loss of these high molecular weight injurious factors, which reflects the combination of loss of size selectivity and magnitude of proteinuria. It was suggested that in diabetic nephropathy a reduction in negative membrane change in the glomerular filter i.e. the number of sulphated groups of glycosamino glycans have been argued to lead to an increase in excretion of negatively charged molecules such as albumin and IgG (37). However albuminuria and an increased excretion rate of IgG could be due to an increase large pore numbers and in charge selectivity may be a pathogenic mechanism for in radius or number of glomerular large pores (38, 39). We obtained a positive correlation between glycosylation of serum IgG and urinary IgG (R^2=0.5995, 0.7114) only in patients with diabetes without complications and with diabetic nephropathy, suggesting the role of excessive glycosylation of IgG in their vascular clearance and progression of diabetic nephropathy.

The results of the present study thus suggest that IgG excretion might be a good marker of renal injury because it combines information on the level of proteinuria and loss of size selectivity and the urinary IgG might be more useful in detection of the onset and/or early stage of diabetic nephropathy, however, requires further detailed studies.

ACKNOWLEDGEMENT

Authors are grateful to University Grant Commission, New Delhi for financial support.

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