N-Acetyl-β-D-Glucosaminidase, Alanine Aminopeptidase and Protein : Creatinine Ratio as Early Indicators of Diabetic Microangiopathy.

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ABSTRACT

The urinary excretion patterns of N-acetyl-β-D-glucosaminidase (NAG), alanine aminopeptidase (AAP) and protein/creatinine ratio (UP/UCR) were studied in 133 diabetic subjects under treatment, 7 patients with established diabetic nephropathy (DN) and 79 carefully selected (age-matched) healthy subjects. NAG, AAP and UP/UCR were highly elevated in DN, while in diabetics urinary NAG levels correlated well with the degree of long-term metabolic control indicated by glycosylated hemoglobin (GHB or Hba1). Both AAP and UP/UCR were found to be more sensitive than NAG, but less specific. Urinary NAG and AAP assays thus offer simple, sensitive and non-invasive techniques for prognostic indication of the onset of microangiopathic changes in long-term diabetic subjects.

KEY WORDS : glycosylated haemoglobin, diabetes, protein, N-acety-β-D-glucosaminidase, alanine aminopeptidase.

INTRODUCTION

Glomerular disease is still widely believed to be the primary cause of diabetic nephropathy (DN), with changes in the interstitium contributing to the etiology of renal failure (1). Studies suggest that a certain correlation exists between the degree of diabetic control and development of renal microvascular disease. The strict control of blood glucose has been shown to reduce development of microangiopathic changes in the kidney (2,3). Indicators are required to predict an incipient diabetic nephropathy which may be reversed by strict glycemic control (14). Such indicators or parameters should be accessible by non-invasive simple tests and should be reliable.

The report that urinary LDH activity is raised in patients with renal parenchymal disease, the clinical era was introduced to the use of urinary enzymes for non-invasive diagnostic purposes. A considerable number of urinary enzymes have since been reported to be of value in the detection of renal disease (4). Lysosomal acid hydrolases, β-glucuronidase (β-Glu, EC 3.2.1.31) and N-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30) have been mainly studied in serum and urine of diabetics (5-8), for the fact that (a) Changes in the enzyme activities may reflect biochemical adjustments to increased deposition of mucopolysaccharides in blood vessles in diabetics and (b) Urinary NAG is a sensitive index of renal cell damage. Early functional changes in kidney have been shown to cause augmented excretion of urinary proteins (9). Reports on correlation between serum enzyme activities and glycemic control are contradictory and inconclusive. The present investigation was therefore undertaken to relate urinary NAG, alanine aminopeptidase (AAP, EC, 3.4.11.2) and protein : creatinine ratio (UP/UCr) to an overall diabetic control so that an early indication of onset of diabetic microagngiopathy is made possible.

MATERIAL AND METHODS

Subjects :

(a) One hundred thirty three diabetic subjects, of whom 22 were insulin-dependent (IDDs) and 111 were non-insulin dependent (NIDDs), were booked cases in diabetic clinic of our hospital and were on regular surveillance. Diabetes was diagnosed based on high-fasting or elevated post-prandial plasma glucose level. The duration of diabetes in these patients was from 18 months to 15 years. Glycemic control was monitored based on glycosylated hemoglobin (GHb, HBA_1) level and other routine tests.

(b) Seven patients with established diabetic nephropathy were selected. Diagnosis was based on renal biopsy, persistent heavy proteinuria and other routine renal function tests.

(c) Forty seven normal healthy subjects of both sexes in an age range of 17-43 years and 32 normal subjects in an age range of 46-63 years were chosen as controls.

Materials :

Trizma base, creatinine, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, L-alanine-4nitroanilide were obtained from Sigma Chem Co. U S A. All other chemicals were of

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high analytical grade quality. Glycosylated hemoglobin affinity chromatography Kit and controls were purchased from Pierce Co. Rockford. IL. USA. All measurements were made on CECIL CE-202 UV/VIS spectrophotometer.

Methods :

Overnight urine samples collected at the first voiding in the morning were stored at + 4°C for not more than 48 hours before assay. pH of all urines was measured immediately after collection.

Creatinine was measured by the Jaffe reaction on Hitachi 704 discrete selective analyser. Protein was estimated by a modified method of Pesce and Strande (10). NAG and AAP enzymes were assayed by methods established by us previously (11,12). Glycosylated hemoglobin was measured colorimetrically using glycogel affinity columns. Glycosylated variants retained on the column were eluted with sorbitol and expressed as precent of total hemoglobin. GHb controls supplied by the company were used in all the batches as a quality control check.

RESULTS :

The pH of urine samples of both normal and patient samples was in a range of 6.0-7.8 well within the prescribed limits for stability of enzymes in native urine (13). Table I. shows the mean and ranges of NAG. AAP and UP/UCr obtained in the two control groups and a group of 7 patients with DN. A comparison of the three analytes between the control groups I and II showed that NAG was unaltered in both groups while AAP and UP/UCr were significantly high ($p < 0.001$) in the older control group II suggesting that declining renal function may be the cause for the elevation of these two sensitive parameters in the older population. The rise in the three analytes in the DN group was very high (2-8 fold for NAG. 2-10 fold for AAP and 4-30 fold for UP/UCr) cornpared to their corresponding levels in control group II.

Table-1

Urinary NAG, AAP and protein : creatinine ratio (mean ±SD) in normal subjects and patients with diabetic nephropathy

Subjects (Age range)	n	Enzyme activities U/m mol NAG	AAP	UP/UCr mg/m mol
Control group I*	47	10.0±4.8	32±14	6.9±3.3
(17-43 yrs.)		(0-20)	(5-60)	(0.4-13.4)
Control group II	32	8.9±3.5	55 ±17	12.6±7.2
(46-63 yrs.)		(2-16)	(22-88)	(0-27)
Diabetic Nephropathy * *	7	80.6±36.7	399±198	418±275
(50-75 yrs.)		(44-129)	(140-800)	(109-874)

* Control gp I vrs II : NAG p = NS; AAP p < 0.001; UP/UCr p < 0.001

* * DN Vrs Control group I or II p < 0.001

Table 2 depicts the screening of the 133 diabetic subjects for NAG. AAP and Up/UCr. Out of 133 subjects. in 20 patients NAG was elevated by 2-7 fold. in 47 cases AAP showed 2-11 fold elevation and in 35 UP/UCr was elevated by 3-10 fold compared to control subjects. If NAG is considered as a specific parameter then 20/47 and 20/35 patients showed simultaneous increase in AAP and UP/UCr respectively. From this observation we subscribe to the view that NAG is a specific parameter while AAP is a sensitive enzyme of the proximal tubular brush border. Table 3 gives the actual ranges of enzyme activities and Protein : Creatinine indices obtained in the 20 diabetic subjects who showed a simultaneous elevation in NAG activity.

Table-2

Screening of diabetic subjects

Subjects	n	No.of Patients showing elevated levels of NAG	AAP	UP/UCr
Insulin dependent diabetics	22	8 (36%)	14 (64%)	8 (36%)
Non-Insulin dependent diabetics	111	12 (11%)	33 (30%)	27 (24%)

Table-3

Comparison of the three parameters in the diabetic subjects (n = 20) showing elevated NAG levels

n = 20* (8 + 12)	NAG	AAP	UP/UCr
Mean ± SD	52.4 ± 33.4	263.2 ± 153.5	208.3 ± 373.6
Observed range	(32-140)	(126-703)	(31-1639)
Fold elevation over upper limit of normal (ULN)	2-9	2-8	2.5-122

* (n = 20) GHb 10.86 ± 2.57 Vrs (n = 113) GHb 5.70 ± 1.47 P < 0.001.

To further substantiate our view that NAG would be a more specific parameter than AAP or UP/UCr, we compared the HbA_1 levels in all the 133 diabetics with NAG and found that in the 20 subjects with elevated NAG (with simultaneous elevation in AAP and UP/UCr) activities the glycemic control was poor (Table 3) as evidenced by HbA_1.

DISCUSSION :

Chronic disease of the microcirculation and consequent renal failure due to long standing diabetes mellitus contributes significantly to morbidity and mortality in both juvenile and adult-onset diabetes (15). In chronic diabetes mellitus the microvascular complications affect both glomerular and peritubular capillaries wherein increased permeability of peritubular capillaries to plasma proteins causes interstitial protein concentration to increase with a corresponding fall in the colloid osmotic pressure gradient across the capillary wall resulting in interstitial fluid accumulation and increased interstitial and hydrostatic pressures (16). Raised Interstitial pressure also has several detrimental effects. Tubules are compressed and intrarenal diameters are diminished; a similar effect on capillaries may interfere with nutritive blood supply (17). The brush border of the proximal tubule is especially susceptible to ischaemic and toxic injury. Shedding of vacuolar fragments of the plasma membrane is a common feature of a variety though not all cells, yet an acute rise of vacuolar blebs in urine indicates more severe cell disintegration, and may lead to necrosis especially of the most vulnerable straight proximal tubule segment (18). N-acetyl-β-D-glucosaminidase is enriched in lysosomes and alanine aminopeptidase in the brush border of the proximal tubular epithelial cells. Any ischaemic or toxic injury leads to release of these enzymes into the ultrafiltrate and thus appear in urine. On the contrary proteins and enzymes of low molecular weight such as albumin, β2-microglobulin, lysozyme, ribonuclease, etc. are freely filtered through glomerulus and completely removed by the cells of the proximal tubule and metabolised (19). When the proximal tubular cells are damaged the levels of these proteins and enzymes also increase in urine. Earlier view (20) that tubular function is not altered in diabetic subjects in the early stages of diabetic nephropathy needs to be revised since that conclusion was drawn from β2-microglobulin measurements which indicated that the tubule was intact. Recent results (21,22) obtained with other low molecular weight proteins viz lysozyme, albumin, retinol binding protein and our own results with NAG and AAP indicate that tubular damage is imminent.

Shimojo et al (23) in their study have shown that AAP is a better parameter than NAG but this opinion is deferred by Jung et al (18) who claim that Shimojo et al evaluated the means and not frequencies of abnormal values for the two enzymes. We agree with Jung et al and wish to add that comparison of all the three parameteres viz : NAG, AAP and UP/UCr in our study has shown that those patients who had high levels of NAG activity also had high activities of AAP and increased concentration of protein. Also, these diabetic patients with elevated activities of NAG suffered from poor control of glycemia as evidenced from the raised percent of glycosylated hemoglobin compared to that of diabetics under good control.

Recently Severini et al (24) have shown that both total and B-isoenzyme of NAG were elevated in urines of diabetic subjects with vascular complications.

Based on these observations we feel that increased activity of N-acetyl-β-D-glucosaminidase could be regarded as reflecting lysosomal enzyme activation in proximal tubular cells, occurring in response to the metabolic need to degrade either various constituents of the cells themselves, in a situation involving increased tissue catabolism, or mucopolysacchyarides and glycoproteins that have accumulated in tissues as in the case in diabetics with vasculopathies.

We, therefore, recommend the inclusion of NAG and AAP enzyme measurements in urine as non-invasive parameters alongside to microalbumin and HbA_1 for continuous monitoring of diabetic subjects with inclination to vasculaar microangiopathy leading to diabetic nephropathy. For long term monitoring they are more economical than microalbumin determination and can easily be automated

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