Association of reduced red blood cell deformability and diabetic nephropathy

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Background. Impaired red blood cell deformability may play a key role in the pathogenesis of chronic vascular complications of diabetes mellitus and progression of renal failure. The present study was conducted to test whether impaired red blood cell deformability is indeed associated with development of diabetic nephropathy.

Methods. We studied 57 adult type 2 diabetic patients divided into three groups according to serum creatinine concentration. Group I comprised 28 diabetic patients with normal renal function (serum creatinine concentration <1.5 mg/dL, mean 1.0 ± 0.3 mg/dL). Group II comprised 10 diabetic patients with renal insufficiency (serum creatinine concentration ranging from 2 to 6 mg/dL, mean 3.9 ± 1.54 mg/dL). Group III consisted of 19 diabetic subjects with end-stage renal disease (ESRD) on hemodialysis (serum creatinine concentration ranging from 7.7 to 14.6 mg/dL, mean 10.1 ± 2.4 mg/dL). In addition, 11 nondiabetic individuals, matched renal function for the diabetic groups (group II and III, respectively) served as control. Red blood cell deformability, measured by filtration technique, is defined as the filtration rate of erythrocyte suspension through a micropore filter divided by the filtration rate of a physiologic buffer solution.

Results. In the diabetic cohort, we found substantially impaired red blood cell deformability in those with normal renal function (group I). With further renal function loss, an increased impairment in red blood cell deformability was observed. Diabetic patients with renal insufficiency (group II) when compared to non-diabetic controls (renal insufficiency) had a significantly greater impairment in red blood cell deformability (P = 0.01). The nondiabetic cohort (renal insufficiency), on the other hand, manifested significant impairment in red blood cell deformability. Their degree of impairment was statistically higher than that in diabetic patients with normal renal function (P = 0.0005). Interestingly, there was a progressive increase in red blood cell deformability impairment, along with progression of renal insufficiency, and thus no significant difference in the degree of red blood cell deformability impairment was observed between diabetic and nondiabetic patients with ESRD (P = 0.52). There is significant correlation between serum creatinine and impairment in red blood cell deformability in both diabetic (group II plus III) (r = 0.43, P = 0.02) and nondiabetic (r = 0.62, P = 0.003) cohorts.

Conclusion. In diabetic patients, early impairment in red blood cell deformability appears in patients with normal renal function, and progressive impairment in red blood cell deformability is associated with renal function loss in all patients regardless of the presence or absence of diabetes.

Diabetic nephropathy, a serious microvascular complications of diabetes mellitus, is the leading cause of end-stage renal disease (ESRD) [1]. Hyperglycemia is the main metabolic perturbation causing irreversible kidney damage in diabetes [2, 3], but the mechanism by which hyperglycemia results in nephropathy is not well defined. Multiple glucose metabolites and reaction products accumulate as a consequence of hyperglycemia. The aldose reductase pathway leading to toxic levels of sorbitol and activation of isoform(s) of protein kinase C (PKC) [4] is proposed to explain how hyperglycemia damages tissues [3]. Accruing evidence supports a key role for advanced glycation end-products (AGEs) formed by nonenzymatic glycation and oxidation (glycoxidation) reactions in the pathogenesis of diabetic nephropathy [5–8].

Impaired red blood cell deformability, is a hemorheologic perturbation induced by diabetes and renal failure. Its effect on the microcirculation have also been implicated in the pathogenesis of diabetic vascular complications [9–11]. Recently, a number of studies have provided evidence that impaired red blood cell deformability is linked to AGEs accumulation. Nonenzymatic glycation of several proteins, especially red cell-membrane glycoproteins and hemoglobin has been found in patients with diabetes [12, 13], and such a biochemical modification of the erythrocyte is one factor that may account for altered rheologic properties of human erythrocytes in diabetes [14]. This hypothesis was confirmed by our recent demonstration that pimagedine, an agent known
to specifically block AGEs formation [15, 16], effectively corrects impaired red blood cell deformability in an animal model of diabetes. Conversely, in this study, red blood cell deformability deteriorated and returned to pretreatment impairment by week 10 following pimagedine withdrawal [17]. This finding was supported further by the course of red blood cell deformability noted in diabetic subjects with ESRD treated with pimagedine [18] and by the significant and independent correlation of AGE carboxymethyllysine (CML) with red blood cell deformability [abstract; Brown et al, Am Soc of Nephrol 14:609A, 2003]. The present study was designed to test the hypothesis that impaired red blood cell deformability correlates with development and progression of diabetic nephropathy.

METHODS

Subjects

Fifty-seven diabetic patients, divided into three groups according to serum creatinine concentration, participated in this study. Eleven (renal insufficiency) and ten (ESRD) nondiabetic patients served as controls. Group designation, patients’ demographics, and clinical features are listed in Table 1.

Group I comprised 28 diabetic patients whose serum creatinine was <1.5 mg/dL (mean 1.0 ± 0.3 mg/dL). Group II comprised 10 diabetic patients with renal insufficiency whose serum creatinine ranged from 2 to 6 mg/dL (mean 3.9 ± 1.5 mg/dL) and group III consisted of 19 diabetic patients with ESRD on hemodialysis, with a serum creatinine ranging from 7.7 to 14.6 mg/dL (mean 10.1 ± 2.4 mg/dL). In the nondiabetic cohort, 11 patients with renal insufficiency (mean serum creatinine of 4.2 ± 1.5 mg/dL) and 10 with ESRD (mean serum creatinine of 11.5 ± 3.6 mg/dL) were controls for diabetic groups II and III, respectively. In addition, there was no significant group difference in mean plasma cholesterol concentrations, approximately 5% of diabetic and nondiabetic patients actively smoked cigarettes, and three patients in group I, two in group II, and two in group III had clinical evidence of cardiovascular disease. No patients in the nondiabetic group had clinically evident cardiovascular disease.

Table 1. Patient demographics and clinical features

<table>
<thead>
<tr>
<th></th>
<th>Normal renal function (N = 28)</th>
<th>Renal insufficiency (N = 21)</th>
<th>End stage renal disease (N = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I Diabetic (28)</td>
<td>Group II Diabetic (10)</td>
<td>Group III Diabetic (19)</td>
</tr>
<tr>
<td>Age</td>
<td>61.2 [8.6]</td>
<td>53.3 [10.8]</td>
<td>61.5 [9.6]</td>
</tr>
<tr>
<td>Gender male/female</td>
<td>9/19</td>
<td>7/3</td>
<td>8/11</td>
</tr>
<tr>
<td>Hemoglobin A5 %</td>
<td>8.6 [1.4]</td>
<td>7.6 [1.3]</td>
<td>6.7 [1.5]</td>
</tr>
<tr>
<td>Serum creatinine mg/dL</td>
<td>1.0 [0.3]</td>
<td>3.9 [1.5]</td>
<td>10.1 [2.4]</td>
</tr>
<tr>
<td>Mean corpuscular volume FL</td>
<td>87.6 [6.5]</td>
<td>88.4 [7.0]</td>
<td>87.7 [6.6]</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>36.1 [3.1]</td>
<td>34.2 [4.0]</td>
<td>34.6 [4.7]</td>
</tr>
<tr>
<td>Urea reduction ratio</td>
<td>—</td>
<td>—</td>
<td>72.3 [6.5]</td>
</tr>
</tbody>
</table>

|                         | Control (11)                  | Non-Diabetic (11)           | Control (19)                    |
| Age                     | 53.3 [10.8]                   | 58.7 [14.0]                 | 44.3 [11.6]                     |
| Gender male/female      | 8/3                            | 8/3                         | 5/5                             |
| Hemoglobin A5 %         | Not assessed                   | Not assessed                |                                  |
| Serum creatinine mg/dL  | Not assessed                   | Not assessed                |                                  |
| Mean corpuscular volume FL | Not assessed                  | Not assessed                |                                  |
| Hematocrit %            | Not assessed                   | Not assessed                |                                  |
| Urea reduction ratio    | Not assessed                   | Not assessed                |                                  |

Mean [SD].

aP = 0.005 significantly higher homoglobin A5 noted in diabetic patients with normal renal function compared to diabetic patients with end-stage renal disease.
bP = 0.0005 significantly higher mean hematocrit level observed in diabetic subjects with normal renal function compared to diabetic and non-diabetic patients with renal insufficiency and end-stage renal disease.

Determination of erythrocytes deformability

Red blood cell deformability, measured by filtration technique, is defined by the rate of filtration of a dilute (hematocrit of 4.0%) suspensions of washed erythrocytes in phosphate-buffered saline (PBS) solution through polycarbonate membranes with straight channels of 3 μ pore diameter (Nucleopore; Corning, Acton, MA, USA) under a constant negative pressure (−20 cm H2O), compared to the rate of filtration of an equal volume of buffer. This technique used in our laboratory is a modification of the method of Reid et al [19], and has been described previously [17, 20]. A major problem with this method (especially those with pore diameters of 3 micron) is its relationship to mean cell volume (MCV) [21], and a substantial occlusion of the pores and decrement in flow rate due to the presence of hyperproteinemia and contaminating leukocytes [22, 23]. The technique was designed to reduce confounding factors: (1) suspensions of washed erythrocytes in PBS instead of whole blood were used to eliminate hyperglycemia and hyperviscous plasma, (2) a polycarbonate membrane with pore size of 3 μ was chosen because it is to be more sensitive to small changes in erythrocyte rheology than filtration through a 5 μ pore [24], (3) Imugard® IG 500 cotton wool (Terumo, Tokyo, Japan) prefiltration method was used to reduce leukocyte contamination below the critical level of <0.025 × 109/L [22] and to remove 99% of platelets and various cell-protein interactions [25], (4) strict adherence to buffer (PBS), pH 7.4, and osmolality (295 to 300 mmol/kg) was maintained to avoid the influence of buffer on the MCV of red blood cells, and (5) pH and osmolality remained constant from batch to batch [21]. This system used in our laboratory for nearly a decade has a high degree of sensitivity in detecting abnormal red blood cell deformability.
A pure suspension of washed erythrocytes was prepared as follows: whole blood from heparinized venous blood was filtered through Imugard® IG 500 cotton wool [22], the effluents were centrifuged at 12,500 rpm for 10 minutes; the plasma, buffy coat, and uppermost red blood cell layer were removed. Packed red blood cells were washed three times in PBS solution. Washed erythrocytes, aspirated from the middle of the packed erythrocyte column, were resuspended in isotonic PBS to a final concentration of 4%. In each filtration experiment, a paired test was included. The flow time required for 5 mL of buffer to pass through the filter was determined as a blank value. The same measurement with the 4% red cell suspensions was performed. A qualitative measurement of red blood cell deformability was expressed as a deformability index (DI) defined as the time required for 5 mL of red cell suspension to filter, divided by the time required for an equal volume of buffer to filter. The DI is reported as the average of two to three repeated tests. DI was standardized to a normal reference mean obtained from a group of age matched healthy adults (N = 30, mean DI of healthy adults 4.33 ± 0.14, DI = 1.0).

To avoid any influence of MCV on red blood cell deformability results, MCV was measured for subjects in each cohort, and there is no statistical significant difference in mean MCV values between diabetic and nondiabetic patients in both renal insufficiency and ESRD cohorts (Table 1). Although mean MCV in diabetic patients with renal insufficiency (group II) was 4 fl greater than that of nondiabetic control subjects, red blood cell filtration through a 3 μm pore filter of the same lot and batch (in the range of MCV measured in this study) will result in small changes in red blood cell filtration that would not significantly influence our results [26].

**Statistical analysis**

Statistical analysis was performed using the unpaired two-tailed Student t test. Linear regression was used to determine correlation between variables. Statistical significance is indicated by P < 0.05. Data are expressed as mean ± standard deviation (SD).

**RESULTS**

As shown in Figure 1, in the diabetic group, a substantially greater impairment in red blood cell deformability was noted early in patients with normal renal function (group I) (compared with normal healthy control, P = 0.0005). Subsequently, an increased impaired red blood cell deformability was found with further renal function loss, especially in diabetic patients with renal insufficiency (group II) when compared with nondiabetic subjects with matched renal insufficiency (P = 0.01). In the nondiabetic cohort marked impaired red blood cell deformability was also noted in patients with renal insufficiency, their degree of impairment was statistically much higher than in diabetic patients with normal renal function (P = 0.0005). In advanced renal failure there was no significant difference between diabetic and nondiabetic patients with ESRD (P = 0.52). A strong and significant correlation between serum creatinine concentration and red blood cell deformability was evident in both diabetic (groups II and III) (r = 0.43, P = 0.02) and nondiabetic (r = 0.62, P = 0.003) patient controls (Fig. 2).

**DISCUSSION**

Red blood cell deformability, generally accepted as the passive change in the shape of red blood cells in response to shear forces, is a pivotal determinant of blood flow and function in the microcirculation [9]. Several hypotheses to explain impaired red blood cell deformability proposed include elevated blood glucose concentration and hyperosmolarity [9]; hypoinsulinemia [27]; alterations in red blood cell membrane lipid composition [28]; increased
Taken together, these studies suggest that AGE does not correlate with red blood cell deformability through serum creatinine, conversely, they suggest that AGE is independently associated with impaired red blood cell deformability.

It is important to note that in the rabbit and human studies, the pimagedine-mediated improvement in red blood cell deformability occurred despite persistence in elevation of blood glucose and hemoglobin A1c (HbA1c). These findings suggest that hyperglycemia and HbA1c, per se, may have little importance in directly influencing red blood cell deformability. An association between AGEs and progression of renal disease is afforded by the report that tissue and serum AGEs levels rise as renal function declines in diabetic patients with nephropathy [7]. In the present study, early impaired red blood cell deformability was noted in diabetic patients with normal renal function (Fig. 1) and a strong correlation between serum creatinine and red blood cell deformability impairment was observed in the diabetic cohorts (groups II and III) ($r = 0.43, P = 0.02$). These observations prompt the hypothesis that impairment in red blood cell deformability observed in diabetic patients (with or without renal failure), may be induced by excessive accumulation of AGEs and/or AGE mediated action such as oxidative stress. Although not measuring serum AGEs directly is a weakness of our study, we do not think that this detracts from our finding of a strong and significant relationship between red blood cell deformability and renal function.

We showed that in a diabetic cohort early impairment in red blood cell deformability appears in patients with normal renal function, and a persistently increased impairment in red blood cell deformability is associated with renal function loss. Additionally, more impairment in red blood cell deformability was observed in diabetic patients with renal insufficiency, compared with matched renal function of nondiabetic subjects with renal insufficiency ($P = 0.01$) (Fig. 1). These findings mean that hyperglycemia and impaired renal function could be pathogenic factors that are important for both the development of impairment in red blood cell deformability and accumulation of AGEs.

As shown in Figure 1, an appreciable impairment in red blood cell deformability was also noted in the nondiabetic patients with renal insufficiency. Furthermore, their degree of impairment was much more severe, when compared to diabetic patients with normal renal function ($P = 0.0005$). There was a strong and significant correlation between serum creatinine concentration and impairment of red blood cell deformability ($r = 0.62, P = 0.003$) in the nondiabetic cohort as well (Fig. 2). The clinical significance of these findings has not yet been defined. Whether or not impaired red blood cell deformability as seen in nondiabetic controls also contributes to development of
uremia or extrarenal comorbidity is unknown. While any difference in AGEs accumulation between diabetic and nondiabetic ESRD patients must be determined, some difference in red blood cell deformability impairment between diabetic and nondiabetic patient cohorts is noted by comparing areas under the bars in Figure 1. In diabetic patients, red blood cell deformability is profoundly altered early, prior to the onset of renal insufficiency and ESRD. By contrast, the impairment in red blood cell deformability is initially observed in nondiabetic patients with the onset of renal insufficiency. Red blood cell deformability impairment in nondiabetic patients reaches a maximum once ESRD develops. Apparently, impaired red blood cell deformability observed in the nondiabetic cohort seems to be influenced by renal functional impairment alone.

The role of impaired renal function in the formation of AGEs has been investigated. It was demonstrated that nonenzymatic modifications in uremia are not only related to carbohydrate-derived adducts generated by the Maillard reaction, such as AGE-peptides [7, 30], pentosidine [31], and CML [32], but may also be stimulated by carbonyl intermediates resulting from the oxidation of glucose, ascorbic acid, and lipid peroxidation [33, 34]. As shown in Figure 1, impairment in red blood cell deformability was much less in nondiabetic patients with renal insufficiency compared with diabetic subjects with a similar degree of renal impairment (P = 0.01), but there is no significant difference in the degree of red blood cell deformability impairment between diabetic and nondiabetic patients once ESRD develops (P = 0.52). Miyata et al [34] measured CML, pentosidine, and malondialdehyde (MDA)-lysine levels in diabetic and nondiabetic hemodialysis patients and found no significant difference in the levels of all three AGEs. This observation suggests that impaired red blood cell deformability observed in nondiabetic cohort patients may be associated with the accumulation of AGEs and may explain why we noted no significant difference in the degree of impairment in red blood cell deformability between diabetic and nondiabetic patients once ESRD was manifested. Although one report at variance with this line of thinking showed greater elevation of AGE peptide levels (low-molecular-weight) in diabetic patients compared to nondiabetic hemodialysis patients [35].

Whether impaired red blood cell deformability results from renal function decline, and therefore serves as a marker for nephropathy, or is a major factor directly contributing to nephropathy in diabetic and nondiabetic patients are issues that are now under study.

REFERENCES

2. FRIEDMAN EA: Diabetic nephropathy is a hyperglycemic glomerulo-
3. PORTE DJ Jr, SCHWARTZ MW: Diabetics complications: Why is glu-
4. CRAVAN PA, STUDER RK, NEGRETE H, DERUBERTIS FR: Protein ki-
5. VLASSARA H: Recent progress on the biologic and clinical signifi-
13. MAKITA Z, VLASSARA H, BUCALA R: Hemoglobin AGE: A cir-
16. EDELSTEIN D, BROWNLEE M: Mechanistic studies of advanced glyco-
sylation end product inhibition by aminoguanidine. Diabetes 41:26–
29, 1992
17. BROWN CD, ZHAO ZH, DE ALVARO F, et al: Correlation of ery-
257, 1992
21. STUART J, STONE PCW, BARFORD D, BILT0 YY: Effect of pore di-
22. STUART J, STONE PCW, BARFORD D, et al: Evaluation of leuco-
23. GREGEROSEN MI, BRYANT CA, HAMMELE W, et al: Flow charac-
25. KENNY MW, MEAKIN M, STUART J: Methods for removal of leuco-
26. BROWN ET AL: Association of reduced red blood cell deformability and diabetic nephropathy

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