Detection of autoantibodies against glycosylated-DNA in diabetic subjects: Its possible correlation with HbA1c

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Abstract. Aims: The vascular tissues have a long memory of their previous glycemic control and intervention studies have demonstrated that microvascular complications are highly correlated with mean glycemic control as measured by glycolated hemoglobin A1C (HbA1c). The present study was carried out to evaluate the autoantibodies against glycosylated DNA (DNA-AGEs) in diabetic sera to see if the level of HbA1c has correlation with the activity of DNA-AGEs, another marker of chronic glycemia.

Methods: Glucose-6-phosphate induced glycosylation of native DNA was studied in 150 diabetic sera (T1DM = 9, T2DM = 141) by spectroscopic techniques (UV and fluorescence) and agarose gel electrophoresis. Direct binding and inhibition enzyme immunoassays were carried out to evaluate binding and specificity of anti-glycated-DNA autoantibodies (anti-DNA-AGE autoantibodies) in sera of diabetes patients. A quantitative estimation of HbA1c was carried out in normal and diabetes sera.

Results: Anti-DNA-AGEs autoantibodies in 55% of diabetic sera (85/150) showed more binding with glycated-DNA compared to native DNA which was subjected to inhibition ELISA indicating true interaction of these autoantibodies in diabetes with glycated DNA. Higher binding with glycosylated-DNA against native-DNA was observed in subjects with HbA1c of 9.8 ± 3.3% compared to those with HbA1c of 7.7 ± 1.7% (p < 0.001). Linear correlation analysis showed that mean absorbance difference was significantly related to HbA1c (r = 0.486, p < 0.001), nephropathy (r = 0.239, p < 0.003), retinopathy (r = 0.165, p < 0.05).

Conclusions: Autoantibodies against glycosylated DNA were correlated with HbA1c and microvascular complications and may be useful as another biomarker for assessment of chronic glycemia.

Keywords: Autoantibodies, glycosylated-DNA, diabetes mellitus

1. Introduction

Reducing sugars react non-enzymatically with amino groups in proteins, lipids, and nucleic acids through a series of reactions forming Schiff base and Amadori product to produce AGEs – advance glycation end products [1–3]. Some of these factors that influence protein glycation also contribute to the non-enzymatic glycation of DNA nucleobases and nucleosides [4,5]. In the cell, glucose has the slowest rate of glycation, mostly phosphorylated to glucose-6-phosphate (G16-P) that is more relevant DNA-AGE precursor forms AGEs at a faster rate [6]. Many AGEs fluoresce under ultraviolet light and some are capable of intra- and intermolecular cross linking [7]. The formation of DNA-AGEs is not only limited to reactions of isolated DNA in-vitro, but also takes place under cell culture conditions, in-vivo. The carboxyethylatedguanaine (CEdG) has been reported to be detected in healthy human urine [8] and
also in the genomic DNA of human smooth muscle cells and bovine aorta endothelium cells in-vitro [9]. Thus, CEdG is the only DNA-AGE to be detected in vivo, however, information on the nuclear concentration of sugar and sugar degradation product is rare. In the nucleus, DNA itself may be considered as an important carbonyl precursor. The oxidation reaction releases sugar degradation products from the DNA backbone, which then, in reverse, may directly glycate the DNA [10]. The presence of nucleotide AGEs in DNA is also associated with increased mutation frequency, DNA strand breaks and cytotoxicity [11]. The AGEs formed during the glycation of DNA/ protein possess unique epitopes capable of triggering an autoimmune response [12], and AGEs autoantibodies have also been reported in the sera of diabetic patients [13]. A casual relationship between chronic hyperglycemia and diabetic microvascular disease, long inferred from clinical studies, has now been definitely established by data from the Diabetic Control and Complications Trial [14], the United Kingdom Prospective Diabetes Study [15], the Kumamoto Study [16], and HbA1c is considered to be the gold standard for assessment of chronic glycaemia. New biochemical markers related to AGEs have also been described, however, the predictive value of a single marker and combinations of markers need to be tested in long term clinical Studies [17]. Furthermore, role of AGEs in microvascular complications has been clearly defined and thus AGEs may prove authentic marker for assessment of microvascular complications in diabetes [18–21].

In the present study, we investigated the presence of anti-glycated-DNA autoantibodies (anti-DNA-AGE autoantibodies) in diabetes sera, its correlation with HbA1c and microvascular complications. An attempt was also made to evaluate, if DNA-AGEs emerge as additional biomarker for assessment of chronic glycaemia in diabetes.

2. Patients and methods

The study was conducted at the Centre for Diabetes and Endocrinology and Department of Biochemistry, J.N. Medical College, Aligarh Muslim University, Aligarh (India). Blood samples from 150 diabetic patients (64 males and 86 females; 9 cases of Type1 and 141 cases of Type2) were collected in a fasting state after an overnight fast of 10–12 hrs. Fasting blood (n = 45) from apparently healthy, age- and sex-matched, individuals were also collected and served as controls. An informed consent was obtained from each subject at the time of enrollment and clearance was obtained from the Institutional Ethics Committee.

A detailed history and physical examination was carried out for each subject. Age, sex, anthropometric measurements (body mass index), duration of diabetes, history of smoking, glycemic control at the time of participation in the study, lipid profile, presence of retinopathy, nephropathy (creatinine > 1.5 mg% or presence of micro- or macroalbuminuria), neuropathy (absence of perception of the Semmes-Weinstein monofilament at 2 of 10 standard plantar sites on either foot), peripheral vascular disease (ischemic symptoms and intermittent claudication of rest pain, with or without absence of pedal pulses or posterior tibial pulses) and hypertension were noted in every patient. Patients were diagnosed as having cardiovascular disease on the basis of history of angina, previous myocardial infarction, coronary artery bypass graft or balloon angioplasty, ECG changes and echocardiography.

2.1. Glycation of DNA

Commercially available native DNA (purified from calf thymus) was purchased from Sigma Chemical Company, USA (product no D-1501 “DNA Sodium salt” type 1, highly polymerized). Pilot experiments were carried out with different concentrations of native DNA (10–100 μg/ml) and a fixed concentration of Gl 6-P to decide the optimum concentration of DNA to be taken for generation of advanced glycation end products. Glycation of native DNA was carried out in vitro. Native DNA (10 μg/ml) was incubated at 37°C with a fixed amount of Gl 6-P (130 mM/ml) in phosphate buffer (0.2 M, pH 7.4) for 3, 7, 10 and 14 days. Controls were devoid of glucose-6-phosphate. We have chosen 130 mM Gl 6-P to glycate the native DNA because (i) potency of Gl 6-P as a glycat ing agent is larger than that of glucose, as suggested by previous workers [22] (ii) the above concentration of Gl 6-P has previously been shown to correspond to the minimum concentration for an in vitro glycation of proteins, DNA etc. [23–25].

Structural changes in glycated DNA were investigated by following techniques.

2.2. Spectroscopic (UV & fluorescence) studies on glycated-DNA

Absorption profile of native DNA and its glycated counterparts were recorded in the wavelength range of 200–400 nm using quartz cuvette of 1 cm path
length. Fluorescence emission spectra of native DNA and glycated-DNA samples were also recorded. Equal amounts of native- and glycated-DNA samples were excited at 370 nm and emission profile was recorded.

2.3. Agarose gel electrophoresis

Nuclease free agarose (0.6%) was dissolved in electrophoresis buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) and poured in a comb fitted transparent plastic tray. The molten agarose was allowed to solidify at room temperature. Comb was carefully re-moved and equal amounts of native and glycated-DNA samples (pre-mixed with tracking dye) were loaded in separate wells. Electrophoresis was carried out for 2 h at 30 mA constant current. The gel was then stained with ethidium bromide (0.5 μg/ml distilled water) and bands were visualized under UV light.

2.4. Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out on flat bottom polystyrene maxisorp modules as described elsewhere [26]. Briefly, microtitre wells were filled with 100 μl of native/glycated-DNA antigens (2.5 μg/ml) in TBS buffer (10 mM Tris, pH 9.6 containing 150 mM NaCl) and incubated for 2 h at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the wells served as control devoid of antigen coating. Unbound antigens were washed thrice with TBS-T (TBS buffer containing 0.05% Tween-20) and unoccupied sites were blocked with 150 μl of 2% fat free milk (dissolved in TBS buffer) for 4–6 h at 37°C. At the end of incubation, wells were washed four times with TBS-T. Sera derived from normal human and diabetes patients were serially diluted (starting from 1:100 dilutions) in TBS-T and added to antigen coated wells (100 μl/well). Antigen-antibody interaction was allowed for 2 h at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The absorbance (A) of each well was monitored at 410 nm on an automatic microplate reader. Each sample was run in duplicate. Results were expressed as a mean of \( A_{\text{test}} - A_{\text{control}} \).

2.5. Inhibition ELISA

The antigenic specificity of autoantibodies in diabetes sera was determined by inhibition ELISA. Varying amount of inhibitors (0–10 μg/ml) was mixed with fixed amount of 100 μl of 1:100 diluted sera of selected diabetic cases. The mixture was incubated at room temperature for 2 h and overnight at 4°C and added in the wells instead of serum. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the formula:

\[
\text{Percent inhibition} = 100 \times \frac{A_{\text{inhibited}} - A_{\text{uninhibited}}}{A_{\text{uninhibited}}}
\]

where, \( A_{\text{inhibited}} \) and \( A_{\text{uninhibited}} \) stand for microtitre wells’ absorbance in presence and absence of inhibitor.

2.6. Estimation of HbA1c

Kit(s) purchased from of Bio-Rad (USA) was used for the estimation of HbA1c in diabetes sera using HPLC based D-10 HbA1c program. In this procedure, sample is automatically diluted and injected into the analytical cartridge. The D-10 device delivers buffer gradient of increasing ionic strength to the cartridge, and the hemoglobins are separated on the basis of their interaction(s) with the cartridge material. The separated hemoglobins pass through the flow cell of the photometer and the absorbance is recorded at 415 nm. The software performs reduction of raw data collected from each analysis. Two-level calibration is used for quantitation of HbA1c values. A sample report and chromatogram is generated for each sample. The HbA1c area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak area from the A1c peak area. The normal HbA1c reference range of the kit used in this study was 4.27–6.07%.

2.7. Statistical analysis

Results are expressed as mean ± SEM. All statistical calculations were performed using SPSS software (version 16). A \( p \) values of < 0.05 was considered as statistically significant. Linear correlation analysis using absorbance difference as dependent variables was also performed.
Table 1
Demographic profile and clinical characteristics of diabetic subjects

| No. | Variables                      | Total        | Male          | Female         |
|-----|--------------------------------|--------------|---------------|----------------|
|     |                                | Mean S.D.    | Mean S.D.     | Mean S.D.      |
| 1   | Age (years)                    | 50.5±11.5    | 53.0±12.5     | 48.5±10.0      |
| 2   | Body mass index (kg/m²)        | 25.5±5.6     | 24.4±6.4      | 26.3±4.8       |
| 3   | W.C. (cm)                      | 93.2±10.9    | 94.6±12.0     | 92.2±9.8       |
| 4   | H.C. (cm)                      | 95.8±9.7     | 95.6±10.2     | 96.0±9.4       |
| 5   | Waist: hip ratio               | 0.97±0.06    | 0.98±0.06     | 0.96±0.06      |
| 6   | Duration of diabetes (years)   | 7.0±5.0      | 6.5±4.5       | 7.0±5.0        |
| 7   | Blood sugar (mg%), Fasting     | 138.0±53.0   | 139.0±61.0    | 136.0±47.0     |
|     |                                | Post prandial |              |                |
| 8   |                                | 180.0±68.0   | 183.0±72.0    | 177.0±65.0     |
| 9   | HbA₁c (%)                      | 8.8±2.9      | 9.1±3.3       | 8.6±2.6        |
| 10  | S. creatinine (mg%)            | 1.0±0.3      | 1.1±0.4       | 1.0±0.3        |
| 11  | Total cholesterol (mg/dl)      | 183.0±43.0   | 178.0±40.0    | 186.0±44.0     |
| 12  | LDL-cholesterol (mg/dl)        | 102.0±30.0   | 102.0±29.0    | 103.0±30.0     |
| 13  | Triglycerides (mg/dl)          | 132.0±46.0   | 130.0±42.0    | 133.0±49.0     |
| 14  | HDL-cholesterol (mg/dl)        | 44.0±7.0     | 44.0±7.0      | 45.0±7.0       |
| 15  | VLDL-cholesterol (mg/dl)       | 30.0±13.0    | 29.0±14.0     | 31.0±17.0      |
| 16  | Systolic B.P. (mmHg)           | 138.0±18.0   | 139.0±18.0    | 137.0±17.0     |
|     |                                | 87.0±11.0    | 87.0±11.0     | 86.0±11.0      |

Table 2
Application of "t" test on various glycemic parameters and disease duration

| Variable                     | Value with native DNA | Value with DNA-AGEs | t∗   | p**  |
|------------------------------|-----------------------|---------------------|------|------|
|                              | Mean S.D.             | Mean S.D.           |      |      |
| Fasting blood sugar (mg/dl)  | 120.0±36.0            | 151.0±60.0          | −2.60| <0.011|
| Post prandial blood sugar (mg/dl) | 155.0±50.0        | 199.0±74.0          | −3.15| <0.002|
| HbA₁c (%)                    | 7.6±1.7               | 9.8±3.3             | −3.70| <0.001|
| Disease duration (years)     | 7.0±5.5               | 6.5±5.0             | 0.78 | >0.437|

t∗ is the value of student’s ‘t’ test.
p** indicate difference (independent sample ‘t’ test).

3. Results

The demographic and clinical characteristics of 150 diabetic subjects included in the study are shown in Table 1. The mean age of diabetic subjects was 50.5±11.5 years. Females predominated (85%). Majority of the subjects (141/150, 94%) had Type 2 DM. The mean duration of diabetes was 7.0±5.0 years. Forty two patients (28%) had retinopathy, 14 (9.3%) neuropathy, 50 (33.3%) nephropathy, 24 (16%) coronary artery disease, 2 (1.3%) peripheral vascular disease and 51 (34%) were hypertensive.

Table 2 shows the glycemic parameters in diabetic subjects on the basis of relative absorption to native DNA and DNA-AGEs. There was significant difference on the basis of fasting & post prandial blood sugar and HbA₁c (p < 0.011, p < 0.002, p < 0.001 respectively) but no significant difference on the basis of disease duration.

3.1. Characterization of glycated-DNA

Native DNA (10 µg/ml) was mixed with 130 mM of Gl 6-P and incubated for 3,7,10 and 14 days and then subjected to spectrophotometric analysis (Fig. 1). Native DNA showed the characteristic peak at 260 nm. Upon glycation, the absorbance values corresponding to 260 nm showed hyperchromicity. Compared to 260 nm peak of native DNA, the hyperchromicities shown by 3,7,10 and 14 days old glycated-DNA samples were 74.8%, 80.7%, 81.6% & 88.1% respectively. The findings suggest structural perturbations in DNA as a consequence of Gl 6-P mediated glycation.

Possible generation of fluorogenic AGEs in glycated-DNA samples were detected using excitation wavelength (λex) of 370 nm [27]. Under identical conditions, native DNA did not show any fluorescence intensity. However, glycated-DNA samples showed maximum fluorescence emission intensity around 430 nm which increased with incubation time (Fig. 2). The results indicate time dependent generation of fluorophores in DNA samples treated with glucose.

Equal amounts of native and glycated-DNA samples were subjected to agarose gel electrophoresis. The migration pattern of ethidium bromide stained bands has been shown in Fig. 3. The pattern shown by native DNA is typical of genomic DNA (lane 1). The
ethidium assisted fluorescence of glycated-DNA samples were found to be decreasing with increasing incubation time (lanes 2–5). Furthermore, faster mobility of glycated-DNA samples were also observed. It may be due to generation of single strand breaks by glycation induced reactive intermediates. Furthermore, the heavily glycated-DNA sample (lane 5) migrated as a short stretch (or band) of lower intensity. The results reiterate Gl 6-P induced modifications.

3.2. Detection of anti-glycated-DNA autoantibodies (anti-DNA-AGEs autoantibodies) in type 2 diabetes sera by ELISA

Anti-glycated-DNA autoantibodies in diabetes sera were detected by enzyme immunoassay on microtitre wells coated with native- and two week old glycated-DNA. Binding profile of diabetes sera with native and glycated-DNA antigens has been shown in Fig. 4.
Autoantibodies in fifty five percent of diabetes sera (83/150) showed more binding with glycated-DNA compared to native DNA. Sera from healthy individuals included as control did not show significant binding with either of the coated antigens. The diabetes sera which showed high binding with glycated-DNA were subjected to inhibition ELISA to evaluate true interaction of autoantibodies in diabetes with glycated-DNA. Results of inhibition ELISA are given in Table 3.

### Table 3

| Serum no. | Max. % inhibition with native DNA | Max. % inhibition with DNA – AGEs |
|-----------|-----------------------------------|----------------------------------|
| 4         | 36                                | 48                               |
| 16        | 54                                | 70                               |
| 17        | 56                                | 74                               |
| 19        | 40                                | 56                               |
| 21        | 30                                | 64                               |
| 25        | 38                                | 52                               |
| 30        | 22                                | 68                               |
| 37        | 36                                | 50                               |
| 38        | 28                                | 40                               |
| 40        | 34                                | 50                               |
| 41        | 34                                | 66                               |
| 42        | 34                                | 63                               |
| 48        | 36                                | 78                               |
| 51        | 38                                | 57                               |
| 52        | 26                                | 38                               |
| 53        | 38                                | 64                               |
| 56        | 24                                | 48                               |
| 57        | 32                                | 62                               |
| 58        | 32                                | 68                               |
| 59        | 30                                | 54                               |
| 60        | 30                                | 60                               |
| 62        | 34                                | 50                               |
| 63        | 54                                | 70                               |
| 64        | 42                                | 84                               |
| 65        | 28                                | 78                               |
| 77        | 26                                | 45                               |
| 94        | 20                                | 34                               |
| 134       | 40                                | 68                               |
| 143       | 18                                | 46                               |
| 150       | 30                                | 59                               |

Note: The serum numbers in Table 3 correspond to serum number in Fig. 4

The data clearly indicates presence of autoantibodies against glycated-DNA in a sub-population of diabetes patients.

### 3.3. Correlation between HbA1c and anti-glycosylated-DNA autoantibodies

Linear correlation analysis was performed using absorbance difference as dependent variable and HbA1c%, nephropathy, neuropathy, blood pressure and lipids as independent variables (Table 4). It was found that the absorbance difference was significantly related to HbA1c (r = 0.486, p < 0.001), nephropathy (r = 0.239, p < 0.003), retinopathy (r = 0.165, p < 0.05) and serum triglycerides and VLDL-cholesterol (r = 0.186, p < 0.001 and r = 0.190, p < 0.001 respectively).
Table 4
Linear correlation using absorbance difference as dependent variable in diabetic subjects, \((n = 141)\)

| Variable       | \(r\)  | \(r^2\) | \(t\)     | \(p\)     |
|----------------|--------|---------|-----------|-----------|
| HbA1c          | 0.486  | 0.236   | 6.55      | < 0.001   |
| Nephropathy    | 0.239  | 0.057   | 2.90      | < 0.003   |
| Neuropathy     | 0.034  | 0.001   | 0.401     | > 0.05    |
| Retinopathy    | 0.165  | 0.027   | 0.027     | < 0.05    |
| Triglycerides, mg% | 0.186  | 0.034   | 2.230     | < 0.001   |
| HDL, mg%       | 0.065  | 0.004   | 0.768     | > 0.05    |
| LDL, mg%       | 0.013  | 0.001   | 0.153     | > 0.05    |
| VLDL, mg%      | 0.190  | 0.036   | 2.282     | < 0.001   |
| Systolic BP, mmHg | 0.108  | 0.011   | 1.280     | > 0.05    |
| Diastolic BP, mmHg | 0.135  | 0.018   | 1.607     | > 0.05    |

4. Discussion

Our understanding of the relationship between glycemic control and the chronic complications of diabetes has been greatly enhanced by the results of several recent seminal intervention studies [14–16]. These studies demonstrated that microvascular complications are highly correlated with mean glycemic control, as measured by glycated hemoglobin (HbA1c), and that improvement in glycemic control results in reduction in all microvascular complications. In contrast, they failed to show a significant reduction in macrovascular complications with improved glycemic control, suggesting that macrovascular complications are the results of multiple metabolic abnormalities. A direct relationship between chronic hyperglycemia and micro- and macro-vascular complications in type 2 diabetes has been emphasised, over and again [28–30]. Persistent hyperglycemia can produce structural changes in long-lived macromolecules which may persist even after restoration of euglycemia [31].

In this study, glycated-DNA was prepared by incubating native DNA with glucose-6-phosphate (cellular metabolite of glucose). Generation of DNA-AGEs was confirmed by fluorescent studies. Recent studies have demonstrated that nucleobases can also be a target for non-enzymatic attack by reactive carbonyl compounds and that the presence of these nucleobase adducts may also play role in vivo [5]. In the present study, higher binding with glycosylated-DNA against native-DNA in diabetic patients with HbA1c of 9.8 ± 3.3% compared to those with HbA1c of 7.7 ± 1.7% (\(p < 0.001\)) indicates the effects of DNA are marked in diabetes. The results are in agreement with earlier report [32], where the study has shown that like protein, nucleic acids can also undergo non-enzymatic modification by reducing sugars and produce spectral/structural changes similar to those reported for non-enzymatic browning of proteins. The DNA glycation marker, N2-carboxyethyl-2’-deoxyguanosine, has been reported in kidney and aorta of diabetic and uremic patients [33].

In view of the immunogenic nature of AGEs, it may not be out of context to think that persistence of similar structures in vivo can initiate/propagate autoimmune response [13]. In the present study, the presence of anti-DNA-AGEs autoantibodies in 55% of diabetic sera may be as a consequence of auto immune response against persistent DNA-AGEs in long term hyperglycemia. A significant correlation was also observed with HbA1c and absorbance different between DNA-AGEs and native DNA. Anti-DNA-AGEs autoantibodies were al-
so correlated with diabetic retinopathy and nephropathy. It is quite possible that the apoptotic DNA might stay in the blood circulation of diabetic patients for a longer period and may undergo heavy glycation to generate DNA-AGEs in long term hyperglycemia. The AGEs of DNA nucleobases have receive little attention, perhaps due to the fact that adenine, guanine, cytosine and thymine do not dissolve under mild pH conditions. To maintain nucleobases in solution, alkaline pH conditions are necessary. Udayan et. al., 2006, evaluated non-enzymatic glycation of DNA nucleobases under alkaline pH [34] and demonstrated that N2-carboxyethyldeoxyguanosine formed during the glycation of DNA in vitro causes increased depurination of DNA. Nucleotide glycation and related effects are expected to be most marked in diseases associated with the accumulation of glycating agents to high concentrations, diabetes and uremia [35]. It also suggests that increased formation of α-oxoaldehydes in hyperglycemia and oxidative stress are associated with diabetes [36].

Findings from the present study indicated that DNA-AGEs and autoantibodies against glycosylated DNA are correlated with HbA1c and microvascular complications like diabetic retinopathy and nephropathy and may serve as additional biomarkers for assessment of chronic glycermia. However, a large prospective multicentric study is required to establish usefulness of DNA-AGEs and its autoantibodies in the assessment of glycemic control and its correlation with microvascular complications in diabetic subjects.

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