

Antibodies against Gluco-Oxidatively Modified Human Serum Albumin Detected in Diabetes-Associated Complications

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Key Words

Autoantibodies • Diabetes • Diabetes-associated complications • Enzyme-linked immunosorbent assay • Gluco-oxidation • Reactive oxygen species-modified glycated human serum albumin

Abstract

Background: Glycated proteins present new immunological epitopes on their surface against which autoantibodies are generated that have a possible role in immunopathogenesis in diabetic complications. **Methods:** In the present study, in vitro glycation- and reactive oxygen species (ROS)-modified human serum albumin (HSA) has been studied by different spectroscopic techniques (UV and fluorescence) and thermal denaturation profiles. The binding characteristics of circulating autoantibodies in diabetic patients and diabetic patients with secondary complications against native HSA (N-HSA) and ROS-modified glycated HSA (RG-HSA) were assessed by direct and competition enzyme-linked immunosorbent assay (ELISA). In another approach, antibodies against RG-HSA (RG-HSA-Abs) induced in experimental animals were used as an immunochemical probe for the detection of gluco-oxidative lesions in blood proteins of patients (n = 8) with diabetic retinopathy. **Results:** Modified RG-HSA showed marked structural changes. High recognition of RG-HSA was shown by diabetic serum autoantibodies. Diabetic

patients with retinopathy, nephropathy and atherosclerosis showed significantly ($p < 0.001$) stronger binding to RG-HSA over N-HSA. Normal human sera exhibited negligible binding with either antigen. Competitive inhibition ELISA results show significantly high binding of RG-HSA-Abs to albumin, immunoglobulin G and red blood cell membrane isolated from diabetic retinopathic patients. **Conclusion:** In conclusion, these results suggest that hyperglycemia together with ROS may contribute to the immunopathogenesis of diabetes-associated complications. Copyright © 2010 S. Karger AG, Basel

Introduction

Diabetes mellitus is a group of metabolic disorders characterized by glucose homeostasis imbalance, chronic hyperglycemia and disturbances of carbohydrate, fat and protein metabolisms [1]. It is now well recognized that long-term exposure of proteins to sugars in hyperglycemia results in nonenzymatic glycation of proteins generating advanced glycation end products (AGEs) [2]. Glycation is considered to be an important pathophysiological mechanism in the development of diabetes-associated complications [3]. Diabetes-specific complications eventually lead to irreversible, lasting microvascular (retinopathy, nephropathy and neuropathy) and

macrovascular (atherosclerosis) changes [4]. Immunohistochemical studies have demonstrated the presence of AGE-modified proteins in several human tissues under pathological conditions, including the kidney of patients with diabetic nephropathy [5], atherosclerotic lesions of arterial walls [6] and diabetic retinopathy [7]. These results indicate that AGEs accumulated in vivo serve as immunological epitopes which generate autoantibodies that may be used as a potential marker for diabetic complications [8].

With the onset of diabetes, persistent and chronic hyperglycemia causes increased production of free radicals through autooxidation of glucose, via nonenzymatic protein glycation and enhanced flux of glucose through the polyol pathway [9]. The generation of reactive oxygen species (ROS) and protein glycation are strictly interconnected [10].

It has recently been established that ROS causes extensive damage to human serum albumin (HSA) and other proteins, which in turn renders it highly immunogenic in experimental animals and recognized by antibodies in patients with diabetes [11, 12]. In light of this, the purpose of the present study was to investigate the possible involvement of gluco-oxidative damaged HSA in diabetes and the pathogenesis of associated complications. To test this hypothesis, we studied the presence of circulating autoantibodies against gluco-oxidatively modified HSA in the sera of diabetic patients and diabetes-related complications (retinopathy, nephropathy and atherosclerosis) that might serve as a potent immunological marker of concomitant cellular oxidative stress with glycation of blood proteins. On the other hand, experimentally induced antibodies against ROS-modified glycated HSA (RG-HSA) were used as a probe to detect gluco-oxidative blood protein lesions in diabetic complications.

Materials and Methods

Human Serum Samples

In the present study 98 diabetic patients (68 males and 30 females) and 60 normal human (41 males and 19 females) control subjects were investigated and divided into six groups on the basis of clinical examinations (table 1). The categories for diabetic complications were mutually exclusive. Patients with diabetic retinopathy had been smokers for the last two decades. Patients with diabetic nephropathy and diabetic atherosclerosis had a history of hypertension for the last 15 ± 4.5 years. Normal human subjects served as controls, were age- and sex-matched with no family history of diabetes. All groups underwent periodic examinations. All subjects gave informed consent to the analysis and the study had been approved by the Ethics Committee. All the serum samples of patients were collected from the Laboratory of Endocrinology, Department of Medicine (J.N. Medical College and Hospital, A.M. University, Aligarh, India) and their clinical features are shown in table 1. Patient classification is summarized as follows:

Patients without Complications (Type 1 and Type 2). These patients (n = 30 each) had type 1 and type 2 diabetes and remained free of any complications (retinopathy, nephropathy and atherosclerosis). These patients were negative for proteinuria.

Nephropathic Patients. These patients (n = 12) had type 1 diabetes and all had proteinuria (urinary protein excretion rates >0.5 g/24 h) in the absence of hematuria or infection on mid-stream urine samples.

Retinopathic Patients. These patients (n = 12) had retinopathy defined as more than five dots or blots per eye, hard or soft exudates and vitreous hemorrhage.

Atherosclerosis. These patients (n = 12) were diagnosed with macrovascular disease and showed narrowing of arterial walls accompanied by chronic inflammation and injury.

Modification of HSA

Native HSA (N-HSA; Sigma, St. Louis, Mo., USA) was glycated according to a published procedure [13]. An aqueous solution of glycated HSA (1 mg/ml in 20 mM phosphate-buffered saline, pH = 7.4) was further modified by hydroxyl radical [12]. Thermal denaturation studies of native and modified HSA were also recorded at 280 nm on a spectrophotometer (Shimadzu UV-240; Shimadzu, Tokyo, Japan) by increasing the temperature in the range of 30–96°C [13].

Table 1. Clinical characterization of patients and normal control subjects

Subjects	Subjects n	Age, years	Duration of disease years	Fasting blood glucose, mg/dl	HbA _{1c} , %
Type 1 diabetic	30	36 ± 14	9 ± 3.1	254 ± 32	7.4 ± 0.6
Type 2 diabetic	30	44 ± 11	7 ± 3.6	263 ± 28	7.1 ± 0.4
Diabetic retinopathy	12	68 ± 2.9	21 ± 4.9	434 ± 11	9.0 ± 0.4
Diabetic nephropathy	12	62 ± 4.3	18 ± 3.3	394 ± 13	9.2 ± 0.6
Diabetic atherosclerosis	12	59 ± 3.6	17 ± 3.6	390 ± 15	8.8 ± 0.3
Normal human controls	60	32 ± 9.5	–	88 ± 9.8	5.8 ± 0.4

Data shown as mean ± SD. For blood glucose estimations, blood was collected in oxalated fluoride containers and the assays were performed immediately.

Fluorometry

The fluorescence analysis of AGE-specific pentosidine was also taken with excitation wavelength 370 nm and the peak was observed in the range of 380–500 nm [14]. Excitation and emission slit widths were 10 nm. All protein solutions were of the same concentration (60 μM).

Immunization and Immunoglobulin G Purification

Antibodies against N-HSA and RG-HSA (RG-HSA-Abs) were raised in female New Zealand white rabbits following the standard protocol and using Freund's complete/incomplete adjuvants (Sigma). The animals were immunized by subcutaneously injecting 0.5 ml antigen preparations (400 mg/l) in 0.02 M sodium phosphate buffer emulsified with Freund's adjuvant. Booster doses of 0.3 g/l antigens were administered weekly for 3 weeks after resting the animals for 15 days. The animals were finally bled through a marginal ear vein. The serum was separated and de complemented by keeping the serum samples at 56°C for 30 min; they were preserved at -20°C with sodium azide. Preimmune sera were also collected from the animals. Formation of antibodies was monitored by direct enzyme-linked immunosorbent assay (ELISA) [15]. Immunoglobulin G (IgG) was purified by Protein A-Agarose column (Sigma) [16], and antibody concentration was determined by protein assay (Bio-Rad Laboratories, Munich, Germany). Experiments on animals were performed in accordance with university ethical guidelines.

Direct ELISA

Direct binding ELISA was performed on flat-bottom 96-well polystyrene immunoplates (Maxisorp™; Nalge Nunc International, Roskilde, Denmark), as described previously [11, 15, 17]. Briefly, plates were coated with 100 μl of respective antigen (20 $\mu\text{g}/\text{ml}$) for 2 h at room temperature and overnight at 4°C. The plates were washed with TBS-T (20 mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4, containing 0.05% Tween-20) and unoccupied sites were blocked with 150 μl of 1.5% bovine serum albumin in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h at room temperature. The test serum (diluted 1:100) in TBS-T (100 μl per well) was adsorbed for 2 h at room temperature and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using *p*-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader (Multiskan EX; Labsystems, Helsinki, Finland).

Competition ELISA

The antigenic specificity of the modified HSA was determined by competition ELISA [11, 12, 15, 17]. Varying concentrations of inhibitors (0–20 $\mu\text{g}/\text{ml}$) were allowed to interact with a constant amount of antiserum or IgG for 2 h at room temperature and overnight at 4°C. The immune complex thus formed was incubated in the wells (instead of the serum/IgG taken in direct binding ELISA) and the bound antibody levels were detected as in direct binding ELISA.

The percent inhibition was calculated using the formula:

$$\text{Percent inhibition} = [1 - (A_{\text{inhibited}}/A_{\text{uninhibited}})] \times 100$$

where $A_{\text{inhibited}}$ is the absorbance at 20 $\mu\text{g}/\text{ml}$ of inhibitor concentration and $A_{\text{uninhibited}}$ the absorbance at zero inhibitor concentration.

Isolation of Serum Albumin

HSA was isolated from the serum samples of healthy controls and diabetic retinopathic subjects by gel exclusion chromatography on Sephacryl S-200 HR (Pharmacia Fine Chemicals, Mount Holly, N.J., USA) [18]. The purity of the HSA samples was checked by SDS-PAGE [19].

Isolation of Red Blood Cells

Blood samples from normal and diabetic retinopathic subjects were collected in heparin tubes. Plasma was separated by centrifugation. Red blood cells (RBC) were washed 3 times with a cold 0.9% NaCl solution, and RBC hemolysate was prepared as described previously [20].

Purification of IgG

IgGs were isolated from the sera of normal controls and diabetic subjects with retinopathy by Protein A-Agarose [16] and the homogeneity of isolated IgG was checked on 7.5% polyacrylamide gel electrophoresis and silver staining was performed for visualization of protein bands [19].

Determination of Protein-Bound Carbonyl Groups

Protein-bound carbonyl groups from sera of subjects were analyzed according to Levine et al. [21] and the results were expressed as the number of nanomoles of carbonyl per milligram of sample protein using $\epsilon_{379\text{ nm}} = 22,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ [12].

Statistical Analysis

All results were expressed as the mean $\pm$ SD. Multiple comparisons between data were done using software OriginPro 6.1 followed by Student's *t* test. The *p* value for significance was set at <0.05 .

Results

Characterization of RG-HSA

RG-HSA showed 32.3% UV hyperchromicity at 280 nm, revealing marked conformational alterations due to ROS and glucose-induced modifications (table 2). Thermal stability of both the samples was evaluated and a substantially higher melting temperature of 9°C in RG-HSA over N-HSA was observed. In vitro formation of AGE products was confirmed by the presence of pentosidine, a cross-link structure between lysine and arginine residues, one of the major stable fluorescent AGE products [22]. Significantly higher fluorescence pentosidine-specific intensity was observed in RG-HSA (310 ± 11.5) over N-HSA (4 ± 3.1) (table 2).

Antigenicity of N-HSA and RG-HSA

Direct binding ELISA of serum samples from experimental animals showed that RG-HSA is a potent immunogen and induced high titer ($\geq 1:25,600$) (fig. 1a) serum IgG as compared to native antigen ($\approx 1:12,800$) (fig. 1b).

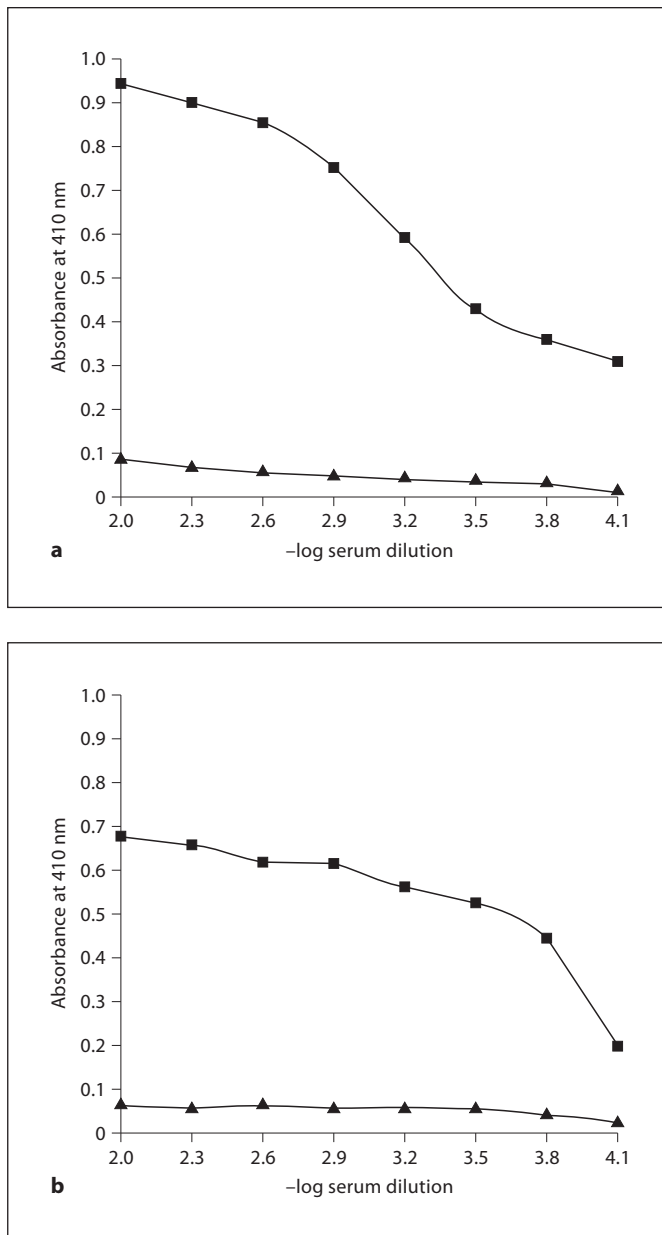


Fig. 1. Direct binding ELISA of RG-HSA (a) and N-HSA (b) with preimmune (▲) and immune (■) sera. Microtiter plates were coated with their respective antigens (20 µg/ml).

On the other hand, preimmune sera from experimental animals did not show any reactivity for both native and modified HSA (fig. 1a, b).

Recognition of RG-HSA by Circulating Autoantibodies

To probe the presence of circulating autoantibodies against RG-HSA, sera from six different subject groups

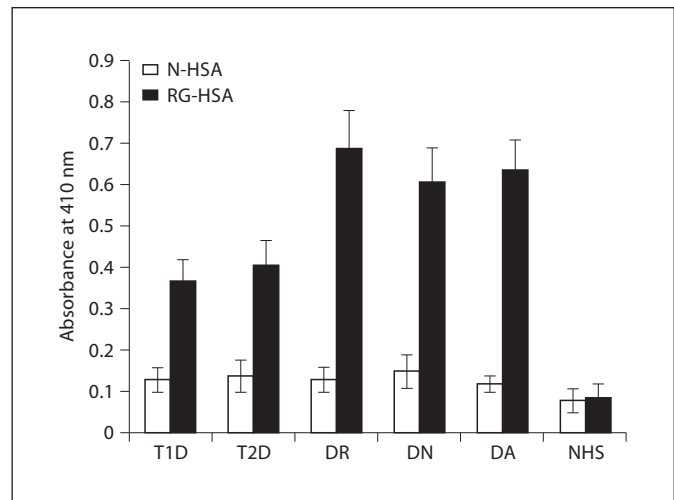


Fig. 2. Direct binding ELISA of 1:100 diluted type 1 diabetes (T1D), type 2 diabetes (T2D), diabetic retinopathy (DR), diabetic nephropathy (DN), diabetic atherosclerosis (DA) and control NHS. Serum IgG reactivity of all six groups was checked towards N-HSA and RG-HSA. Microtiter plates were individually coated with N-HSA and RG-HSA (20 µg/ml). Each histogram represents the mean $\pm$ SD values.

Table 2. Characterization of N-HSA and RG-HSA under identical conditions

Parameters	N-HSA	RG-HSA
Hyperchromicity, at 280 nm	–	32.3%*
Melting temperature (T_m), °C	55.0 ± 3.2	$64.0 \pm 2.7^*$
Pentosidine specific fluorescence (Ext. at 370 nm; ems. at 440 nm)	4 ± 3.1	$310 \pm 11.5^{**}$

N-HSA was considered as standard in percent hyperchromicity estimations. Each value represents arithmetic mean $\pm$ SD of three independent assays. Ext. and ems. represent excitation and emission wavelengths. * $p < 0.05$; ** $p < 0.001$.

were tested by direct ELISA (fig. 2). RG-HSA exhibited significantly higher ($p < 0.001$) optical density (OD) in diabetic subjects with secondary complications (retinopathic, OD 0.69 ± 0.09 ; nephropathic, OD 0.61 ± 0.08 , and atherosclerotic, OD 0.64 ± 0.07) as compared to diabetic alone (type 1, OD 0.37 ± 0.05 , and type 2, OD 0.41 ± 0.06) or normal control subjects (OD 0.09 ± 0.03). Conversely, no effective binding was observed in all six groups for N-HSA (fig. 2).

Table 3. Detection of N-HSA-Abs and RG-HSA-Abs and the estimation of carbonyl contents as oxidative stress in the sera of various diabetic groups and controls

Groups	Sera positive for RG-HSA ¹	Sera positive for N-HSA ²	Sera positive for both antigens	Carbonyl content nmol/mg protein
Type 1 diabetes (n = 30)	21 (52 ± 5.5)	–	1 (43 ± 4.7 ¹ ; 51 ± 5.2 ²)	2.9 ± 0.35
Type 2 diabetes (n = 30)	16 (48 ± 4.7)	–	2 (47 ± 4.7 ¹ ; 43 ± 5.2 ² , 45 ± 3.3 ¹ ; 41 ± 4.7 ²)	2.8 ± 0.4
Diabetes retinopathy (n = 12)	8 (76 ± 4.5)*	–	–	3.9 ± 0.5*
Diabetes nephropathy (n = 12)	7 (69 ± 3.1)*	1 (41 ± 4.7)	–	3.5 ± 0.35*
Diabetes atherosclerosis (n = 14)	9 (67 ± 4.0)*	–	1 (55 ± 4.1 ¹ ; 55 ± 2.8 ²)	3.3 ± 0.55*
Normal human controls (n = 60)	–	–	–	2.3 ± 0.42

The ELISA plate was coated with the respective antigens (N-HSA and RG-HSA) at 20 µg/ml. 'Sera positive' means serum samples which gave inhibition ≥30%. n denotes the number of sera tested. Values in parentheses are means ± SD of maximum percent inhibition serum samples at inhibitor concentrations of 20 µg/ml. Values with superscripts 1 and 2 indicate the maximum percent inhibition of serum sample(s) with RG-HSA and N-HSA as inhibitors, respectively.

* p < 0.001.

The binding specificity of circulating autoantibodies was further investigated by inhibition ELISA using N-HSA and RG-HSA as inhibitors (table 3). Out of 30 sera of type 1 diabetic patients, 21 were found to react toward RG-HSA. Among these 21 sera, 20 recognized RG-HSA to a greater extent as competitor, whereas 1 serum sample showed a better recognition of N-HSA as inhibitor compared to modified HSA (51 vs. 43%, respectively). The circulating antibodies of 21 serum samples showed mean maximum percent inhibition (MMPI) of 52 ± 7.5 with modified antigen. Sixteen sera out of 30 from type 2 diabetic patients also showed recognition of RG-HSA (48 ± 8.7 MMPI). Out of 16 serum samples, 2 recognized N-HSA (43 and 41% inhibition) and also reacted with RG-HSA to an even higher degree (47 and 45% inhibition).

The results of diabetic subjects with complications (table 3) are summarized as follows. None of the serum samples from diabetic retinopathic patients (n = 12) showed reactivity toward N-HSA. However, 8 sera exhibited significantly higher binding to RG-HSA (76 MMPI, p < 0.001). Seven sera out of 12 from diabetic nephropathic patients showed greater binding to RG-HSA (69 MMPI, p < 0.001), whereas 1 serum sample exhibited recognition of only N-HSA. In case of 14 sera from patients with diabetic atherosclerosis, 1 sample exhibited equal inhibition of 55% with both N-HSA and RG-HSA, whereas 8 serum samples preferentially recognized RG-HSA (67 MMPI, p < 0.001). No reactivity was noted in the plasma samples of controls (normal human samples, NHS) with either of the antigens.

Gluko-Oxidative Stress in Diabetes Mellitus

Carbonyl content observed in patients with type 1 and type 2 diabetes was higher (2.9 ± 0.35 and 2.8 ± 0.4 nmol/mg proteins, respectively) as compared to NHS (2.3 ± 0.42 nmol/mg protein). The highest carbonyl content (3.9 ± 0.5 nmol/mg protein) was observed in diabetic retinopathic patients. Moreover, significant increases were also observed in diabetic nephropathic and atherosclerotic patients (3.5 ± 0.35 and 3.3 ± 0.4 nmol/mg protein, respectively, table 3).

Probing in vivo Gluko-Oxidative Damage of Blood Proteins

To define the effect of gluco-oxidation on human blood proteins, we compared the activity of RG-HSA-Abs induced in experimental animals with blood proteins (albumin, RBC membrane hemocyte and IgG) isolated from 8 blood samples of diabetic retinopathic subjects. We selected diabetic retinopathic subjects as they exhibited highest recognition among the diabetic groups with our modified antigen. Blood proteins isolated from 5 NHS served as control. In inhibition ELISA blood proteins were used as inhibitors between the binding reactivity of RG-HSA and RG-HSA-Abs. All 8 albumin samples showed significant recognition (70.3 MMPI) towards RG-HSA-Abs (fig. 3a). RBC membrane hemocyte and IgG isolates exhibited almost similar reactivity (64.4 and 63 MMPI, respectively) towards the anti-RG-HSA-Abs (fig. 3b, c). The samples of albumin, RBC membrane hemocyte and IgG isolated from 5 NHS showed negligible recognition of RG-HSA-Abs (fig. 3a–c, respectively).

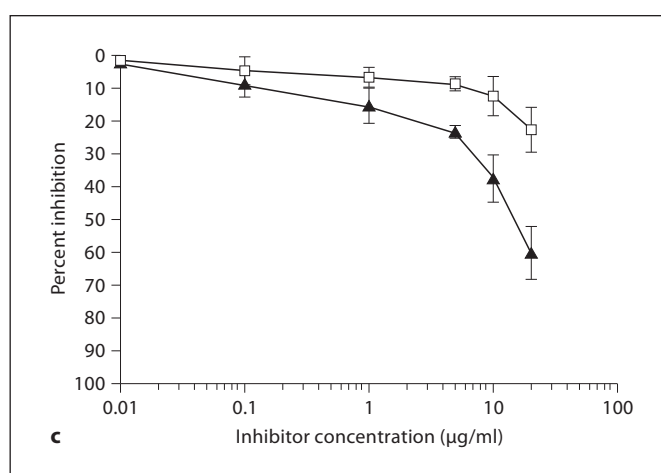
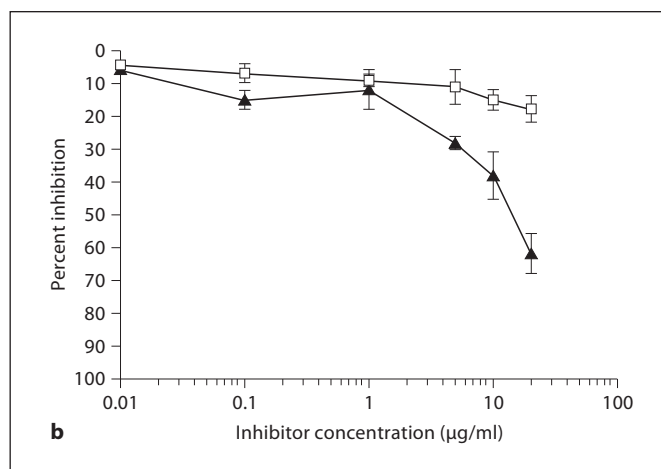
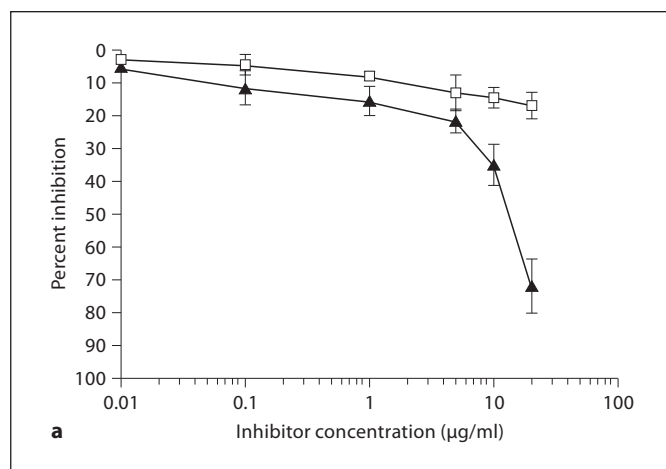


Fig. 3. Inhibition of experimentally induced RG-HSA-Abs binding to RG-HSA. Isolated blood proteins from diabetic retinopathic patients (▲) and normal human subjects (□) were used as inhibitors albumin (a), RBC membrane hemolyate (b) and IgG (c). The microtiter plates were coated with RG-HSA (20 µg/ml).

Discussion

In this study, in vitro modification of N-HSA with glucose and ROS led to marked conformational structural deformations. Increase in UV absorbance is due to the exposure of chromophobic groups. The nonenzymatic glycation of HSA induces refolding of globular protein accompanied by formation of cross- β links, which convert glycated albumin to fibrous or amorphous aggregate forms [23]. These aggregates are high molecular weight stable molecules formed due to AGE cross-linking agents like pentosidine that persist in the circulation for a long duration [24] and are substantiated by results of increased melting temperature and pentosidine-specific fluorescence of RG-HSA.

Induction of high titer RG-HSA-Abs due to glycation and oxidative modifications induces structural alterations in the native protein and leads to the generation of new epitopes, rendering them more immunogenic.

Significantly high serum autoantibodies against RG-HSA were detected in diabetic patients with complications. Increased levels of anti-AGE albumin antibodies in the sera of diabetic patients have been reported previously [8] and are supported by our results (fig. 2). Comparing figure 2 and the data from table 1 suggests that diabetic patients with complications are older and had longer duration of disease. With long duration of disease and increased age there would be more chances of protein gluco-oxidation and formation of new epitopes that are immunogenic and generate RG-HSA-Abs production.

It is well accepted that formation of carbonyl groups is a hallmark of protein gluco-oxidation due to glycation [24, 25]. Hyperglycemia and high carbonyl content, an index of free radical generation, are interrelated factors. Combination of these can cause severe damage to the native conformation of in vivo blood protein and autoantibody generation [26]. Significantly increased levels of carbonyl in diabetic patients with complications suggest

in vivo blood protein glycation, exhibiting severe gluco-oxidative toxicity of blood proteins.

The binding of RG-HSA and serum autoantibodies of patients with type 1 and 2 diabetes, further ascertained by competition ELISA, confirmed that circulating autoantibodies from these diabetic subjects recognize modified antigen with high affinity.

Interestingly, diabetics with associated complications (retinopathy, nephropathy and atherosclerosis) generated more autoantibodies against RG-HSA than controls and diabetic subjects without secondary complications. The above contention supports that AGEs are toxic and highly immunogenic [2]. Patients with diabetic nephropathy and atherosclerosis exhibited increased carbonyl content and higher binding (69 and 67 MMPI, respectively) affinity of autoantibodies to modified antigen. Both these groups had a history of uncontrolled chronic hyperglycemia and hypertension coupled with age. Thus hypertension [27] and chronic hyperglycemia with advanced age has been considered a potential risk factor in the development of autoantibodies in diabetic nephropathy and atherosclerosis [28]. In the overall cohort of diabetic patients, the highest recognition of RG-HSA by autoantibodies (76 MMPI) and maximum amount of carbonyl content were apparent in diabetic retinopathic patients, which showed a significant correlation between a history of poor blood glucose control (>423 mg/dl), smoking for over 2 decades and older age, as given in table 1. AGEs are a product of the oxidative modification of glycated proteins, which damage proteins with ageing, high oxidative stress and toxic blood glucose levels [2]. These are found to be the common factors behind the generation of high autoantibodies and the progression of disease complications.

AGEs may produce adverse effects on tissues and cells via a variety of mechanisms involving the alteration of tissue protein structure and function [29]. Our results suggest that these conditions contributed to excessive in vivo protein glycation and oxidation [2, 30] against which high titer autoantibodies were generated in diabetic secondary complications. A high degree of reactivity of autoantibodies to RG-HSA suggests that gluco-oxidative damage might have a potential role in the immunopathogenesis of diabetes-associated complications.

Gluco-oxidation leads to toxicity of blood proteins [30] as confirmed by cross-reaction of these isolated proteins (albumin, RBC membrane and IgG) from diabetic retinopathic patients towards RG-HSA-Abs induced in experimental animals. This suggests that considerable amounts of AGEs such as pentosidine were formed in blood proteins that would develop immune complexes with anti-AGE antibodies [31]. The raised RG-HSA-Abs could be considered a potent immunological tool for the detection of the development of diabetes-associated complications.

In vitro treatment of N-HSA with ROS and glucose synergistically causes biophysical and biochemical alterations that confer immunogenicity. Interestingly, far greater levels of RG-HSA-Abs were observed in the sera of patients with diabetic complications than in normal controls and patients with diabetes alone. Induced RG-HSA-Abs in experimental animals have been shown to be a potential alternative immunochemical probe that detects in vivo gluco-oxidative blood protein damage and deterioration. RG-HSA-Abs have a significant role in the diagnosis/prognosis of disease complications.

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