

Circulating Soluble Receptor for Advanced Glycation End Products and Total Antioxidant Status in Serum of Diabetic Patients with and without Retinopathy

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Abstract: The interaction of advanced glycation end products, with their cellular receptor (RAGE) and oxidative stress is implicated in the pathogenesis of diabetic vascular complications including diabetic retinopathy. RAGE has a circulating secretory receptor form, soluble RAGE(sRAGE) which neutralizes the action of advanced glycation end products and exerts a protective role against the development of cardiovascular disease. The objective of this study was to investigate whether serum sRAGE levels were associated with glycemic control and total antioxidant status (TAO) in serum of patients with diabetic retinopathy. ELISA (enzyme linked immunosorbent assay) was used to determine the serum levels of sRAGE, while total antioxidant status was determined by a colorimetric method. This study included 45 diabetic patients (15 diabetic patients without retinopathy, 15 with non proliferative diabetic retinopathy (NPDR) and 15 with proliferative diabetic retinopathy (PDR) compared to 15 non-diabetic control subjects. The serum levels of sRAGE were significantly higher in all diabetic groups compared to controls. Also sRAGE serum levels were significantly higher in PDR patients compared to diabetic patients without retinopathy and NPDR. There was a significant correlation between serum sRAGE and HbA_{1c} % levels in diabetic patients. Total antioxidant status was decreased in serum of patients with diabetes compared to controls and in patients with PDR compared to diabetics without retinopathy. Furthermore, sRAGE levels were inversely correlated with the total antioxidant status in diabetic patients. **Conclusion:** These findings suggest that plasma level of sRAGE is up-regulated in chronic hyperglycemia. Endogenous sRAGE level may be elevated in diabetes as a counter-system against endothelial cells damage and could reflect enhanced RAGE expression in the diabetic vasculature. Also the decreased antioxidant levels could enhance the deleterious effects of advanced glycation end product on diabetic retinopathy through the overproduction of sRAGE.

Key words:

INTRODUCTION

Diabetic retinopathy is a common and potentially devastating microvascular complication in diabetes and is a leading cause of acquired blindness among the people of occupational age. Chronic hyperglycemia is a major initiator of diabetic retinopathy (Bailey C.C. *et al.* 1999). Among various biochemical pathways implicated in the pathogenesis of diabetic retinopathy, the process of formation and accumulation of advanced glycation end products (AGEs) and their mode of action are most compatible with the hyperglycemic theory (Yamagishi S. *et al.* 2003).

Driven by hyperglycemia and oxidant stress, advanced glycation end products (AGEs), including *N*-(carboxymethyl) lysine-protein adducts (CML), are formed in a greatly accelerated degree in diabetes (Basta G *et al.* 2004). Accumulation of AGEs and the enhanced tissue expression of the main receptor for AGEs (RAGE) may contribute to vascular damage (Yonekura H. *et al.* 2005).

Two classes of RAGE ligands, AGEs and S100A12 proteins, appear to drive receptor-mediated cellular activation and, potentially, perturbation of a variety of homeostatic functions of the vasculature (Hofmann M.A *et al.* 1999). S100A12, also called EN-RAGE (extracellular newly identified receptor for AGE binding protein), is a proinflammatory cytokine expressed mainly by granulocytes and is involved in Ca⁺-dependent signal transduction events (Bierhaus, A., S *et al.* 2001). Recently it has been reported that human vascular cells express several RAGE variant proteins, including three novel human RAGE transcripts (Dell, Angelica E.C. *et al.* 1994), all encoding truncated soluble forms of RAGE (sRAGE), consisting of only the extracellular ligand

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binding domain and lacking the cytosolic and transmembrane domains. Due to these characteristics, the sRAGE represents a naturally occurring competitive inhibitor of the signaling pathways induced by the interaction of AGEs with its cellular receptor; by functioning as a decoy, sRAGE may contribute to the removal/neutralization of circulating RAGE-ligands (Yonekura H.Y. *et al.* 2003). There is growing evidence that AGEs and their receptor (RAGE) are implicated in the pathogenesis of diabetic vascular complications. Furthermore, AGEs-RAGE interaction-mediated oxidative stress generation plays an important role in diabetic retinopathy (Asi, H. & Perlman, I. *et al.* 1992).

The aim of this study was to determine sRAGE levels and total antioxidant status in patients with and without diabetic retinopathy. Also we examined whether serum levels of sRAGE correlated with glycemic control and total antioxidant status in those patients.

Subjects and Methods:

The studied subjects were 45 diabetic patients with type 2 diabetes, their age ranged from 56-65 years. They were classified into 3 groups:

- Group1: 15 diabetic patients without retinopathy.
- Group2: 15 diabetic patients with non proliferative diabetic retinopathy (NPDR).
- Group3: 15 diabetic patients with proliferative diabetic retinopathy (PDR). They were compared to 15 normal non diabetic control subjects.
- Full ophthalmological examination and medical history was taken for each subject including:
- Intraocular pressure measurement by applanation tonometry.
- Slit lamp examination to determine anterior chamber depth and presence of iris neovascularization . Indirect ophthalmoscopy and biomicroscopy to evaluate the grade of vitreous proliferation and determine the presence and nature of macular oedema.
- The pre-operative findings were recorded and the clinical disease severity was classified, according to the presence and extent of active fibrovascular tissue, vitreous hemorrhage, tractional retinal detachment (with or without retinal tears). Recent vitreous hemorrhage was excluded to avoid affecting the vitreous samples.
- Fundus fluorescein angiography was done using a 50 field fundus camera, 5 ml of 10% sodium fluorescein was injected in the anti cubital vein and photography was carried out.
- Angiography was performed in patients with diabetic retinopathy to differentiate between non proliferative and proliferative retinopathies.
- Electroretinogram ERG, Flash ERG used RETI system, program ISCEV-ERG GF parameter of background and stimuli of the steps correspond with minimum standard ISCEV.
- Flash ERG was subnormal in amplitude and oscillatory potentials was found in all PDR patients.
- Oscillatory potentials (OPs) are very sensitive to ischemia in localized retinal areas. Therefore, in situations where the a- and b-waves remain normal in waveform and amplitude, OP recordings can indicate mild retinal ischemia in the inner retina.(Asi, H. & Perlman, I., *et al.* 1992).

Exclusion criteria:

Only those patients who did not have any hepatic or GIT and renal diseases were selected. Any patient with serum creatinine < 1.2 mg/dl or urinary albumin excretion < 150 mg/24hrs was not included in this study. Also any patient with local eye disease such as, cataract, glaucoma or uveitis was excluded from the study.

Samples of venous blood was collected, part on EDTA to estimate HbA_{1c}, and the other part centrifuged and serum was separated and stored at - 80°C until assayed.

Laboratory assays of:

sRAGE:

Concentrations of sRAGE(pg/ml) in serum samples were measured by Quantikine enzyme-linked immunosorbent assay (ELISA). This test employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for RAGE (extracellular domain) has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells and any RAGE present is bound by immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for RAGE is added to the wells. After wash, a substrate solution is added to the wells and color develops in proportion to the amount of RAGE present (Huttunen H.J., *et al.* 2000).

Total antioxidant status:

Total antioxidants concentrations mmol/L in serum samples were measured by a new calorimetric assay based upon microplate technology. Antioxidant activity is detected by the reduction of Cu²⁺ to Cu⁺ followed

by complex formation between Cu⁺ and bathocuprin. This complex is stable and shows absorption in the range 480-490nm (Santini S.A., *et al.*, 1997).

Measuring HbA_{1c}:

With a cation exchange chromatography method assessed recent glycaemic control. The procedure is a micro-chromatographic methodology for the quantitation of glycosylated hemoglobin (non diabetic reference 5.5%- 7.7%) Glyco-HbQuick Column procedure (Helana) (Maquart F.X. *et al.* 1980).

Statistical analysis:

Data was expressed as mean SD. The four groups were compared using the Anova; single factor test. The degree of association between the variables was assessed using Pearson's correlation coefficient (r), where values of p > 0.05 were considered significant.

Results:

Clinical characteristics of the patients are presented in table 1. The results of this study are illustrated in table 2 and figures 1-4.

The mean serum levels of sRAGE were significantly elevated in all diabetic groups compared to controls. Also serum levels of sRAGE in PDR patients (group 3) were significantly elevated compared to NPDR (group 2) and diabetics without retinopathy (p>0.001).

The mean serum levels of TAO were significantly decreased in all diabetic groups compared to controls. Also serum levels of TAO in PDR patients (group 3) were significantly decreased compared to diabetics without retinopathy (p<0.001). While no significant difference between PDR (group 3) and NPDR (group 2) in TAO serum levels (p<0.05).

Serum levels of sRAGE were significantly inversely correlated with serum levels of TAO (r= -0.51, p>0.05) in diabetic patients. Also, Serum levels of sRAGE were significantly inversely correlated with levels of HbA_{1c} % (r= -0.54, p>0.01) in diabetic patients.

Table 1: Characteristics of diabetic patients and controls included in this study

	Controls	Group 1	Group 2	Group 3
Number (n)	15	15	15	15
Sex (M/F)	5/10	8/7	7/8	9/6
Mean age ± SD	55.7±7.6	63.6± 9.2	63± 8.8	64.1 ± 8.3
Duration of diabetes	-	12.6 ± 8.4	12.6 ± 8.4	12.6 ± 8.4
Serum Glucose (mg/dl)	99.9± 3.4	180±5.5	190±6.1	227±10.2

Table 2: Comparison of the different studied parameters among all diabetic groups.

	Controls	Group 1	Group 2	Group 3	p value
Number (n)	15	15	15	15	
HbA _{1c} %	6.9±0.8 a	9.4±1.2 b	9.7±1.1 b	10.7±1.5 b	p<0.001
sRAGE (pg/ml)	137±14.9 a	382±90.8 b	527±116 c	710±130 d	p<0.001
TAO (mmol/l)	6.6±1.8 a	2.9±0.9 b	1.8±0.58 c	1.68±0.6 c	p<0.001

p> 0.05 is statistically significant. Groups with different letters have a statistically significant difference.

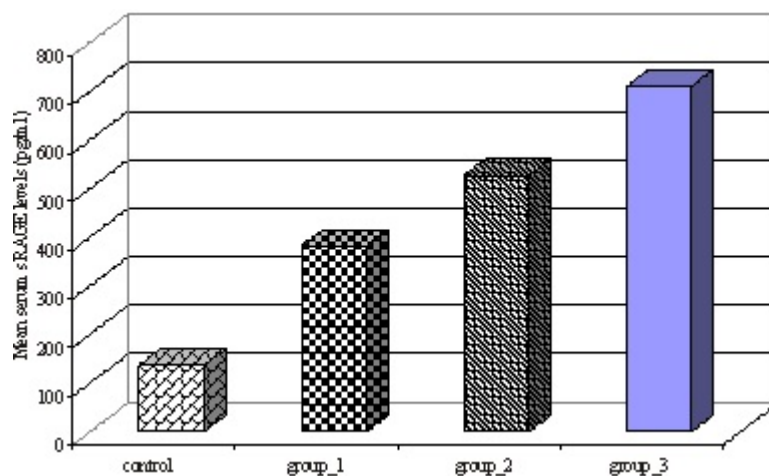


Fig. 1: Comparison of mean serum sRAGE among the different studied groups.

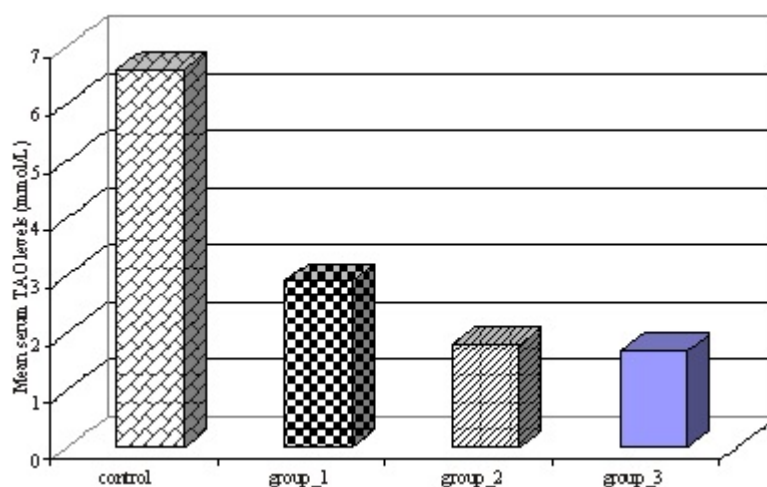


Fig 2: Comparison of mean serum total antioxidant status among the different studied groups.

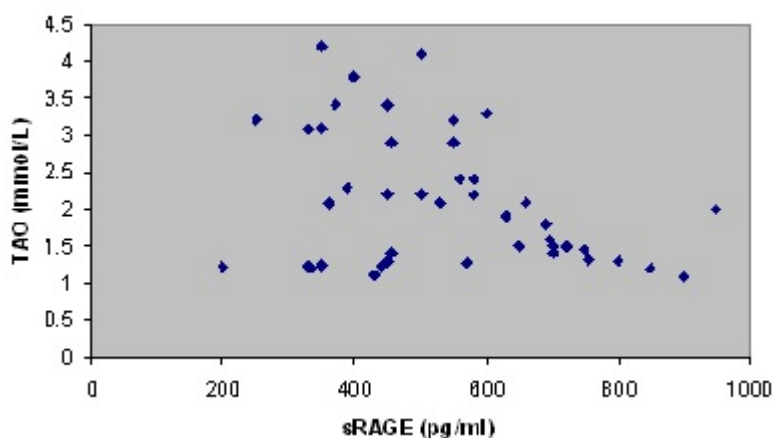


Fig 3: Correlation between serum sRAGE and total antioxidant status in diabetic patients. ($r=-0.51$, $p> 0.05$)

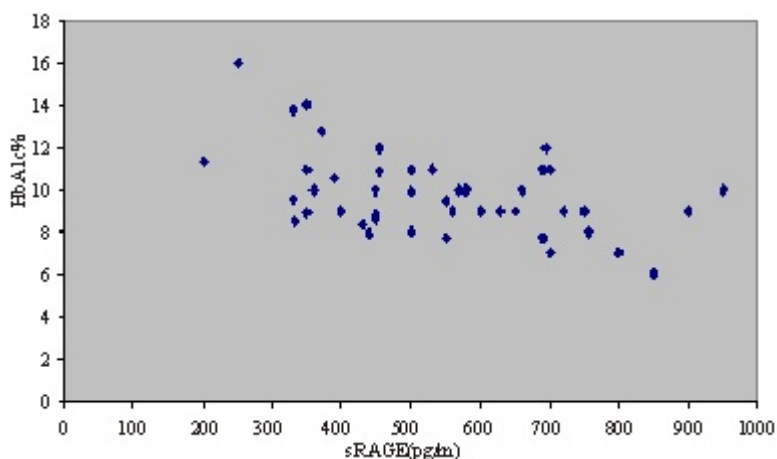


Fig. 4: Correlation between serum sRAGE and HbA_{1c}% in diabetic patients. ($r= -0.58$, $p> 0.01$)

Discussion:

RAGE has a circulating secretory receptor form, soluble RAGE (sRAGE), which by neutralizing the action of advanced glycation end products, might exert a protective role against the development of diabetic vascular

disease (Schmidt, A.M., D. Stern *et al.* 2000). A growing body of evidence suggests that RAGE is a signal transducing receptor for AGEs and that engagement of RAGE elicits vascular inflammation, thus being involved in accelerated atherosclerosis in diabetes (Schmidt, A.M. *et al.*, 1999). Moreover, RAGE expression in the vasculature is enhanced in diabetes, and sRAGE could be generated from the cleavage of cell surface RAGE in endothelial cells (ECs) (Yamagishi, S., T. Imaizumi *et al.* 2005).

The results of this study demonstrated that sRAGE serum levels were significantly elevated in all diabetic groups. Also sRAGE serum levels were significantly higher in diabetic patients with PDR compared to diabetic patients without retinopathy. Previous reports (Katakami, N., *et al.* 2005) (Koyama, H., *et al.* 2005). have reported that AGEs are positive regulators of cell expression of RAGE, and serum sRAGE levels are positively associated with circulating AGE levels in humans. Also several reports (Koyama, H., *et al.* 2005) (Pachydaki, S.I, *et al.* 2006) showed that RAGE is up-regulated in atherosclerotic plaques in diabetes. Others showed that the vitreous levels of sRAGE are increased in proliferative retinal diseases, reflecting enhanced RAGE expression in epiretinal membranes of the eyes in retinopathy (Cipollone, F., *et al.* 2003). These observations suggest that circulating endogenous sRAGE could reflect tissue RAGE expression and may increase as a counter-system against ECs injury in diabetic patients.

On the other hand, Basta (Katakami, N., *et al.* 2005). reported that plasma levels of sRAGE were decreased in diabetic patients. They postulated that hyperglycemia inhibits sRAGE production directly or via increased cytokines and/or hyperglycemia-induced AGEs. Alternatively, lower sRAGE levels could be due to an increased clearance of AGE ligand/sRAGE complexes (Koyama, H., *et al.* 2005).

This study showed that HbA1c is a significant independent factor inversely associated with serum sRAGE levels in diabetic subjects. This agreed with the findings of Basta (Basta G., *et al.* 2006). who reported that sRAGE was inversely associated with HbA1c. Recently another study has described HbA1c as major factor determining serum sRAGE but only in non diabetic subjects (Koyama, H., *et al.* 2005). Other previous observation showed that serum sRAGE levels were positively associated with circulating AGE levels in non diabetic subjects because HbA1c is one of the early glycation products (Yonekura, H., *et al.* 2003). However, these studies confirm that sRAGE levels are associated with glycemic control.

In this study, it was found that total antioxidant status was decreased in serum of diabetic patients with and without retinopathy. Also sRAGE serum levels were inversely correlated with the total antioxidant status in patients with diabetic retinopathy. The formation and action of AGE not only induces oxidative stress generation, but also inactivate a superoxide scavenging enzyme, Cu-Zn superoxide dismutase, both of which could lead to impairment of antioxidant defence systems (Yamagishi S., *et al.* 2008). AGE may also contribute to the decreased total antioxidant status in diabetic retinopathy (Yamagishi S., *et al.* 2003).

Oxidative stress also has an effect in diabetic retinopathy: it regulates vascular inflammation, gene expression of growth factors and cytokines (Kowluru R.A., A. Kennedy., *et al.* 2001). Therefore, it is conceivable that the decreased antioxidant levels could further enhance the deleterious effects of AGE on diabetic retinopathy through the overproduction of sRAGE. Moreover, the formation, accumulation, and action of AGE are enhanced under oxidative stress conditions (Yokoi M., *et al.* 2005).

These observations suggest that AGE and oxidative stress generation are correlated to form a positive feedback loop, thus having an important role in the development and progression of diabetic retinopathy. So, down-regulation of RAGE expression or blockade of the RAGE downstream signaling may be a promising target for therapeutic intervention in diabetic vascular complication (Yamagishi S., *et al.* 2008).

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