

Could Angiotensin II, Soluble Advanced Glycation End Product Receptors (sRAGE) Levels Implicate the Progression of Proliferative Diabetic retinopathy

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Abstract: Various hyperglycemia induced metabolic derangements; include increased formation of advanced glycation end products (AGEs), enhanced production of reactive oxygen species (ROS) and renin-angiotensin system, implicate in pathogenesis of diabetic vascular complications, including diabetic retinopathy. AGEs elicit their effects through interaction with their receptor (RAGE). This study tries to elucidate the implication of vitreous levels of angiotensin II, soluble RAGE and total antioxidant status in progression of proliferative diabetic retinopathy. This study, was conducted on 43 diabetic patients with and without proliferative retinopathy who were subjected to vitrectomy in the Research Institute of Ophthalmology. Eight age matched controls were included in this study. Plasma and vitreal angiotensin II were measured by ELISA Kits. Also serum and vitreal sRAGE were estimated by ELISA Kits. Serum and vitreal total antioxidant status levels were detected colorimetrically. Significantly elevated vitreal angiotensin II was detected in diabetic patients compared to controls. Meanwhile, significant reduction of both vitreal sRAGE and total antioxidant status in all diabetics compared to controls. All vitreal results were more evident in active PDR cases, than non proliferative patients. This study pointed out to interrelation of various parameters in the molecular pathology for progression of retinopathy.

Key words: sRAGE, Angiotensin II, oxidative stress, diabetic retinopathy.

INTRODUCTION

Diabetic retinopathy (DR) is one of the miserable microvascular complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (Yamagish and Imaizumi, 2005). Various hyperglycemia induced metabolic and hemodynamic derangements, including increased formation of advanced glycation end products (AGEs), enhanced products of reactive oxygen species (ROS), activation of protein Kinase C (PKC) and the renin - angiotensin system (RAS), contribute to the characteristic histopathological changes observed in diabetic vascular complications (Yamagishi and Imaizumi, 2005).

Angiogenesis is the major feature in the pathogenesis of proliferative diabetic retinopathy (PDR) (Battagay, 1995). Retinal neovascularization has a catastrophic effect on vision by causing vitreous hemorrhage, retinal detachment with formation of fibrovascular membrane and eventual blindness.

There is circumstantial evidence that vasoactive hormone angiotensin II was involved in stimulation of the growth of retinal blood vessels (Danser *et al.*, 1994). Angiotensin II has a proliferative effect to regulate the growth of smooth muscle cells and to stimulate the induction of various growth factors (Delafontaine & Lou, 1993).

Various studies have suggested that abnormalities of RAS may also play part in the progression of diabetic retinopathy (Danser *et al.*, 1994 & Wagner *et al.*, 1996). The hypothesis that an ocular RAS is involved in development of PDR is supported by evidence that all components of RAS are present in retina and that angiotensin II the effector molecule of this system has angiogenic activity (Lonchampt *et al.*, 2001).

The formation and accumulation of advanced glycation end products (AGEs) is one of several pathogenic mechanisms that contribute to diabetic microvasculopathy (Moore *et al.*, 2003). AGEs can form on the amino groups of proteins lipid and DNA through several complex pathways including non - enzymatic glycation by glucose and reaction with metabolic intermediates and reactive dicarbonyl intermediates. These reactions lead to formation of complex cross-links Metal catalyzed oxidative reactions enhancing irreversible formation of

AGEs that accumulate at increased rate in diabetes (Moore *et al.*, 2003).

AGE engagement of cell surface receptor for AGE (RAGE) results in cellular signaling, including activation of nuclear factor $\kappa\beta$, increases expression of cytokines and adhesion molecules and induction of oxidative stress (Schmidt *et al.*, 2000). Similarly, the accumulation of AGEs has been proposed to have an important role in development of diabetic retinopathy (Kaji *et al.*, 2007).

The biological effect of AGEs is in part mediated by the specific cell surface receptor. Several AGE receptors are known such as RAGE, galectin-3, CD 36 and macrophage scavenger receptor (Kaji *et al.*, 2007). Soluble RAGE (sRAGE), the extracellular two-third of the receptor, bind up AGEs and interfere with their ability to bind and activate cellular RAGE (Park *et al.*, 1998), and lack cytosolic and transmembrane domain. Endogenous secretory RAGE has been identified as a novel splice variant that direct the synthesis of RAGE proteins carrying all of the extracellular domains but devoid of the transmembrane and intracytoplasmic domains. Endogenous secretory RAGE was found to be released outside the cell and binds AGE ligands and to be capable of neutralizing AGE actions on endothelial cell culture (Koyama *et al.*, 2005).

The sRAGE represents a naturally occurring competitive inhibitor of signaling pathway induced by the interaction of AGEs with its cellular receptor. The sRAGE may contribute to the removal /neutralization of circulating RAGE ligands (Yonekura *et al.*, 2003). The cross talk between the AGEs – RAGE and renin-angiotensin system has been proposed in the pathogenesis of PDR (Yamagishi *et al.*, 2005). Furthermore AGEs-RAGE interaction mediate oxidative stress generation has also been reported to have a role in diabetic retinopathy. However, the interrelation between vitreous levels of angiotensin II, sRAGE and total antioxidant status in diabetic with retinopathy remains to be elucidated.

MATERIAL AND METHODS

This study was conducted on 43 patients performing pars plana vitrectomy in Research Institute of Ophthalmology. Eight age matched controls were included in this study. They were performing vitrectomy because of macular hole or giant tear as long as there was no vitreoretinal proliferation. Vitrectomy was indicated in these cases because of the sites and/or the state of the break. Patients and controls are age matched (ranged 47-65 years), and with duration of diabetes 10 years. All diabetics are controlled by oral hypoglycemic drugs. Full ophthalmological examination and medical history was taken for each subject including:

- Intraocular pressure measurement with Goldman applanation tonometry.
- Slit lamp examination.
- Fundus examination by a binocular indirect ophthalmoscope and a slit lamp biomicroscopic examination with a 90 D lens to evaluate the grade of vitreous proliferation and detect the presence and nature of macular oedema.
- Fundus fluorescein angiography was done using topocon fundus camera TRC .50 EX on image –net, 5 ml of 10% sodium fluorescein was injected in antecubital vein and photography was carried out.
- Preoperative findings were clarified and clinical severity was assayed; according to presence and extent of fibrovascular tissues, vitreous hemorrhage, tractional retinal detachment.
- Recent vitreous hemorrhage was excluded to avoid affecting the vitreous samples.
- Undiluted vitreous fluid samples were harvested at the start of vitrectomy after informed consent was obtained from each subject following an explanation of the purpose and potential adverse effects of the procedure. Vitrectomy was performed on the 36 patients with PDR, seven diabetic patients without retinopathy and eight non diabetics with ocular diseases as controls.
- The seven diabetic patients without retinopathy included four with macular hole and three with epiretinal membrane.
- Exclusion criteria for this study were:
 1. Previous ocular surgery.
 2. History of ocular inflammation.
 3. Treatment with angiotensin converting enzyme inhibitor or an angiotensin II receptor antagonist.
- Angiography was performed to differentiate proliferative and non proliferative retinopathy.
- PDR was classified as active (26) eyes if there were new preretinal capillaries and as quiescent (10) eyes if the vasoproliferation only consisted of large vessels within the membrane at the time of surgery (Moravski *et al.*, 2000).
- Pars plana vitrectomy was done by a standard technique using three pars plana sclerotomy incisions. The undiluted samples of vitreous fluid (0.2 – 0.5 ml) were aspirated under standardized conditions directly

- from the mid-vitreous at the beginning of surgery and were immediately transferred to sterile tubes.
- Vitreous samples were centrifuged at 10.000 rpm for 5 minutes to remove contaminating cells. The supernatants were frozen at -80°C until assay.
 - Blood samples were collected in two tubes part on EDTA, the other part centrifuged and serum was separated and stored at -80°C until assay.
 - Routine laboratory investigations were performed including estimation of fasting blood glucose, creatinine, and complete lipid profile using commercial available kits.
 - Serum fructosamine was estimated by commercial kits provided by QCA (Quimica clinica Aplicada) TARRAGCNA – Espania.
 - The concentration of angiotensin II in plasma ng/ml in vitreous and plasma samples were measured by Enzyme immune sorbent assay Kit (ELISA), which is designed to detect a specific peptide and related peptides based on principle of competitive enzyme immunoassay by kits of DRG International Inc, USA.
 - The blood samples on the EDTA tube were centrifuged at 16.000 rpm at 4°C and collected plasma, plasma which was kept at -70°C till time of assay.
 - The quantitative determination of extracellular domain of human receptor for advanced glycation end product (RAGE) concentration (pg/ml) in vitreous and serum by sandwich enzyme linked immunosorbent assay (ELISA) kits Quankikine R&D systems, Ins. Minneapolis, USA. the minimum detectable levels of sRAGE ranged from 1.23 – 16.14 pg/ml.
 - Estimation of total antioxidant levels m mol/L in vitreous and serum by colorimetric assay kits provided by Antoxit, Fujirebio Diagnostic AB. This colorimetric 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 show absorption in the range 480 – 490nm (Ceriello, 1997).

Statistical Analysis:

Data was expressed as mean ± SD. The four groups were compared using the ANOVA; single factor test. The degree of association between variables was assessed using Pearson’s correlation coefficient (r). P values<0.05 were considered significant.

RESULTS AND DISCUSSION

The results of this study were illustrated in Table 1, 2.

Table 1: The mean levels ± SD of angiotensin II, sRAGE and total antioxidant status in vitreous of all studied groups:

	Control	Diabetic without retinopathy	Quiescent PDR	Active PDR	P value
Number	(8)	(7)	(10)	(26)	
Angiotensin II (ng/mL)	8.2 ±5.2	12.3±5.4	20.2±6.1	31.4±6.2	< 0.001
sRAGE (pg/mL)	40.2±7.9	28.4±6.4	20.7±5.9	13.2±5.7	< 0.01
Total antioxidant status (mmol/L)	8.6±2.7	4.6±1.2	2.3±0.9	1.87±0.8	< 0.001

P<0.05 was significant.

P< 0.001 highly significant.

Table 2: The mean levels ± SD of plasma angiotensin II, serum levels of sRAGE, total antioxidant status and fructosamine in all studied groups.

	Control	Diabetic without retinopathy	Quiescent PDR	Active PDR	P value
Number	(8)	(7)	(10)	(26)	
Angiotensin II (ng/mL)	7.9 ±2.5	8.7±3.1	8.7±2.9	9.1±2.8	NS
sRAGE (pg/mL)	71.8±20.1	39. 2±4.6	29.9±4.5	20.7±5.2	< 0.001
Total antioxidant status (mmol/L)	16.7±1.6	7.1±1.9	3.2±0.9	1.8±0.7	< 0.001
Fructosamine (mmol/L)	1.77±0.2	4.1±0.3	4.3±0.4	4.4±0.5	< 0.01

NS: not significant

P<0.001 highly significant

- The mean vitreous levels of angiotensin II were significantly elevated in all diabetic groups compared to controls and this elevation was more pronounced in PDR groups compared to diabetics without retinopathy P<0.001 Table(1). The active stage of PDR show higher vitreous angiotensin II levels compared to quiescent PDR p<0.05.
- The vitreous sRAGE and total antioxidant status levels were significantly decreased in all diabetic groups

compared to controls and this decrease was more evident in PDR groups compared to diabetics without retinopathy $P < 0.001$ Table (1). Meanwhile, the mean vitreous sRAGE levels were significantly decreased in active stage of PDR compared to quiescent PDR patients $p < 0.05$. On the other hand, the total antioxidant status levels in vitreous were not significantly different in active or quiescent PDR.

- The mean plasma levels of angiotensin II were not significantly different in all studied groups Table (1).
- The mean serum levels of both sRAGE and total antioxidant status levels were decreased in all diabetic groups. This decrease was more aggravated in PDR patients than diabetics without retinopathy $p < 0.001$ Table (2). Meanwhile, both serum sRAGE and total antioxidant status revealed significant decrease in active PDR patients compared to patients in quiescent stage of PDR $p < 0.05$.
- A significantly elevated mean serum fructosamine level in all diabetic groups compared to controls $P < 0.001$ Table (2). No significant difference was detected in fructosamine levels among all diabetic groups.
- The mean angiotensin II levels in vitreous were significantly elevated compared to its plasma levels $P < 0.001$. Plasma angiotensin II showed a significant correlation with its vitreous levels $r = 0.59$ $p < 0.001$.
- Furthermore angiotensin II was inversely correlated with sRAGE and total antioxidant status levels in vitreous fluid $r = -0.48$, $p < 0.01$, $r = -0.39$, $p < 0.05$ respectively.
- Significant positive correlation between vitreal sRAGE and total antioxidants status $r = 0.46$, $p < 0.01$.
- No significant correlation was elicited between vitreal angiotensin II and serum sRAGE $r = 0.21$, $p > 0.05$ or serum fructosamine $r = 0.22$, $p > 0.05$
- Mean while, the mean serum total antioxidant vitreal levels were not correlated to serum sRAGE or serum fructosamine, $r = 0.22$, $p > 0.05$, 0.23 , $p > 0.05$ respectively.

Discussion:

The present study showed that angiotensin II levels were increased in vitreous fluid of diabetic patients especially PDR patients and were correlated with the severity of diabetic retinopathy, also, angiotensin II vitreal level was elevated in active stage of PDR, these results agree with that of Funatsu *et al.*, (2002, 2004). Angiotensin II has been shown to promote the growth of capillary vessels in the chorio-allantoic membrane and to stimulate new vessel formation in the rabbit cornea (Le Noble *et al.*, 1991). Angiotensin II not only had a growth promoting effect but also stimulate the induction of many cytokines and growth factors thus, angiotensin II may affect neovascularization in combination with other cytokines (Funatsu *et al.*, 2002). Receptors for Angiotensin II are present on endothelial cells, and angiotensin II act to stimulate endothelial cell growth and upregulate vascular endothelial growth factor (VEGF), VEGF mRNA expression. Moreover, angiotensin -II may potentiate VEGF induced angiogenic activity in the retina through increased expression of the VEGF receptor FLK-1 / KDR. The possibility that angiotensin II might influence VEGF elicited signal transduction or post-transcriptional regulation of KDR (Otani *et al.*, 1998). The capacity of VEGF to act as a potent angiogenic agent suggests that angiotensin II induce increased production of VEGF production which could have a key role in the occurrence of neovascularization in PDR.

In the present study, vitreal angiotensin II level was higher than the plasma levels. Furthermore both levels were significantly correlated. Breakdown of the blood retina barrier may facilitate diffusion of angiotensin II from the blood into vitreous fluid. Since the vitreous can be considered the repository for products originating from the retina, a high level of angiotensin II might will be explained by its production and secretion from the retina. Accordingly, angiotensin II may be produced locally in ocular tissues but little may leak into the ocular fluid under normal condition and only when the blood retinal barrier is disrupted, angiotensin II reach the vitreous cavity in high concentration (Danser *et al.*, 1994). It may be possible that angiotensin II in vitreous fluids derived from both production in ocular tissues (such as retinal pigment epithelium – choroid complex) and via disruption of blood retinal barrier (Funatsu *et al.*, 2002).

The elevated vitreal angiotensin levels in patients with active PDR than quiescent PDR, suggests that the measurement of vitreous level of angiotensin II may be useful to predict neovascularization activity. Moreover it could be potential predictor of the postoperative progression of PDR.

In this study, significant decrease of vitreal and serum sRAGE levels were detected in all diabetics compared to controls, meanwhile, the reduction was more evident in active PDR patients compared to quiescent stage. To the best of our knowledge few articles estimating the vitreal sRAGE. To the contrary of our results Pachydaki *et al.*, (2006) showed that vitreous sRAGE is increased in proliferative retinal diseases reflecting enhanced RAGE expression in epiretinal membranes of eye in retinopathy. While, Barile *et al.*, (2005) detected that the accumulation of advanced glycation end product within the vitreous cavity in diabetic patients, result in characteristic structural alteration referred to as diabetic vitreopathy. The biological effects of AGEs are mediated by specific cell receptor RAGE.

The interaction of AGEs and RAGE lead to the increased oxidative stress and activation of nuclear factor $\kappa\beta$ (NF $\kappa\beta$). The activation of NF $\kappa\beta$ is known to induce the expression of various cytokines included vascular endothelial growth factors, interleukin I, and various adhesion molecules including ICAM, VCAM and CD 18 (Kaji *et al.*, 2007).

The blockade of AGEs – RAGE axis by administration of soluble form of RAGE (sRAGE) ameliorates neuronal dysfunction and reduces the development of cellular capillaries and pericytes ghosts in hyperglycemic, hyperlipidemic mice (Barile *et al.*, 2005). Furthermore, Kaji *et al.*, (2007), have shown that attenuation of the RAGE axis with sRAGE inhibits retinal leukostasis and blood retinal barrier breakdown in RAGE transgenic mice which were accompanied by decreased expression of VEGF and ICAM in the retina. These observations suggest that exogenously administered sRAGE may capture and eliminate circulating AGEs, thus protecting the AGE – elicited tissue damage by acting as a decoy. Thus antagonism of RAGE axis by sRAGE is novel therapeutic target for diabetic vascular complication (Yamagishi *et al.*, 2008).

Our results of decrease sRAGE in serum of diabetic retinopathy patients especially active PDR, are accepted by (Koyama *et al.*, 2005) and (Falcone *et al.*, 2005). Koyama *et al.*, (2005) results revealed that plasma endogenous secretory RAGE is not. Only involved in pathophysiology of diabetic microangiopathy, but also act as potential factor protecting against metabolic syndrome and atherosclerosis. On the other hand, Abdel Hamid *et al.*, (2008) demonstrated significantly higher sRAGE in serum of PDR patients compared to those without retinopathy. Cipollone *et al.*, (2003) have reported that RAGE is up-regulated in atherosclerotic plaques in diabetes.

These observations suggested that circulating endogenous sRAGE could reflect tissue RAGE expression and may increase counter system against endothelial injury in diabetics. Antagonism of RAGE also, reduces the progression of vascular lesion of diabetic retinopathy in experimental animals. The perturbation in retinal vascular endothelial cells also suggests that antagonism of circulating serum AGEs with sRAGE may reduce these perturbations and the resultant anatomic disease (Barile *et al.*, 2005).

This study detected significant decrease in vitreal total antioxidant status in all diabetics, especially active PDR compared to quiescent cases. These results were in agreement with that of Yokoi *et al.*, (2005). They suggested that vitreous levels of AGEs not only includes oxidative stress generation, but also inactivate a superoxide scavenging enzyme, Cu-Zn superoxide dismutase, both of which could lead to impairment of antioxidant defense system, (Arai *et al.*, 1987). AGEs may also contribute to decreased total antioxidant status in diabetic retinopathy. However, although it is not known whether the decrease of total antioxidant status is a cause, consequence or epiphenomenon, it is conceivable that the decreased antioxidant levels could further potentiate the deleterious effect of AGEs on diabetic retinopathy, through the overproduction of VEGF, (Uokoi *et al.*, 2005). Also in this study, a decrease in serum total antioxidant status in all diabetics with and without retinopathy was found. These results go with that of Abdel Hamid *et al.*, (2008).

In this study, the inverse correlation of vitreal angiotensin II with sRAGE and total antioxidant status vitreal levels could be verified by the study of (Yamagishi *et al.*, 2008). They suggested the blockade of the renin-angiotensin system by olmesartan, an angiotensin II type I receptor blocker, may play a protective role against PDR by attenuating the deleterious effects of AGEs. They found out that selective angiotensin II receptor blocker (olmesartan) inhibits the AGEs include NF- $\kappa\beta$ activation by blocking oxidative stress generation in endothelial cells. Moreover, reactive oxygen species was involved in angiotensin II – induced RAGE gene expression in endothelial cells (Nakamura *et al.*, 2005). Since RAGE mediated NADPH oxidase driven reactive oxygen species generation was a key mediator of AGEs elicited angiogenesis in endothelial cells (Yamagishi *et al.*, 2008). NADPH Oxidase activity may be a molecular target formation for angiotensin II receptor blocker on AGEs – exposed endothelial cells. Moreover, the administration of sRAGE fully suppressed accelerated diabetic atherosclerosis, these data support the hypothesis that sRAGE may also prevent interaction of AGEs with other putative AGE – binding proteins (Park *et al.*, 1998).

This study detected elevated fructosamine levels in all diabetics compared to controls. Meanwhile no significant differences were detected in fructosamine levels between neither proliferative nor non-proliferative patients. These results were also suggested by (Tawadrous *et al.*, 1994).

The present study indicates that sRAGE may not only a new tool to describe the molecular mechanisms involved in accelerated diabetic vascular diseases, but also a prototypic structure for design of agent to prevent microvascular complications in diabetes. However, the exact molecular mechanism and the interrelation of angiotensin II, sRAGE and oxidative stress in PDR remain to be elucidated on wide range of patients.

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