The role of methylglyoxal and angiopoietin2 assay in pathogenesis of proliferative diabetic retinopathy

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ABSTRACT

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound, where it form advanced glycation end product. MGO increases the expression of Angiopoietin 2 (Ang2) in diabetic retina. Ang2 may potentiate vascular endothelial growth factor (VEGF) – induced angiogenesis. This study tries to clarify the complex relationship of MGO, Ang2, VEGF and hypoxia inducible factor 1α (HIF1α) in initiation and progression of diabetic retinopathy. This study conducted on sera collected from 16 diabetic patients (type2) with proliferative diabetic retinopathy (PDR), 20 diabetic patients with non-proliferative diabetic retinopathy NPDR, ten diabetic patients without retinopathy and 15 healthy subjects served as controls. Detection of serum MGO, Ang2, VEGF and HIF-1α by immune enzymatic ELISA technique. The mean serum MGO, Ang2 were elevated in both diabetics with PDR and NPDR patients compared to controls. While Ang2 elevated in diabetics without retinopathy not statistically significant higher compared to controls. Both serum VEGF and HIF-1α were significantly higher in diabetics without retinopathy compared to control. Their elevation was more evident in PDR groups compared to NPDR patients. This study clarifies an association between retinopathy and increased serum MGO in PDR patients. Given the apparent importance of Ang2 in modulating VEGF induced vascular permeability as well as angiogenesis. Thus Ang2 is clearly a therapeutic target of considerable interest of diabetic retinopathy (DR). However, future studies are required to elucidate the specific role of HIF-1α in pathogenesis of PDR in diabetics.

Key words: Proliferative diabetic retinopathy, Methylglyoxal, angiogenesis, Angiopoietins, Oxidative stress.

Introduction

Progressive microvascular complications is a main feature of diabetes and is associated with impairment of angiogenic response, (Bento et al, 2010). Although late stages of proliferative diabetic retinopathy (PDR) are associated with neovascularization, the disease originates with endothelial cell damage, extensive capillary regression and widespread retinal ischemia (Cai & Boulton. 2002 & Hammes et al, 2002).

Diabetic retinopathy is closely associated with long term hyperglycemia. Advanced glycation end-products (AGEs) can form from non-enzymatic glycation of proteins was claimed to play a major role in pathogenesis of diabetic retinopathy, (Cai & Boulton, 2002). Methylglyoxal (MGO) is a highly reactive dicarbonyl compound, it reacts with arginine, lysine and cysteine residues of proteins in a reversible manner, whereas, it form AGEs by future irreversible reaction. The main product formed when MGO reacts with arginine in protein is hydroimidazoleone. Increased level of MG – arginine adducts, have been found in lens of diabetic patients, and in retina of streptozotocin induced diabetic experimental rats (Sit et al, 2005).

Also, Piskorska et al, (1998) suggested that periodic hyperglycemia may contribute to the development of diabetic complications through Methylglyoxal-mediated changes in protein solubility and aggregation characteristics.

Methylglyoxal is a high reactive α oxoaldehyde formed as a byproduct of glycolysis and it productions is enhanced by exposure of cells to high glucose levels. Increased level of MGO has been demonstrated in serum of type 2 diabetics (Vlassaraet al. 1994).

Diabetes was the first disease in which evidence emerged for the increased formation of MGO in the cell and in serum. MGO has a toxic effect on insulin secretion from pancreatic beta cells and on modification of proteins and nucleic acid. Moreover, MGO is one of the major precursors of advanced glycation end products. (Kender et al, 2012). Recently, Kim et al, (2012) hypothesized that increased levels of MGO disrupt the tight junction protein known as occluding protein by matrix metalloproteinase (MMPs) leading to breakage of the blood retinal barrier.
MGO is detoxified via the glyoxalases enzyme system by reducing AGE adduct accumulation. Enhanced glycolytic metabolism in diabetics may overpower detoxification capacity and lead to AGE related pathology (Berner et al., 2012). Also, Kim et al., (2004) suggested that elevation of MGO levels observed in diabetes may cause apoptosis in bovine retinal pericytes through oxidative stress mechanism and suggested that nuclear activation of NF kappa B are involved in apoptotic process.

MGO as well as hydrogen intracellular PH can and augment the production of reactive oxygen species leading to apoptosis. These processes play a role in diabetic retinopathy (Kniep et al., 2006). Interestingly, MGO increases the expression of angiopoietins 2 in diabetic retinas (Yao et al., 2007).

Angiopoietins represent a family of inflammatory growth factor that bind to the receptor tyrosine kinase Tie, and are modulator of angiogenesis. (Rangasamy et al., 2012). Angiopoietin 2 (Ang2) may potentiate vascular endothelial growth factor VEGF – induced Angiogenesis and causes neovascularization. Ang2 was increased in retina during pathologic angiogenesis in experimental mouse model of ischemic retinopathy (Hackett et al., 2000). Angiopoietins and Tie2 (Tyrosine Kinase) receptor constitute a crucial role an angiogenesis (Takagi et al., 2003). Angiopoietin 1 counteracts the potential harmful actions of Ang2, (Loukovaara et al., 2004).

Feng et al (2008) detected that the function of Ang2 appears to be highly dependant on the presence of VEGF. Also, Yancopoulos et al., (2000) suggested that VEGF and possibly Ang2 plays an important role in the development of diabetic macular oedema. Also, it has been found that VEGF up-regulates Ang2 expression on endothelial cells, (Peters et al., 2007). They detected also an evidence of implication of VEGF and Ang2 in increased vascular permeability which is hallmark of diabetic retinopathy (Peters et al., 2007). Meanwhile, exposure of retinal pigment epithelial cell to MGO leads to destabilization of hypoxia inducible factor which is a key regulator of VEGF.

Thus, the molecular mechanisms that underlie endothelial dysfunction in diabetics are still enigma. This study tries to clarify the complex relationship of Ang2, MGO, VEGF and HIF1α in initiation and progression of diabetic retinopathy.

Subjects and methods

This study was conducted on sera collected from 16 diabetic patients (type 2 diabetes) with proliferative diabetic retinopathy, 20 diabetic patients with non proliferative diabetic retinopathy, 10 diabetics without retinopathy and 15 healthy subjects age matched (55-69 years) served as controls.

Written consent was obtained from each case in this study according to ethical committee instructions. The duration of diabetes ranged from 10-15 years (mean was 12.1± 1.6).

Full ophthalmological examination and medical history was taken for each subject examination including:

- Intraocular pressure measured by Goldman applanation tonometry.
- Slit lamp examination to determine anterior chamber depth and the presence of iris neovascularization.
- Indirect ophthalmoscopy and biomicroscopy to evaluate the grade of vitreous proliferation and determine the presence and nature of macular oedema.
- Retinopathy was diagnosed on the basis of fundoscopy to differentiate between NPDR & PDR.
- Fundus fluorescein angiography was done using Topcon fundus camera TRC 50 Ex on image net. 5 ml of 10% sodium fluorescein was injected in the antecubital vein and photography was carried out.
- Exclusion criteria: only those patients who did not have hepatic or renal diseases were selected.
- Any patient with serum creatinine >1.2 mg /dl or urinary albumin excretion > 150 mg / 24 hrs was not included in this study. Also any patients with local eye disease such as cataract, glaucoma or uveitis was excluded from the study.
- Routine laboratory investigations were preformed to all cases and controls (fasting and 2 hours blood glucose; liver function test; kidney function tests and lipid profiles were performed).
- Samples of venous blood were collected part on EDTA tube to estimate HbA1c and the other part of the whole blood on plain tubes was centrifuged and serum was separated and stored at -70°C until assayed.
- Measuring HbA1c: with cation exchange chromatography method assessed recent glycogenic control. The procedure is a micro chromatographic methodology for the quantitation of glycosylated hemoglobin (non diabetic reference 5.5% - 7.7%) by commercial Kit provided by Human, Wiesbaden Germany.
- Detection of serum MGO-adduct, Ang2, VEGF and HIF1α by immunoenzymatic ELISA technique provided by commercial kits of cell bio labs, Inc, USA; Abcam, UK and R&D systems, Abingdon, UK respectively.

Statistical Analysis:

Analysis of data was done via SPSS version 9 (Statistical Package Social Science). Different tests were applied. Mean and standard deviations were used for data description. ANOVA (analysis of variance) was done to compare mean ranks of different parameters for more than two groups.
P < 0.05 considered significant. Pearson's correlation was done to detect association correlation coefficient (r) where p<0.05 was considered significant.

Table 1: Serum levels of MGO, Ang2, VEGF & HIF1α in all studied groups (mean ± SD).

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>MGO µg/ml</th>
<th>Ang2 ng/ml</th>
<th>VEGF pg/ml</th>
<th>HIF1α ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (15)</td>
<td>58±13</td>
<td>2.72±1.7</td>
<td>37.9±7.1</td>
<td>23±10.7</td>
<td>0.28±0.07</td>
</tr>
<tr>
<td>Diabetics without retinopathy (10)</td>
<td>59±12</td>
<td>6.9±1.5</td>
<td>42.7±6.1</td>
<td>69.2±12.9</td>
<td>0.78±0.1</td>
</tr>
<tr>
<td>NPDR (20)</td>
<td>60±10</td>
<td>18.1±3.2</td>
<td>89.1±8.7</td>
<td>139.7±21.1</td>
<td>0.13±0.09</td>
</tr>
<tr>
<td>PDR (16)</td>
<td>61±8</td>
<td>23.1±3.7</td>
<td>97.9±9.1</td>
<td>269±37.7</td>
<td>0.30±0.06</td>
</tr>
</tbody>
</table>

p<0.05 ns (not significant)
p<0.05 significant
p< 0.01, P<0.001 is highly significant.

Results:

Table (1) illustrates that:

- The mean serum level of MGO was significantly higher in diabetics without retinopathy compared to controls. This elevation was more evident in PDR patients compared to NPDR patients p<0.01.
- The mean level of Ang2 was significantly higher in diabetics in both PDR and NPDR groups p<0.01.
- The mean VEGF level was significantly elevated in diabetics without retinopathy compared to controls. This elevation was more obvious in PDR patients compared to NPDR groups. p<0.001.
- The mean HIF1α level was significantly higher in diabetics without retinopathy, the elevation was more pronounced in PDR group compared to NPDR patients p<0.001.
- There were significant positive correlations between Ang2 and VEGF in NPDR and PDR patients were found r= 0.63, 0.61, p<0.001 respectively.
- Also there was significant positive correlations between VEGF & HIF1α in NPDR and PDR patients were detected r=0.57 p<0.05, r=0.61 p<0.01 respectively.
- There was significant positive correlations between MGO & Ang2 and VEGF in both PDR and NPDR patients were detected r=0.61 p < 0.01, r=0.64 P<0.01 respectively.

Discussion:

Diabetic retinopathy is a debilitating complication of diabetes mellitus. The retina is a vital tissue of the eye with higher metabolic role than any other tissue in the body. The retina capillary wall is lined by endothelial cells and pericytes which maintain a blood retinal barrier. Loss of pericytes and formation of cellular capillaries are early morphological changes in diabetic retina (Cai & Boulton 2002 & Hammes et al, 2002). Later stage of diabetic retinopathy disturbed homeostasis to retinal cells occur leading to neovascularization. Hypoxia due to retinal ischemia trigger angiogenic growth factors VEGF, Ang2 leading to neovascularization and proliferative retinopathy (Bento et al, 2010). Also, there is increasing evidence that accumulation of Methylglyoxal (MGO) and formation of advanced glycation end products (AGEs) may contribute to impair Angiogenic response (Stitt et al, 2005).

In this study, a highly statistical significant increase in serum level of MGO in diabetics compared to controls. This elevation is more evident in proliferative diabetic compared to NPDR patients. These results agree with that of (Kim et al, 2004, Bento et al, 2010. Kim et al, 2010). MGO increases oxidative stress by inactivating glutathione reductase, thus reactive oxygen species increased in dose dependant manner through increased activity of nuclear factor –κB (NF-κB). Moreover, Kim et al, 2010 detected elevated levels of MGO in early diabetic changes in the retina, loss of retinal pericytes, because pericytes is insulin independent cell for glucose transport, thereby allowing entrance of glucose into even in mild hyperglycemic states.

Also, intracellular proteins including antioxidant enzymes, transcription factors and mitochondrial protein are target of dicarbonyl modification and this can modify their functional properties and thus compromise a cellular physiology. Likewise, modification of extra cellular protein by MGO can impair cell adhesion and generate ligand that can potentially bind to surface AGE receptors activate pro-inflammatory signaling pathways. (Milne & Brownstein, 2011).

In this study, highly significant increase in serum levels of Ang2 in both PDR and NPDR patients compared to controls & diabetics without fundus changes. To the contrary, Loukovaara et al (2004) found out that Ang2 levels were not related to the progression of retinopathy.
However, Angiopoietins are family of inflammatory growth factors that bind to receptor tyrosine Kinase2 Tie2, and important modulator of angiogenesis; vitreous levels of Ang2 are significantly elevated in patients with macular oedema, indicating the role of Ang2 in alternation of the blood retinal barrier (Patel et al, 2008). Also, increased expression of Ang2 mRNA in retina of diabetic animal were detected (Rangasamy et al, 2011).

While, Takagi et al (2003) found that Ang2 are more expressed in retinal vascular proliferative membranes in human ischemic retinal diseases, such as PDR than in nonischemic retinal diseases. Ang2 is also upregulated by hypoxia and VEGF (Takagi et al, 2003) and ischemia in retina of an animal model. Feng et al, (2008) suggested that the function of Ang2 appears to be highly dependant on the presence of VEGF. Thus in high levels of VEGF induce proliferation and angiogenesis.

In this study, a highly significant increase in VEGF levels in diabetics compared to controls were detected. This elevation was more pronounced in PDR compared to NPDR. These results agreed with Moemen et al, (1999).

Katsura et al (1998) reported high VEGF in active PDR compared to quiescent PDR suggesting that VEGF plays a major role in mediating active intraocular neovascularization. VEGF has been identified as primary initiator of proliferation of DR and mediator of non PDR (Aiello & Wong, 2000) VEGF not only angiogenic but also has been suggested to play an important role in an early stage of DR as stimulator of microaneurysm formation, capillary occlusion with ischemia as well as promoter of increased vascular permeability. On the other hand, Loukovaara et al (2004) found out no significant changes in VEGF among different groups.

Peters et al (2007), concluded that VEGF alone is twice potent in interrupting tight junction in an endothelial cell monolayer as Ang2. However, both growth factors acting together increasing vascular permeability, three times as much as VEGF alone. Which explains the result of the significant positive correlation detected in this study between Ang2 & VEGF.

Also, it has been found that VEGF up regulates Ang2 expression in endothelial cells. Furthermore, VEGF can also bind to the Angiopoietin receptor, VEGF could influence Angiopoietin signaling pathway directly (Jones, 2003).

It appears that VEGF and Angiopoietins coordinate vessel proliferation. While, Rangasamy et al, (2012) assumed that hyperglycemia cause up regulation of adhesion molecules as vascular adhesion molecule-1 in the endothelial lining of retinal microvasculature. Further activation of leukocytes in diabetes leads to their attachment to the endothelial cells resulting in microvascular damage through secretion of VEGF, Ang2 and tumor necrosis factor α which leads to alteration of blood retinal barrier and retinopathy.

Okamoto et al, (2002) investigated the role of culture microvascular endothelial cell with methyl glyoxal, growth and tube formation; the key steps of angiogenesis were significantly stimulated. They suggested that advanced glycation end product & MGO increase the transcriptional activity of NFkB and then upregulate mRNA levels of VEGF and Ang2. This could explain the result of the present study of significant positive correlation of these parameters.

The last parameter studied in this study is HIF-1α highly significant increase in their levels in diabetics compared to controls. Also, this elevation is more evident in PDR than NPDR patients suggesting a significant role of HIF1α in neovascularization and PDR.

HIF-1α is an alpha- beta heterodimeric transcription factor that mediates cellular responses to ischemic or hypoxic conditions via the transcriptional activation of VEGF, (Wang et al, 2009). However, they detected elevated vitreous levels of both HIF-1α & VEGF in PDR but not in non diabetics. To the contrary of the results of the present study. They did not observe significant difference in serum levels of VEGF & HIF-1α either in diabetic with or without PDR. However, intravitreous levels could lead to misinterpretation. Firstly, the disruption of the blood retina barrier that occurs in DR produce increase of proteins in vitreous fluid. Secondly, the high serum levels of specific protein could influence their intravitreous concentrations due to disruption of blood retina barrier. Finally, vitreous hemorrhage which occurs in PDR can produce an influx of serum proteins such as growth factors into the vitreous.

Moreover, Wang et al, (2009) suggested that HIF-1α does not play a leading role as big as VEGF does in the angiogenesis of PDR. However, it should be noted that HIF-1α increase the expression of VEGF in human endothelial cells, and HIF-1α enhances VEGF induced angiogenesis in vitro and in vivo (Forsythe et al, (1996) and Kim et al 2008).

There is evidence strongly suggested cellular and tissue dysfunction associated with diabetes is related to the lack of cellular ability to adopt and survive in hypoxic conditions. Also Bento et al, (2010) suggested increase MGO production is likely to be a link between hyperglycemia and destabilization of HIF-1α. Moreover they suggested that hyperglycemia by increasing MGO is likely to disturb the VEGF – Ang2 balance resulting in characteristic features of early stage of diabetic vascular complications (Bento et al, 2010).

In conclusion: an association between retinopathy and increased serum MGO in PDR were detected.

Given the apparent importance of Ang2 in modulating VEGF induced vascular permeability as well as angiogenesis; this Ang2 is clearly a therapeutic target of considerable interest for diabetic retinopathy.
However, further studies are required to elucidate the specific role of HIF-1α in pathogenesis of PDR diabetics.

References


