ROLE OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL DYSFUNCTION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

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ABSTRACT

BACKGROUND: Oxidative stress, inflammation and endothelial dysfunction are commonly found in persons with type II diabetes mellitus (DM), but their role in the pathogenesis of diabetic retinopathy (DR) is not fully elucidated. Therefore, the present study investigates the relationship and the role of these factors in the incidence and progression of different stages of DR. METHODS: This study included 85 subjects divided into four groups. First group consisted of 20 healthy subjects who served as controls. The second group consisted of 23 patients with type II DM without retinopathy, while the third group consisted of 20 patients having non-proliferative diabetic retinopathy (NPDR), and finally the last group consisted of 22 patients having severe proliferative diabetic retinopathy (SPDR). For all subjects in all groups, the levels of glycated hemoglobin (HbA1c %), lipid profiles, malondialdehyde (MDA) and nitric oxide (NO) were measured spectrophotometrically, while tumor necrosis factor-alpha (TNF-α) and soluble Eselectin (sE-selectin) were measured using ELISA technique. RESULTS: All the above measured parameters were significantly elevated in all diabetic patients with or without retinopathy when compared to control subjects, with the most significant increase in case of the SPDR group. There was a significant positive correlation between plasma MDA with both TG & HbA1c%, NO & TNF-α and finally s-Eselectin & HbA1c%. CONCLUSION: Oxidative stress, inflammation and endothelial dysfunction have a fundamental role in the pathogenesis of DR.

Key words: Endothelial dysfunction; reactive oxygen species; oxidative stress; anti oxidants; nitric oxide; drug toxicity

INTRODUCTION

Diabetic retinopathy (DR) is among the most common microvascular complications of diabetes [1]. The prevalence of DR is about 4-28% and about 2% of diabetic population is blind as a result of DR [2], thus DR is regarded as one of the leading causes of blindness worldwide [3].

Diabetic retinopathy can be principally classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). In the former type, the earliest clinical signs are microaneurysms, small outpourings from retinal capillaries, and dot intraretinal hemorrhages [4]. As the NPDR progresses from mild, moderate to severe, patients have an increase in the number and size of intraretinal hemorrhages [5]. This increase may be accompanied by cotton wool spots; both of these signs indicate regional failure of retinal microvascular circulation, which results in ischemia [1, 6]. Proliferative diabetic retinopathy occurs when further retinal ischemia is characterized by the growth of new blood vessels on the surface of the retina or the optic disc [6]. These abnormal vessels may bleed, resulting in vitreous hemorrhage, subsequent fibrosis, and tractional retinal detachment [1] leading to severe and often irreversible vision loss [7, 8].

The retina has high content of polyunsaturated fatty acids and has the highest oxygen uptake and glucose oxidation relative to any other tissue. This phenomenon renders retina more susceptible to oxidative stress [9] and lipid peroxidation [10]. It has been suggested that the correlation between hyperglycemia, changes in the redox homeostasis and oxidative stress are the key events in the pathogenesis of DR [1]. Oxidative stress, besides creating a vicious cycle of damage to macromolecules by amplifying the production of more reactive oxygen species (ROS), also activates other metabolic pathways that are detrimental to the development of DR [9]. These pathways are mostly dependant on excessive transport of glucose into retinal cells resulting in increased intracellular glucose levels [11]. These pathways include the polyol pathway [9], production of advanced glycation end products (AGEs) [12] and protein kinase C pathway[13].Free radicals are continuously formed in all aerobic cells, and consist of the superoxide radical, hydrogen peroxide and hydroxyl radical. These metabolites are responsible for lipid peroxidation, which is described as a conglomeration reaction of the polyunsaturated fatty acids found in the cell membrane to various products such as peroxides and hydroxy fatty acids[14]. Some lipid peroxidation
products such as MDA may bind to proteins and amplify glyco and oxidation generated lesions [15]. Several studies have been made measuring the degree of lipid peroxidation in DR using MDA. Recently, Pan et al. [16] reported a significant increase in the serum MDA levels of type II diabetics with retinopathy when compared to those diabetics without retinopathy and control subjects.

Inflammation is a prominent component of many diseases [17]. Chronic inflammation is characterized by increased vascular permeability, edema, inflammatory cell infiltration, cytokine and chemokine expression, tissue destruction, neovascularization, and attempts at repair [18], and DR exhibits most of these features such as increased blood flow and vascular permeability, tissue macular edema, macrophage infiltration [19], microglial cell activation [20], accelerated cell death [21], acute phase response protein expression [22], increased cytokine expression [23], increased leucocyte adhesion [24], neovascularization, and acute phase response protein expression [22]. Several studies have been made on human subjects to postulate the role of TNF-α in the pathogenesis of DR. Doganay et al. [25] reported that TNF-α levels in the serum of patients with PDR was significantly higher than in patients with NPDR, type II DM and controls.

Nitric Oxide is synthesized from its precursor, L-arginine by the enzyme nitric oxide synthase (NOS). There are three major isomers of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [26]. The consequences of increased levels of NO in retinas from subjects with diabetes could be twofold: neurotoxicity and angiogenesis. Nitric oxide can be beneficial in its role as a vasodilator, but high concentrations of NO produced by iNOS are neurotoxic [27]. The toxicity of NO has been attributed to multiple mechanisms, including DNA damage, peroxynitrite mediated oxidative damage, and energy failure [28]. Ozden et al. [29] compared the basal serum levels of NO in patient with type II DM without retinopathy and different stages of DR with the levels in non-diabetic control subjects and the results showed a significant increase in serum NO levels in patients with different stages of DR than in type II diabetics which were both significantly higher than control subjects.

Recently, leucocyte activation and adhesion to the endothelium have been considered as a cause of capillary occlusion in DR [30]. Leukocytes of individuals with DR show decreased deformability and increased adhesion to retinal capillaries leading to leukostasis, which appears to play a role in retinopathy, in diabetic patients [31].

Leukocytes are present within microaneurisms and may play roles in the development of these abnormal vessels. E-selectin is an adhesion molecule which is selectively synthesized by activated endothelial cells [32]. Increased levels of serum sE-selectin were reported to be associated with endothelial cell dysfunction in previous studies [33], however, the relationship of sE-selectin to DR is currently not fully explained. Olsen et al. [30] measured the serum concentrations of soluble E-Selectin molecules in serum of diabetic patients with different stages of DR and compared it with healthy control subjects. Results have shown a significant increase in the level of this marker being the highest in case of severe NPDR.

The aim of this study is to determine the role of these inflammatory and oxidative stress markers as well as adhesion molecules in the pathogenesis of DR.

[II] MATERIALS AND METHODS

2.1. Study Subjects

The study compromised 85 subjects divided into 46 males and 39 postmenopausal females aged 45-69 years. All of the study subjects were non-smokers. Twenty of them were healthy volunteers serving as the control group. Twenty three patients suffering from type II DM without retinopathy were recruited from the Department of Endocrinology of El Matariah Hospital, Cairo, Egypt and these were representing the second group. Forty four patients were recruited from the Research Institute of Ophthalmology, Giza, Egypt. Those patients were divided into 20 patients suffering from NPDR representing third group and 22 patients suffering from PDR representing the fourth group as shown in Table 1. All diabetic patients with or without retinopathy were under treatment of oral hypoglycemics or insulin and the level of DR was determined by fundus findings where all the diabetic patients underwent a complete ocular examination including visual field testing, slit lamp biomicroscopy and indirect ophthalmoscopy. Exclusion criteria included: age over 70 years, ischemic cardiovascular disorders, hepatic disorders, history of malignancy, presence of hematological diseases and renal disorders. The study protocol was approved by the local university committee and informed consent was obtained from all subjects in accordance with the principles of the Helsinki Declaration.

2.2. Sample collection

For all subjects, blood samples (5-10 ml) were collected in the morning after overnight fasting. Samples were divided into three portions: First portion of blood was collected on vacutainer tubes containing Na2EDTA for assay of HbA1c. The second portion was collected on vacutainer tubes containing Na2EDTA for assay of MDA in plasma. Plasma samples were separated after 20 minutes by centrifugation at 2500 rpm for 15 minutes. The last portion was collected on plain vacutainer tubes for serum preparation used for the assay of the lipid profiles, NO, TNF-α and sE-selectin. Sera were separated from clotted blood after 30 minutes by centrifugation at 4000 rpm for 15 minutes. Plasma and sera samples were kept frozen in aliquots at -80 °C until assayed.

2.3. Assays

Fresh blood aliquots were used for the measurement of HbA1c, while sera aliquots were used for the measurement of total cholesterol (TC) and triglycerides (TG) by standard enzymatic techniques using commercially available kits [34, 35]. High density lipoprotein-cholesterol (HDL-C) was determined after the precipitation of apolipoprotein B-containing lipoproteins [36]. Low density lipoprotein-cholesterol (LDL-C) was calculated according to Friedewald equation [37]. All spectrophotometric measurements were done by UV/Visible spectrophotometer, Jenway, model no. 6305.
2.3.1. MDA Measurement

Levels of MDA were determined as thiobarbituric acid-reactive substances (TBARS) following a protocol described previously by Uchiyama and Mihara [38].

2.3.2. NO determination

Levels of NO were determined based on the conversion of nitrate to nitrite by Vanadium (III) chloride according to the method of Cox [39]. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction according to the method of Griess [40].

2.3.3. TNF-α and sE-selectin determinations:

These two markers were assayed by a validated ELISA technique using commercial kits provided by R&D Systems, Inc., USA. All ELISA procedures were done by Hyprep ® automated ELISA system, USA, according to the instructions of the manufacturer.

2.3.4. Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 9 software. Data were represented as mean ± SEM. Differences between groups were compared using a one-way analysis of variance (ANOVA) followed by LSD post-hoc analysis. A P value < 0.05 was considered statistically significant. Pearson correlation coefficient was used to determine correlation between different parameters.

[III] RESULTS

With regard to the levels of HbA1c % and the lipid profiles (TC, TG, HDL-C and LDL-C), all the diabetic groups with or without retinopathy showed a significant increase when compared to the control group with the exception of HDL-C level which was significantly lower as shown in Table 2. The levels of MDA, NO, TNF-α and sE-selectin were significantly higher in all diabetic groups when compared with the healthy controls as shown in Table 3. In case of MDA, the levels were 5.28 ± 0.34, 5.57 ± 0.43 and 6.81 ± 0.49 nmole/ml, respectively, for type II DM, NPDR and SPDR groups whereas, the normal control was 2.15 ± 0.19 nmole/ml. As for NO, the levels were 41.5 ± 2.29, 40.43 ± 3.51 and 49.76 ± 3.0 mmole/l for the former three groups, respectively while the normal control was 16.1 ± 0.68 mmole/l. The levels of TNF-α in type II DM, NPDR and SPDR groups were 21.7 ± 0.63, 22 ± 0.51 and 25.8 ± 1.23 pg/ml, respectively while the control group was 16.1 ± 0.68 pg/ml. As for the sE-selectin levels in these diabetic groups, they were 30.28 ± 1.96, 37.74 ± 2.67 and 63.7 ± 4.65 ng/ml, respectively while the control group was 10.86 ± 0.74 ng/ml.

3.1. Correlation data

Evaluation of the correlation coefficient of the biomarkers in all diabetic patients, comprising type II DM, NPDR and SPDR groups, revealed a positive and significant association of MDA with HbA1c % (fig1a ), MDA with TG [Figure – 1 b], NO with TNF-α [Figure – 2] and sE-selectin with HbA1c % [Figure – 3].

Table 1. Clinical characterization of the study subjects. Data are expressed as mean ±SEM

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (n=20)</th>
<th>Type II DM (n=23)</th>
<th>NPDR (n=20)</th>
<th>SPDR (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M / F )</td>
<td>12 / 8</td>
<td>11 / 12</td>
<td>9 / 11</td>
<td>14 / 8</td>
</tr>
<tr>
<td>Age (year)</td>
<td>M: 51.59 ± 0.70</td>
<td>M: 54.65 ± 2.24</td>
<td>M: 54.11 ± 2.23</td>
<td>M: 59.65 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>F: 55.63 ± 1.11</td>
<td>F: 57.92 ± 1.11</td>
<td>F: 58.82 ± 1.05</td>
<td>F: 59.13 ± 1.30</td>
</tr>
<tr>
<td>Duration of Diabetes(years)</td>
<td>—</td>
<td>6.87 ± 0.39</td>
<td>7.08 ± 0.38</td>
<td>8.98 ± 0.51</td>
</tr>
</tbody>
</table>

Fig: 1. Correlation between (a) MDA and HbA1c, (b) MDA and TG in all diabetic patients
Table 2. Levels of glycated hemoglobin (HbA1c), triglycerides (TG), total cholesterol (TC), high density-lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) in all diabetic patients and in healthy controls. Results are expressed as mean ±SEM

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Type II DM</th>
<th>NPDR</th>
<th>SPDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>5.178 ± 0.22</td>
<td>8.37 ± 0.28 a</td>
<td>7.86 ± 0.32 a</td>
<td>9.59 ± 0.29 a,b,c</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>78.63 ± 4.92</td>
<td>188.6 ±16.85 a</td>
<td>199.6 ± 14 a</td>
<td>233.49 ± 15.57 a,b</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>142.32 ± 4.79</td>
<td>225.24 ± 9.73 a</td>
<td>242 ±13 a</td>
<td>258.73 ± 11.54 a,b,c</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>50.75 ±1.65</td>
<td>41.63 ± 0.97 a</td>
<td>41.26 ± 0.85 a</td>
<td>39 ± 0.75 a</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>75.85 ± 4.57</td>
<td>145.9 ± 4.57</td>
<td>160.88 ±12.1 a</td>
<td>173 ± 10.96 a,b,c</td>
</tr>
</tbody>
</table>

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the type II DM group at P ≤ 0.05.
c: Significantly different from the NPDR group at P ≤ 0.001.

Table 3. Levels of malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), soluble E-selectin (sE-selectin) in all diabetic patients with or without retinopathy and in healthy controls. Results are expressed as mean ±SEM

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Type II DM</th>
<th>NPDR</th>
<th>SPDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmole/mL)</td>
<td>2.15 ± 0.19</td>
<td>5.28 ± 0.34 a</td>
<td>5.57 ± 0.43 a</td>
<td>6.81 ± 0.49 a,b,c</td>
</tr>
<tr>
<td>Nitric Oxide (mmole/L)</td>
<td>20.77 ± 1.24</td>
<td>41.5 ± 2.29 a</td>
<td>40.34 ± 3.51 a</td>
<td>49.76 ± 3.00 a,b,c</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>16.1 ± 0.68</td>
<td>21.7 ± 0.63 a</td>
<td>22 ± 0.51 a</td>
<td>25.8 ± 1.23 a,b,c</td>
</tr>
<tr>
<td>sE-Selectin (ng/mL)</td>
<td>10.86 ± 0.74</td>
<td>30.28 ± 1.96 a</td>
<td>37.74 ± 2.67 a</td>
<td>63.7 ± 4.65 a,b,c</td>
</tr>
</tbody>
</table>

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the type II DM group at P ≤ 0.03.
c: Significantly different from the NPDR group at P ≤ 0.03.

[IV] DISCUSSION

Complications of DM, which are the cause of major morbidity and mortality, are related mainly to chronic level of glycemia [41]. The risk of DR is increased with poor glycemic control [42]. Early epidemiologic studies have shown a consistent relationship between HbA1c% levels and the incidence of DR. This important observation has been confirmed in large randomized clinical trials demonstrating that tight glycemic control reduces both the incidence and progression of DR [6]. Early in the course of diabetes, hyperglycemia is responsible for many of the functional retinal vascular changes, including impairment of retinal blood flow, increased leukocyte and monocyte adhesion in the retinal micro vessels, and capillary closure resulting in localized hypoxia [43]. In addition, retinal neuronal function may also exhibit abnormalities early in the course of the disease. One of the earliest and most specific retinal changes induced by hyperglycemia is the death of pericytes [44]. The death of pericytes and the loss of vascular intercellular contacts may predispose to endothelial cell proliferation, facilitating the development of microaneurysms [45]. Alterations in hemodynamics and vascular autoregulation that are characteristic of the diabetic state [46] can produce venous dilatation and beading as well as intraretinal microvascular abnormalities that represent dilated small vessels [47]. Impairments of vascular cell-to-cell contacts and altered barrier permeability function can lead to small intraretinal hemorrhages and fluid leakage. When water is reabsorbed, the plasma lipids and proteins precipitate as hard exudates [43].

Fig. 2. Correlation between NO and TNF-α in all diabetic patients
major reasons for vision loss in DR, in addition to elevation in the blood viscosity[48]. Moreover, the incorporation of TGs into the cell membrane leads to changes in membrane fluidity and leakage of plasma constituents into the retina resulting in haemorrhage and edema in the retina [49].

On the other hand, iNOS in particular is known to release a great deal of NO continuously compared with nNOS and eNOS, especially in patients without active neovascularization [56]. The two fundamental abnormalities in DR are increased retinal vascular permeability and progressive retinal vessel closure, which leads to tissue hypoxia and ischemia which in turn induce iNOS. This induction leads to microenviromental changes in diabetic retinas resulting in sustained and high NO production [57]. This increased NO release can cause oxidation and overproduction of peroxynitrite, a ROS mediated by NO, that has been reported to cause vascular endothelial cell dysfunction and breakdown of the blood-retinal barrier, which are important components of the development of DR [25].

Several studies have addressed the recent hypothesis that the angiogenesis of PDR is due to the release of growth factors and interleukins from the ischaemic retina [58]. Abnormal production of cytokines such as TNF-α [25] may also be important in the progression of DR. The mechanism of TNF-α contribution to DR is not fully elucidated. It has been suggested that hyperglycemia may lead to the activation of proinflammatory cytokines that are crucial for micro- and macroangiopathy developments [59]. In diabetic patients, an increased synthesis of the macrophage’s RAGE receptors, which bind final glycation products, has been noted [60]. The RAGE receptors signalize the proinflammatory cytokines’ cascade induction, including TNF-α, interleukin-6, and interleukin-12 [59, 60]. These cytokines may mediate the synthesis of acute phase proteins which are able to initiate and support inflammatory process in the vascular wall. In our study, the significant correlation between NO and TNF-α may be explained by the fact that NO mediates the angiogenic activity of platelet-activating factor and TNF-α [61]. Moreover, diabetes causes microangiopathy in retina and causes hypoxia. Transcription factor kappa (NF-KB) is activated by hypoxia and controls the expression of many gens, some involved with angiogenic factors [62].

Adhesion molecules such as E-selectin have been implicated in the pathogenesis of DR [63]. Neovascularization, a process involved in the pathogenesis of DR, is a result of microvascular thrombi leading to retinal ischaemia. E-selectin and cell adhesion molecules, being expressed on retinal vascular endothelium, may take part in this process [64]. E-selectin is expressed on activated endothelial cells and initiates rolling and tethering of leucocytes on the endothelium [65]. This leucocyte recruitment may be the first step in the ensuing endothelial dysfunction resulting in increased permeability of the vessel wall, capillary occlusion, retinal ischemia and ultimately new vessel formation, all characteristics of various stages of DR [66]. The positive correlation between E-selectin and HbA1c may be explained by the fact that massive hyperglycemia and subclinical tissue injury as well as increased fat mass seen in DM elevate blood levels of inflammatory cytokines, especially TNF-α, which in turn stimulate an acute phase response marked by elevated levels of C-reactive protein [67]. Localization of this inflammatory cascade by vascular endothelial cells is mediated by cellular adhesion molecules including E-selectin whose surface
expression is a common endothelial response to a variety of toxic stimuli.

[V] CONCLUSION

From our results we conclude that dyslipidemia, oxidative stress and inflammation as well as endothelial dysfunction are all involved in the pathogenesis of DR.

[VI] REFERENCES

[37] Friedwald WT, Levy RI, Fredrickson DS. [1972] Estimation of the concentration of low density lipoprotein cholesterol in


