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ORIGINAL ARTICLE

APOPTOSIS AND INFLAMMATION FORM THE MYSTERY OF PATHOGENESIS OF DIABETIC RETINOPATHY

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ABSTRACT: Faster anaerobic glycolysis in type 2 diabetes mellitus generates increased lactate and NADH. Uncoupling of NADH produces excessive reactive oxygen species which form the main stream of apoptosis of vascular and neural cells in retina. Increased lipid peroxidation and extracellular glutamate toxicity further augment this process. Vascular endothelial growth factor is up regulated to resist the apoptotic destruction or in response to non-perfusion of acellular capillaries. Deleterious effects of excessive vascular endothelial growth factor under an adverse biochemical circumstances causes microangiopathy in diabetic subjects. Blood levels of lactate, glutamate, malondialdehyde and vascular endothelial growth factor were seen significantly higher in mild non-proliferative diabetic retinopathy in comparison to diabetic subjects with no retinopathy.

KEYWORDS: Apoptosis, Inflammation, Anaerobic glycolysis, vascular endothelial growth factor, Diabetic retinopathy.

INTRODUCTION: The most intrinsic findings of diabetic retinopathy are the vascular abnormalities that are apparent by retinal examination. There is ample evidence that diabetes causes apoptosis of vascular and neural cells in the retina.[1-3] As diabetic retinopathy is gradually initiated even in uncontrolled diabetes, there is good reason to define diabetic retinopathy as a form of chronic neurovascular degeneration.[4,5]

Another school thought inflammation to be the prime pathology for the development of diabetic retinopathy. Several studies have documented increased concentrations of cytokines, particularly vascular endothelial growth factor (VEGF), interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and monocyte chemoattractant (MCP)-1 in the vitreous of patients with proliferative diabetic retinopathy and diabetic macular edema.[6-10]

However, an inflammatory component was supported by the finding that vascular apoptosis was blocked by the systemic administration of antibody to Fas ligand, preventing leukostasis.[11]

On the other hand, clearance of apoptotic cell prevents release of toxic contents from dying cells, promotes resolution of inflammation and produces growth factors that enhances macrophases clearance of apoptotic cells.[12] Anti-inflammatory effects of apoptotic cells has been documented in recent study.[13]

Under this background of aetiopathologic confusion, analysis of principal biochemical derangement of diabetes mellitus i.e anaerobic glycolysis in some essential organs, may answer this mysterious aspect.

Our present biochemical study on clinical background attempts to shower some new light on the aetiopathogenesis of diabetic retinopathy.

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MATERIALS AND METHODS: Ninety patients of type 2 diabetes mellitus (DM) with mild non-proliferative diabetic retinopathy (NPDR) were recruited as study subjects and 64 patients of type 2 DM of similar duration were included as diabetic controls e.g. diabetics without retinopathy (DNR).

All the subjects underwent detailed fundus examination by slit-lamp biomicroscopy with +90 D lens and dilated fundus photography. Grading of DR was based on modified Airlie House classification system. Mild non-proliferative diabetic retinopathy (mild NPDR) is characterized by presence of few microaneurysms only in retina.

Presence of coronary artery disease, strong family history of coronary artery disease, hypertension, peripheral vascular disease, recent acute infection, thrombotic event, urinary microalbumin >300 mg/dl, prediabetes (fasting blood glucose >100 mg/dl but <126 mg/dl) and ocular disorders (glaucoma, Eales' disease, branch retinal venous occlusion, etc) were considered as exclusion criteria of this study.

Then samples were collected from the recruited subjects attending the retina clinic of the Regional Institute of Ophthalmology, Kolkata and the diabetic clinic of the Institute of Post-Graduate Medical Education & Research, Kolkata. All the subjects enrolled in the study were belonging to the same geographical area, eastern India. Written informed consent was collected from each patient according to the declaration of Helsinki and was approved by the ethics committee of the Institute.

Age, sex and blood pressure were matched within the study groups. Diabetes mellitus was diagnosed according to World Health Organization criteria. Glycemic status of all diabetic subjects was investigated by measuring oral glucose tolerance test and glycosylated haemoglobin A1c%. None of the diabetic subjects was on metformin drug therapy and insulin treatment during the study period.

Sample collection and Processing: Venous blood samples (5 ml in EDTA vial and 5 ml in clot vial) were collected after 12 hour fast and 30 minutes physical rest and centrifuged at 3000 r.p.m for 10 minutes at 4^0C temperature to separate cellular components and plasma. Plasma samples were collected in cryocube vials for glucose, lactate, pyruvate and glutamate assay. Serum samples were collected in cryocube vials for estimation of malondialdehyde (MDA) and vascular endothelial growth factor (VEGF).

Measurement of Lactate and Pyruvate: Plasma lactate was measured by lactate oxidase and peroxidase enzymatic method using a kit (Randox, United Kingdom) with a linearity of 12.21 mmol/L. Lactate present in the sample was oxidized to pyruvate by the enzyme lactate oxidase. The hydrogen peroxide so produced as byproduct of this reaction was subjected to react with 4-aminoantipyrine catalysed by peroxidase and produced a purple color product. The absorbance of this color product was measured spectrophotometrically (Halo DB-20; Dynamica) by using 550 nm filter against the reagent blank within 30 minutes after incubation.

Pyruvate level from plasma was determined by using a kit (Bio Vision, Mountain view, USA) with a detection limit 1 to 1000μM/L. In the assay pyruvate was oxidized by pyruvate oxidase via enzyme reaction to generate color and the intensity of the color was directly proportional to the pyruvate content of the sample. Plasma samples was deproteinized by 10 K-Da cut off spin filter (Bio Vision) to prevent conversion of lactate to pyruvate, due to presence of LDH in the samples. Pyruvate probe was dissolved with 220μL of dimethyl sulphoxide and the pyruvate enzyme mix was dissolved

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in 220µL pyruvate assay buffer just before the experiment. Fifty microliters of samples in pyruvate assay buffer was mixed with 50µL reaction mixture containing pyruvate probe and pyruvate enzyme mix, in 96 well plate. The reaction mix was incubated at room temperature for 30 minutes. The absorbance of color product was read at 570 nm in a Bio-Rad multiplate reader (Bio Rad, Model 680). Concentration of pyruvate in unknown sample was determined from the slope of the standard curve ranging from 0.2 to 1.0 nmol / well.

Measurement of plasma Glutamate: Plasma glutamate was measured by enzyme linked spectrophotometric assay as described previously (Wang 2006) in the presence of 1 U / µL glutamate dehydrogenase and 40 mM β nicotinamide adenine dinucleotide phosphate ($NADP^+$). Plasma glutamate was oxidized to α-ketoglutarate with the fluorimetric production of NADPH in Krebs-Ringer-Hepes reaction buffer (145 mM NaCl, 1.2 mM $CaCl_2$, 10 mM dextrose, 1.5 mM KCl, 1.3 mM $MgCl_2$, 1.2 Na_2HpO_4, H_2O). After 30 minutes incubation of reaction mixture at 37^0C, the fluorescence generated by reduction of $NADP^+$ to NADPH was monitored spectrophotometrically (Model FP 6300, JASCO, Essex, UK) at excitation and emission wave length of 366 nm and 455 nm respectively. The concentration of glutamate in plasma was determined from linear standard curve established by 25-150 µmol of L-glutamate standard.

Measurement of MDA: Lipid peroxidation in serum was measured by MDA estimation as described previously (Satoh 1978). Lipoproteins in serum were precipitated by adding 20% trichloro acetic acid and 8.1% sodium dodesyl sulphate. There after 0.8% aqueous solution of thiobarbituric acid was added to this precipitate, mixed well and finally was heated at 95^0C for 1 hour for coupling of lipid peroxide with thiobarbituric acid. The resulting chromogen was extracted from the precipitate by adding n-butanol and pyridine mixture (15:1). The orange mixture was separated by centrifugation and the intensity of the organic layer was measured spectrophotometrically (Halo DB-20 Dynamica, Salzburg-Marywise, Austria) by using 530 nm filter against water bank. The concentration of MDA in serum samples was determined from linear standard curve established by 1 to 8 nm of 1, 1, 3, 3 tetramethoxy propane.

Serum Vascular Endothelial Growth Factor (VEGF) Estimation: Human serum VEGF was estimated by ELISA, using the kit of Ray Biotech (catalogue ELH-VEGF-001, Ray Biotech, Norcross, USA). In the assay, an antibody specific for human VEGF was coated on a well plate. A series of standards ranging from 8.23pg/ml to 6000pg/ml and samples (serum samples were two fold diluted with assay diluents) were added into the wells. VEGF protein present in the sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated antihuman VEGF antibody was added. After Buffer wash HRP-conjugated streptavidin was pipette into the wells. Further the wells were subjected to wash and TMB substrate solution was added into the wells and were placed in incubation at room temperature for 30 minutes. Intensity of final color product was proportional to the concentration of VEGF protein present in the samples and the absorbance of the color product was measured colorimetrically by using 450 nm filter in an ELISA plate reader (BIO-Rad, Model 680). The concentration of VEGF in the samples was determined by a standard curve and the assay detects less than 10pg / ml of VEGF from the sample.

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RESULTS: Study and control subjects enrolled in our present study revealed no statistically significant difference for sex distribution, age, duration of diabetes, body mass index (BMI), glycemic status and blood pressure (Table 1).

Biochemical investigations revealed elevated levels of lactate (3.14±1.14 mmol/L in comparison to 1.1±0.76 mmol / L) in diabetic patients with mild NPDR compared to diabetic patients without diabetic retinopathy (DNR). Pyruvate levels (58.24±2.6 µmol /L in comparison to 87.50±3.5 µmol /L) were seen lowered in mild NPDR group compared to DNR.

Malondialdehyde (MDA) and glutamate levels were also significantly elevated in mild NPDR subjects compared to DNR.

Above all serum endothelial vascular growth factor (VEGF) showed significantly higher concentration in mild NPDR group in comparison to DNR group (182.61±49.36 pg/ml versus 96.66±37.35 pg/ml).

Table 1: Clinical characteristics of DNR and mild NPDR subjects

Parameters	DNR	Mild NPDR	P value
Sex Male	44(68.75%)	52(57.77)	0.615
Female	20(31.25%)	38(42.23%)	
Age (years)	49.5±8.3	51.7±8.1	0.207
Duration of diabetes (years)	15.2±6.45	16.4±5.26	0.372
Body mass index (kg/M ^{2})	26.4±4.62	25.3±4.24	0.088
Blood pressure (mm of Hg)			
Systolic	128.2±7.7	130±8.8	0.127
Diastolic	82±6.2	83.5±6	0.12
Blood glucose (mg/dl)			
Fasting	172±22.9	188.8±24.22	0.001
Post prandial	246±29.4	292.7±33.65	0.001

Table 2: Showing levels of different biochemical parameters among DNR and Mild NPDR

Parameters	Sample type	DNR	Mild NPDR	P value
Lactate (mmol/L)	Plasma	1.9±0.76	3.14±1.14	0.0001
Pyruvate (µmol/L)	Plasma	87.50±3.5	58.24±2.6	0.001
MDA (nmol/L)	Serum	3.1±1.83	4.65±1.79	0.004
Glutamate (µmol/L)	Plasma	55.13±20.8	67.94±20.29	$p < 0.05$
VEGF (pg/ml)	Serum	182.61±49.36	96.66±37.35	0.0001

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DISCUSSION: It is well established that diabetes leads to loss of vascular cells in retina.[1,2] The increase in vascular cell apoptosis also suggest a potential mechanism for the appearance of acellular capillaries in diabetic retinopathy.[3] Apoptosis may also account for appearance of pericyte ghosts frequently noted in trypsin digest samples.

Studies using terminal dUTP nick-end labeling in histologic sections of retinas of diabetic rats and postmortem human retinas revealed that diabetes increased apoptosis in neurons of inner retina where ganglion cells are located.[4,5]

So from these studies, it is evident that apoptosis play the key role in the initiation of diabetic retinopathy.

According to opinion of other schools, inflammation is considered to play the key role in the pathology of diabetic retinopathy.[6,7] Several studies have demonstrated increased concentrations of cytokines, particularly vascular endothelial growth factor (VEGF), Interleukin (IL)-1β, IL-6, IL-8, Tumour necrosis factor (TNF)-α, and monocyte chemoattractant (MCP)-1 in the vitreous of patients of proliferative diabetic retinopathy and macular edema.[8,9] So a causal link between cytokine expression and retinal apoptosis in diabetes has not been well established.

However one study documented that injection of IL-1 into the vitreous of normal rats induced TUNEL labeling and increased numbers of acellular capillaries in trypsin digest retinas, whereas a diet rich in multiple antioxidants reduced the amount of IL-1 and prevented vascular apoptosis in diabetic rats.[10]

Similarly another study showed that a TNF-α antagonist also reduced the number of apoptotic vascular cells.[11]

The mechanism by which these cytokines may contribute to retinal vascular and neural apoptosis is not clear but may invole the induction of excitotoxicity, oxidative stress, or mitochondrial dysfunction.[12]

In our present study we have demonstrated higher blood levels of lactate, glutamate and malondialdehyde (MDA) and lower levels of pyruvate in type 2 diabetic patients with mild NPDR in comparison to diabetic patients without any retinopathy. It is assumed that main metabolic derangement is shifting of glycolysis to anaerobic pathway leading to increased production of lactate. Increased lactate makes the medium surrounding retinal vascular and neural cells acidic which follows some adverse consequences.

Glutamate, the major excitatory amino acid acting as the principal neurotransmitter of brain and retina, is toxic to retinal neurons when present in high concentration.[13] Transport of glutamate from extracellular space is pH dependent and lowering pH slows or even reverses glutamate uptake.[14] So increased anaerobic glycolysis impairs glutamate uptake from extracellular space, resulting in a potential accumulation of extracellular glutamate and leading to cell excitotoxicity. In this cascade excess glutamate stimulation causes an uncontrolled intracellular calcium concentration, contributing to one way of apoptosis of vascular and neural cells of retina in diabetes mellitus.

The high level of intracellular Ca^{++} activates Ca^{++} dependent phospholipase which causes degradation of membrane phospholipids producing arachidonic acid and eicosanoids. Metabolism of eicosanoids leads to formation of oxygen free radicals, which cause peroxidative damage of the lipids of neural membrane, producing the byproduct, malondialdehyde. This lipid peroxide is toxic to cell.[15]

As glycolysis occurs faster in those tissues like retina, neurons, nephons, lens epithelium and erythrocytes, which are insulin independent for intracellular transport of glucose, the intermediates

of glycolytic system e.g. glyceraldehydes-3 phosphate and dihydroxyacetone phosphate are produced excessively. These two molecules ultimately generate inositol triphosphate and diacylglycerol which cause increased accumulation of intracellular calcium. The enzyme glyceraldehydes 3 phosphate dehydrogenase, responsible for oxidation of glyceraldehydes 3 phosphate, has been suspected to be one signaling pathway of apoptosis.[16]

So increased anaerobic glycolysis, glutamate induced cell excitotoxicity and increased lipid peroxide are suspected to be related to apoptosis of neurovascular cells of retina which invite increased secretion of VEGF to resist this process. It has been demonstrated that VEGF confers endothelial resistance to apoptosis through poly (ADP-ribose) polymerase.[17]

Efficient clearance of apoptotic cells by macrophases prevents release of toxic contents from dying cells, promotes the resolution of inflammation, and produces growth factors that support tissue including vascular endothelial growth factor (VEGF) and hepatocyte growth factor.[18]

The unknown mechanism between cytokine expression and retinal apoptosis has not been revealed. In our previous studies we have demonstrated increased up regulation of VEGF, IL-10, and TNF-α in proliferative diabetic retinopathy and upregulation of these cytokines is associated with specific polymorphism in the promoter regions of the genes.[19,20]

The Mechanism by which these cytokines may contribute to retinal vascular and neural apoptosis is not clear. It may involve the induction of excitotoxicity, oxidative stress, or mitochondrial dysfunction.[21]

It is suggested from this study that hyperglycemia induced oxidative stress induces cellular senescence or cellular aging. In response to DNA damage cell either age or go into apoptosis, if the damage cannot be repaired. Expression of inflammatory cytokines and adhesion molecules is up regulated in senescent endothelial cells.

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