j ocul biol dis inform (2010) 3:85–87
DOI 10.1007/s12177-011-9059-5

Elevated lipid peroxides induced angiogenesis in proliferative diabetic retinopathy

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Received: 2 April 2011 / Accepted: 11 May 2011 / Published online: 25 May 2011

Abstract Oxidative stress is associated with causation of diabetic vascular complications. A case–control study was undertaken to evaluate the association of platelet thiobarbituric acid reacting substances (TBARS) with the severity of diabetic retinopathy for the first time. Platelet TBARS levels were estimated using standard protocol. Platelet TBARS levels in the cases with non-proliferative diabetic retinopathy, proliferative diabetic retinopathy, and healthy controls were 0.56±0.09, 0.69±0.11 and 0.41±0.1 nmol/h/10^8 platelets, respectively. A significant increase in platelet TBARS levels was observed in the cases as compared to controls ($p<0.001$). Elevated TBARS levels were observed to significantly increase further during the proliferative stage of the disease ($p<0.01$). The increase in platelet TBARS levels, and thereby at retinal level, is associated with angiogenesis in diabetic retinopathy. Supplemental anti-oxidant therapy in diabetic retinopathy may prevent ocular angiogenesis resulting as a consequence of oxidative stress.

Keywords Oxidative stress · Platelets · Thiobarbituric acid reacting substances · Proliferative diabetic retinopathy · Anti-oxidant therapy

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Introduction

Ocular angiogenesis is a complex pathophysiologic process. Factors have been isolated that play key role in the regulation of angiogenesis [1]. The involvement of peptide growth factors, namely, insulin-derived growth factor and transforming growth factors alpha and beta have been documented in several vascular retinopathies [2]. The influence of stimulating growth factors is counter balanced by a number anti proliferative agents. The net result of these opposing factors in the vascular endothelial cells determines the outcome of angiogenesis homeostasis. Many angiogenesis-dependent diseases including those of ocular neovascularization may be driven by stimuli including cytokine derived growth factors, inflammation, hypoxia, and angiogenic mediators including reactive free radical nitric oxide [1].

Retinal hypoxia is known to stimulate neovascularization in ischemic proliferative retinopathies [3]. Retina because of its high content of polyunsaturated fatty acids (PUFA) is prone to oxidative stress. Platelets, like retina, also contain high proportion of PUFA, which serve as potent substrate for oxidants [4]. Role of oxidative stress in causation of diabetic vascular complications is well recognized [5–9].

A tertiary care center-based case–control study was undertaken to evaluate the association of platelet thiobarbituric acid reacting substances (TBARS) with the severity of diabetic retinopathy for the first time.

Material and methods

Thirty consecutive cases of non-insulin-dependent diabetes mellitus retinopathy (non-proliferative diabetic retinopathy

(NPDR), $n=15$ and proliferative diabetic retinopathy (PDR), $n=15$) presenting at the retina clinic of our tertiary care center were included. Sixteen subjects, of similar age group, presenting for refraction were included as healthy controls. Body mass index (weight in kilograms divided by the square of the height in meters (kg/m^2); BMI), of all the cases and the controls, was assessed using BMI calculator. Smokers, alchoholics, and patients with diabetic nephropathy and with any other chronic diseases were excluded. All the cases underwent detailed ophthalmological examination including LogMAR visual acuity, slit lamp biomicroscopy, indirect ophthalmoscopy, and fluorescein angiography. Cases with previous laser photocoagulation, and/or vitrectomy were excluded from this study.

Assay of lipid peroxidation

Blood samples were collected from all the cases and controls after informed consent. Five milliliters of blood was drawn by venipuncture using a plastic disposable syringe and a 22-gauge needle. Disodium citrate (3.89%) was used as an anticoagulant. As a measure of lipid peroxidation, malonaldialdehyde formation was estimated, using the level of TBARS, according to the method of Bohme et al. [10]. Equal volumes 120 μl of EDTA (10 mM), ascorbate (10 mM), and mixture of EDTA (16.7 mM) and $FeSo_4$ (16.7 mM) were mixed and to this, platelet suspension (840 μl) was added. The controls were incubated at 37°C for 0 and 90 min. The reaction was stopped by adding 1 ml of ice cold 10% trichloroacetic acid (TCA). The mixture was centrifuge at 2,000×*g* for 10 min. and supernatant was aspirated out. Platelet suspension was incubated at 37°C for 0 and 90 min, and the reaction was stopped by adding 10% ice cold TCA using same incubation medium. Deproteinized homogenates were centrifuged at 2,000×*g* for 10 min and supernatant was aspirated out. Supernatant (2 ml) was mixed with equal amount of 0.67% TBA and kept in boiling water bath for 15 to 20 min. The intensity of pink color developed was read at 532 nm on a spectrophotometer.

Data was statistically analyzed by two sample *t* test and the statistical significance was set at $p<0.05$. Results are presented as mean±SD.

Results

Body mass index of all the cases and the controls ranged between 19 and 23 kg/m^2. Mean age of the cases with NPDR was 50.7±3.8 years. There were six males and nine females. Median duration of disease was 8 years. Mean age of the cases with PDR was 51.2±2.3 years. There were eight males and seven females. Median duration of disease was 13 years. Mean age of the controls was 53.9±3.7 years. There were five males and ten females. No significant difference was observed, in age and sex, between the study groups ($p>0.05$). Two cases in NPDR and five in PDR groups had hypertension. One case in NPDR and four in PDR groups had associated diabetic neuropathy. Platelet TBARS levels in the cases with NPDR, PDR, and healthy controls were 0.56±0.09, 0.69±0.11 and 0.41±0.1 nmol/h/10^8 platelets, respectively. A significant increase in platelet TBARS levels was observed in the cases as compared to controls ($p<0.001$). Elevated TBARS levels were observed to significantly increase further during the proliferative stage of the disease ($p<0.01$).

Discussion

Significantly elevated platelet TBARS levels were noted in cases with NPDR which were observed to be further elevated in cases with PDR, highlighting the association of increased lipid peroxides with the severity of diabetic retinopathy, for the first time.

It is now well recognized that the normal vascular state reflects a complex and exquisite balance of endogenous positive and negative mediators of angiogenesis, a concept referred to as the angiogenesis model of homeostasis [1]. Loss of this balance in favor of neovascularization predominates in many pathologic conditions. In PDR, this balance is also disturbed in favor of neovascularization. The concept of angiogenic homeostasis in the eye and the consequences of its disruption have increasing operational value in understanding the mechanisms underlying ocular angiogenesis. Substantial data now implicates vascular endothelial growth factor as a dominant factor in proliferative retinopathies [1]. An angiogenic stimulus, such as vascular endothelial growth factor induced by hypoxic conditions, directs vascular endothelial cell orientation, migration, proliferation, and adhesion. Lumen formation and blood flow through these channels complete this process, which is regarded as a type of vasculogenesis because new vessels form from precursor cells [1]. Multiple cytokine signals govern the proliferative response of vascular endothelial cells in angiogenesis. Emerging data suggest that Interleukin-6, Interleukin-8, and chemokine-monocyte chemoattractant protein-1 levels in the vitreous are significantly higher in cases with PDR [11].

The human body has a complex anti-oxidant defense system that prevents the initiation of free radical chain reactions. Increased levels of lipid peroxides have been implicated in the pathogenesis of diabetic complications [12]. The increase in serum TBARS have been associated

with the occurrence of microvascular complications in NIDDM patients [13]. Increased serum lipid peroxides suggesting increased free radical activity is associated with retinopathy and this may have pathogenetic implications [14]. Increased lipid peroxide levels have also been observed in the vitreous of patients suffering from proliferative diabetic retinopathy [15]. Patients with diabetic nephropathy and retinopathy, but not neuropathy, have been found to have elevated plasma TBARS levels but no statistically significant difference was observed when compared with diabetic patients without microangiopathy. Additionally, no correlation was observed between TBARS levels and blood glucose and HbA_{1C} levels [16].

Our data highlight that the increase in platelet TBARS levels, and thereby at retinal level, [4] is associated with angiogenesis in diabetic retinopathy. Lipid peroxides induce the synthesis of cytokines, particularly tumor necrosis factor alpha. These in turn lead to increase in transforming growth factor alpha and beta which is associated with neovascularization in the retina [17]. Thiobarbituric acid reacting substances activity has been found to be significantly elevated in proliferative membranes of eyes suffering from PDR. Inflammatory cells and oxidative metabolites lead to oxidative reactions in PDR [18]. A variety of processes might increase the production of reactive oxygen species at the retinal level. One is the oxidative stress caused by conditions of transient hypoxia-hyperoxia [19, 20]. The oxidative alterations of retinal tissue in diabetic patient may also be attributed to the chronic deficiency of antioxidants in reduced form as a consequence of monosaccharide auto oxidation, NADPH oxidation, and formation of protein glycation products [21, 22].

Our findings have implications for supplemental antioxidant therapy in diabetic retinopathy for preventing ocular angiogenesis resulting as a consequence of elevated lipid peroxides.

Financial disclosures None.

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