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Increased protein carbonylation of red blood cell membrane in diabetic retinopathy

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ABSTRACT

We investigated the protein carbonylation of red blood cell (RBC) membrane in type 2 diabetic patients and the potential implication of carbonyl/oxidative stress in reflecting disease severity. Sixty-four diabetic patients with or without retinopathy of variable clinical severity (Groups DR and DM, respectively) and 20 healthy controls were included in the study. Protein carbonyls were determined in RBC membranes by immunoblotting. Compared to healthy volunteers, the RBC membranes of diabetic patients were characterized by significantly increased levels of carbonylated proteins. The carbonylation of Group DR was higher compared to that of Group DM. The subgroup of patients with proliferative retinopathy exhibited a trend towards a significant increase in protein carbonyls, compared to both free-of-retinopathy diabetic cases and non-proliferative diabetic retinopathy cases. The correlation between the chemical modifications of the erythrocyte membrane proteins and the clinical severity of diabetic retinopathy suggests a potential utility of membrane carbonylation as a marker and risk factor in the development of retinopathy.

Introduction

The oxidative stress, defined as an imbalance between oxidants and antioxidants, is considered a hallmark of the pathophysiology of diabetes mellitus. Different mechanisms seem to be related to the glucose-based reactions that account for the genesis of oxidative stress in this disease, including glucose autoxidation (Wolff and Dean, 1987), protein glycation, and formation of advanced glycation end-products (AGEs) (Brownlee et al., 1988). The red blood cells (RBC) have been reported to undergo oxidative stress in diabetes, reflected by the disturbed expression or activity of free radical scavenger systems (Martin-Gallan et al., 2003) and by the free radical-associated modifications in their ultrastructure (Straface et al., 2002) and in RBC membrane components (Straface et al., 2002; Ahmed et al., 2006).

The increased levels of reactive oxygen species (ROS) in diabetic RBC worsen their compromised functions, leading to diminished lifespan and deformability (Schwartz et al., 1991) and to increased microviscosity (Jones and Peterson, 1981), aggregation, and adhesiveness to the endothelial layer, facilitating thrombotic events (Wautier et al., 1981). As a result, in diabetes there is an intrinsic erythrocyte abnormality that is related to vascular disease (Wautier et al., 1981). Diabetic retinopathy remains the most common microvascular complication suffered by diabetic patients (Frank, 2004). It is classified into two clinical forms: non-proliferative and proliferative, the first often progressing to the sight-threatening second one. Non-proliferative diabetic retinopathy is manifested by abnormal capillary permeability, microaneurysm formation and microhemorrhages. In the proliferative stage, capillary closure causes widespread retinal ischemia, which in turn drives neovascularization, the hallmark of proliferative diabetic retinopathy. Although various pathogenic mechanisms have been proposed for diabetic retinopathy, the underlying molecular pathways remain largely unveiled. Increased blood viscosity (Lowe et al., 1980) and the rheological/biomechanical disorders of the diabetic erythrocytes mentioned above have been implicated in the development of diabetic retinopathy, while major clinical trials have established that hyperglycemia is a principal and underlying cause of this disease (The Diabetes Control and Complications Trial Research Group, 1993).

Hyperglycemia is also associated with a rise in the levels of reactive carbonyl compounds in diabetic cells, serum and tissues, a phenomenon known as “carbonyl stress” (Baynes and Thorpe, 1999; Constantin et al., 2005). The increase in reactive carbonyls, derived from both ROS-mediated and non-oxidative reactions (like the glycation Amadori product reaction), leads to various pathogenic consequences, including accelerated non-enzymatic modification of proteins, loss of protein functions and finally oxidative stress and tissue damage. Either as a consequence or as a contributor, protein carbonyls are considered sensitive indices of oxidative injury and stress to proteins, cells and tissues in many diseases (Levine et al., 1994; Aksenov et al., 2001; Dalle-Donne et al., 2003). Therefore, the determination of protein carbonyls has been used for the assessment of oxidative stress in diabetes, although the findings, at least regarding

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Table 1
Classification of diabetic retinopathy according to the Early Treatment Diabetic Retinopathy Study (ETDRS) grading system.

Severity level of DR	Definition
No retinopathy	Absence of diabetic lesions
Mild NP-DR	Microaneurysms only, or microaneurysms plus hard exudates, soft exudates (cotton-wool spots) and/or mild retinal hemorrhages
Moderate NP-DR	Microaneurysms plus mild or moderate IRMA, moderate or severe retinal hemorrhages, or venous beading in 1 quadrant only
Severe NP-DR	Severe retinal hemorrhages in 4 quadrants, or venous beading in at least 2 quadrants, or severe IRMA in at least 1 quadrant
Mild P-DR	NVE<1/2 disc area in 1 or more quadrants
Moderate P-DR	NVE≥1/2 disc area in 1 or more quadrants, or NVD<1/4–1/3 disc area
High-risk P-DR	NVD≥1/4–1/3 disc area and/or vitreous hemorrhage

NP-DR = non-proliferative diabetic retinopathy; P-DR = proliferative diabetic retinopathy; IRMA = intraretinal microvascular abnormalities; NVE = new vessels elsewhere; NVD = new vessels on or within 1 disc diameter of optic disc.
Based on the *Abbreviated Summary of the ETDRS Final Scale of Diabetic Retinopathy Severity for Individual Eyes* (Diabetes Control and Complications Trial Research Group, 1995).

the plasma proteins, have often been controversial. Whereas some studies (Telci et al., 2000; Martin-Gallan et al. 2003) showed pathologically increased plasma protein carbonylation in complication-free type 1 and type 2 diabetic patients, other studies reported opposite results (Odetti et al., 1999; Kalogerakis et al., 2005). Furthermore, an interrelation between protein carbonyl levels and the development of diabetic complications has been reported for microangiopathy cases (Konukoglu et al., 2002; Adaikalakoteswari et al., 2006) but rarely for a diabetic retinopathy group of patients (Pan et al., 2008), further subdivided according to the clinical forms of the disease. Thus, in the present study we estimated the RBC membrane protein carbonyls in type 2 diabetic patients with or without retinopathy and we searched for a potential relationship between this biochemical marker and the clinical severity of retinopathy in type 2 diabetes mellitus.

Methods

Subjects

In this prospective, cross-sectional study, a total of 64 type 2 diabetes mellitus patients and 20 healthy volunteers were included and were grouped as follows: (a) patients with diabetic retinopathy of variable clinical severity (Group DR, $n=45$); (b) diabetic patients without retinopathy (Group DM, $n=19$); and (c) healthy non-diabetic age-matched volunteers who served as controls (Group C, $n=20$). The patients of Group DR were further subdivided into two subgroups: (a) patients with non-proliferative diabetic retinopathy (Group NP-DR, $n=19$) and (b) patients with proliferative diabetic retinopathy (Group P-DR, $n=26$). The classification of diabetic retinopathy was performed according to the Early Treatment Diabetic Retinopathy Study (ETDRS) grading system (Table 1), based on seven-field stereoscopic color fundus photographs (Early Treatment Diabetic Retinopathy Study Research Group, 1991; Diabetes Control and Complications Trial Research Group, 1995).

All study subjects had normal renal and hepatic function and had no evidence of any acute infection or active inflammatory process. All of Group DM diabetic patients were clinically free of complications, except for one subject that had coronary heart disease. Exclusion of this subject did not affect the comparative significance of the measurements. The demographic, clinical and laboratory characteristics of the subjects are presented in Table 2.

In Group DR, 58% of the patients had already developed mild (8%), moderate (10%) or high risk (40%) proliferative diabetic retinopathy, whereas the remaining patients were diagnosed with non-proliferative diabetic retinopathy (mild, 4%; moderate, 22.5%; severe, 15.5%). All the proliferative diabetic retinopathy patients had active untreated proliferative diabetic retinopathy; panretinal photocoagulation was offered to all of them after the collection of blood. As expected, the duration of diabetes was higher in Group DR compared to Group DM (Table 2). None of the diabetic or control subjects were receiving vitamin E or C supplementation as part of their routine management. The proportion of smokers was small and equally distributed within Groups C, DM and DR. The proportion of obese and hypertensive patients did not differ significantly between Groups DM and DR. All diabetic groups (DM, total DR, NP-DR and P-DR) presented similar HbA1c values that were statistically different only against those of healthy subjects (Table 2). The study gained approval from the local ethics committee and informed consent was obtained from all subjects. The investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Preparation of ghosts

Peripheral venous blood was collected from all subjects into acid citrate/EDTA-containing tubes and was centrifuged (1.000 ×*g*, 10 min) to remove plasma and buffy coat. Erythrocytes were washed three times with isotonic saline and white ghosts were prepared by hypotonic lysis of RBC in phosphate buffer at 4 °C as previously described (Dodge et al., 1963), with the addition of 0.3 mM phenyl-methyl-sulfonyl-fluoride, 4 μg/ml leupeptin and 0.5 mg/ml diisopropylfluorophosphate to the lysis buffer to inhibit protease activity. Protein concentration was assayed using the Bradford protein assay

Table 2
Demographic, clinical and laboratory characteristics of study subjects.

Subject characteristic	Group C	Group DM	Group DR	Subgroup NP-DR	Subgroup P-DR
Number (*n*)	20	19	45	19	26
Gender (male/female)	13/7	13/6	24/21	8/11	16/10
Age (range) (year)	33–84	45–84	52–81	60–81	52–73
Age (mean) (year)	62.1 ± 17.0	68.3 ± 10.5	66.1 ± 6.9	69.8 ± 5.9	63.3 ± 6.4
Duration of diabetes (year)	–	10.1 ± 8.3	16.7 ± 9.1*	18.1 ± 11.0*	15.6 ± 7.5
Non-insulin/insulin treated	–	16/3	16/29**	8/11**	8/18**
HbA_{1c} (%)	5.6 ± 0.7***	9.0 ± 1.7	8.6 ± 1.5	8.6 ± 1.8	8.7 ± 1.4
Cholesterol (mg/dl)	237.7 ± 54.3	229.7 ± 41.8	227.7 ± 47.4	218.5 ± 44.5	233.4 ± 49.7
Triglycerides (mg/dl)	142.1 ± 65.5	193.3 ± 71.8	238.8 ± 167.8	222.1 ± 87.7	249.3 ± 204.9
RBC count (10^6/μl)	4.77 ± 0.71	5.15 ± 0.74	4.61 ± 0.67	4.63 ± 0.77	4.60 ± 0.60
Hematocrit (%)	41.8 ± 4.3	41.6 ± 3.8	38.2 ± 4.5	37.6 ± 4.8	38.6 ± 4.4
Hemoglobin (mg/dl)	14.2 ± 1.8	14.1 ± 1.5	12.9 ± 1.7	12.7 ± 1.7	13.1 ± 1.7

Group C: controls; Group DM: diabetes mellitus without retinopathy; Group DR: diabetic retinopathy; Group NP-DR: non-proliferative diabetic retinopathy; Group P-DR: proliferative diabetic retinopathy. Values are expressed as the mean ± SD.
* $p<0.05$ vs. Group DM (ANOVA with Tukey HSD test).
** $p<0.05$ vs. Group DM (chi-square test).
*** $p<0.05$ vs. all the other groups (ANOVA with Tukey HSD test).