Oxidative Stress in Families of Type 1 Diabetic Patients

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OBJECTIVE — The link between hyperglycemia and the complications of diabetes is unknown. It is still discussed whether oxidative stress precedes or merely reflects diabetic complications. To search for a familial predisposition to oxidative stress, we investigated indexes of glucose and lipid metabolism, markers of plasma and cell lipid oxidation, a marker of oxidant-induced protein damage, and the effects of oxygen radicals on erythrocytes (or red blood cells [RBCs]) of patients with type 1 diabetes and their relatives.

RESEARCH DESIGN AND METHODS — We recruited 30 type 1 diabetic subjects (10 without diabetic complications, 10 with retinopathy, and 10 with nephropathy), 36 nondiabetic siblings, 37 nondiabetic parents of type 1 diabetic subjects, and 3 control groups of healthy subjects without a family history of diabetes. Levels of blood creatinine, glucose, HbA_{1c}, cholesterol, triglycerides, lipoprotein(a) (Lp[a]), fibrinogen, malondialdehyde (MDA), and advanced oxidation protein products were determined. The RBC response to oxidative stress (3-h incubation at 37°C with or without a radical generating system) was evaluated by measuring RBC glutathione (GSH), RBC-MDA, and hemolysis.

RESULTS — Diabetic patients had higher levels of blood glucose ($P < 0.001$), HbA_{1c} ($P < 0.001$), Lp(a) ($P < 0.01$), and fibrinogen ($P < 0.05$) than control subjects. Siblings of diabetic patients had higher Lp(a) levels ($P < 0.001$). Parents had higher levels of plasma glucose ($P < 0.05$) and Lp(a) ($P < 0.01$). Plasma and RBC-MDA were significantly elevated in diabetic subjects and relatives compared with control subjects. Basal RBC-GSH was lower in diabetic subjects ($P < 0.01$). In diabetic subjects, incubations of cells caused a decrease in RBC-GSH of a lesser degree than that in control subjects, but they caused a significant increase in hemolysis. Among relatives, hemolysis was increased both at baseline and after incubation. Plasma MDA levels were associated with blood glucose, creatinine, and fibrinogen levels (multiple $r = 0.5$, $P < 0.001$), and basal RBC-MDA levels were associated with plasma Lp(a), fibrinogen, and plasma MDA levels ($r = 0.6$, $P < 0.001$). Basal RBC-GSH content correlated with serum glucose and RBC-MDA production ($r = 0.3$, $P < 0.01$).

CONCLUSIONS — Our study is the first to present evidence that markers of lipoprotein metabolism (Lp[a]), oxidative stress (plasma and RBC-MDA), and cellular fragility (hemolysis) are abnormal in nondiabetic relatives of type 1 diabetic subjects, thereby supporting the view that familial elements of diabetes even precede the onset of diabetes. It seems reasonable that the same biological markers considered major predictors of cardiovascular disease can also trace familial susceptibility to type 1 diabetes, just as they have been associated with the development of type 2 diabetes.

Diabetes Care 23:1182–1186, 2000

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Received for publication 18 January 2000 and accepted in revised form 13 April 2000.

Abbreviations: AOPP, advanced oxidation protein product; CV, coefficient of variation; GADA, antibody to GAD65; GSH, glutathione; ICA, islet cell antibody; IL-6, interleukin-6; Lp(a), lipoprotein(a); MDA, malondialdehyde; OGTT, oral glucose tolerance test; RBC, red blood cell; UAER, urinary albumin excretion rate.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

There is evidence for a role of inflammatory mediators in cytokine-induced pancreatic β-cell dysfunction (1). Moreover, the pathogenetic link between hyperglycemia and the complications of diabetes is unknown. Oxidative stress has been suggested to play a primary role (2). However, investigators still discuss whether oxidative stress precedes the appearance of complications or whether it merely reflects the presence of complications (3). We have previously observed that nondiabetic normotensive relatives of type 1 diabetic patients and relatives of patients with nephropathy, compared with healthy control subjects, showed increased erythrocyte (red blood cell [RBC]) sodium-hydrogen exchange activity and increased albuminuria, respectively (4). Because type 1 diabetes and its complications are assumed to arise through a complex interaction of genetic and environmental factors (5,6), a third hypothesis is equally tenable: oxidative stress even precedes diabetes. In that case, indirect evidence for increased oxidative stress could be detectable also in nondiabetic relatives of type 1 diabetic patients. To provide evidence of a familial imbalance between radical production and antioxidant defenses, we investigated indexes of glucose and lipid metabolism, markers of plasma and cell lipid peroxidation, a novel marker of oxidant-induced protein damage, and the effects of oxygen radicals on RBCs of patients with type 1 diabetes and their relatives.

RESEARCH DESIGN AND METHODS

Selection of patients

A total of 30 type 1 diabetic patients with at least one living biological sibling were recruited from the outpatient clinic. The mean duration of diabetes was 20 ± 8 years. The group consisted of 10 patients without diabetic complications, 10 patients with retinopathy (background or proliferative determined by fluorescein angiography after fundus exam), and 10 patients with nephropathy. All patients with nephropathy also had diabetic retinopathy. Among them, one was on hemodialysis (serum creatinine levels >133 μmol/l), 4 had persistent macroalbuminuria (urinary albumin excretion rate

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[UAER] >200 µg/min and serum creatinine <133 µmol/l), and 5 had persistent microalbuminuria (defined as a UAER >20 µg/min in 2 of 3 consecutive 24-h urine collections within 6 months in the absence of urinary tract infection or heart failure). All patients had been treated from time of diagnosis with at least 2 daily insulin injections and were now receiving at least 4 daily insulin injections. No patients received medical treatment, except insulin (0.6 ± 0.1 U/kg) and possibly antihypertensive drugs. A total of 36 nondiabetic normotensive siblings of type 1 diabetic patients were also studied. Only 37 parents of the diabetic patients could be recruited: of the potential 60 parents, 16 had died and 7 were excluded because of essential hypertension (2 cases), type 2 diabetes (1 case), autoimmune/inflammatory diseases such as rheumatoid arthritis and systemic lupus erithematosus (3 cases), or because they were aged >80 years (1 case). None of the relatives had clinical evidence of illness or was taking any drugs. If fasting plasma glucose levels were ≥7 mmol/l, a 75-g oral glucose tolerance test (OGTT) was performed. Three groups of healthy subjects with similar age and sex distribution as the diabetic, sibling, and parent populations, yet without family history of diabetes, were selected as control subjects. All recruited subjects gave informed consent to the study. They were examined between 8:00 and 9:00 A.M.; history was recorded, and sitting systolic and diastolic blood pressures (Korotkoff V) were measured twice and averaged after a 10-min rest. Fasting venous blood and 24-h urine collection were drawn and immediately processed for measurement of the following analyses. All measurements were performed in freshly obtained material immediately after blood withdrawal, except insulin and autoantibodies, which were measured on samples frozen at −20°C.

Biochemical measurements

Creatinine, glucose, total cholesterol, and triglycerides were measured with the BM/Hitachi system (model 717) and reagents from Boehringer Mannheim (Mannheim, Germany). HDL cholesterol was measured after precipitation with phosphotungstic acid. Albumin and lipoprotein(a) (Lp[a]) were determined by the kinetic immunonephelometric method with reagents and an automated Behring Institute nephelometer (Scoppitto, L'Aquila, Italy). Plasma fibrinogen concentrations were assayed by the thrombin-initiated clotting rate assay according to the method of Clauss (7). HbA_{1c} was evaluated by a Bio-Rad Diamat fully automated glycosylated hemoglobin analyzer system (Bio-Rad, Milan, Italy). Immunoreactive insulin and antibodies to GAD65 (GADAs) were measured by commercial radioimmunoassay kits (Anti-GAD [Medgenix Diagnostics, Fleurus, Belgium] and Anti-GAD [Biochem Immuno Systems, Milan, Italy], respectively). Islet cell antibodies (ICAs) were determined by indirect immunofluorescence tests on human pancreases. The lower limit of detection for ICAs was 5 Juvenile Diabetes Foundation units with values of 80, 91, and 88% for validity, consistency, and specificity, respectively, according to the 12th International Proficiency Program for the Standardization of ICA.

The RBC response to oxidative stress was determined according to Davies and Goldberg (8). Washed RBCs (9) were incubated at 37°C for 3 h with or without the superoxide and hydrogen peroxide generating system (2.5 mmol/l xanthine and 0.2 U xanthine oxidase/3.2-ml reaction volume). At baseline and after a 3-h incubation, aliquots of the cell suspensions were drawn to measure RBC glutathione (GSH), RBC malondialdehyde (MDA), hemolysis, and methemoglobin accumulation. RBC-GSH was estimated in the RBCs by the method of Beutler et al. (10). The peroxidation of plasma (plasma MDA) and membrane (RBC-MDA) lipids was measured according to Esterbauer and Cheeseman (11). Hemolysis was assessed by measuring the percent of hemoglobin released from incubated cells, relative to the total RBC hemoglobin content. Hemoglobin concentration of supernatants was measured according to the methods described by Van Kampen and Zijlstra (12). Methemoglobin concentration (in micromoles per liter) was calculated according to the methods described by Winterbourn (13) and was expressed as the percent of total hemoglobin content. Plasma advanced oxidation protein products (AOPPs) were measured according to the methods of Witko-Sarsat et al. (14), expressed in chloramine T equivalents, and corrected by serum albumin concentrations. The intra- and interassay coefficients of variation (CVs) resulted in the following determinations: 2 and 4%, respectively, for GSH; 6 and 9% for MDA; 1 and 5% for AOPPs; and a 2% intra-assay CV for hemolysis.

Statistical analysis

All data were expressed as means ± SD. Because of the skewed frequency distribution of urinary albumin, serum glucose, triglycerides, Lp(a), and hemolysis, their medians have been represented. Results were analyzed by a commercial software package (Systat 5 for the Macintosh) that used one-way analysis of variance for multiple comparisons and unpaired Student's *t* test (2-tailed) for single comparisons when data were normally distributed. Data that were not normally distributed were log-transformed before analysis. The χ^2 test was used to compare prevalence among groups. Correlations were sought by stepwise regression analysis and multiple linear regression. Statistical significance was defined as $P < 0.05$.

RESULTS — As shown in Table 1, the study groups were well matched for age, sex, and BMI distribution with their respective control groups. The patients with type 1 diabetes had significantly elevated levels of fasting blood glucose and HbA_{1c}. Although the diabetic patients had lipid levels similar to the control subjects, serum Lp(a) concentrations were significantly higher. The average fibrinogen level increased from the level of the control group to that of the type 1 diabetic group. The UAER was increased in the diabetic group (12 µg/min, range 4–255, vs. 6 µg/min, range 3–12, in the diabetic vs. the control group, respectively, $P < 0.01$) due to the presence of 10 subjects with nephropathy. Siblings of type 1 diabetic patients had higher circulating levels of Lp(a) than the control subjects. Parents had higher plasma concentrations of glucose and Lp(a) than control subjects. Fibrinogen, although borderline, did not reach any statistical significance in the relatives. With respect to the markers of autoimmunity, 11 diabetic patients, 2 siblings, 6 parents, and none of the control subjects were positive for circulating ICAs and/or GADAs (only diabetic patients differed significantly from the control subjects, $P < 0.001$).

With respect to the markers of oxidative stress, plasma and RBC levels of MDA were significantly elevated in type 1 diabetic patients and their siblings and parents in comparison with control subjects (Table 2). Among patients with type 1 diabetes, RBCs contained a lower concentration of GSH, the levels of which were normal in relatives. Incubations of cells with the radical generating system caused a decrease in RBC-GSH of a lesser degree in diabetic patients than in control subjects (ΔGSH −0.06 vs. −0.16, $P < 0.05$). Increases in RBC-MDA (ΔMDA

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Table 1—*Clinical characteristics of type 1 diabetic patients, siblings, relatives, and healthy matched control groups*

	Type 1 diabetic patients	Control subjects	Siblings of diabetic patients	Control subjects	Parents of diabetic patients	Control subjects
F/M (*n*)	19/11 (30)	16/14 (30)	23/13 (36)	19/17 (36)	20/17 (37)	12/18 (30)
Age (years)	34 ± 10	36 ± 10	39 ± 13	39 ± 10	58 ± 9	57 ± 9
BMI (kg/m^2)	24 ± 2	24 ± 3	25 ± 4	25 ± 4	27 ± 4	26 ± 3
Mean blood pressure (mmHg)	92 ± 13	90 ± 11	92 ± 14	93 ± 11	99 ± 11	97 ± 10
Serum glucose (mmol/l)	15.5 ± 7.2*	4.9 ± 0.5	5.3 ± 0.9	5.0 ± 0.6	5.8 ± 1.7†	5.2 ± 0.6
HbA_{1c} (%)	8.4 ± 1.5*	5.4 ± 0.3	5.4 ± 0.5	5.4 ± 0.4	5.6 ± 0.7	5.6 ± 0.4
Cholesterol (mmol/l)	5.1 ± 1.0	5.0 ± 1.0	5.2 ± 1.1	5.3 ± 1.0	5.7 ± 1.1	5.8 ± 1.0
HDL (mmol/l)	1.6 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.3
Triglycerides (mmol/l)	0.8	0.9	0.9	0.9	1.2	1.2
Lp(a) (mg/dl)	14‡	10	17*	10	11‡	10
Fibrinogen (mg/dl)	311 ± 66†	269 ± 65	312 ± 105	277 ± 65	323 ± 107	293 ± 57
Immunoreactive insulin (U/ml)	—	—	10	10	11	9

Data are *n* or means ± SD, except for triglycerides, Lp(a), and immunoreactive insulin, which are medians. *$P < 0.001$ vs. control subjects; †$P < 0.05$ vs. control subjects; ‡$P < 0.01$ vs. control subjects.

between 3-h incubation and baseline RBC-MDA values) after RBC exposure to xanthine oxidase did not differ among the populations examined. Increased hemolysis was detected after exposure of RBCs of diabetic patients to xanthine plus xanthine oxidase. Among relatives of diabetic patients, lysis of RBCs was observed to be increased above that of control subjects both at baseline and after 3 h of incubation. Significant differences among groups were not observed with respect to RBC-methemoglobin content either at baseline or after incubation (data not shown).

ICA^+ relatives did not differ from the ICA^- relatives in any clinical or biochemical variable (plasma MDA 0.5 ± 0.3 vs. 0.6 ± 0.4 μmol/l, AOPP/albumin 10 ± 5 vs. 8 ± 7, RBC-GSH 0.9 ± 0.1 vs. 0.8 ± 0.2, RBC-MDA 0.4 ± 0.3 vs. 0.4 ± 0.2, and hemolysis 0.3 vs. 0.3, respectively).

In all of the study subjects, the plasma MDA concentration was positively associated with blood levels of glucose, creatinine, and fibrinogen (multiple $r = 0.5$, $P < 0.001$); basal RBC-MDA correlated with plasma concentrations of Lp(a), fibrinogen, and plasma MDA ($r = 0.6$, $P < 0.001$). Details of multiple regression analysis are in Table 3. RBC breakdown at baseline positively correlated with plasma HDL cholesterol and Lp(a) ($r = 0.3$, $P < 0.01$), and 3-h hemolysis correlated with 3-h RBC-MDA ($r = 0.2$, $P < 0.01$). Basal RBC-GSH content correlated negatively with serum glucose but positively with ΔMDA ($r = 0.3$, $P < 0.01$).

CONCLUSIONS

Enhanced levels of free radicals found in diabetes (3,15) and impaired glucose tolerance (16) have long been assumed to be related to chronically elevated glucose levels. On the contrary, our study is the first to present evidence that an abnormal redox status clusters in families and even precedes diabetes. Indeed, markers of oxidative stress (plasma and RBC-MDA), cellular fragility (hemolysis), and lipoprotein metabolism (Lp[a]) were abnormal in nondiabetic relatives of type 1 diabetic patients. Abnormal biomarkers, which also include borderline fibrinogen levels, seemed to be mutually and positively related. Interestingly, recent studies have shown supporting evidence that links predisposition to diabetes with inflammation, which in turn is related to oxidative damage.

First, plasma Lp(a) concentrations have been shown to be elevated in type 1 diabetes (17) and to be correlated with glycemic control and proteinuria (18). In addition, plasma Lp(a) concentrations have been shown to be elevated in living parents

Table 2—*Plasma markers of oxidative stress for lipid peroxidation (MDA) and oxidant-mediated protein damage (AOPP) and the effect of 3-h exposure to xanthine oxidase on RBC-GSH, RBC-MDA, and osmotic fragility*

	Type 1 diabetic patients	Control subjects	Siblings of diabetic patients	Control subjects	Parents of diabetic patients	Control subjects
Plasma MDA (μmol/l)	0.7 ± 0.4*	0.2 ± 0.1	0.5 ± 0.3†	0.3 ± 0.2	0.7 ± 0.4‡	0.5 ± 0.3
AOPP/albumin	6.3 ± 2.9	6.1 ± 3.6	9.4 ± 10.0	7.5 ± 5.0	7.5 ± 4.0	7.0 ± 3.5
RBC-GSH (baseline)	0.76 ± 0.12†	0.88 ± 0.18	0.85 ± 0.17	0.86 ± 0.18	0.8 ± 0.2	0.9 ± 0.2
RBC-GSH (3 h)	0.70 ± 0.17	0.72 ± 0.18	0.76 ± 0.18	0.72 ± 0.18	0.7 ± 0.1	0.8 ± 0.1
RBC-MDA (baseline)	0.38 ± 0.17*	0.20 ± 0.07	0.37 ± 0.21*	0.22 ± 0.14	0.4 ± 0.2†	0.3 ± 0.2
RBC-MDA (3 h)	0.44 ± 0.19*	0.29 ± 0.09	0.49 ± 0.34†	0.30 ± 0.15	0.5 ± 0.2†	0.3 ± 0.2
Hemolysis (basal)	0.2	0.1	0.3*	0.1	0.4‡	0.2
Hemolysis (3 h)	0.9†	0.6	0.9‡	0.7	0.9‡	0.7

Data are means ± SD or medians. Tubes contained 3.2 ml of a 6% suspension of RBCs in Krebs-Ringer phosphate buffer containing glucose and 2.5 mmol/l xanthine. In control tubes, incubations were performed in the absence of both xanthine and xanthine oxidase. *$P < 0.001$ vs. control subjects; †$P < 0.01$ vs. control subjects; ‡$P < 0.05$ vs. control subjects.

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Table 3—*Details of multiple regression analysis*

	Slope*	SEM†	Student's *t* test
Plasma MDA			
vs. fibrinogen	0.002	0.0003	5.7
vs. serum creatinine	0.002	0.0004	4.1
vs. serum glucose	0.01	0.005	2.7
RBC-MDA			
vs. plasma MDA	0.2	0.03	6.0
vs. fibrinogen	0.0005	0.0001	3.6
vs. Lp(a)	0.05	0.02	2.9

*Intercept = −0.2; †r = 0.5; ‡P = 0.0001.

of type 1 diabetic patients with nephropathy (19). The higher plasma Lp(a) concentrations in our group of relatives would confirm a familial clustering of elevated Lp(a). Furthermore, new potential roles for Lp(a) in autoimmunity and fibrinolysis have been suggested (20–22).

Second, levels of Lp(a) may play an important role as acute-phase reactants in the repair of tissue injury, such as fibrinogen levels, which have been observed to be positively associated with parental history of diabetes and Lp(a) concentrations (23). Thus, a continuous and complex relationship of lipid metabolism has been suggested to occur physiologically (24). Indeed, the association between high Lp(a) serum levels and high plasma concentrations of the proinflammatory cytokine interleukin-6 (IL-6) suggested that genetic control of Lp(a) levels may be modulated by age and environmental factors, such as the chronic subclinical inflammatory process (25). Similarly, the concentrations of IL-6 and fibrinogen correlated (26–28). Thus, markers of cardiovascular risk are also acute-phase reactants (29).

Third, associations were previously seen between inflammatory markers (at concentrations lower than those characteristic of acute inflammation) and the development of diabetes in middle-aged adults (31). The suggested interpretation for these findings was that markers of inflammation probably reflect the pathogenesis of type 2 diabetes. In accord with the methods of Schmidt et al. (30), we can exclude the following variables in our study relatives: *1*) undetected diabetes, because relatives with borderline glycemia also underwent an OGTT; *2*) insulin resistance, based on fasting plasma glucose and insulin concentrations; and *3*) latent autoimmune diabetes, which we assessed differently than Schmidt et al. (30). No association was detectable between markers of inflammation and those of autoimmunity.

A few minor points also deserve attention. Membrane lipid peroxidation has previously shown a significant positive correlation with increased osmotic fragility in human RBCs (31). In the present study, basal hemolysis correlated better with HDL cholesterol and Lp(a) concentration, whereas post-stress hemolysis correlated with RBC-MDA accumulation. Lower RBC-GSH characterized type 1 diabetic patients only. The deficit depended on glycemia and seemed to be a contributing factor to RBC-MDA accumulation under oxidative stimulus. Minor changes in RBC-GSH of diabetic patients after cell incubation with a radical generating system could be due to the augmented GSH reductase activity that has been observed in type 1 diabetic patients (32). A compensatory increase in the activity of antioxidant enzymes secondary to higher oxidative stress could explain the near-normal basal hemolysis of diabetic patients (33).

In conclusion, in nondiabetic relatives of type 1 diabetic patients, we found indirect manifestations of increased oxidative stress (i.e., the detection and measurement of oxidative damage as estimated from the accumulation of oxidation products in plasma and cells). These biochemical abnormalities were significantly associated with supposed markers of inflammation, which could reflect the effects of cytokines. To our knowledge, there have been no previous reports on the presence of elevated circulating markers of lipid peroxidation and increased cellular fragility in nondiabetic relatives of type 1 diabetic patients. Is inflammation the source of oxidative damage? Alternatively, a cluster of cardiovascular risk factors, including emerging noninvasive biomarkers (34), is detectable in relatives of type 1 diabetic patients and presumably should also have been detected in the probands before the appearance of diabetes. It seems reasonable that the same biological markers considered major predictors of cardiovascular disease can also trace familial susceptibility to type 1 diabetes, regardless of their precise mechanisms.

Acknowledgments — We acknowledge the superb technical assistance of Vincenzo Cinapri and Stefano Quilici.

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