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Evaluation of chromosome aberrations, sister chromatid exchange and micronuclei in patients with type-1 *diabetes mellitus*

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ABSTRACT

Oxidative stress-induced DNA damage seems to play a role in the pathogenesis of type-1 *diabetes mellitus* and its complications. Several *in vitro* assays have been used to measure the DNA damage produced by oxidative stress. In the present study, we aimed to investigate the frequency of sister chromatid exchange (SCE), chromosomal aberrations (CA) and micronuclei (MN) in type-1 *diabetes mellitus* patients compared with healthy controls. SCE, CA and MN tests were carried out with the blood-cell cultures from 35 type-1 diabetic patients and 15 healthy, age- and sex-matched control subjects. The mean age of the type-1 diabetic patients was 31.89 ± 10.01 years, with a mean duration of the diabetes of 7.8 ± 6.02 years. The mean level of HbA1c of the type-1 diabetic patients was 8.37 ± 1.36%. Only three (8.5%) patients with type-1 *diabetes mellitus* had an HbA1c level below 7%. Patients with type-1 *diabetes mellitus* showed a higher frequency of SCE compared with controls (5.44 ± 1.47 and 2.54 ± 0.82, respectively, $p < 0.001$), but there was no significant correlation between the duration of diabetes, HbA1c and SCE. No significant difference was found in CA or MN frequency in type-1 diabetic patients compared with controls. In conclusion, these results suggest that type-1 *diabetes mellitus* is a condition with genomic instability characterized by an increased level of SCE. Hyperglycemia-induced oxidative stress may be the underlying factor of the increased SCE frequency.

1. Introduction

Oxidative stress plays a crucial role in the cellular and molecular mechanisms of tissue injury in a wide spectrum of disease states [1]. Type-1 *diabetes mellitus* is a complex multifactorial disorder caused by absolute insulin deficiency due to destruction of the pancreatic β-cells [2]. Pancreatic damage caused by oxidative stress has been involved in the pathogenesis of type-1 *diabetes mellitus* [3]. Systemic oxidative stress is present upon early onset of type-1 *diabetes mellitus* and becomes stronger over time [1]. Production of reactive oxygen species (ROS) and lipid peroxidation are increased in diabetic patients, especially in those with poor glycemic control and hypertriglyceridemia [4]. Previous studies have clearly shown that ROS, including O_2^-, $OH^\bullet$, and H_2O_2, are highly reactive and capable of damaging cellular macromolecules, including proteins, lipids and DNA [5].

During the last decades, several *in vitro* assays have been developed for measurement of genotoxicity. The sister chromatid exchange (SCE) test, analysis of chromosomal aberrations (CA) and the cytokinesis-block micronucleus (CBMN) assay in lymphocytes are being used extensively to evaluate the presence and extent of chromosomal damage in human populations [6]. There are limited reports on the association between diabetes and the occurrence of genotoxic effects. Sheth et al. [7] reported that there was a significant increase in SCE frequency in type-2 diabetic patients compared with healthy controls. However, there is no knowledge about SCE, CA and micronuclei (MN) in type-1 diabetic patients. Since free radical-induced DNA damage can be measured *in vitro*, we sought in this study to evaluate type-1 diabetes-associated genotoxicity by use of three cytogenetic assay systems. We evaluated CA, SCE and MN in cultured lymphocytes from individuals with type-1 *diabetes mellitus* and compared the results with those in healthy controls.

2. Materials and methods

2.1. Subjects

A total of 35 patients with type-1 *diabetes mellitus* and 15 age- and sex-matched healthy control subjects were studied. Patients were recruited from pre-screened type-1 diabetic patients from an endocrinology outpatient clinic of a tertiary referral center. Type-1 *diabetes mellitus* was clinically defined as a diagnosis made prior to age 30, with a continuous need for insulin therapy within one year of diagnosis

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and a C-peptide level <0.5 ng/ml. The controls had normal glucose metabolism and none had a family history of diabetes. The use of antioxidant vitamins or drugs that are able to induce the production of ROS was an exclusion criterion. The general characteristics of the study participants and lifestyle factors were collected by means of a questionnaire. Informed consent was obtained from all participants, and the study was performed in accordance with the Declaration of Helsinki and with the approval of the local ethics committee.

2.2. Lymphocyte cultures and cell harvesting

Peripheral blood samples were drawn by venipuncture into sodium-heparin tubes. For each donor, two blood cultures were set up for each genotoxicity parameter (SCE, CA and CBMN). A 0.5-ml whole blood sample was added to a culture medium (5 ml) containing RPMI 1640 medium (pH 6.8–7.2), 20% fetal calf serum, 6 μg/ml phytohemagglutinin L (PHA-L), 0.5 mg/ml L-glutamine, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) for 72 h (h) at 37 °C.

2.2.1. SCE assay

The SCE assay was performed following the addition of 5-bromo-2′-deoxyuridine (BrdUrd) at a final concentration of 10 μg/ml for the entire incubation period of 72 h. The cultures were carefully shielded from light in all steps until cell harvest. Colcemid (0.2 μg/ml) was added 70 h after initiation of all cultures. The cells were harvested and processed through treatments with a hypotonic solution (0.075 M KCl) and fixative (3:1 methanol:glacial acetic acid). Chromosome slides were identified by the fluorescence-plus-Giemsa technique [8].

2.2.2. Chromosome aberration assay

For the CA analysis, we employed the same conditions as specified above, but BrdUrd was not added to the cultures. Chromosome slides were stained with 5% Giemsa solution (pH 6.8) for 15 min.

2.2.3. CBMN assay

The CBMN assay was performed following the addition of cytochalasin B at a final concentration of 6 μg/ml after 44 h of incubation at 37 °C. The cells were harvested after 72 h and treated with hypotonic solution at room temperature for 10 min. Then, the fixation step with 5:1 methanol:glacial acetic acid was repeated four times. Slides were stained with 8% Giemsa for 15 min [9].

2.3. Microscopic evaluation

2.3.1. SCE analysis

Scoring criteria for SCE were as described by Carrano and Natarajan [10]. A total of 30 well-spread and complete ($2n = 46$) second-division metaphases per culture were scored for SCE and the frequency of SCE per cell was recorded.

2.3.2. Chromosomal aberrations

The analysis of cells with CA was performed on 50 metaphases for each culture and 100 metaphases per individual. Gaps were both included and excluded from total CA counts. CA were classified according to the recommendation of EHC 51 (Environmental Health Criteria) for short-term test for mutagenic and carcinogenic chemicals [11]. For the analyses of the CA, chromatid and isochromatid gaps and breaks, acentric fragments, and exchanges were scored. The numerical CA were not assessed in this study. The mitotic index was also calculated as the number of metaphases in 2000 cells analyzed per culture for each donor.

2.3.3. CBMN analysis

We used the protocol described by Fenech and Moorley [9] for CBMN analysis. Only cells with well-preserved cytoplasm were scored. We used the criteria for selection of bi-nucleated cells and identification of MN as reported in the HUMN project website [http://www.humn.org]. Briefly, cells having two distinct nuclei of approximately equal size, which may be connected by a fine nucleoplasmic bridge (NPB), overlapping slightly or touching each other at the edges, were selected. The number of MN (with size varying from 1/16 to 1/3 of the mean diameter of the main nucleus) located within the cytoplasm of bi-nucleated cells were scored and recorded. NPB and nuclear buds (NBUD) per 1000 bi-nucleate cells were also scored and recorded. The numbers of bi-nucleated cells with one, two, three or more MN were recorded. The data for each treatment were expressed as the frequency of MN per 2000 bi-nucleated cells. The nuclear division index (NDI) was calculated as a measure of cytotoxicity according to the following formula:

$$\mathrm{NDI} = \frac{\mathrm{MI} + 2 \times \mathrm{MII} + 3 \times \mathrm{MIII} + 4 \times \mathrm{MIV}}{500}$$

where MI–MIV represent the number of cells with one (I) to four (IV) nuclei per 500 cells, respectively [9].

2.4. Statistical analysis

The significance levels of SCE, CA and MN in the cultures of diabetic patients and controls were determined by use of the Mann–Whitney *U*-test. One-sided tests were found appropriate, considering the hypothesis of the study. Further analysis of the relative importance of the different explaining variables was done with multiple linear regression analysis. All calculations were performed using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and Statistica 6.0 statistical software packages and shown as mean ± standard deviation. A p-value of less than 0.05 was considered to correspond with statistical significance.

Table 1
General characteristics of the study participants.

	Controls	Type-1 diabetic patients
Number of subjects	15	35
Gender (males/females)	9/6	20/15
Age (years) (mean ± SD)	38.76 ± 9.12	31.89 ± 10.01
Duration of diabetes (years)	–	1–22
Smoke habit (n)		
Non-smokers	8	16
Smokers	7	19
Alcohol habit (n)		
Drinkers	0	0
Non-drinkers	15	35

3. Results

The general characteristics of the study participants are listed in Table 1. The mean age of the type-1 diabetic patients was 31.89 ± 10.01 years, with a mean duration of the diabetes of 7.8 ± 6.02 years (range: 1–22 years). Six diabetic patients (17%) had a family history of type-1 diabetes, and 12 (34%) had a family history of type-2 diabetes. The prevalences of dyslipidemia (13 patients) and thyroid disease (4 patients) were higher in patients with type-1 *diabetes mellitus*, while none of control subjects had either. All patients were using intensified insulin-therapy regimens with multiple daily injections. Insulin glargine was used as long-acting insulin for basal insulin replacement, whereas insulin lispro and insulin aspartate were used for bolus injections. Four type-1 diabetic patients were taking angiotensinogen-converting enzyme inhibitors for the presence of microalbuminuria and only one patient was using statins. There was no other medication use or chemical substance exposure among the type-1 diabetic patients or the healthy controls.

Table 2 shows the frequencies of total CA, types of aberrations and MI in the two study groups. MN and SCE test results of the study participants are shown in Table 3. There were no differences in CA and the frequency of MN between patients and controls. However, patients with type-1 *diabetes mellitus* showed a higher frequency of SCE (5.44 ± 1.47) than the controls (2.54 ± 0.82) ($p < 0.001$). NDI, NPB and NBUD per 1000 bi-nucleate cells did not differ significantly when compared with the control group. The frequency of SCE in patients and controls according to age strata and the relationship between frequency of SCE and glucose level are shown in Table 4.

Table 2
The frequencies of chromosome aberrations and mitotic index in cultured human lymphocytes from diabetic patients and control groups.

	Controls	Type-1 diabetic patients
Mitotic index	4.55 ± 1.75	4.50 ± 2.12*
Chromatid gap	0.004 ± 0.006	0.003 ± 0.008*
Isochromatid gap	0.003 ± 0.002	0.002 ± 0.003*
Chromatid break	0.000 ± 0.000	0.002 ± 0.001*
Isochromatid break	0.000 ± 0.000	0.000 ± 0.000*
Chromatid acentric fragment	0.000 ± 0.000	0.002 ± 0.001*
Isochromatid acentric fragment	0.000 ± 0.000	0.00 ± 0.000*
Chromatid exchange	0.000 ± 0.000	0.000 ± 0.000*
TCA	0.007 ± 0.009	0.009 ± 0.012*
TCA-(G)	0.000 ± 0.000	0.004 ± 0.007*

TCA: total chromosome aberrations, TCA-(G): total chromosome aberrations excluding gaps, *: $p > 0.05$; data are expressed as mean ± standard deviation. 100 lymphocytes were counted per donor for chromosome aberrations.