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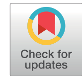
Unusual shape and structure of lymphocyte nuclei is linked to hyperglycemia in type 2 diabetes patients



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ABSTRACT

Type 2 diabetes is an endocrine disorder characterized with hyperglycemia, hyperinsulinemia and insulin resistance. Morphological changes in cell nuclei in diabetes were recently detected. The aim of this study was to compare electron microscopic features of lymphocyte nuclei in type 2 diabetes and healthy individuals using conventional computer assisted methods, fractal analysis and gray level co-occurrence matrix (GLCM) analysis of nuclear chromatin. Mononuclear cells taken from the peripheral blood of newly diagnosed type 2 diabetes patients, metformin treated type 2 diabetes patients and healthy individuals were analyzed with transmission electron microscope. Irregular nuclear contours and lower amount of heterochromatin in lymphocytes were detected with conventional computer assisted methods in type 2 diabetes. Fractal analysis of chromatin structures and GLCM angular second moment (ASM) analysis detected differences in nuclear structure between metformin treated type 2 diabetes and two other groups. Irregularities in lymphocyte nuclei correlated with blood glucose, but not with cholesterol and triglyceride levels. Decrease in fractal dimension, indicating lower level of complexity, increase in GLCM ASM, indicating higher texture uniformity, and higher amount of euchromatin that we found in metformin treated type 2 diabetes could be indicators of higher transcriptional activity in these cells.

1. Introduction

Diabetes is a common endocrine-metabolic disorder (World Health Organization, 2014). One of the main hallmarks in diabetes is hyperglycemia, accompanied with disturbances in the metabolism of carbohydrate, fat, and proteins due to the absolute or relative insulin deficiency (World Health Organization, 2014). The general categories of diabetes are type 1 (previously known as insulin-dependent diabetes) and type 2 (previously known as non-insulin-dependent diabetes) diabetes (Kasper et al., 2017). Autoimmunity is a well-known pathogenic component in type 1 diabetes (Clark et al., 2017) and recent findings of circulating autoantibodies against beta cells in type 2 diabetes (Subauste et al., 2014; Irvine et al., 1977), as well as the self-reactive T cells (Brooks-Worrell et al., 2011) led to the assumption that the pathogenesis of type 2 diabetes also includes autoimmune aspects.

Metformin is an oral drug used in type 2 diabetes to treat high blood glucose levels (Qaseem et al., 2017). The mechanisms of action of metformin are diverse (He and Wondisford, 2015). Metformin activates 5′ AMP-activated protein kinase (AMPK) through the inhibition of the respiratory chain complex I (Viollet and Foretz, 2013; Owen et al., 2000). On the other hand, there are also many effects that metformin has on cell metabolism that are not mediated by AMPK, such as control of main metabolic pathways in T cells, and consequently control of T cell growth and proliferation (Miller et al., 2013; Ben Sahra et al., 2011; Foretz et al., 2010).

Irregularities in nuclear morphology were first published in recent exfoliative cytology studies of oral mucosa cells in patients with type 1 and type 2 diabetes (Oz et al., 2014; Hallikerimath et al., 2011). Authors of these publications indicated that diabetes could produce morphological changes like binucleation and nuclear membrane irregularities in the nuclei of exfoliated buccal epithelial cells (Oz et al., 2014; Hallikerimath et al., 2011). Unusual appearance of nuclei, with deep

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indentations of nuclear membrane, open nuclear pores and irregularity in heterochromatin location were also detected in the electron microscopic study of ciliary epithelium in the experimental diabetes animals (Machowicz-Matejko et al., 2002).

To the best of our knowledge, there are no published data proving that nuclear irregularities were found in peripheral blood lymphocytes of patients with type 2 diabetes. Lymphoid cells with highly convoluted cerebriform nuclei and condensed chromatin along the nuclear membrane were for the first time described in Sezary syndrome (Lutzner and Jordan, 1968). Sezary-like cells have been also found in benign inflammatory-skin diseases, dermopathic lymphadenopathy, and in a small percentage of normal peripheral blood lymphocytes (McNutt and Crain, 1981; Meyer et al., 1977). It has been proposed then that nuclear contour irregularity in normal human lymphocytes could result from T cell activation (Reinhold et al., 1994).

Previously published morphological analyses of nuclei in diabetes were done with conventional methods that focus on the size of the nuclei and irregularities in its shape (Oz et al., 2014; Hallikerimath et al., 2011; Machowicz-Matejko et al., 2002). However, more modern and accurate methods, like fractal analysis that assess the complexity of nuclear structures have not been applied in the analysis of nuclei in diabetic patients before. We applied these methods in the morphological analysis of nuclei in peripheral blood lymphocytes of patients with type 2 diabetes.

The aim of this study was to evaluate the ultrastructural features of peripheral blood lymphocyte nuclei in type 2 diabetes, with computer-aided image analysis, fractal analysis and gray level co-occurrence matrix analysis, and to investigate the effects of metformin therapy on the ultrastructural appearance of lymphocyte nuclei in type 2 diabetes patients.

2. Materials and Methods

2.1. Study population

Subjects were assigned into three groups; newly diagnosed type 2 diabetes patients, metformin treated type 2 diabetes patients and healthy control group. Newly diagnosed type 2 diabetes patients ($n = 7$) were selected from patients that were previously not treated with antidiabetic therapy. Patients in the second group were type 2 diabetes patients ($n = 5$) that were previously treated with metformin for more than one year (5.6 ± 3.5 years). Type 2 diabetes patients who had other systemic diseases or taking medications other than the metformin were excluded. The control group ($n = 6$) was selected from healthy, age and sex matched individuals with no risk factors for diabetes. The general characteristics of patients and healthy individuals are shown in Table 1. This study was conducted with the permission of the Ethical Committee of the Clinic for Endocrinology, Diabetes and Metabolic Disease, Clinical Center of Serbia and the Ethical Committee of the School of Medicine, University of Belgrade, and it was conducted in accordance with the Declaration of Helsinki (1975). The type 2 diabetes patients were selected according to the American Diabetes Association criteria (American Diabetes Association, 2017). Fasting glucose, glycosylated hemoglobin (HbA_{1c}), cholesterol (high-density lipoproteins (HDL), low-density lipoprotein (LDL), total) and triglyceride levels were measured for each patient attending Clinic for Endocrinology, Diabetes and Metabolic Disease, Clinical Center of Serbia (Table 1). Informed consent was obtained from all patients and healthy volunteers before blood samples were taken.

2.2. Laboratory analysis

The blood sample for determination of glucose and lipid parameters was drawn from the antecubital vein, in the morning, at 08:00, after 12 h overnight fast. Plasma glucose level was determined by glucose oxidase method on Beckman glucose analyzer (Beckman Instruments, Fullerton, USA), while HbA_{1c} was determined by capillary electrophoresis (Sebia Capillarys, Evry Cedex, France). Lipid parameters, cholesterol (total, HDL) and triglyceride concentrations were analyzed using commercial enzymatic kit (Roche Diagnostics GmbH, Mannheim, Germany), while LDL-cholesterol levels were calculated by using standard Friedewald formula.

2.3. Isolation of peripheral blood lymphocytes

Peripheral blood from type 2 diabetes patients and healthy volunteers was taken in heparin containing tubes (10 ml). Isolation of mononuclear cells was done by density gradient centrifugation, using LymphoPrep (Axis Shield, Oslo, Norway). After separation, cells were washed 3 times in phosphate-buffered saline (PBS) and fixed in 3% glutaraldehyde in cacodylate buffer.

2.4. Transmission electron microscopy (TEM) morphometry

After 24 h fixation cells were postfixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epoxy medium (45345, Sigma-Aldrich, St. Louis, USA). Thin sections were cut, mounted on copper grids (G4901, Sigma-Aldrich, St. Louis, USA) and stained with uranyl acetate and lead citrate for examination on an electron microscope (Morgagni 268D, FEI, Hillsboro, OR). The sections and micrographs for the analysis were selected by using Systematic Uniform Random Sampling procedure, and the characteristics of lymphocyte nuclei were determined in 60 cells per blood sample (Lucocq and Hacker, 2013). Electron micrographs of lymphocytes were taken at the same magnification (8900x), coded, analyzed and used for quantitative evaluation. Only the sections of the cells cut through its center with the average size of 7.30 ± 1.00 µm were analyzed. Subnuclear structures that were recognizable on the above mentioned micrographs,

Table 1
General characteristics of patients and healthy individuals.

Characteristic	Healthy individuals	Newly diagnosed type 2 diabetes	Metformin treated type 2 diabetes	p
n	6	7	5	
Sex (male/female)	3/3	3/4	2/3	0.950
Age (years)	55 ± 5	59 ± 9	51 ± 9	0.285
Fasting plasma glucose (mmol/l)	5.46 ± 0.18	7.19 ± 1.73	11.6 ± 4.23 ^{a,d}	0.002
HbA_{1c} (%)	5.2 ± 0.26	6.4 ± 1	11.3 ± 2.5 ^{a,c}	0.000
HbA_{1c} (mmol/mol)	36.1 ± 2	55.2 ± 18.9	103.2 ± 46.2 ^{a,d}	0.002
Triglycerides (mmol/l)	1.5 ± 0.6	1.6 ± 0.6	3.3 ± 1.5^{b,d}	0.030
Total cholesterol (mmol/l)	4.9 ± 1.5	5.9 ± 1.8	6.2 ± 1.4	0.415
LDL-cholesterol (mmol/l)	3.3 ± 1.1	4.1 ± 1.8	3.8 ± 1.1	0.570
HDL-cholesterol (mmol/l)	1.4 ± 0.3	1.3 ± 0.4	0.9 ± 0.2	0.305

Differences between groups were analyzed by two-way ANOVA followed by Tukey.
$^{a}p < 0.01$ healthy vs metformin treated; $^{b}p < 0.05$ vs healthy vs metformin treated, $^{c}p < 0.01$ newly diagnosed vs metformin treated; $^{d}p < 0.05$ vs newly diagnosed vs metformin treated.

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