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ORIGINAL ARTICLE

Activities of Neutrophil Membrane-bound Proteases in Type 2 Diabetic Patients

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Background and Aims. Hyperglycemia and oxidative stress in type 2 diabetes (T2DM) provoke neutrophil overstimulation and the release and/or translocation of proteases from granules to the cell surface. Although the expression of neutrophil membrane-bound elastase (MLE) is well documented, the presence of the membrane-bound form of cathepsin B (MCB) is unknown. The aim of our study was to evaluate the neutrophil MLE and MCB activities in T2DM patients and their associations with the metabolic and clinical parameters of the disease.

Methods. Neutrophils were obtained from 47 T2DM patients and 20 control subjects. The activities of MLE and MCB and the intracellular activities of the examined proteases (ILE and ICB, respectively) were measured using fluorometric substrates. Additionally, the percentage equivalents of the activities, namely, MLE_{tot}/ILE_{tot} and MCB_{tot}/ICB_{tot}, were calculated. The susceptibility to inhibitors of both forms of the studied proteases was also determined.

Results. A significant increase in the activities of MLE, MCB, ILE, and ICB was found in neutrophils from T2DM patients compared with the control group. The percentage equivalent (contribution of the total membrane-bound activities to the total intracellular activities) was also higher. A partial resistance of the membrane-bound forms toward their inhibitors was revealed. Higher activities of both the membrane-bound and the intracellular proteases were also observed in patients with poor glycemic and metabolic control. The differences between subgroups with different therapeutic schemes were also revealed.

Conclusions. The pathophysiological implications of the neutrophil membrane-bound forms of leukocyte elastase and cathepsin B are of great importance in the development of T2DM and its complications. © 2014 IMSS. Published by Elsevier Inc.

Key Words: Neutrophils, Membrane-bound proteases, Cathepsin B, Leukocyte elastase, Diabetes mellitus.

Introduction

Although neutrophil proteases are the main weapon in the immune defense directed against invading pathogens, these proteases constitute a potential risk of host tissue injury. Normally, a diversified pool of proteases is stored intracellularly in granules and participates in the last stage of phagocytosis, remaining innocuous in the tissues. After neutrophil activation, these proteases may be released into the extracellular space and contribute to extracellular matrix (ECM) degradation. A major amount of the secreted proteases are rapidly entrapped by naturally appearing inhibitors, which prevent excessive proteolysis (1). In diabetes, it is known that degradation of the ECM by proteases occurs even in the presence of their inhibitors. The mechanism through which proteases avoid the effects of their inhibitors has not been precisely elucidated. It has been suggested that, in addition to the soluble forms of neutrophil proteases, which act extracellularly in plasma and tissues, their membrane-bound forms also exist. These forms of enzymes show similar catalytic activity to that of

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the soluble forms but are also considered as exhibiting more active hydrolytic potential. Moreover, these enzyme forms are postulated to be persistently active due to their partial resistance to inhibitor action (2,3).

Leukocyte elastase (LE; E.C. 3.4.21.37) is one of the proteases that have been shown to be expressed on the surface of various cells (macrophages and neutrophils) and that exhibits activity in its membrane-bound form. LE is a serine protease that is abundantly found in azurophilic granules and participates in non-specific host defense by killing pathogens. This protease is also capable of degrading most of the ECM and basement membrane components and of modulating inflammation through cleavage of a number of various agents (e.g., cytokines, chemokines, growth factors, and cell surface receptors) (4,5). Cathepsin B (CB; E.C.3.4.22.1) is a cysteine protease that has been described in its membrane-bound form, mainly in tumor cells. CB also occurs in the azurophilic granules of neutrophils. In addition, CB is engaged in protein turnover and also participates in tissue remodeling and degradation of the ECM. This protease is also linked to cellular signal transduction, antigen presentation, prohormones, proenzymes, and growth factors activation (6,7).

The increased expression of membrane-bound leukocyte elastase (MLE) is reported mainly on neutrophils (e.g., in inflammatory lung diseases), whereas membrane-bound cathepsin B (MCB) is mostly found in tumor cells (8,9). There is no information of their presence and activity on the surface of neutrophils in diabetes, which motivated us to address this lack. It is known that the functions of neutrophils are disturbed in diabetes, particularly type 2 diabetes. Under diabetic conditions, neutrophils (also called PMNs – polymorphonuclear leukocytes) participate in vascular endothelium injury and deepen the metabolic and clinical disorders of the disease. However, the detailed mechanism is still unclear and remains a very interesting research topic (2,10).

Due to the increased intracellular activity of leukocyte elastase and cathepsin B in type 2 diabetic neutrophils, which was revealed previously (11,12), and the lack of information on their membrane-bound forms in these cells, the aim of this study was the evaluation and comparison of neutrophil MLE and MCB activities in patients with type 2 diabetes mellitus (T2DM) and the estimation of their relationship to the metabolic and clinical parameters of the disease, which may be of interest for the effective management of diabetes.

Materials and Methods

Clinical and Biochemical Characteristic of Diabetic Patients and Control Subjects

Forty-seven patients with type 2 diabetes mellitus who were treated in the Clinic of Angiology, Hypertension, and Diabetology of Wroclaw Medical University were enrolled in this study. The patients were in a stable clinical state and exhibited no symptoms of acute infection. In accordance with the Polish Diabetes Association (PDA) clinical practice guidelines, the diabetic patients were divided into subgroups based on their levels of plasma glucose (good and poor short-term glycemic control), glycated hemoglobin (good and poor long-term glycemic control), total cholesterol (well and poorly controlled hypercholesterolemia), and triglycerides (well and poorly controlled hypertriglyceridemia) (13). A differentiation into subgroups of patients based on the HDL- and LDL-cholesterol levels was not performed because of their near-normal range. All of the above-mentioned parameters were measured using routine assays upon admission to the hospital. Moreover, the diabetic patients were divided into subgroups based on their BMI (normal weight, overweight, and obese), blood pressure (normalized blood pressure and hypertension), and type of vascular diabetic late complications (micro-, macro-, and both micro- and macro-angiopathy) according to PDA guidelines (13). Based on the pharmacological treatment of hyperglycemia, the patients were divided into two subgroups: those treated with insulin alone and those treated with insulin and oral agents (metformin and/or glibenclamide).

The control group consisted of 20 healthy adults with no abnormalities in their carbohydrate and lipid metabolisms and with no inflammatory states as determined through a routine medical check-up. All participants were informed of the aim of the study and gave their permission to enter the study. The use of human blood was approved by the local Bioethics Committee of Wroclaw Medical University. The main clinical features of the diabetic patients and control subjects are provided in Table 1.

Polymorphonuclear Leukocyte Isolation

Venous blood was collected after overnight fasting in standard vacuum tubes with heparin (16 IU/mL). Polymorphonuclear leukocytes were isolated immediately according to Zemann et al. (14) with some modifications. Briefly, all of the blood was carefully overlaid on Gradisol G (d = 1.115 g/mL, Aqa-Medica, Poland) at a ratio of 3:2 and centrifuged at 380 g and 4°C for 30 min to obtain three layers. The middle layer containing the PMNs was transferred into a separate tube, washed twice with phosphate-buffered saline (PBS), and centrifuged (380 g for 10 min at +4°C). If necessary, the remaining impurities (i.e., red blood cells) were removed by 10-min incubation with cold hypotonic lysing buffer (containing NH_4Cl, Na_2EDTA, and $KHCO_3$). PMNs were then collected by centrifugation and washed once again with PBS under the conditions as above. The isolated PMNs were suspended in PBS and counted under a microscope with the use of a Bürker chamber. The high purity and viability of the isolated PMNs were confirmed by

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