

REDUCING THE PHAGOCYtic ABILITY OF MONOCYTES IN PATIENTS WITH MULTIPLE SCLEROSIS

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SMANJENJE FAGOCITNE SPOSOBNOSTI MONOCITA KOD PACIJENATA OBOLELIH OD MULTIPLE SKLEROZE

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ABSTRACT

Multiple sclerosis (MS) is an immune-mediated disease of the CNS that is characterised by inflammation, demyelination, and axon loss. It is an autoimmune disorder involving inflammatory T cells (CD4+, CD8+) and auto-antibodies against myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). During the process of the autoimmune inflammatory attack in the CNS a large amount of apoptotic T cells is generated. Microglia and blood monocyte-derived macrophages play the most important part in the efficient clearance of these cells. Their ability to engulf the apoptotic cells efficiently is accompanied by an array of anti-inflammatory effects that are important in reaching the remitting phase.

We observed the ability of monocytes to efficiently clear apoptotic T cells in individuals with MS. It was found that the percentage of monocyte-induced phagocytosis of apoptotic lymphocytes as well as the phagocytic potential of monocytes significantly decreased ($p = 0.000$) in people with MS compared to healthy controls. Our results suggest that the reduction in the ability of monocytes to efficiently engulf a large number of apoptotic cells is connected to the inflammatory process in diseases such as MS.

Keywords: phagocytosis, monocytes, T cell, apoptosis, multiple sclerosis

SAŽETAK

Multipla skleroza (MS) je imunski posredovano oboljenje centralnog nervnog sistema (CNS) koju karakteriše zapaljenje, demijelinizacija i gubitak aksona. To je autoimunska oboljenja u kome učestvuju zapaljenjske T ćelije (CD4+, CD8+) i autoantitela prema antigenima mijelina, kao što su mijelinski bazni protein (MBP), proteolipidni protein (PLP) i mijelinski oligodendrocitni glikoprotein (MOG). Tokom ovog autoimuskog zapaljenjskog procesa stvara se veliki broj apoptotičnih T ćelija u CNS-u. Mikroglia i makrofagi (koji potiču od monocita) imaju najvažniju ulogu u efikasnom uklanjanju ovih ćelija. Njihova sposobnost da fagocituju apoptotične ćelije je praćena nizom antizapaljenjskih efekata. što je značajano za ulazak bolesti u fazu remisije.

U ovoj studiji smo pratili sposobnost monocita kod ljudi oboljelih od multiple skleroze (MS) da efikasno uklanjaju apoptotične T ćelije. Pronašli smo da je procenat monocitne fagocitoze apoptotičnih limfocita i fagocitni potencijal monocita značajno smanjen ($p = 0,000$) kod pacijenata oboljelih od MS-a. Ovakvo smanjenje sposobnosti monocita da efikasno fagocituju veliki broj apoptotičnih ćelija je povezano sa zapaljenjskim procesom u oboljenju kao što je MS.

Cljučne reči: fagocitoza, monocit, T limfocit, apoptoza, multipla skleroza

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INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated disease of the CNS that is characterised by inflammation, demyelination, and axon loss. Traditionally, MS is considered to be a CD4⁺ T helper 1 (Th1)-mediated disease (1, 2), but these T cells alone are not sufficient to produce the typical neuropathology of the disease (3, 4). It has been shown that MS is an autoimmune disease with a heterogeneity of pathogenetic mechanisms responsible for myelin destruction (5, 6). Myelin is damaged due to an autoimmune attack consisting of several pathways and molecules. The most impor-

tant myelin antigens are myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) (7-9).

In actively demyelinating lesions, T cells (CD4+, CD8+) and macrophages are dominant cells that participate in the inflammatory reaction (10 - 12). Apoptotic cell death of inflammatory T cells is an established mechanism to terminate an autoimmune inflammatory response in the rodent or human CNS. The apoptosis leukocytes lose the ability to release toxic components through receptor signals so that they are confined with-



in an intracellular protein membrane (13). The efficient clearance of apoptotic cells in the CNS prevents them from going into secondary necrosis (14, 15). When apoptotic cells go into secondary necrosis, they release their own intracellular contents, which releases many harmful substances and causes damage to the surrounding tissue and induces inflammatory disease (16-18). Cationic protein release following secondary cell necrosis further inhibits the phagocyte clearance of apoptotic cells and causes lower interstitial pH that could activate lysosomal enzymes (16). Furthermore, proteinase inhibitors may be inactivated by oxidation in tissues with dying cells (19).

In addition, the efficient clearance of apoptotic cells prevents the presentation of neoantigene determinants that are formed under the influence of caspases. This can trigger autoimmune disease in sensitive individuals.

Microglia and blood monocyte-derived macrophages constitute the primary phagocytic cells in the brain and they are the primary candidate for the clearance of apoptotic T cells (20). It is important to mention that no quantitative data has been published and no clear distinction has been made between macrophages and microglia. We therefore investigated the ability of monocytes to efficiently clear apoptotic T cells in individuals with MS. Our data demonstrate the lower ability of monocytes to engulf apoptotic T cells in patients with MS compared to healthy controls.

MATERIALS AND METHODS

Patients and controls

Twenty patients with MS were recruited at the Department of Neurology of the Medical Faculty at the University of Kragujevac. All patients had been diagnosed to have relapsing-remitting MS according to the revised diagnostic criteria (21). Patients were not given corticosteroids or immunomodulatory therapy for three months prior the blood collection. Seven healthy staff members of the same institution represented the control group. The clinical features of the subjects are summarised in Table 1.

Table 1. Clinical data of patients with multiple sclerosis (MS) and healthy controls

	Controls	Patients with MS
Total number (males)	7 (3)	20 (9)
Age	40.1 ± 9.1	43± 11.8
MS duration (years)	/	11.4± 8.1

Preparation of monocytes

To test their phagocytotic ability, monocytes were obtained from 10 ml of heparinised whole blood of patients with MS and healthy controls. Mononuclear leukocytes were separated by density gradient cen-

trifugation (Histopaque 1077, Sigma, Germany) (14, 22). Cells were washed three times and suspended in Haemacel (Hoechst Marion Rousel, Germany). Mononuclear leukocytes were put in petri dishes with autologous plasma and kept at 37°C in a 5% CO₂ atmosphere for 30 min. After incubation, non-adherent lymphocytes were washed out with Haemacel. Adherent monocytes were slowly removed using a scraper and were then washed with Haemacel. Cell number and viability were determined using AO/EB staining (Sigma, Germany).

Preparation of apoptotic lymphocytes

We used peripheral blood lymphocytes obtained from the patients with chronic lymphocytic leukaemia (CLL) to obtain apoptotic cells. After separation, CLL mononuclear leukocytes were washed three times in RPMI 1640 culture medium (Sigma, Germany) and suspended in RPMI 1640, supplemented with 20% of autologous plasma, and kept at 37°C in a 5% CO₂ atmosphere until further use. Cells were incubated in 100 µg/ml of CHX (Oncogene, Germany) for up to 24 hr at 37°C in 5% CO₂ atmosphere to induce apoptosis. Cell number and viability were determined using AO/EB staining (Sigma, Germany).

In vitro phagocytosis assay

Apoptotic lymphocytes were collected, washed two times in Haemacel to remove CHX, and suspended in 1 ml of Haemacel. To differentiate between tested and apoptotic cells, we induced an increase in the membrane permeability of apoptotic cells by incubating them in a water bath at 80°C for 10 min. As a result, the membranes of early and late apoptotic cells became permeable to EB. Apoptotic cells stained with AO/EB emitted red fluorescence and were later easily distinguished from the green viable cells. After finishing this stage, apoptotic cells were centrifuged and stained with 10 µl of AO/EB. Because of the fast absorption of stains, the excess of stains were immediately removed by washing the cells in 5 ml of Haemacel. The assay was performed by adding 0.1x10⁶ of the tested monocytes and 20 µl of autologous serum. Haemacel was added to make a final volume of 150 µl that was added to 4x10⁶ apoptotic cells. The suspension was then centrifuged for 5 min at 200 g and incubated at 37°C for 1 hr. After the incubation period, the cell suspension was washed once with ice-cold 0.02% EDTA (Merck). Cells were resuspended in 30 µl of cold EDTA and kept at 4°C until analysis.

Fluorescent microscopy analysis

Samples of 10 µl were taken and stained with 0.5µl AO/EB. Apoptotic cells emitted red fluorescence and viable monocytes emitted green fluorescence. Phagocytosed apoptotic cells were visualised as red cells engulfed by tested monocytes with a green nucleus. When using light



microscopy, engulfed cells were seen as circles surrounded by the cell membrane of the monocytes. The percentage of monocytes engulfing at least one apoptotic cell defined the percentage of phagocytosis (PP). The percentage of phagocytosis was determined by the examination of at least 1000 green cells. The absolute number of phagocytes (AN) was calculated by multiplication of PP and the number of monocytes in 1 ml of whole blood. This value was the measurement of phagocytic potential of the tested monocytes.

Statistical analysis

Results are expressed as mean \pm standard deviation (SD). Statistical significance of the differences between the groups ($p < 0.05$) was evaluated using an unpaired independent samples t-test.

RESULTS

Phagocytosis of apoptotic cells by monocytes

As the previous studies showed, monocytes were capable of engulfing apoptotic cells (23, 24), so a comparative measurement of the percentage of phagocytosis of apoptotic lymphocytes by monocytes and the absolute phagocytic potential of monocytes in the individuals with multiple sclerosis and in the healthy control was conducted in this experiment.

Furthermore, by measuring the percentage of the phagocytosis of apoptotic lymphocytes by monocytes a significant decrease ($p = 0.000$) was found in the patients with MS ($6.551 \pm 2.665\%$) in comparison to the healthy control ($14.143 \pm 3.437\%$) (Fig.1). Considering the fact that the better evaluation of the disorder in monocyte-induced phagocytosis is obtained by measuring their absolute phagocytic potential, a comparative analysis of the absolute phagocytic potential of monocytes was conducted. We found that the phagocytic potential of monocytes significantly decreased ($p = 0.000$) in people with MS (0.208 ± 0.083) compared to healthy controls (0.427 ± 0.051) (Fig. 2).

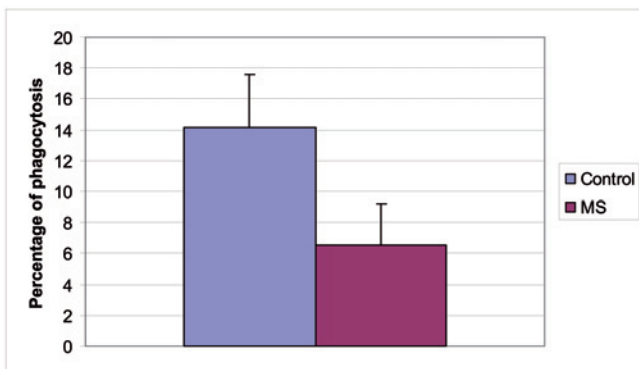


Figure 1. Percentage of phagocytosis of apoptotic lymphocytes by monocytes.

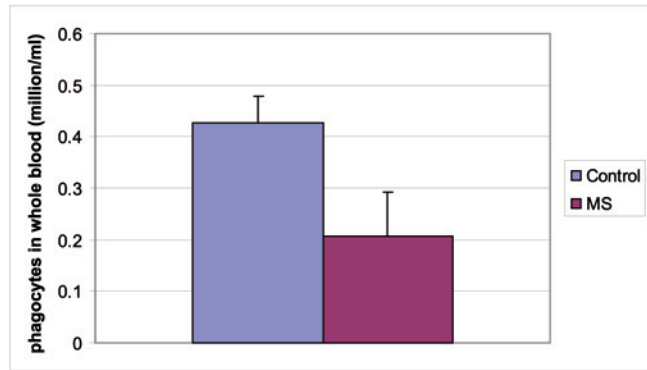


Figure 2. Phagocytic potential of monocytes in 1 ml of whole blood

DISCUSSION

A large amount of apoptotic cells is generated during the process of the autoimmune inflammatory attack of the CNS in people with MS. One of the most important mechanisms to the resolution of inflammation in the CNS is the efficient clearance of the large number of apoptotic inflammatory T cells (15) as well as other apoptotic cells such as oligodendrocytes and neurons (25) that are generated during this process. It has been shown that clinical recovery in patients with MS is connected to the loss of inflammatory cells in the CNS and in the peripheral immune system (26, 27).

Brain microglia, along with blood monocyte-derived macrophages, play the most important part in the clearance of apoptotic cells produced in this way (20). The reduction in their ability to efficiently engulf a large number of apoptotic cells in the CNS causes the relapse in MS. We have shown a significant decrease in the phagocytic potential of monocytes in people with MS compared to healthy controls ($p = 0,000$). The importance of this process in MS is also documented in the fact that the phagocytosis of apoptotic cells significantly alters the macrophages' states of immune activation, which is accompanied by an array of anti-inflammatory effects.

Many studies have shown that phagocytosis of apoptotic cells by macrophages influences their cytokine secretion. After engulfing the apoptotic cells, macrophages secrete considerably less pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-12, tromboxane B2, leucotriene C4, and granulocyte-monocyte colony-stimulating factor (GM-CSF) (28). This process, however, results in the increased secretion of anti-inflammatory cytokines such as transforming growth factor (TGF)- β 1, prostaglandin (PG)E2, and platelet-activating factor (PAF) (28- 30). It is important to note that at the same time, the secretion of IL-10 is decreased. In addition, the release of TGF- β 1, PGE2, and PAF affect the macrophages, causing the decrease in pro-inflammatory cytokine secretion (28).

Following the phagocytosis of apoptotic cells, macrophages / microglia reduce the B7-2 co-stimulatory ex-



pression, leading to the inhibition of T cell proliferation (14).

Our findings suggest that the importance of the ability of monocytes to engulf apoptotic cells is fairly signifi-

cant in disorders such as MS. Future studies investigating the interaction of monocytes and apoptotic cells may provide new important insights into such disorders.

ABBREVIATIONS

AO- acridine orange, EB- ethidium bromide, CHX- cycloheximide, CLL- chronic lymphocytic leukaemia, CNS- central nervous system, EDTA- ethylene diaminetetraacetic acid, IL- interleukin, MS- multiple sclerosis

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