ORIGINAL ARTICLE

THE VIABILITY OF LEUKOCYTES AND REACTIVE OXYGEN SPECIES GENERATION BY THEM IN RATS WITH CHRONIC COLITIS

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ABSTRACT

The aim: To assess reactive oxygen species production by leukocytes and their viability in rats with chronic colitis.

Materials and methods: Reactive oxygen species production was estimated in leukocytes, isolated from rats with Dextran Sulfate Sodium-induced chronic colitis and control rats, by flow cytometry using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate. Leukocyte viability and apoptosis stages were assessed by flow cytometry using annexin V and 7-aminoactinomycin D staining. White blood cell counting was carried out with using Hematology Analyzer.

Results: The increased fluorescence intensity of 2',7'-dichlorofluorescein in viable leukocytes by 36.7% was revealed in rats with chronic colitis compared control rats. A significant decrease in the percentage of viable cells and an increase in apoptotic cells were found compared to intact animals. Leukocytes, granulocytes, monocytes, lymphocytes counts in blood of experimental group animals were significantly higher compared to control those.

Conclusions: Our findings indicate that Dextran Sulfate Sodium-induced chronic colitis increases an intracellular production of reactive oxygen species by leukocytes. Despite of increased leukopoiesis it reduces viability of white blood cells and promotes their apoptosis via stimulation of oxidative stress.

KEY WORDS: leukocytes, rats, reactive oxygen species, apoptosis, colitis

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of inflammatory bowel diseases (IBD). Oxidative stress is considered to be one of the main mechanisms involved in the pathophysiology of IBD [1, 2]. However, it should be noted that most studies that examined the interrelation between IBD severity and oxidative stress were not able to show a significant correlation between them [3].

Ulcerative colitis is also associated with excessive neutrophil infiltration [4]. In inflammation the main source of reactive oxygen species (ROS) in tissue are neutrophils and monocytes. Reactive oxygen species formed by neutrophils are necessary not only to eliminate the pathogen, but also for neutrophil apoptosis [5]. Neutrophil apoptosis plays an important role in an inflammation resolution [6]. Delayed neutrophil apoptosis leads to chronic inflammatory diseases [7]. Although ROS directly promotes and/or regulates neutrophil apoptosis, there is no consensus on the role of ROS on their lifespan [6].

Data on the relationship between ROS levels and severity of IBD are contradictory. Thus, according to Delson L A et al. (2018), mutations in genes encoding NADPH oxidases in children with CD are associated with reduced ROS production and a more aggressive course of the disease [8]. On the contrary, the reduced colonic inflammation during DSS-induced colitis and less ROS generation by bone marrow neutrophils after lipopolysaccharide stimulation are observed in dendritic cell immunoreceptor 1-knockout mice compared to wild type [9]. In addition, the blood plasma of patients with UC has attenuated inhibitory effects on the formation of ROS compared with the blood plasma of healthy people [10].

The study of ROS generation by blood leukocytes, their counts and apoptosis in DSS-induced colitis rats will not only clarify the relationship between ROS production and apoptosis, but also assess the possibilities of environmental influences (blood plasma) on these processes and an involvement the changes in these processes in leukocytes to extragastrointestinal manifestations of colitis.

THE AIM

The aim is to assess reactive oxygen species production by leukocytes and their viability in rats with chronic colitis.

MATERIALS AND METHODS

The study used 12 adult WAG rats, divided into two equal groups. Control group rats were fed on a standard diet. Water was provided ad libitum. Experimental group included the rats with chronic colitis induced by oral administration of 2.5% solution (weight/volume) Dextran Sulfate Sodium (DSS) (molecular weight: 40 kDa; PanReac AppliChem, Germany) in drinking water according the scheme: from

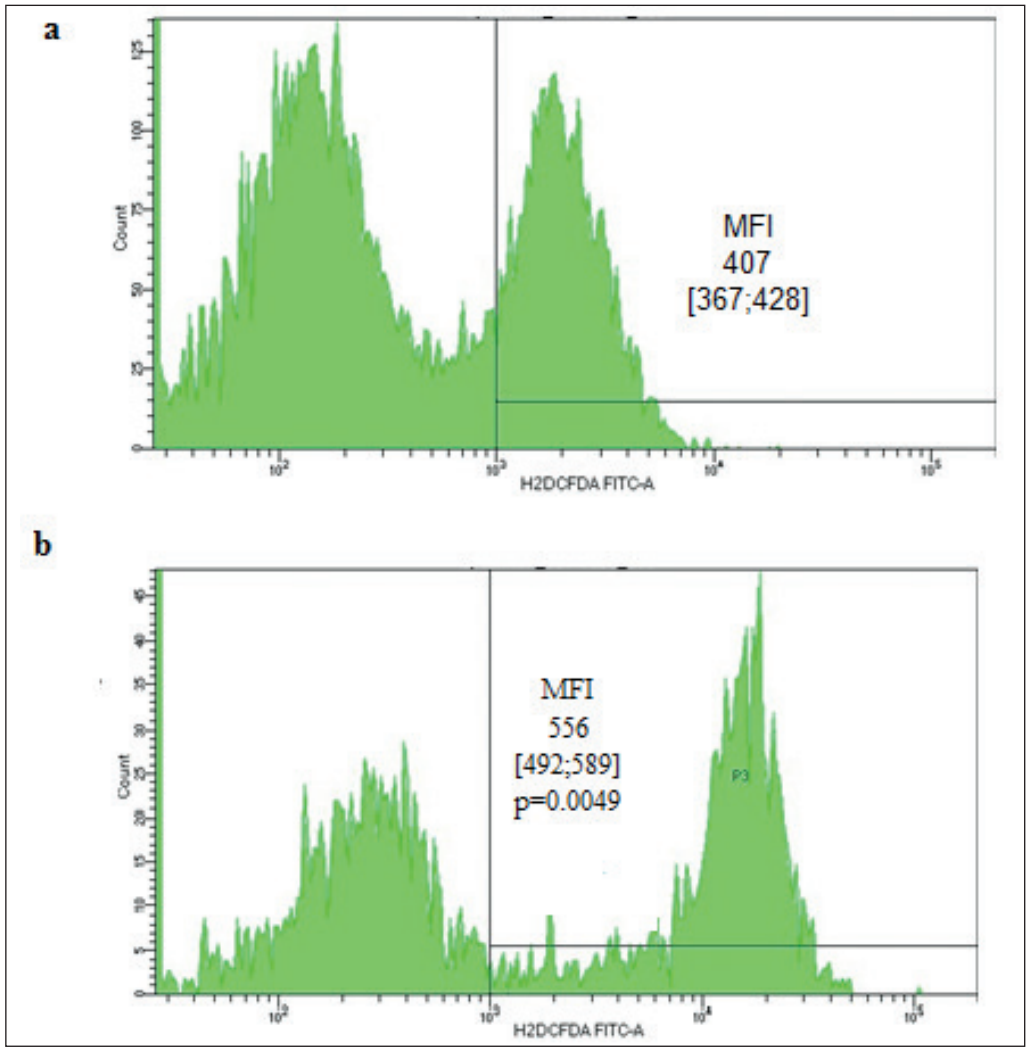

Fig. 1. Representative SSC/FL1 (2',7'-dichlorofluorescein diacetate, H2DCFDA) histograms of a control animal (a) and a rat with DSS-induced chronic colitis (b). Mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) in CD45+, 7-AAD– cells is represented as Me [25%; 75%].

1st to 5th days, from 13th to 17th days, from 25th to 29th days the animals from the experimental group were orally administered DSS solution in drinking water; from 6th to 12th days, from 18th to 24th, from 30th to 38th days they received drinking water. On day 39, the animals were removed from the experiment with a guillotine knife [11-12].

Blood samples were collected to sterile vacutainer test tubes (IMPROVACUTER Evacuated EDTA K2 Spray Dried PET Tubes, Guangzhou, China) containing ethylenediaminetetraacetic acid dipotassium salt (K2EDTA). Blood samples were used for clinical blood test and leukocyte suspension preparation. To prepare leukocyte suspensions, aliquots of blood (100 µl) obtained from each animal were lysed and washed twice with Pharmlyse solution (Becton Dickinson, San Jose, USA) and phosphate-buffered saline (PBS), respectively. Leukocyte suspensions were used for further evaluation of ROS levels in living leukocytes and for analysis of leukocyte viability and their death modes.

To detect intracellular ROS concentration, the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was employed. It is cleaved by intracellular esterases to form 2',7'-dichlorodihydrofluorescein, which is transformed by ROS into highly fluorescent 2',7'- dichlorofluorescein (DCF). Fluorescence degree of last reflects the intracellular ROS levels. According to the staining protocol used, in order for discrimination of leukocytes their suspensions in 100 µl PBS were stained with 10 µl APC-Cy™ 7 mouse anti-rat CD45 (BD Pharmingen, USA) for 15 minutes. The viable cells in the $CD45^+$ cells regions were then selected by staining with 5 µl 7-aminoactinomycin D (7-AAD, BD Pharmingen, USA) for 15 minutes. When cell membrane integrity is impaired, 7-AAD can enter the cell and bind to double-stranded DNA. The viable leukocytes were negatively stained with 7-AAD. So, $CD45^+/7AAD^-$ cells were incubated with freshly prepared 5 µM H2DCFDA (InvitrogenTM, USA) working solution in PBS at 37°C for 30min. The FL-1 channel was used to detect the fluorescence of DCF. The mean fluorescenceintensity (MFI) of DCF was analyzed using BD FACSDiva™software (Becton Dickinson, USA) for quantitative assessment of intracellular ROS production.

The viability of leukocytes and cell death modes were assessed with flow cytometry. Leukocyte suspensions (100 µL) were incubated for 15 min in the dark at room temperature with 10 µL APC-CyTM 7 mouse anti-rat CD45 (Becton Dickinson, USA), 5 µL FITC Annexin V, and 5 µL 7-AAD (Becton Dickinson, USA). Then 400 µL of 1 × binding buffer was added to each test tube. Flow cytome-

Table I. Mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) in ROS intermediate/high CD45+, 7-AAD– cells of rats with DSS-induced chronic colitis

Groups of animals	Median	25% Percentile	75% Percentile
Control group	401.0	367.0	427.8
Experimental group	555.5 **	492.3	589.0

Note: ** - P <0.01 compared to the control group

Table II. Viability and cell death modes of leukocytes in rats with DSS-induced chronic colitis

Parameters	Control group			Experimental group		
	Median	25% Percentile	75% Percentile	Median	25% Percentile	75% Percentile
Viable leukocytes (%)	97.60	96.95	98.30	66.90 **	65.25	69.75
Early apoptotic cells (%)	0.65	0.40	0.83	25.40 **	24.00	26.93
Late apoptotic/ necrotic cells (%)	0.95	0.40	1.30	4.75 **	3.15	5.35
Dead necrotic leukocytes (%)	0.80	0.80	0.90	2.85 **	2.55	4.83

Note: ** - P <0.01 compared to the control group

try was performed with a FACSCanto™ II flow cytometry system (Becton Dickinson, USA) counting 10,000 events per measurement. FACSDiva™software (Becton Dickinson, USA) was used to assess the flow cytometry results. First of all, the region of $CD45^+$ cells was gated. Then, Annexin V and 7-AAD stained $CD45^+$ cells were analyzed. Dye-conjugated annexin V is able to label phosphatidylserine (PS), an early marker of apoptosis, on the extracellular membrane. In late-stage apoptosis, when cell integrity is damaged, it binds PS on the inner side of plasma membrane. 7-Aminoactinomycin D is fluorochrome which binds to double stranded DNA and can be used to stain non-viable cells. This staining is used to identify four possible states of leukocytes: 1, viable leukocytes (Annexin V^-, 7-AAD^- cells); 2, early apoptotic cells (Annexin V^+, 7-AAD^- cells); 3, late apoptotic/necrotic cells (Annexin V^+, 7-AAD^+); 4, dead necrotic cells (Annexin V^-, 7-AAD^+).

Lymphocyte, monocyte, granulocyte and total leukocyte counts were determined with using ABX Micros 60 ES Hematology Analyzer, France (10 µl of whole blood were used for analysis).

Statistical data processing was carried out using GraphPad Prism 5 Software (GraphPad Software, USA). Comparisons between two independent groups of variables were performed using a non-parametric Mann–Whitney U test. Results are represented as medians and interquartile ranges. Differences were considered significant at $p<0.05$.

The research was carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123). The Commission of Ethics and Bioethics approved the study design (Kharkiv National Medical University, Kharkiv, Ukraine; Protocol #6 d.d. 2 October 2019).

RESULTS

Analysis of DCF fluorescence in $CD45^+$, 7-AAD^- cells (viable leukocytes) revealed the fluorescence intensity increase in rats with chronic colitis by 36.7% compared control rats (fig. 1, table I). This indicates significantly elevated ROS levels in such subpopulations of WBC, as monocytes and granulocytes, in rats with DSS-induced chronic colitis compared to the control.

At the same time, the study of viability and apoptosis of leukocytes in the blood of experimental rats showed a significant decrease in the percentage of viable cells and an increase in apoptotic cells compared to intact animals (table 2). In the percentage the viable $CD45^+$ cells decreased by 1.5-fold ($p<0.01$), apoptotic cells increased (early apoptotic cells by 38.6-fold, $p<0.01$; late apoptotic/necrotic cells by 5-fold, $p<0.001$; dead necrotic leukocytes by 3.6-fold, $p<0.01$) in blood of experimental group rats compared to control group. This argues a greater predisposition to apoptosis and less stability of leukocytes in rats with chronic colitis compared with controls.

Nevertheless, the total number of leukocytes and counts of every of their fractions (granulocytes, monocytes, lymphocytes) in blood of experimental group animals were significantly enhanced compared to control those. Thus, the number of leukocytes increased by 62.6% (8.05×10^9 vs. 4.95×10^9, $p<0.01$), lymphocytes by 58% (5.85×10^9 vs. 3.7×10^9, $p<0.01$), granulocytes by 33% (1.4×10^9 vs. 1.05×10^9, $p< 0.01$), monocytes by 216.7% (0.95×10^9 vs. 0.3×10^9, $p<0.01$) in the blood of rats with colitis compared with control group rats.

DISCUSSION

Neutrophils are the most numerous fraction of phagocytic white blood cells; therefore, it is neutrophils that are the main

source of ROS in our study. Neutrophil-produced ROS are involved not only in the pathogen neutralization, but also in the neutrophil apoptosis regulation [5, 13]. ROS generation is triggered by NADPH oxidase. The induction of NADPH oxidase assembly in the plasma membrane leads to release of superoxide to the extracellular compartment or into preformed phagosome. Contrary, the induction of of NADPH oxidase assembly in the intracellular membrane (probably the granule membrane) results in intracellular production of superoxide [14]. According to the review work of Karsten Kruger, the sustained excessive ROS production can result in apoptotic cell death by the intrinsic pathway of apoptosis [15]. In our study namely intracellular ROS were determined. We found elevated levels of ROS in the blood leukocytes of the experimental group animals compared with the controls. And the increased ROS generation was accompanied by enhanced apoptosis and reduced viability of leukocytes.

We speculate that the intensive production of ROS by leukocytes of these animals is due to their activation in the bloodstream under the influence of pro-inflammatory cytokines and/or the programed altering expression of related to apoptosis proteins (death receptors, proapoptotic or antiapoptotic proteins) on differentiaton and maturation stages. This assumption is confirmed by literature data. Thus, according to study of Avdagić N et al. [16], TNF elevated levels were found in patients with CD and patients with UC as compared healthy persons. According to the literature, the effect of TNF on the survival of neutrophils depends on its concentration: at low concentrations it increases the survival of neutrophils, at high - aggrevates their apoptosis [17]. The opposite TNF-α effects are caused by involving different elements of antiapoptotic machinery: caspase-dependent turnover of the antiapoptotic protein Mcl-1 in high concentrations and TNF-α - mediated activation of Bfl-1 expression in low concentrations [18].

In addition to that, neutrophil subpopulations are distingueshed by their sensitivity to the same TNF-α concentration: one of them rapidly losses the mitochondrial membrane potential and is involved into apoptosis; the other one has elevated mitochondrial membrane potential that facilitates an apoptosis delay [18]. In a review article [13] it is noted that the reduction of transmembrane potential of mitochondria promotes the release of proapoptotic proteins such as: cytochrome c, second mitochondria-derived activator of caspases (Smac)/DIABLO (direct IAP-binding protein with low pl), apoptosis-inducing factor (AIF), and endonuclease G.

So, our data indicate increased myelopoiesis and lymphopoiesis in animals with chronic DSS-induced colitis. However, the ratio between the individual fractions of leukocytes is practically unchanged, with the exception of monocytes. The intensified ROS generation and the increased leukocyte apoptosis were observed in rats with chronic DSS-induced colitis.

CONCLUSIONS

Our findings indicate that DSS-induced chronic colitis increases an intracellular production of ROS by leukocytes. Despite of increased leukopoiesis it reduces viability of white blood cells and promotes their apoptosis via stimulation of oxidative stress.

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Conflict of interest:

The Authors declare no conflict of interest.

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