



Apoptosis and secondary necrosis of neutrophils and monocytes in the immunopathogenesis of rheumatoid arthritis: a cohort study

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Abstract

Rheumatoid arthritis (RA) is a progressive chronic inflammatory and autoimmune joint disease. Neutrophils and monocytes are the main target cells of innate immune defense that modulate the course of inflammatory rheumatic diseases. Dysfunctional phagocytosis is a common feature in RA. The aim of this study was to evaluate the diagnostic value of apoptotic changes in neutrophils and monocytes and their relationship with rheumatoid activity measured by the DAS28 score. We used the APOLECT flow cytometric assay for evaluating primary necrotic, apoptotic, and secondary necrotic neutrophils and monocytes determination in RA patients compared with healthy controls. The apoptotic granulocytes were greater in RA patients compared to healthy controls ($0.76 \pm 0.15\%$ vs. $0.58 \pm 0.17\%$, $P < 0.05$). The percentage of primary necrotic granulocytes was significantly elevated in RA patients compared to healthy controls ($3.84 \pm 0.5\%$ vs. $1.96 \pm 0.33\%$). No significant difference was noted for primary necrotic monocytes. The number of secondary necrotic granulocytes and monocytes was high in RA patients ($0.94 \pm 0.15\%$ vs. $0.4 \pm 0.06\%$ and $4.83 \pm 1.06\%$ vs. $1.8 \pm 0.33\%$, respectively). The obtained results suggest that neutrophils and monocytes undergo apoptotic modifications which are accompanied by secondary necrotic cells formation in RA. These shifts may lead to autoantigen accumulation that results in the progressive course RA.

Keywords Rheumatoid arthritis · Neutrophils · Monocytes · Apoptotic cells

Introduction

Rheumatoid arthritis (RA) is a progressive inflammatory rheumatic disease which is characterized by synovial hyperplasia, joint cartilage destruction, and extra-articular manifestations [1]. Neutrophils are primary target cells that infiltrate the sites of tissue inflammation in RA and attract other inflammatory cells such as monocytes [1]. Neutrophil activation leads to the degranulation and release of bioactive agents that damage collagen matrix within the cartilage [2].

These agents aid in removing dead cells or apoptotic particles and send signals about invading foreign substances to other innate immune cells [3].

Cell survival, apoptosis, primary necrosis, and regulated necrotic phenomena may take place simultaneously in the same tissues [4], which are characterized by a variety of changes in the cellular morphology and imbalance between cell proliferation and apoptosis [5].

Apoptotic cells disintegrate into apoptotic bodies and small membrane vesicles. The fragmented chromatin is packing into smaller structures that can be easily engulfed by phagocytes [6]. Neutrophils trigger oxidative damage of vital cellular components and induce cell death [1, 7]. At that stage, neutrophils undergo apoptosis and ingestion by macrophages, clearing the inflamed sites. Phagocytes recognize and internalize various damaged and dead cells (i.e., early apoptotic and necrotic cells), which may lead to either anti- or proinflammatory responses [8–10]. In inflammatory conditions, the clearance of apoptotic bodies by polymorphonuclear (PMN) cells is often impaired, resulting in the damage of surrounding tissues, progressive course of inflammation, and autoimmunity [6].

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Apoptotic cells contain receptors on their membranes that facilitate their recognition, absorption, and clearance by phagocytes of surrounding tissues before the cell content is released [4]. Wyllie et al. named this cell lysis “secondary necrosis” to distinguish it from primary (or “accidental”) necrosis [4]. Manuel Silva noticed that secondary necrosis is the natural outcome of complete apoptosis of cells before their membrane disintegration [4]. In rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), defective or delayed apoptosis, as well as persistent or massive secondary necrosis, plays crucial pathophysiological roles implicated in adaptive immunity and autoimmune responses [4, 11].

It is unknown whether the attenuated survival of neutrophils at sites of inflammation is due to various intracellular mechanisms of apoptosis or due to the possibility of external mediators of survival in the inflammatory environment [1]. To the apoptotic cells are better connected α -D-mannose-specific lectins from *Narcissus pseudonarcissus* (NPL) than to the intact cells. The level of cell staining with NPL can be a reliable marker of apoptosis, “at least as sensitive as Annexin V binding assay” [11]. Altered sugar moieties on dying cells are recognized by lectin molecules on phagocytes. Pre-stages or immature glycosylated macromolecules may be available by detecting cryptic sugar fragments (e.g., mannosyl residues) that are typically masked in mature surface molecules of viable cells [5, 12]. Finally, lectins can be used for efficacious and reversible binding to dying cell glycoproteins (GPs) and can be novel plasma membrane markers of apoptotic cells [11, 13].

The aim of this study was to evaluate the diagnostic value of apoptotic changes in neutrophils and monocytes and their relationship with rheumatoid activity measured by the 28-joint disease activity score (DAS28).

Materials and methods

Participants

Twenty-one RA patients (4 men and 17 women) and 15 healthy controls (HC) were enrolled in this study. This study complies with the Declaration of Helsinki and has been approved by the ethics committee of the DanyloHalyskyLviv National Medical University (approval protocol number 5, May 16, 2016). Written informed consents were obtained from all examined subjects.

All RA patients fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for RA [14]. In 2016 and 2017, RA patients were enrolled at the Rheumatology Unit of Lviv Regional Clinical Hospital and at ambulatory clinics of the Regional Center for Clinical Immunology and Allergology. The inclusion criteria were: age 18–65 years; DAS28

–5.1–9.4; disease duration >2 years; ESR >20 mm/h; anti-CCP >35 U/mL; swelling of soft tissues, articular demineralization, bone erosions on joint X-ray images.

The exclusion criteria were: age <18 years, pregnancy, reactive arthritis, spondyloarthritis, osteoarthritis, acute viral hepatitis, current infection, AIDS, mental illness.

HC were hospital staff or their relatives who were matched with patients in terms of age and gender and underwent venous puncture.

Clinical and laboratory parameters

The DAS28-CRP score was recorded for all patients. The simplified disease activity index (SDAI) and the clinical disease activity index (CDAI), health assessment questionnaire (HAQ) were also reported. The routine laboratory tests included complete blood count, urinalysis, biochemistry, immunoglobulin levels (IgG, IgM, IgA).

Blood sampling

Blood sampling for laboratory tests was carried out by venipuncture at the time of the patients’ hospital admission (before the treatment) and processed within 2 h of the collection.

Whole blood APOLECT assay

The assay was performed according to the manufacturer’s instructions [5]. Briefly, to 100 μ L of heparinized whole blood in tube 10 μ L of propidium iodide (Sigma-Aldrich, USA) was added. The mixture was incubated at 4 °C for 30 min. The sample was washed with PBS (150 mM sodium phosphate; 150 mM NaCl; pH 7.2 \pm 0.1 (25 °C) (Sigma-Aldrich, USA). After centrifugation at 250 \times g for 10 min, buffer was removed and 100 μ L of BSA (bovine serum albumin) (Sigma-Aldrich, USA) was added. Then, solutions A, B, and C were added to the sample successively, and the mixture was vortexed every 15 s. Finally, 2 μ L of NPL-FITC was added and incubated at 4 °C for 30 min [5]. After staining, the sample was analyzed by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). An excitation wavelength of 488-nm and 515-nm bandpass filter for fluorescence emission was used.

Statistical analyses

All tests were repeated three times, with three parallels in each variant. We assessed the differences between groups using two-tailed Student *t* test for unpaired data for normally distributed parameters and Mann–Whitney *U* test for not normally distributed parameters. Normality of data distribution was estimated by Shapiro–Wilk test. Chi-squared

test was used for categorical variables. Pearson correlation coefficient was employed to test associations between apoptotic changes of neutrophils and monocytes and rheumatoid activity parameters. Data were reported as mean $M \pm SD$. The results were processed by the variation statistics methods using StatSoft STATISTICA v.10 and Excel (Microsoft, USA). $P < 0.05$ was considered statistically significant.

Results

Twenty-one RA patients (4 men and 17 women) and 15 HC were enrolled in this study. Demographic and medical history data of RA patients are presented in Table 1. The study groups were matched by gender and age. The characteristics of the disease and current antirheumatic treatments are presented in Table 1.

In the RA group, the vast majority of patients were seropositive (18, 86%). All patients demonstrated high DAS28 that ranged from 7.29 to 7.98 (mean 7.59 ± 0.28). Normochromic mild anemia was diagnosed in one patient, leukocytosis in 6 (28.6%), neutrophilic leukocytosis in 9 (42.9%), thrombocytosis in three (14.3%) patients. In all patients, CRP level was elevated (average concentration 13.52 mg/L). An increased RF was detected in 18 (85.7%) patients (ranging from 13.3 to 472.0 IU/mL, mean 155.5 ± 11.3 IU/mL). Anti-CCP autoantibodies were detected in 14 (66.7%) patients (472 ± 123 U/mL). Levels of IgG, IgM, IgA were elevated in three patients, with one patient experiencing simultaneous IgG and IgM increase.

When analyzing the results of the HAQ questionnaire, it was found that the mean patient pain assessment ($PAIN_{NRS}$) was 6.50 ± 0.78 , subjective disease activity assessment by patient (SGA_{NRS}) 6.71 ± 0.82 , and evaluation of the patient global assessment – 6.85 ± 0.77 (PGA_{NRS}).

The phenotype characteristics of non-viable neutrophils and monocytes in peripheral blood

The phenotype of neutrophils and monocytes presented in peripheral blood was analyzed by two-color flow cytometric analysis, based on forward scatter (FSC) and side scatter (SSC) parameters (Fig. 1). We noticed changes in size and granularity and physiological characteristics of peripheral blood neutrophils and monocytes. As shown in Fig. 2, there was a significant decrease in the number of viable granulocytes in RA patients compared to HC ($94.46 \pm 0.65\%$ vs. $97.06 \pm 0.41\%$, $P < 0.05$). The percentage of non-viable granulocytes (Figs. 2, 3) was greater in RA patients ($5.54 \pm 0.64\%$ vs. $2.94 \pm 0.14\%$, $P < 0.05$).

There was also a significant decrease in viable peripheral blood monocytes in RA patients compared with HC ($88.63 \pm 1.24\%$ vs. $92.98 \pm 0.66\%$, $P < 0.05$). Non-viable

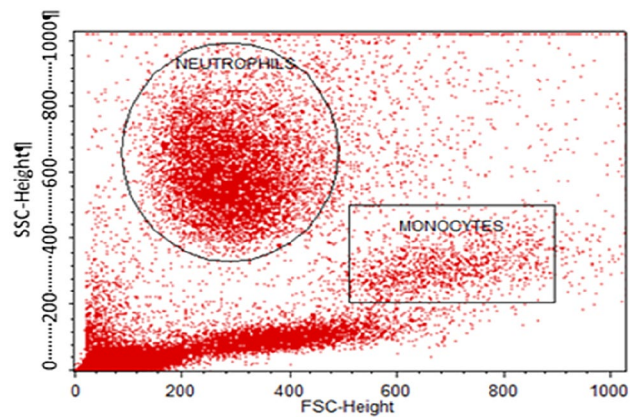


Fig. 1 Flow cytometry discrimination of neutrophils and monocytes based on FSC and SSC (elliptical region, neutrophils; rectangular region, monocytes). Flow cytometry was performed on a BD FACSCalibur™ system

Table 1 Background and disease characteristics in groups of RA patients and healthy controls

	RA patients (n=21)	Healthy controls (n=15)	P value
Age: median (min–max), years	52 (36–64)	48 (32–60)	> 0.05
Sex: ratio female/male	17/4	12/3	> 0.05
Disease duration: median (min–max), years	8 (4–16)	–	–
Swollen and painful joints	21(100)	–	–
C-reactive protein (%)	21 (100)	–	–
RF-positive (%)	18 (86)	–	–
Anti-CCP-positive (%)	14 (67)	–	–
Current treatment with methotrexate (%)	21 (100)	–	–
Current treatment with DMARDs (%)	2 (9.5)	–	–
Current treatment with biologic agents (%)	4 (19)	–	–
Current treatment with glucocorticoids (%)	17 (81)	–	–

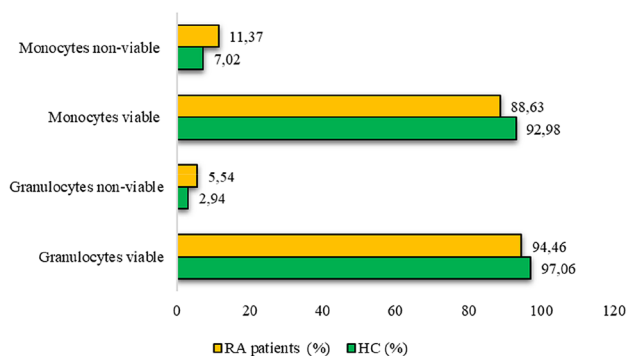


Fig. 2 Viable and non-viable (%) neutrophils and monocytes in blood samples of RA patients and healthy controls (HC)

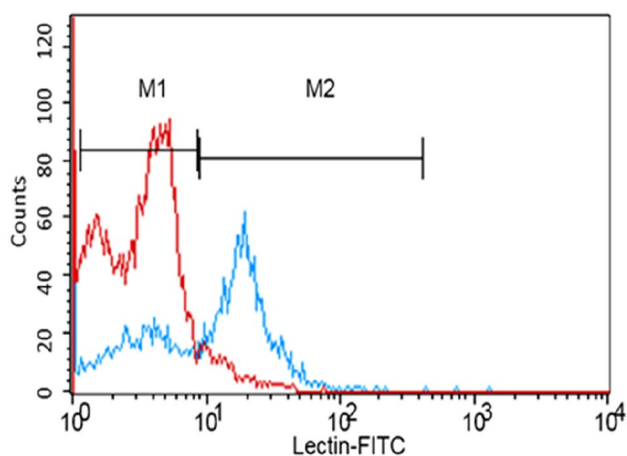


Fig. 3 Flow cytometry histogram of viable and non-viable neutrophils distribution in RA patients. M1 (red color), viable cells, M2 (blue color), non-viable cells. Flow cytometry was performed on a BD FACSCalibur™ system. Fluorescence channel FL1 (lectin-FITC)

monocytes were greater in RA patients ($11.37 \pm 0.1\%$ vs. $7.02 \pm 0.06\%$, $P < 0.05$).

The distribution of non-viable cells to the apoptotic cells, primary necrotic, and secondary necrotic cells is presented in Table 2 and Fig. 4.

We analyzed the difference between non-viable monocytes and granulocytes in RA and HC. No significant difference was noted between the apoptotic monocytes. However, there was a difference between apoptotic granulocytes in RA and HC ($0.76 \pm 0.15\%$ vs. $0.58 \pm 0.17\%$, $P < 0.05$).

The percentage of primary necrotic cells was significantly elevated in granulocytes of RA patients ($1.96 \pm 0.33\%$ vs. $3.84 \pm 0.5\%$, $P < 0.05$), unlike the primary necrotic monocytes, where the significant difference was not observed.

Table 2 Neutrophils and monocytes division in peripheral blood samples of RA patients and healthy controls

	RA patients (%)	Healthy controls (%)
Viable granulocytes	94.46 ± 0.65	97.06 ± 0.41
Apoptotic granulocytes	$0.76 \pm 0.15^*$	0.58 ± 0.17
Secondary necrotic granulocytes	$0.94 \pm 0.15^*$	0.40 ± 0.06
Primary necrotic granulocytes	$3.84 \pm 0.50^*$	1.96 ± 0.33
Viable monocytes	88.63 ± 1.24	92.98 ± 0.66
Apoptotic monocytes	0.98 ± 0.15	0.93 ± 0.13
Secondary necrotic monocytes	$4.83 \pm 1.06^*$	1.80 ± 0.33
Primary necrotic monocytes	5.56 ± 0.60	4.30 ± 0.62

* $P < 0.05$ difference compared to the healthy control group

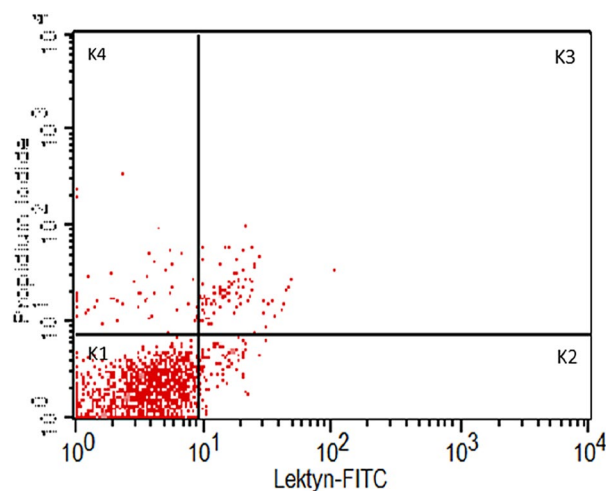


Fig. 4 Flow cytometry dot plot of neutrophils distribution in RA: quadrant K1, viable cells (lectin-/PI-), quadrant K2, apoptotic cells (lectin+/PI-), quadrant K3, secondary necrotic cells (lectin+/PI+), quadrant K4, primary necrotic cells (lectin-/PI+). Fluorescence channels FL1 (lectin-FITC) and FL3 (propidium iodide)

The phenotype characteristics of viable neutrophils and monocytes in peripheral blood

The results are presented in Table 2, and Fig. 2 displays the percentage of viable granulocytes and monocytes, and it was lower in RA patients compared with the healthy controls without statistical differences.

The percentage of secondary necrotic neutrophils and monocytes in peripheral blood

The number of secondary necrotic granulocytes presented in Table 2 and Fig. 4 was higher in RA patients compared to HC ($0.94 \pm 0.15\%$ vs. $0.40 \pm 0.06\%$, $P < 0.05$), pointing to a

significant damage of the surrounding tissue in response to the apoptosis. Inefficient clearance of apoptotic cell material [shrunken apoptotic cells, apoptotic bodies or subcellular membranous particles (scMP)] will result in the accumulation of secondary necrotic remnants that release modified and potentially proinflammatory contents into the extracellular milieu [11].

We also analyzed peripheral monocytes function in association with secondary necrotic cells. The percentage was significantly elevated in RA patients ($4.83 \pm 1.06\%$ vs. $1.80 \pm 0.33\%$, $P < 0.05$), indicating insufficient clearance at the inflammatory area.

Discussion

Neutrophils that migrate into RA joints display molecular changes associated with a delay in apoptosis. These cells have an enhanced potential to mediate host tissue damage because of their extended lifespan [2]. We found that the percentage of apoptotic neutrophils was elevated in RA patients. A significant positive correlation between DAS28 and the percentage of apoptotic neutrophils was also revealed in RA patients ($r = 0.62$, $P < 0.05$). There was also a positive correlation between anti-CCP concentration and percentage of secondary necrotic monocytes ($r = 0.48$, $P < 0.05$).

Apoptotic cells do not disappear after phagocytosis in the autoimmunity process. Autoantigens from apoptotic cells proceed to immune response in a different manner: (1) overload the phagocytic capacity; (2) trigger an autoimmune reaction through its presentation to the immune system [15], (3) show distinct inflammatory properties; (4) have the same capacity to form immune complexes (IC) with autoantibodies and activate the immune system [16].

We also analyzed IgM RF and percentage of secondary necrotic monocytes and neutrophils. There was no correlation between IgM RF and secondary necrotic monocytes ($r = -0.14$, $P > 0.05$). There was an association between IgM RF and secondary necrotic neutrophils ($r = 0.42$, $P < 0.05$).

Finally, we analyzed a link between CRP and apoptotic cells. CRP appears in the blood in response to inflammation and plays a protective role in injury or infection. We detected a positive correlation between CRP and primary necrotic neutrophils ($r = 0.4$, $P < 0.05$) and between CRP and primary necrotic monocytes ($r = 0.38$, $P < 0.05$). CRP can bind to apoptotic cells and opsonize intracellular components from necrotic cells [17, 18].

Apoptosis has long been viewed as a non-inflammatory process that does not elicit an immune response. Apoptotic cells can be involved in (auto) immune processes. They can express autoreactive antigens on their surface, activate

dendritic cells and induce the production of autoantibodies such as RF [3].

Neutrophils express peptidylarginine deiminase enzymes (PAD2 and PAD4) responsible for generating citrullinated proteins, which are the most specific targets of autoimmunity in RA. Patients with autoimmune diseases express target proteins, including those produced by neutrophils. It is unclear why neutrophil-specific proteins are targeted. These molecules may be linked to neutrophils damage at inflammatory sites, resulting in changes in normal immune system antigens [16].

NETs (neutrophil extracellular traps) can be another source of citrullinated autoantigens. RF is involved in the formation of immune complexes (IC), with complement activation being responsible for its consumption and resultant inflammation in RA [19]. The increase in apoptotic neutrophils observed in our RA patient may be due to the release of neutrophilic reactive oxygen (ROS) and nitrogen (RNS) species, proteases, chemotactic factors, all of which attract monocytes/macrophages and immature dendritic cells (DCs) and result in the autoantibodies production.

We revealed no changes in the percentage of apoptotic monocytes in RA patients. The essential functions of monocytes/macrophages and suppression of immune and inflammatory responses could be altered. Also, the disequilibrium in the recognition and clearance of apoptotic cells could result in inflammation and autoimmunity disorders [20].

Apoptosis defects in autoimmunity may have the following consequences. Firstly, it may affect lymphocytes and impair immunological tolerance with the survival of B- and T-cells clones influencing autoimmune responses. Secondly, it can slow down cell apoptosis and/or induce exposure of an abnormal number of apoptosis-related antigens and promote the immune response against them [20].

In this study, we examined secondary necrosis of neutrophils and monocytes in RA patients. We reported delayed apoptosis and impaired phagocytosis. Delayed apoptosis and secondary necrosis may lead to enhanced autoimmune responses [4, 11]. Apoptotic material in complex with autoantibodies shifts the balance toward a proinflammatory response [16].

Neutrophils are present in the synovial fluid of RA patients and at the pannus/cartilage interface [16]. Macrophages modulate synovial apoptosis [21, 22]. The clearance of apoptotic cells by macrophages yields an anti-inflammatory effect. In contrast, macrophage response to necrotic cells, including secondary necrotic cells, derived from unclean apoptotic cells, results in a proinflammatory effect.

Biologic agents targeting inflammatory cytokines, such as anti-TNF therapy, combined with methotrexate (MTX) have significantly improved the efficiency of RA therapies. However, there are patients who are unresponsive to

these therapies. Some immune mechanisms and subsets of immune cells are associated with the pathophysiology of RA and represent targets for therapeutic strategies such as apoptotic cell infusion [19]. The infusion of early-stage apoptotic cells may force macrophage reprogramming with proinflammatory cytokines decreasing.

Conclusion

The apoptotic shifts in peripheral blood of RA patients may affect the course of the disease. These shifts are associated with disease activity. Apoptotic, primary necrotic changes, or secondary necrotic may result in early manifestations. These results are essential to value the leading immunopathological mechanism of RA development in each case of RA patient and require of the treatment strategy.

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