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Modern methods for studying atherosclerosis and coronary artery disease: flow cytometry

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ABSTRACT

The problem of atherosclerosis, which forms the pathological basis of coronary artery disease (CAD), is one of the most discussed ones in development of cardiovascular diseases. This chronic inflammatory disease involves interactions between different cells, and an atherosclerotic plaque is a complex immunological environment. Modern quantitative methods increase the understanding of the pathophysiological processes responsible for progression of atherosclerotic plaques. Flow cytometry is a powerful modern method that allows for a complex and simultaneous cell analysis. This review is devoted to studies on atherosclerosis and CAD performed using flow cytometry.

Key words: flow cytometry, atherosclerosis, inflammation, T-lymphocytes, monocytes.

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Современные методы исследования атеросклероза и ишемической болезни сердца: проточная цитометрия

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РЕЗЮМЕ

Проблема атеросклероза, формирующего патологическую основу ишемической болезни сердца, является одной из наиболее обсуждаемых в развитии сердечно-сосудистых заболеваний. Это хроническое воспалительное заболевание включает комплекс сложных взаимодействий между различными клетками, а атеросклеротическая бляшка представляет собой сложную иммунологическую среду. Современные количественные методы повышают понимание патофизиологических процессов, ответственных за прогресси-

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рование атеросклеротической бляшки. Проточная цитометрия представляет собой мощный современный метод, позволяющий проводить комплексный анализ клеток одновременно. Данный обзор посвящен научным исследованиям атеросклероза и ишемической болезни сердца, выполненным с помощью метода проточной цитометрии.

Ключевые слова: проточная цитометрия, атеросклероз, воспаление, Т-лимфоциты, моноциты.

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INTRODUCTION

More than 50 years ago, such a new diagnostic method as pulse cytophotometry was developed. Since 1978, it has been called “flow cytometry”. Today, flow cytometry is a technique for studying dispersive substances using single particle analysis in the dispersed phase via signals received during fluorescence and light scattering. Flow cytometry is based on a combination of modern cytochemical fluorescent methods for analyzing the structural components of cells and their antigens. It is a modern and highly functional method that allows for a comprehensive analysis of various cell populations [1].

PRINCIPLES OF FLOW CYTOMETRY

The physical principles of flow cytometry are simple: a cell suspension, previously incubated with fluorochromes, is placed in a flow of liquid passed through a flow cell. This generates the effect of hydrodynamic focusing: the cells under investigation line up in a chain and, in this order, are guided through laser beams. This is how an individual cell is analyzed.

The light emitted from fluorochromes is focused using an optical system consisting of several mirrors and lenses, and then decomposed into certain components. The received light signals are converted into electrical impulses and analyzed using special software. In just a few seconds, thousands of cells pass through the flow cell, allowing a researcher to identify the composition and characteristics of the cell suspension.

Flow cytometry is a powerful method with many advantages: rapid analysis of a large number of cells (up to 10^7 cells per second), objective measurement of fluorescence intensity, obtainment of data for a single cell population, simultaneous analysis of different processes, an ability to characterize rare events [1].

FLOW CYTOMETRY IN STUDIES ON CAD AND ATHEROSCLEROSIS

The problem of atherosclerosis and the complications it causes is one of the most discussed ones in the development of cardiovascular diseases (CVDs). Atherosclerosis develops for a long time and forms the pathological basis for CAD. This complex chronic inflammatory disease involves interactions between different cells. New technologies, in particular, flow cytometry, allow to perform a complex analysis of different cells simultaneously.

One of the main pathophysiological mechanisms in the development of atherosclerosis is a disruption of the structural integrity and functional activity of the vascular endothelium. Circulating endothelial cells detached from the endothelial wall in the course of its damage can act as a direct cellular marker of endothelial dysfunction [2, 3].

Using flow cytometry, it was revealed that the presence of more than 3 circulating endothelial cells per 3×10^5 leukocytes in the peripheral blood increased the relative risk of CAD development in young and middle-aged women by 4 times. In women with CAD, it increased the risk of developing acute myocardial infarction by 8 times [2].

Endothelial progenitor cells, which are capable of self-renewal and differentiation, are involved in repair of the endothelium and formation of new blood vessels. Recruitment and migration of endothelial progenitor cells in the body are controlled by cells located directly in the damaged area [4]. Most progenitor cells mature from hematopoietic stem cells, mainly found

in the bone marrow, peripheral blood, and umbilical cord, but they are also present in the spleen, intestines, liver, adipose tissue, and adventitia. Regardless of their source, all hematopoietic stem cells have markers CD34+ and CD133+ [4].

Endothelial progenitor cells are characterized by expression of surface markers, such as vascular endothelial growth factor receptor-2 (VEGFR-2), CD31, endothelial nitric oxide synthase (eNOS), and vascular endothelial cadherin (VE-cadherin). Therefore, to identify endothelial progenitor cells, the following surface markers of hematopoietic and endothelial cell lines are used: CD34, CD133, VEGFR-2, or kinase domain receptor (KDR) [4, 5]. In patients with arterial hypertension with a high level of low -density lipoproteins (LDL), the number and migration capacity of circulating endothelial progenitor cells are reduced [6, 7]. The number and functional activity of circulating endothelial progenitor cells in the blood are independent predictors of morbidity and mortality from CVDs [8].

The involvement of endothelial progenitor cells in atherogenesis is beyond doubt. In the study by S. Ai et al., when evaluating the expression of the vitamin D receptor (VDR), the authors found that the VDR expression on circulating endothelial progenitor cells was significantly reduced in CAD patients and negatively correlated with the glycated hemoglobin levels. Consistently high serum glucose decreased the VDR expression on endothelial progenitor cells, potentially accelerating the pathological process of atherosclerosis. Thus, low VDR expression on circulating endothelial progenitor cells may serve as a potential risk factor for CAD development [9].

Monocytes are cells of the immune system that are involved in the formation of innate and adaptive immunity. Monocytes play a key role in the atherogenesis, since, after being recruited into the lipid and lipoprotein-rich areas of the arterial intima, they differentiate into macrophages under the influence of the macrophage colony-stimulating factor (M-CSF) produced by the activated endothelium [10, 11]. In the peripheral blood of patients with atherosclerosis, monocytes are preactivated and have some features of macrophages [10]. Their adhesion to the endothelium is 1.5 times higher than that of monocytes in healthy individuals, and they express a number of receptors (type I and type II Fcγ-receptor, intercellular adhesion molecule-1 (ICAM-1)) [12]. Monocytes have heterogeneous composition. In atherosclerosis, an increase in the relative count of monocytes of the intermediate (CD14++/CD16+) and nonclassical (CD14+/CD16++) subpopulations is detected – by 2.3 and 1.8 times, respectively [10, 13].

K.A. Arnold et al. investigated correlations of monocyte subtypes and macrophages cultured from them with CAD progression. Groups of study participants were formed after a coronary angiogram in accordance with CAD severity: a group without CAD (minor disturbances in the vascular lumen); a group with CAD with a single-vessel lesion; a group with CAD with a multi-vessel lesion. In CAD patients (with both single-vessel and multi-vessel lesions), the blood levels of both intermediate and non-classical monocytes were elevated compared to patients without CAD ($p < 0.05$). The count of regulatory macrophages (CD206+) was reduced in patients with both single-vessel and multi-vessel lesions ($p < 0.001$) [14].

A relationship between the atherogenic lipoprotein phenotype and innate immunity in atherosclerotic patients with CAD was shown. The atherogenic fraction of LDLP was associated with an increase in the content of non-classical monocytes (CD14+CD16++) and a decrease in the content of the classical subpopulation (CD14++CD16-) [15].

Lymphocytes play a key role in the development of inflammatory responses in CVDs. The content and phenotype of lymphocytes in the peripheral blood, subcutaneous adipose tissue, and epicardial adipose tissue in patients with and without CAD, who had undergone an elective heart surgery, were studied. It was found that the epicardial adipose tissue in CAD patients was characterized by an increased number of T-lymphocytes, B-lymphocytes, a higher total lymphocyte count, and a reduced number of natural killer (NK) cells in comparison with patients without CAD [16].

The complement system is involved in the CVD pathogenesis by inducing inflammation and interacting with the coagulation system [17]. The balance between activation and inhibition of the complement system is critical for controlling the degree of inflammation. Molecules involved in the regulation of the complement system activation include complement receptor type 1 (CR1, CD35), membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55), and membrane inhibitor of reactive lysis (MIRL, CD59). Each protein is different in the mechanism of action for the complement system regulation [18]. Patients with CAD showed lower expression of CD46 and CD55 on the surface of lymphocytes,

monocytes, and granulocytes and higher surface expression of CD35 and CD59 on granulocytes ($p < 0.0001$), compared to healthy donors. High CD59 expression on granulocytes positively correlated with the severity of the disease and may serve as a potential marker of disease progression [18].

FLOW CYTOMETRY IN THE STUDIES ON ATHEROSCLEROTIC PLAQUES

The structure of atherosclerotic plaques is one of the key directions in the study of the pathogenesis of atherosclerosis. There is a large number of experimental studies devoted to formation, maturation, and rupture of atherosclerotic plaques, but the mechanisms of these phenomena remain largely unclear. Traditionally, blood is used as a material for scientific research on atherosclerosis, since collection of tissues for research is associated with certain difficulties. However, for the study of the pathophysiological mechanisms of atherosclerosis, the atherosclerotic lesion itself is of the greatest interest.

Modern quantitative methods can increase the understanding of the pathological processes responsible for plaque progression. It was flow cytometry and development of original protocols for enzymatic isolation of cells from atherosclerotic plaques that allowed to perform the most complete analysis of lymphocytes, their role in the immunological mechanisms of maturation and rupture, and their distribution in atherosclerotic plaques [19, 20].

Earlier there were some attempts to study the role of lymphocytes in an atherosclerotic lesion. However, at the same time, scientists isolated lymphocytes from the tissue into a culture medium with various activators of the lymphocyte migration, i.e. studied individual lymphocytes migrating from the atherosclerotic plaque tissue into the culture medium. This made it possible not to practice the technique of isolating living cells but to study the cellular composition of the atherosclerotic plaque tissue itself [21].

At the same time, attempts were made to accurately determine the cellular composition of the tissue of human atherosclerotic plaques using flow cytometry, where the material obtained during carotid endarterectomy was subjected to enzymatic treatment with collagenase I (250 U / ml) at 37° C. This study showed that about 50% of the cells in the atherosclerotic plaques were mononuclear inflammatory cells (T-lymphocytes and monocytes (macrophages)) [22].

In further research, scientists developed original protocols for isolating cells from the tissue of atherosclerotic plaques and studied the whole complexes of antibodies labeled with fluorochromes. In addition, the use of several fluorochromes simultaneously made it possible to study various processes in the cell. The study by L. Sh. Grievel et al. showed that the phenotypic composition of T-lymphocytes in the plaque differed from that in the blood. When comparing the expression levels of cellular markers CD3, CD4, CD16, CD45, CD45RA, CCR7 CD28, CD27, HLA-DR, and CD38, high levels of CD4 and CD8 T-cells in the plaques were revealed [19].

The dominant inflammatory cell in atherosclerosis is a macrophage. However, interactions with other inflammatory cells may also play a role in the atherogenesis. Using immunohistochemistry, the presence of mast cells in the atherosclerotic lesion, constituting a heterogeneous population, was established. In a 3-year study of 270 patients with carotid stenosis, plasma mast cell levels were shown to be associated with future acute cardiovascular events. Despite the fact that mast cells are present in a plaque in small numbers, they can contribute to destabilization of an atherosclerotic plaque [23].

Mast cells are involved in inflammatory responses in various tissues, including the arterial intima. Mast cells are activated for degranulation, releasing large amounts of inflammatory mediators (histamine, heparin, proteases, and cytokines) stored in their cytoplasm. Activated mast cells in the atherosclerotic lesion can promote leukocyte chemotaxis, adhesion to the activated endothelium, and subsequent transendothelial migration [24].

In order to characterize the population of mast cells in human atherosclerotic lesions in more detail and to determine its activity, E. Kritikou et al. used several markers simultaneously (CD45, CD117, CD63, and IgE, Tryptase/TPSAB1) [25]. The authors confirmed that the main but not the only pathway of mast cell activation inside a plaque is IgE-mediated one, but there is a group of mast cells that are activated without IgE binding [25].

Flow cytometry is a powerful tool for detecting the diversity of leukocyte subsets in the atherosclerotic plaque [26]. The atherosclerotic plaque is a complex immunological environment. Since the discovery of T-cells in atherosclerotic plaques, they have been found to play an important role in the development of atherosclerosis and CVDs. T-helpers can differentiate into several phenotypes (Th1, Th2, Th9, Th17, Treg, etc.), produce various interleukins, and have both pro- and anti-inflammatory mechanisms. The study by

Grönberg et al. suggested that therapeutic inhibition of T-cell differentiation into Th1-cells is a promising strategy for reducing the progression of atherosclerosis [27].

T-lymphocytes play an essential role in atherogenesis, but the atherogenic or atheroprotective role of CD8+ T-cells in late stages of atherosclerosis development remains controversial. The study by J. van Duijn et al. demonstrated the local, protective role of CD8+ T-cells in progressive atherosclerosis by comparing the phenotypes of CD8+ T-cells obtained from plaques from the aorta, spleen, and blood of mice. In progressive atherosclerosis in a mouse model, aortic CD8+ T-cells produced lower amounts of IFNγ and TNFα compared to their systemic counterparts, with a simultaneous increase in the expression of CD39 ectonucleotidase. At the same time, pharmacological inhibition of CD39 in apoE-/- mice partially restored the production of cytokines by CD8+ T-cells. The studies on the samples of atherosclerotic plaques in the human carotid and femoral arteries confirmed these results [28, 29].

Tissue cell death is a characteristic feature of progressive atherosclerotic plaques, including unstable lesions, which are largely responsible for complications of CVDs. The data on the accumulation of cytotoxic lymphocytes in human lesions strongly suggest that these lymphocytes promote cell death in atherosclerotic foci and lead to potential rupture of plaques [30].

NK cells can induce cell death in various ways, using killer activation receptors (KARs) or the cytolytic components perforin and granzyme B to form an immunological synapse, through which the release of cytolytic granules ultimately leads to lysis of target cells [31, 32]. T-cells (CD8, CD4, γδ-cells) and NK cells are involved in the atherogenesis. These cells are present in large numbers in unstable plaques, which indicates that their killer function is important for the progression of atherosclerotic process [30].

Inflammatory cells in the atherosclerotic plaque are derived from hematopoietic stem cells (HSPCs). When analyzing mononuclear blood cells by flow cytometry using antibodies CD38+CD45RA+CD34+HSPCs, the authors found that in CAD patients (coronary stenosis ≥ 50%), the level of circulating HSPCs in the peripheral blood was 1.8 times higher than in individuals without CAD. The level of HSPCs in the blood was associated with the degree of coronary stenosis. In addition, according to the multivariable logistic regression analysis, the level of circulating HSPCs was the only marker that was associated with the odds ratio (OR) of mild to severe (≥ 70%) coronary stenosis (OR 2.08 (95% confidence interval (CI) 1.35–3.21), p = 0.0009). That allowed the authors to propose HSPCs as an important marker for the assessment of atherosclerotic coronary stenosis [33].

CONCLUSION

Flow cytometry has been rapidly developing as a modern method for diagnosing and researching disorders of the immune system. Atherosclerosis is a multi-factorial chronic inflammatory disease that includes complex interactions between the vascular wall and blood cells, and an atherosclerotic plaque is a complex immunological environment.

Scientific knowledge about the atherosclerotic plaque composition and the role of cells found in the lesion focus is constantly growing due to fundamental research. However, a simple and accessible diagnosis of early atherosclerosis remains a problem, since measurement and determination of changes in cell populations in the atherosclerotic focus are impracticable in clinical practice.

It is possible that flow cytometry will become a priority method for determining new prognostic peripheral blood markers for the severity of atherosclerosis.

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