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Review Article

Atherosclerosis is a side effect of cellular senescence

Elena I. Leonova[1,2], Angelina V. Chirinskaite[1], Julia V. Sopova[1,2,3]

1 *Center of Tansgenesic and Genome Editing, Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, 199034, Russia*
2 *Scientific Center for Genetics and Life Sciences, Sirius University of Science and Technology, 1 Olympic Ave, Sochi, 354340, Russia*
3 *Laboratory of Plant Genetics and Biotechnology, Saint-Petersburg Branch of Vavilov Institute of General Genetics, St. Petersburg, 199034, Russia*

Corresponding author: Elena I. Leonova (e.leonova@spbu.ru)

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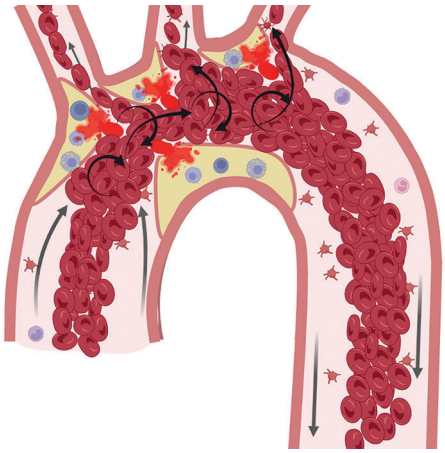
Abstract

Atherosclerosis is a systemic autoimmune disease of the arterial wall characterized by chronic inflammation, high blood pressure, oxidative stress, and progressive loss of cell and organ function with aging. An imbalance of macrophage polarization is associated with many aging diseases, including atherosclerosis. The polarization toward the pro-inflammatory M1 macrophage is a major promoter of the atheroma formation. It is known that efferocytosis, or ingestion of apoptotic cells, is stimulated by M2 macrophage polarization. A failure of efferocytosis leads to the prolongation of chronic pathology in tissue. In addition, fat-laden macrophages contribute to the plague progression by transforming into foam cells in response to excess lipid deposition in arteries. In spite of the generally accepted theory that macrophages capture oxidized low-density lipoprotein by phagocytosis and become foam cells, we postulate that the main source of lipid accumulation in foam cells are senescent erythrocytes. Senescent erythrocytes lose their plasticity, which affects the rheological blood properties. It is known that their membrane contains high levels of cholesterol. There is evidence that senescent erythrocytes play a pathogenic role in the atheroma formation after breaking down during flowing through an artery bifurcation. Here we review the current knowledge on the impact of age-associated immune cells and red blood cells modifications on atherogenesis.

Graphical abstract:



Keywords

Atherosclerosis, cholesterol, erythrocytes, low-density lipoprotein, macrophage polarization, red blood cells, vasa vasorum.

Introduction

Atherosclerosis is a chronic autoimmune disease accompanied by the formation and growth of atherosclerotic plaques on the walls of the main arteries which leads to stenosis and insufficient bloodstream in different organs (Libby et al. 2019). Unbalanced interaction of senescent cells of the immune system and intercellular communication underlie the development of atherosclerosis. A human body consists of a variety of cells all working together to keep up the entire organism, so intercellular communication processes are crucial in their coordinated performance. However, upon aging, mutations are accumulated both in the mitochondrial and cellular DNA that turns out to be a reason for functional disorders. Systems of intercellular communication trigger "danger signals" inducing inflammatory responses by activating the immune system (Bäck et al. 2019). Under normal conditions, the balanced performance of the immune system prevents tissue damage. However, upon its prolonged activation the inflammatory processes become chronic, and under the action of additional factors, a serious pathology develops. Such a scheme is typical for most age-associated diseases, in particular, atherosclerosis. Senescent macrophages lose their ability to adequately complete the efferocytosis process (Elder and Emmerson 2020). Thus, conditions are created for a chronic autoimmune disorder: increased migration of monocytes to the inflammation area and formation of foam cells. Other risk factors for atherosclerosis development are high blood pressure and senescent erythrocytes (red blood cells (RBC)). During laminar flow, erythrocytes move in the middle of the vessels where flow is faster. Blood serum, opposite, flows slower near the artery wall region. Laminar flow is characterized by a parallel flow of layers with no disruption between them. Under turbulent flow, layers are mixed causing either damage of RBC or platelets clot formation, and predisposing them to come in contact with the endothelial lining of the vessel (Gillespie and Doctor 2021). As a result, the content of destroyed erythrocytes is leaked out inside of the arterial wall. Thus, free cholesterol and thrombus are frequently observed features in atherosclerotic plaques. Though it is commonly believed that low-density lipoproteins (LDL) facilitate the growth of plaques, we suggest another hypothesis: the growth of plaques is stimulated by cholesterol, which has an RBC origin. It has been known that RBCs are major cells in the blood (about 99%) (Pawliszyn 2012). Also, they have a significantly bigger size (about 6–8 μm in diameter), compared to that of LDL (about 20–26 nm in diameter). The cell membrane is composed of phospholipids, glycolipids, and cholesterol, the latter being a major component. The membranes of RBC have very high cholesterol content (1.5–2.0-times higher, than any other cell). The level of cholesterol in the RBC membrane is significantly increased with age (Prisco et al. 1991). A high-fat diet leads to further accumulation of cholesterol in RBC membranes of wild-type mice, which promotes macrophage phagocytosis in vitro (O'Brien and Rouser 1964; Singer and Nicolson 1972; Sonnino et al. 2007; Unruh et al. 2015). When the focus of chronic inflammation in a vascular wall has appeared, the plaque growth continues due to various other pathological processes. In this review, we present substantial information supporting our conception of atherosclerosis development.

Effect of fading of immune system cells on the atheroma development

One of the functions of immune cells is to support the homeostasis of the organism. The programmed cell death (apoptosis) underlies this process. Cellular debris after apoptosis is destroyed by different cells performing a phagocytic function, and this process is called efferocytosis. From 200 to 300 billion cells are renewed daily in the human body. In a young healthy organism clearance of apoptotic cells works perfectly without damaging the nearby tissues. But with aging, apoptotic cells are accumulated due to phagocytic cells losing their ability to function properly (Aprahamian et al. 2008). Therefore, the general fading of immune cells leads to the "clogging" of tissues. Taking into account that under normal conditions millions of cells undergo apoptosis every day, it is possible to imagine the scale of the catastrophe. Phagocytes that cannot efficiently complete efferocytosis enhance the synthesis of inflammatory signals, and so the pathological inflammation process becomes chronic (Wang et al. 2021). This phenomenon is associated with a great number of age-associated pathologies, in particular, plaque formation in arterial walls. The main phagocytes of blood vessel arteries, playing an important role in the initiation of atheroma development, are neutrophils and monocytes. Circulating in the blood, monocytes migrate continuously into the tissues, where they differentiate into dendritic cells or macrophages. Neutrophils are the most numerous residents of white blood cells constituting about 60–70% of the common pool (Peiseler and Kubes 2019). As a rule, they are the first to react to pathogens and cell fragments associated with the damage. It is worth noting a quite unusual way of removal of pathogens and damaged molecules which is accompanied by programmed neutrophil death. This process is called NETosis, a process of generation of Neutrophil Extracellular Traps (NETs). NETosis differs essentially from apoptosis or necrosis. First, in addition to the destruction of organelles under NETosis, the nuclear envelope is broken and the cellular DNA is mixed with the cytoplasm. After that, the activated neutrophils discharge the NETs to the extracellular space. These traps include DNAs with histones, a large number of proteins and enzymes as well as active forms of oxygen generated by nicotinamide adenine dinucleotide phosphate (NADPH) – oxidase (Branzk and Papayannopoulos 2013). Under NETosis, antimicrobial proteins LL3 are released in humans (CRAMP-1/2 in mice); these proteins can bind to DNAs, including the mitochondrial DNA, in the case of tissue damage (Caielli et al. 2012). NETs activates macrophages for cytokine release, stimulating T helper 17 (TH17) cells that increase immune cell recruitment

and M1 macrophage polarization (Warnatsch et al. 2015; Song et al. 2019). Neutrophils respond to the appearance of mitochondrial DNA in the tissue as a danger signal because mitochondria are descendants of aerobic bacteria. However, if proteins LL-37/Cramp bind to a mutant mitochondrial DNA (after age-associated changes), the formed complexes cannot be degraded either by DNAses or by means of autophagy (Zhang et al. 2015). Thus, these complexes cannot be destroyed completely by macrophages, resulting in the initiation of age-related inflammation in the arterial wall.

Further activation of the inflammation stimulates the migration of monocytes from the bloodstream to the inflammation region. This process is regulated by cytokines which stimulate endothelial cells to secrete adhesion receptors such as E and P selectins. Leukocytes cannot independently overcome the bloodstream and be fixed on a vascular wall (Kubes and Granger 1996; Langer and Chavakis 2009). The role of selectins is to decrease the rate of leukocyte motion. Leukocytes secrete selective ligands (sialylated oligosaccharides) to which selectins weakly bind, thus slowing down leukocytes until stop. Then leukocytes are capable of permeating between endothelial cells to the area of vascular intima and differentiate into macrophages (Langer and Chavakis 2009). There are two ways of monocyte-to-macrophage differentiation: classic M1 and alternative M2, which perform opposite functions; both ways have been found in the atheroma (Martinez and Gordon 2014; Peled and Fisher 2014). The classification of M1 and M2 macrophage is based on the differentiation of T-helpers (Th1 and Th2). M1 macrophages promote Th1 response activating inflammation, while M2 macrophages are involved in tissue remodeling promotion of Th2 response (Wang et al. 2014). Polarization toward M1 is induced via toll-like receptors and gamma-interferons. Activated M1 macrophage facilitates degradation of the extracellular matrix and induces migration of new monocytes to the inflammation area due to secretion of pro-inflammatory mediators such as nitrogen oxide synthases, tumor necrosis factors, interleukins (IL) IL-1b, IL-6, IL-12, and proteolytic enzymes. M2 macrophages are activated by cytokines IL4 and IL13. Activated M2 macrophages synthesize anti-inflammatory cytokines like the transforming growth factor-beta, the antagonist of receptors IL1 and IL10, and enhance the synthesis of collagen (Peled and Fisher 2014). Namely M1 macrophages trigger plaque rupture, whereas M2 stabilizes the plaque. If a phagocyte cannot completely destroy cell debris after apoptosis, it starts to actively synthesize pro-inflammatory molecules, which facilitates the polarization of M2 to M1. In contrast polarization from M1 to M2 is associated with tissue repair. It is assumed that in a young organism, plaques are not generated because the balanced function of macrophages allows revealing and stopping an inflammation on time. Upon aging, the balance between M1 and M2 gets broken as does their capacity to perform their functions. As a result, M2 cannot stop inflammatory processes in tissues, giving rise to the prolongation of the pathological autoimmune process.

Age-associated alterations in hematopoietic cells lead to clonal hematopoiesis which in turn can be associated with the formation of the inflammatory environment and higher risk of atherosclerotic cardiovascular disease (Perner et al. 2019; Sánchez-Cabo and Fuster 2021). Clonal hematopoiesis is often linked with accumulating with age mutations particularly in DNMT3A, TET2, and JAK2 genes coding for DNA methyltransferase 3 alpha, Tet methylcytosine dioxygenase 2, and Janus kinase 2, respectively (Silver et al. 2021; Cobo et al. 2022). We suggest that mutations in these genes might shift the balance in macrophage polarization, so that increase in M1 amount leads to chronic inflammation (and atherosclerosis), whereas M2 prevalence ends up with cancer.

In addition, mitochondrial DNA mutations are also associated with aging (Cree et al. 2008). As the number of mutations in mitochondria exceeds the critical minimum, they are eliminated, decreasing their per-cell copy numbers (Kim and Lemasters 2011; Gaziev et al. 2014). The main energy source of ATP for M2 macrophages is mitochondrial oxidative phosphorylation. So, reduction of copy number of mitochondrial DNA can impede polarization of macrophages toward M2. M1 macrophages use glycolysis to produce ATP (Ravi et al. 2014). A decrease in the number of copies of mitochondrial DNAs is connected with age and gender, aging women preserving a larger number of copies as compared to that in men (Ashar et al. 2015).

Monocyte-derived dendritic cells also play a crucial role in the adaptive immune system. Under the influence of an inflammatory microenvironment, monocytes also differentiate into inflammatory dendritic cells. The main function of dendritic cells is to capture pathogens, then migrate to lymph nodes and present antigens directly to naive CD_4T cells. Activated CD_4T cells proliferate to type 1 T helpers (Th1) and migrate to the inflammation region where they interact with M1 macrophages (de Jong et al. 2005). As a result, Th1 is additionally stimulated by M1 macrophages to release pro-inflammatory cytokines, in particular, gamma-interferons (Mills 2015). Dendritic cells can be activated by complexes of antimicrobial proteins LL3/CRAMP-1/2 and DNAs formed upon NETosis (Kumar and Sharma 2010).

Effect of hemodynamics and erythrocytes on atherosclerosis

It is known that laminar flow and high shear stress are atheroprotective, neither is atheroma formed in veins, small arteries, and capillaries. In contrast, areas of bends and bifurcation of large and middle-size arteries as well as low shear stress exhibit progressive development of atherosclerosis (Fig. 1). Interestingly, pulmonary artery atherosclerosis is an extremely rare case occurring only under hypertension of the lesser circulation (Cicconi et al. 2012). Hence it follows that hypertension and turbulent bloodstream are necessary and sufficient conditions for determining the atheroma location. At the same time, the process of pathology development will be affected by other molecules.

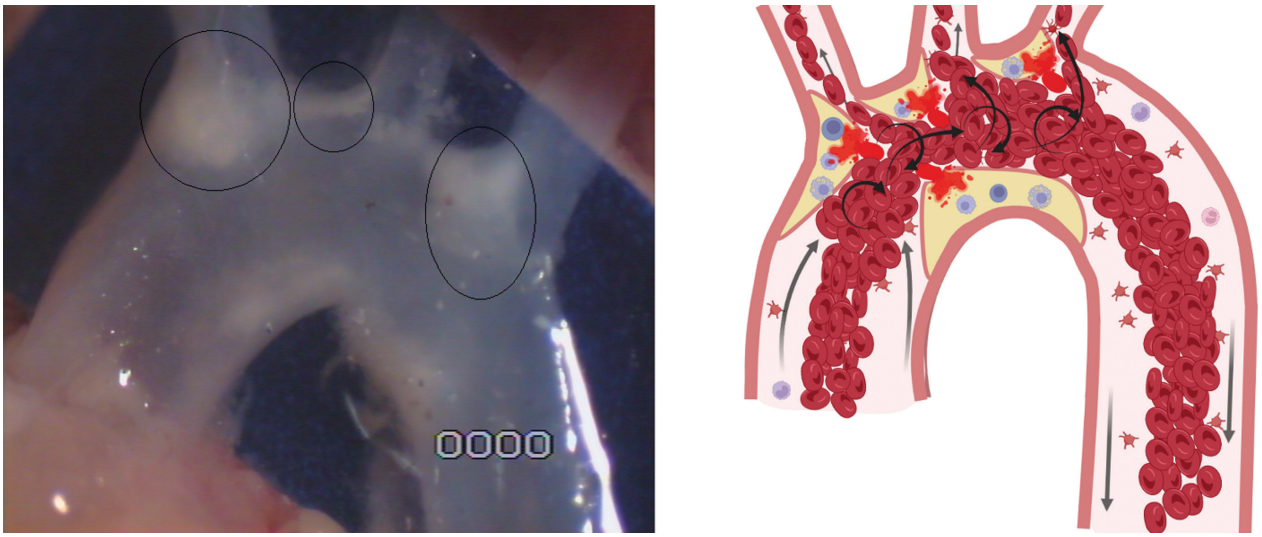


Figure 1. Schematic drawing shows the way of blood circulation through bifurcation in the aortic arch (right) and aortic arch of ApoE-/- mice at 5 months of age (left). The atherosclerotic plaque usually forms in the outer wall opposite to the flow divider in the low-shear stress region. The first destruction of red blood cells may take place in the bifurcation region, then they move to the turbulent flow and stick into the glycocalyx layer, where their collision-induced hemolysis may take place with the following penetration into the vascular. Figures were created with BioRender.com.

Blood is a complex disperse system consisting of cells, thrombocytes, lipids, and a colloidal solution of proteins. Part of the blood volume constituted of cells and thrombocytes is called the hematocrit. Erythrocytes make 99% of the hematocrit; therefore, for convenience blood may be divided into two main fractions – RBC and the plasma (Cokelet 2011). Under laminar flow the two layers move independently, creating shear stress (viscous resistance) between themselves and the vascular wall. In this case, erythrocytes in large arteries distribute closer to the vessel axis, whereas the plasma is closer to vascular walls. When turbulent motion appears, the layers are intermixed ameliorating the collision of erythrocytes with vascular walls. Under normal conditions, the negative charge of RBC simplifies repulsion from the negatively charged endothelial glycocalyx layer. But in aging organisms, the negative charge of RBC decreases, and their membrane loses its elasticity and becomes more rigid. In a turbulent flux, upon touching the vascular wall such RBC can be holed in the glycocalyx layer, where their final destruction may take place with the following penetration of its components into the vascular walls (Fig. 1) (Huang et al. 2011; Mehdi et al. 2012). Previously, we found that in ApoE -/- mice atherosclerotic plaque developed within 6 months of standard diet (Fig. 1) (Leonova et al. 2017).

A high level of cholesterol induces membrane rigidity of RBC (Prisco et al. 1991), in patients with acute coronary syndrome the amount of cholesterol in erythrocyte membranes is higher than in healthy individuals (Tziakas et al. 2007). Moreover, there is some evidence that particular cholesterol from erythrocyte membranes is accumulated in an atherosclerotic plaque (Arbustini 2007). Accumulation of erythrocytes can also facilitate hematoma and thrombus formation in a plaque as well as accumulation of calcium; proliferation of smooth muscle cells precedes all these processes (Stary et al. 1995).

All age-associated changes in erythrocytes occur in parallel with the accumulation of superoxide radicals generated during hemoglobin autoxidation (Silva et al. 2009). The oxidation process has a pathological action on erythrocytes, promoting deformation of membranes, formation of spectrin–hemoglobin cross-links, increased calcium capture, and potassium leaching (Kay et al. 1986; Datta et al. 2006; Minetti et al. 2007). Normal erythrocytes contain a high percentage of oxygen; therefore, they use a glutathione antioxidant system to protect from the active forms of oxygen (AFO). In patients with atherosclerosis, the activity of the enzyme glutathione peroxidase decreases. Glutathione peroxidase reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols (Espinola-Klein et al. 2007). With aging the activity of the glutathione-antioxidant system decreases, leading to the accumulation of AFO and enhancement of oxidative stress (Erden-Inal et al. 2002).

It is interesting that the erythrocytes react to oxidative stress by the distribution of danger signals, which are accurately packed in membrane microvesicles or exosomes. Exosomes are famous for delivering molecules from one cell to another due to their ability to rapidly merge with the recipient cell membrane. That way exosomes activate the Th1 cell response of the immune system via antigen-presenting cells (Buttari et al. 2015). It was found that the numbers of exosomes elaborated by erythrocytes were significantly lower in premenopausal women than age-matched men. But after menopause, this number increases greatly. Recent studies have demonstrated that exosomal miRNAs are capable of regulating macrophage polarization, serving as transcription factors (Montecalvo et al. 2012; Chang et al. 2014; Das and Halushka 2015). It is believed that exosomes, like the homing pigeons, carry information from one cell to another by the given address; therefore their content can be used to determine the pathology of any or-

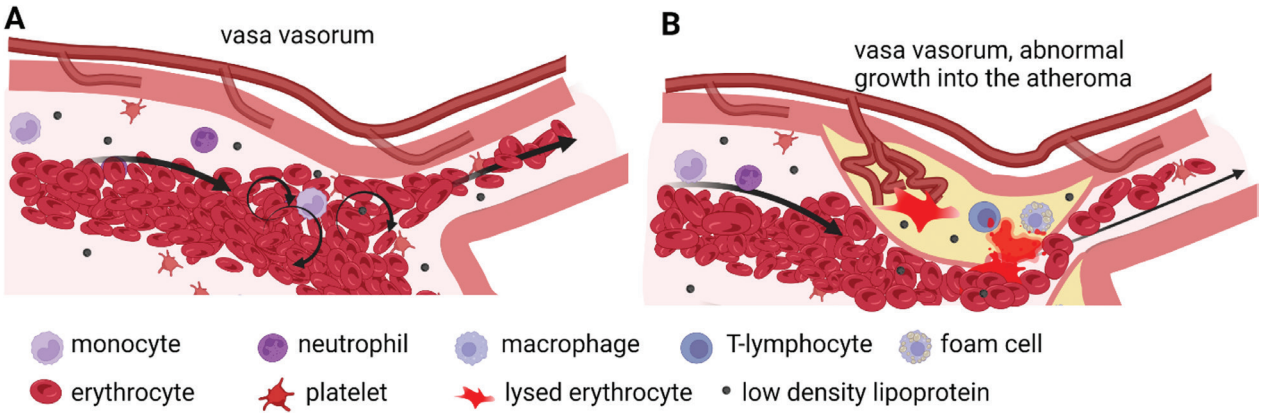


Figure 2. Fragment of aortic arch of healthy mice (**A**) and mice with atherosclerosis (**B**). In a turbulent flow upon touching the vascular wall ageing RBCs lyse, providing additional cholesterol deposition. VV abnormal growth can be an additional source of erythrocytes and leukocytes, migrating to the plaque. Figures were created with BioRender.com.

gan (Choi et al. 2013). The Japanese researchers suggested the coordinated aging process is associated with the capacity of exosomes to distribute signals from senescent cells over the entire organism (Xu and Tahara 2013).

Migration and proliferation of vascular smooth muscle cells

Clinical manifestation of atherosclerosis takes place when the plaque grows into a vascular lumen. This process is associated with the proliferation of smooth muscle cells with the following migration from the middle layer of arterial walls (the media area) to the intima area. It should be noted that the human intima area always contains smooth muscle cells in contrast to rodents (Libby et al. 2011). However, the cells from the media area are associated with the atherosclerosis process, when they start actively dividing in response to the signals of growth factors of fibroblasts, thrombocytes, and endothelium proteins (thrombin and interleukin-1). In this case, the synthesis of nitrogen oxide (NO) and transforming factor-beta is inhibited (Rudijanto 2007). Reactive oxygen species (ROS) facilitate the division of smooth cells which may be considered as an important factor stimulating the development of atherosclerosis. As shown above, the main sources of ROS and inflammation foci are erythrocytes and neutrophils. Moreover, the enzymes of the NADPH-oxidase family contributing to the formation of ROS are expressed in smooth muscle cells, endothelium cells, and macrophages.

The proliferation of smooth muscle cells and the growth of the extracellular matrix are the reasons for restenosis, complication, and repeated vasoconstriction after stenting.

Formation of a fibrous capsule

Intima cells generate extracellular matrix molecules including collagen and elastin, which leads to the formation of a fibrous capsule covering the plaque. When the collagen layer of a fibrous capsule is not yet overgrown, the plaque may be ruptured with the formation of a thrombus capable of vessel plugging (Libby 2008). The formation of a thick fibrous capsule prevents the plaque from rupturing. The plaque rupture and formation of a thrombus is the worst scenario of clinical manifestation of atherosclerosis. At later stages of the disease, the capillaries extend from the external arterial layer to the atheroma. The avascular arterial media needs to be supplemented with oxygen and nutrients that can be transported by diffusion from the lumen of the vessel and from the vasa vasorum (VV). The VV growth is stimulated in hypoxic conditions by hypoxia-inducible transcription factors HIF-1 and HIF-2 (Heistad and Marcus 1979; Pages and Pouyssegur 2005). These vessels can be an additional source of erythrocytes and leukocytes, migrating to the plaque (Fig. 2). Besides, they are a pool for storage of vascular stem cells, which can differentiate to smooth muscle cells, endothelium cells, and fibroblasts (Kawabe and Hasebe 2014). However, the atheroma surrounding supports abnormal vascular stem cell differentiation; as a result, osteoblast-like cells can be formed (Abedin et al. 2004). It is a paradox that the generation of osteoblast-like cells in a plaque is evidence of its stability (Pugliese et al. 2015). Thus, the organism has elaborated a protecting mechanism from undesirable plaque ruptures.

Interpretation of data obtained on mouse models of hypercholesterolemia

The influence of the immune system cells on the atheroma development is supported by numerous studies on mouse models with hypercholesterolemia. Upon switching out genes coding for P selectin and P-selectin glycoprotein ligand-1 in ApoE knockout mice, the atheroma growth was remarkably retarded (Manka et al. 2001; Luo et al. 2012). The same result was obtained in studies of the double knockout of *apoe* genes and the enzyme

α2,3-sialyltransferase IV that links sialic acid to selectin ligands, without which monocyte migration is slowed down (Doring et al. 2014). Crossbreeding of immune-deficient mice with ApoE knockout ones has led to a decrease in the plaque size in the latter by 73% with the cholesterol level remaining unchanged (Zhou et al. 2000). Transplantation of CD4-T cells from ApoE knockout mice to double knockouts with immune-deficient cells, on the contrary, promoted plaque growth in the latter. In this case, the expression of gamma-interferon and MHCII proteins increased, which is evidence of M1 macrophage action. In mice with double-knockout gamma-interferon and *apoe* genes, the plaque size was much smaller than in ApoE-deficient ones (Gupta et al. 1997). The extremely high cholesterol level in mouse models can affect hemodynamic indices of blood and increase plasma viscosity. This will result in a decreased shear stress, i.e., will cause the same effect as upon changes in erythrocyte deformation. The small size of low-density lipoproteins (LDL) (21–26 nm) suggests their penetration into the vascular wall by diffusion avoiding LDL receptors (Lin et al. 1989). In our assumption, this process occurs constantly which is supported by the presence of lipid bands in young organisms. However, the accumulation of LDL can be intensified by some factors, for example, hypertension or upon endothelial cell mitosis as well as at cell death (Lin et al. 1990). Although normally apoptosis of endothelium cells is an infrequent phenomenon, it intensifies with aging (Yildiz 2007). The LDL concentration is higher in the region of low parietal stress and turbulent bloodstream. At the same time, the higher the concentration of LDL, the more actively their infiltration occurs (Deng et al. 1995). At an extremely high concentration, LDL can accumulate over the whole length of a vessel, independent of the bloodstream and parietal stress (Kumar et al. 2016). Since in human blood the LDL concentration is low, it can be suggested that the main source of cholesterol on vascular walls will be accumulated because of penetration and destruction of erythrocytes, the number of which is much higher than normal.

Conclusions

The analysis of the literature data has permitted determining atherosclerosis first of all as a disease of the autoimmune inflammatory character. As a rule, the cells that cannot be "repaired" for the sake of maintaining the integrity of the organisms are programmed for apoptosis. Age-specific changes in the cells of the immune system leads to the accumulation of apoptosis products. Thus, foci of inflammation are formed which under the action of additional factors become a pathology. These processes are the basis for the development of a large number of age-specific diseases, in particular, atherosclerosis. A focus of inflammation is formed in arterial walls provoking "fading" neutrophils, M1 and M2 phages as well as dendritic cells and parallel oxidative stress. At the same time, the action of external factors such as hypertension and turbulent bloodstream in areas of bifurcation and bends determines the site for initiation of formation of an atherosclerotic plaque. Then, the semi-destructed erythrocytes carried by the turbulent bloodstream are the basis for plaque formation. The accumulation of erythrocytes in the plaque is one of the reasons for the accumulation of cholesterol and calcium and for the formation of a thrombus. At later stages of the disease, the growth of capillaries from the external rather layer to the plaque area replenishes the pool of erythrocytes and leukocytes, thus elongating the inflammation. Moreover, capillaries are the source of stem cells with damaged differentiation, which leads to the accumulation of osteoblast-like cells. This way of calcification results in stabilization of the plaque, preventing it from disruption. Aging erythrocytes can produce dangerous signals packed in exosomes which reprogram other cells of the immune system. Thus, inflammation becomes chronic. The fact that in women prior the menopause the number of exosomes produced by erythrocytes is lower than in men of the same age and after menopause, it grows drastically is a reason why atherosclerosis in women does not develop prior to menopause. An important role in the loss of the function of immune cells belongs to the accumulation of mutations in the mitochondrial DNA, which is also characteristic of an aging organism. Age-associated clonal hematopoiesis, which leads to M1/M2 disbalance, also correlates with a higher risk for atherosclerosis development.

More research is needed to identify the relationship between age-associated clonal hematopoiesis, M1/M2 polarization, senescent erythrocytes, and the development of atherosclerosis in order to search for new pharmacological targets.

Conflict of interests

The authors declare no conflict of interests.

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Author contributions

- **Elena I. Leonova**, PhD in Biology, Head of the Center for Transgenesis and Genome Editing of St. Petersburg State University, Scientific Center for Genetics and Life Sciences of Sirius University of Science and Technology, e-mail: e.leonova@spbu.ru, **ORCID ID** https://orcid.org/0000-0002-0236-3302. The author provided the idea of research, analyzed the material, results and made the conclusions, wrote and edited the text of the article, image visualization

- **Angelina V. Chirinskaite,** Junior researcher of Center of Transgenesis and Genome Editing, e-mail: ChirinskaiteA@yandex.ru, **ORCID ID** https://orcid.org/0000-0002-7466-0680 The author analyzed the material, results and conclusions, wrote the text of the article.

- **Julia V. Sopova,** PhD in Biology, Center of Tansgenesic and Genome Editing, Institute of Translational Biomedicine, St. Petersburg State University, Scientific Center for Genetics and Life Sciences, Sirius University of Science and Technology **e-mail:** sopova@hotmail.com, **ORCID ID** https://orcid.org/0000-0002-7825-273X. The author defined the idea of research, analyzed the material, results and conclusions, wrote the text of the article, image visualization