UDC:616 - 008.9 – 06:616.381 – 005.4 – 091.8
DOI: 10.15587/2519-4798.2020.220202

ENDOTHELIUM CONDITION AND ROLE OF IMMUNOCOMPETENT CELLS IN ATHEROSCLEROSIS DEVELOPMENT AS A CAUSE OF ISCHEMIC STROKE

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The aim. *To determine the state of the cerebral vascular endothelium and the role of immunocompetent cells in the ischemic stroke development on the background of atherosclerosis.*
Materials and methods. *We studied cerebral vessels of 50 deaths with ischemic cerebral infarctions, 50 – with severe cerebral atherosclerosis without CVD (cerebrovascular disease) manifestation and 50 deaths, whose cause of death was not related to CVD and atherosclerosis (control group). Histological preparations of vessels were stained with hematoxylin-eosin and Masson Trichrome, and also immunohistochemical study was conducted using CD31/PECAM-1 (Endothelial Cell Marker) Ab-1, CD4 (CD4 Ab-8), CD8 (SP 16), CD20 (CD20 Ab-1) CD68 and (CD68/Macrophage Marker Ab-4) markers.*
Results. *Under ischemic strokes and severe atherosclerosis the cerebral vessels endothelium acquires structural changes in form of rupture, desquamation and exfoliation, formation of desquamated endothelial cells clusters. Speaking of endothelial damage, it should not be supposed that changes should occur at the macroscopic level only, endothelial damage at the cellular level shall be sufficient enough. Immunocompetent cells are of key importance in atherosclerosis development; adhesion on the luminal surface of arteries, presence of a large number of these cells under the endothelium and of more mature macrophages in the intima depth indicates the influx of these cells, which actively potentiate atherosclerosis formation, from the blood into the artery wall.*
Conclusions*. Disorders of the endothelial lining with changes in endothelial cells morphology contribute to the atherosclerotic plaque development. Lymphocytes and macrophages form the molecular basis of many important processes, including the inflammatory response and the immune response*
Keywords*: atherosclerosis, endothelium, immunocompetent cells, macrophages, lymphocytes, ischemic stroke, CD4, CD8, CD20, CD68, CD31*

1. Introduction

Cerebrovascular disorders are one of the leading causes of death in the world. Mortality due to cerebrovascular diseases (CVD) for several decades ranks second in the structure of total mortality. Every 5 minutes one person in Ukraine has a stroke and every 12 minutes one of the patients dies of a stroke; 30-40 % of stroke patients in Ukraine die within 1 month and about half within 1 year, 20–40 % become disabled and only less than 20 % return to full life [1, 2]. Moreover, these diseases are becoming "younger" and more common every year [3].

There are a number of factors, such as hypertension, hyperinsulinemia, dyslipidemia, obesity, which increase the risk of cardiovascular disease, contribute to their earlier development, rapid progression and a greater likelihood of serious complications. Most of them are risk factors for the development of acute and chronic cerebrovascular diseases, and their total effect increases this probability many times over [4, 5].

The main morphological substrate of arterial lesions in the manifestations of cerebrovascular diseases is atherosclerosis, the morphogenesis of which is currently insufficiently studied, despite the large number of theories that try to explain its occurrence [6, 7]. In recent years, the development of atherosclerosis is considered from the standpoint of immune inflammation, where an important role is assigned to immunocompetent cells [8]. Local infiltration by macrophages in the areas of atherosclerotic lesions is often combined with the accumulation of T-lymphocytes, which indicates the inflammatory nature of the process [8, 9]. Macrophages have receptors on their surface for low-density lipoproteins, activation of mononuclear cells through the binding of these receptors to oxidized atherogenic lipoproteins leads to activation of subintimal mononuclear phagocytes, as a result of which they acquire the ability to endocytose lipoproteins.

A significant role in the morphogenesis of atherosclerosis is given to the endothelium, which is reflected in the "theory of endothelial damage", where the cause of atherosclerotic lesions of arteries is defects in the endothelium with platelet adhesion, accompanied by the release of biologically active aggregates. However, in recent years, theories such as "monoclonal" and "immune inflammation" have been proposed, which deny the role of mechanical damage to the endothelium and suggest reliance on changes in endothelial permeability and vascular wall cell proliferation [10–12]. Therefore, it is important to study the role of immunocompetent cells and endothelium in atherogenesis and further development of ischemic stroke.

The aim of the research was to study the state of the vascular endothelium of the brain and the role of immunocompetent cells in the development of ischemic stroke on the background of atherosclerosis.

2. Material and research methods

To solve the goal set in the work, a number of clinical and morphological studies were conducted. The

material was collected on the basis of the centralized pathological anatomical department of the Regional Clinical Hospital in Ivano-Frankivsk. Histological and histochemical examinations were performed on the basis of the histological laboratory of the centralized pathological department of the Regional Clinical Hospital in Ivano-Frankivsk and on the basis of the histological laboratory of the Department of Pathological Anatomy of Ivano-Frankivsk National Medical University. Immunohistochemical studies were performed in the pathomorphological laboratory of the diagnostic and consulting center "CSD Health care", Kyiv. The study was conducted for the period 2016–2020 (5 years).

The cerebral vessels of 50 deaths with ischemic cerebral infarctions, 50 - with severe cerebral atherosclerosis without CVD and 50 deaths from causes not related to CVD and atherosclerosis (comparison group) were studied. Arteries of two structural and functional levels were studied: main – carotid arteries and extra cerebral - arteries of the brain base, where 2–3 segments were taken from the infarct area, with lipid and fibrous plaques, in the comparison group – unchanged areas.

The material for the study was taken in accordance with the moral and ethical requirements of ethics. The Ethics Commission of Ivano-Frankivsk National Medical University did not find any violations (Minutes No. 74/13 of 19.11.2016).

Histological preparations of vessels were stained with hematoxylin-eosin, Mason's trichrome, and also carried out immunohistochemical research using markers CD31 / PECAM-1 (Endothelial Cell Marker) Ab-1, CD4 (CD4 Ab-8), CD8 (SP 16), CD20 CD20 Ab-1) CD68 and (CD68 / Makrophager Marker Ab-4).

The material was fixed in a 10 % solution of neutral buffered formalin, performed according to conventional methods. For immunohistochemical reactions, sections 4–5 μm thick were mounted on Super Frost Plus adhesive slides (Menzel), dewaxed, hydrated, and treated with 3 % hydrogen peroxide solution to block endogenous peroxidase. The Ultra Detection System kit (Thermo Scientific) was used as the second antibody. To separate nonspecific structures, sections were additionally stained with Mayer's hematoxylin.

The results of immunohistochemical reactions of markers CD4 (CD4 Ab-8), CD8 (SP 16), CD20 (CD20 Ab-1) CD68 and (CD68 / Makrophager Marker Ab-4) were evaluated by counting cells with positive staining in 10 randomly selected fields of view of the microscope at a magnification of 400. Immunopositivity of CD31 is observed in the cytoplasm and cell membrane. The results of the immunohistochemical reaction CD31 / PECAM-1 (Endothelial Cell Marker) Ab-1 were evaluated by a semi-quantitative method in points from 0 to 6 according to the conventional method, taking into account the stained cells [1]. 0 points were determined in the absence of staining, 1 point – up to 10 %, 2 points – up to 20 %, 3 points – up to 30 %, 4 points – up to 40 %, 5 points – up to 50 %, 6 points – more than 50 % of stained cells . In addition, the degree of color intensity was evaluated: 0 – no color, 1 (+) – weak color of light brown color, 2 (++) – moderate color of brown color, 3 (+++) – pronounced color of dark brown color.

Histological examination and photography of micropreparations were performed on an AxioScop 40 microscope (Zeiss).

Data from morphometric studies were subjected to statistical processing using a personal computer using the standard program Microsoft Excel, the results were processed by the method of variation statistics and considered reliable at $p < 0,05$.

3. Research results

The internal carotid arteries, middle cerebral arteries and arteries of the base of the brain, the main function of which is the blood supply, as well as the vessels of the microcirculatory tract, which provide metabolic processes in the brain, were studied.

Examination of cerebral arteries of large and medium caliber revealed a significant thickening of the wall with narrowing of the lumen, the presence of atherosclerotic plaques with narrowing, in places with complete closure of the lumen (Fig. 1).

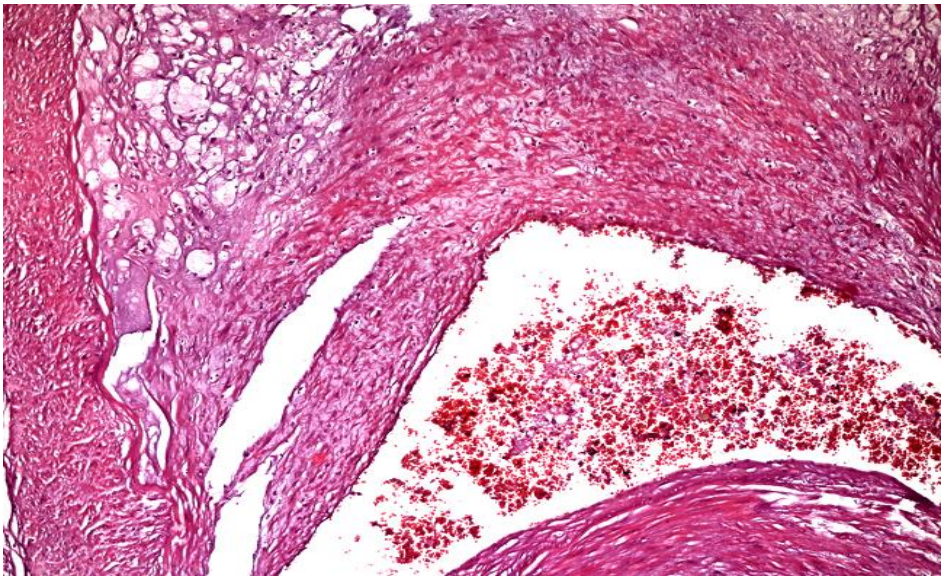

Fig. 1. Significant thickening of the artery wall with narrowing of the lumen, the presence of atherosclerotic plaque with a predominance of lipoidosis and liposclerosis. Staining with hematoxylin-eosin ×400

In the development of atherosclerosis an important role is played by the state of metabolism in the inner and middle membranes of the arteries, given that they are nourished by plasma perfusion. Therefore, in intimal hyperplasia there are conditions for impaired plasma perfusion through the vessel wall, which creates conditions for the development of lipidosis of the intima and the medial vessels of the brain (Buie J., Oates J., 2014). Macrophages with lipid inclusions, so-called xanthoma cells, with a foamy cytoplasm and a round nucleus were observed in the areas of intima proliferation (Fig. 2).

The development of atherosclerosis requires the presence of atherogenic dyslipoproteinemia, the entry of lipoproteins into the inner lining of the arteries, cell rearrangement in the inner lining and capture by cells of low-density lipoproteins coming from the blood, as well as the formation of mesenchymal fibrous cells (Shimada K., 2009). Some substances, such as cholesterol and lipoproteins, and endothelial changes can initiate atherogenesis – activated and damaged endothelium may be involved in atherogenesis. Endothelial activation is also accompanied by the production of cell growth factors (Schäfer A., Bauersachs J., 2008) (Fig. 3).

The study of the marker CD31 in the arterial wall in the group with ischemic stroke revealed uneven deposition of antigen in the endothelium – 3.2±0.3 points, in the group with severe atherosclerosis also observed high expression of the marker and averaged 3.5±0.3 points ($p>0.05$). The endothelium of cerebral vessels in ischemic strokes and severe atherosclerosis acquires structural changes in the form of rupture, desquamation and exfoliation, the formation of clusters of desquamated endothelial cells (Fig. 4, 5).

In the comparison group in the reaction to CD31 revealed uniform antigen deposition – 5.6±0.3 points, which is significantly more than in the study groups (3.2±0.3 points – group I and 3.5±0.3 points – group II, $p>0.05$) (Fig. 6).

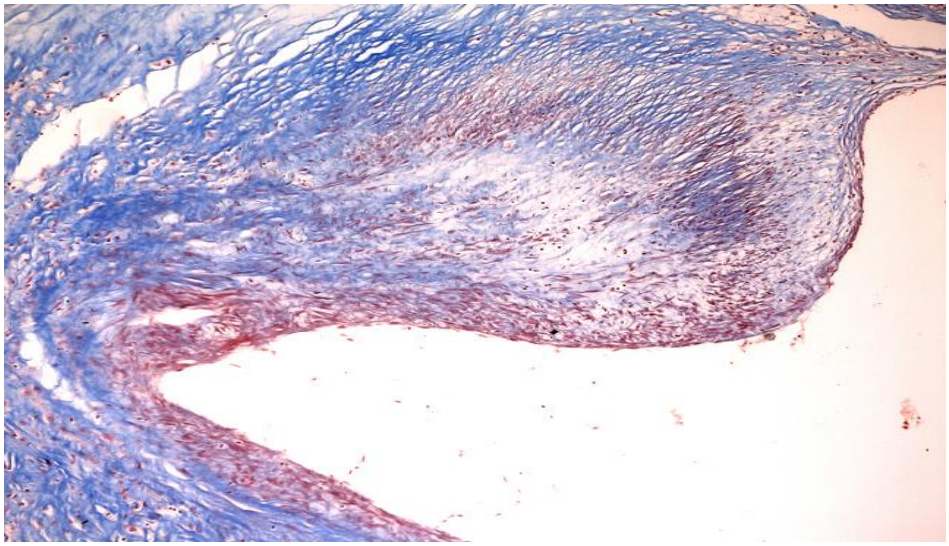

Fig. 2. Proliferation of the intima, the presence of macrophages with lipid inclusions. Trichrome color by Masson × 400

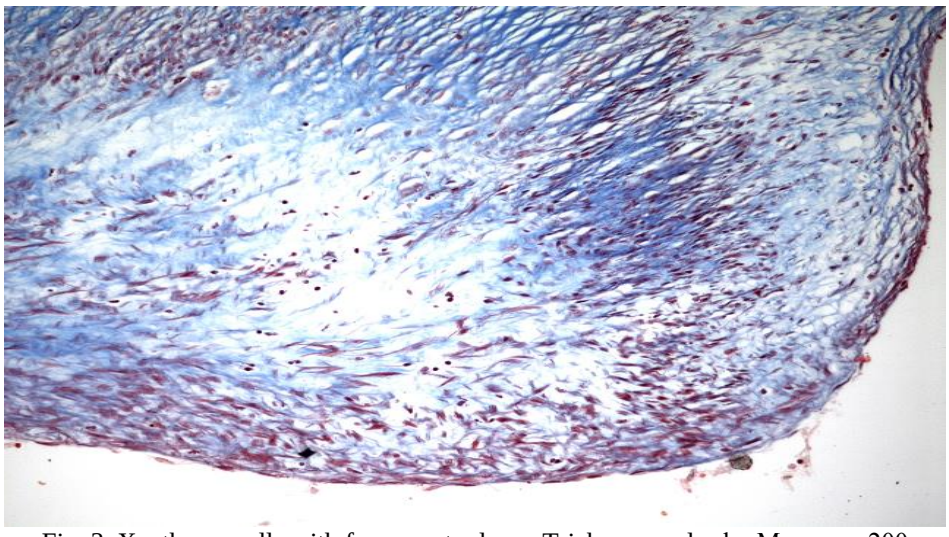

Fig. 3. Xanthoma cells with foamy cytoplasm. Trichrome color by Masson × 200

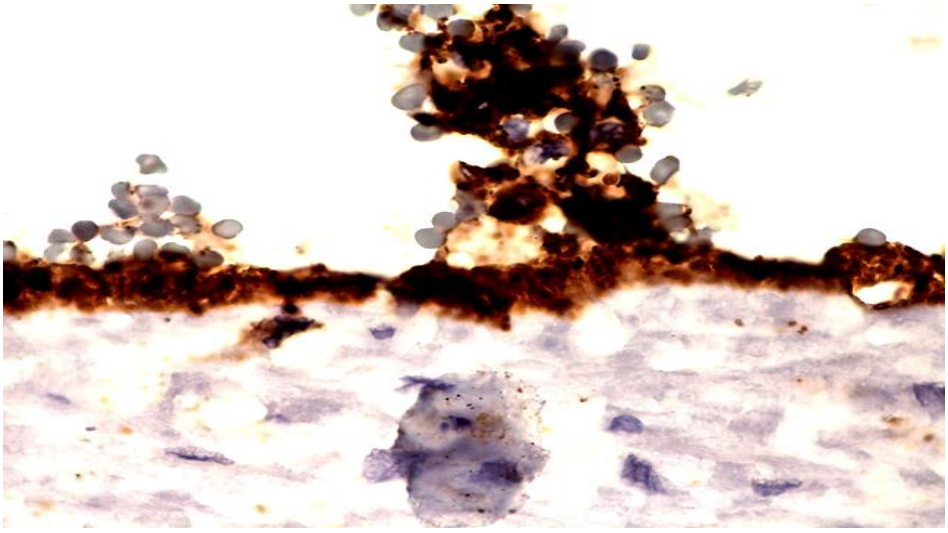

Fig. 4. Intensive expression of the CD31 marker in the endothelium of the artery of a patient with ischemic stroke – the formation of clusters of desquamated endothelial cells ×1000

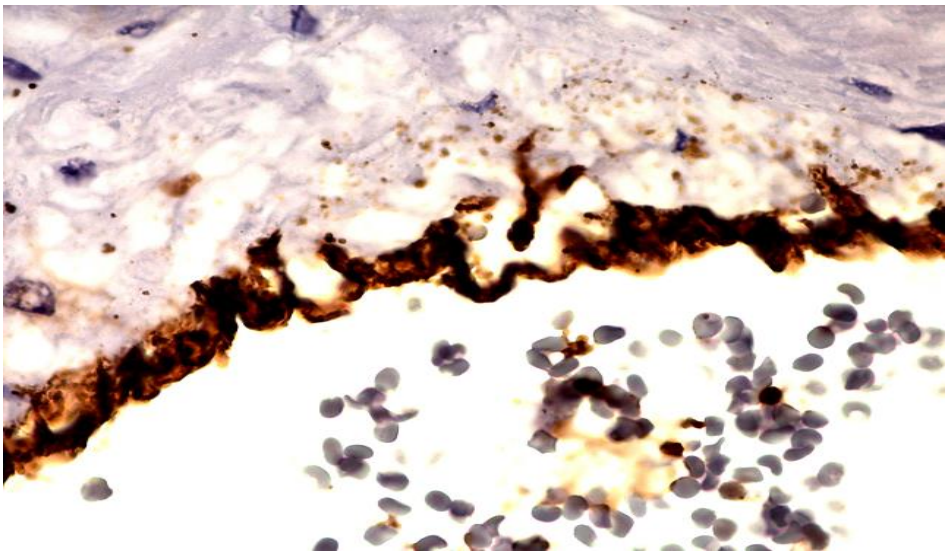

Fig. 5. Intensive expression of the marker CD31 in the endothelium of the artery of a patient with severe atherosclerosis of the vessels of the brain - dystrophic changes, endothelial detachment ×1000

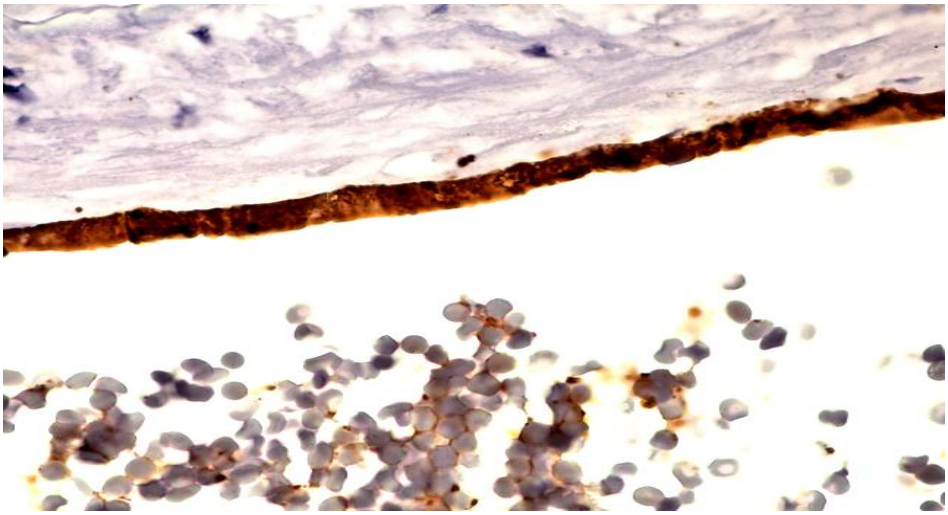

Fig. 6. Intensive expression of the marker CD31 in intact arterial endothelium in the comparison group ×1000

Therefore, speaking of endothelial damage, it should not be assumed that the changes should occur at the macroscopic level, it is enough to damage the endothelium at the cellular level, i.e. changes in the morphology of endothelial cells in places where atherosclerotic plaques may develop later. It is believed that the endothelial barrier plays an active role in the occurrence of atherosclerotic plaque by regulating the penetration of endothelial cover, by local secretion of vasoactive mediators (Vanhoutte P. M., 2009).

In the areas of atherosclerotic lesions, a significant number of CD68 positive cells with marker expression in the cytoplasm was observed (Fig. 7), which was 16.68±1.82 in the group with ischemic stroke, 17.93±1.67 in the group with severe atherosclerosis ($p>0.05$).

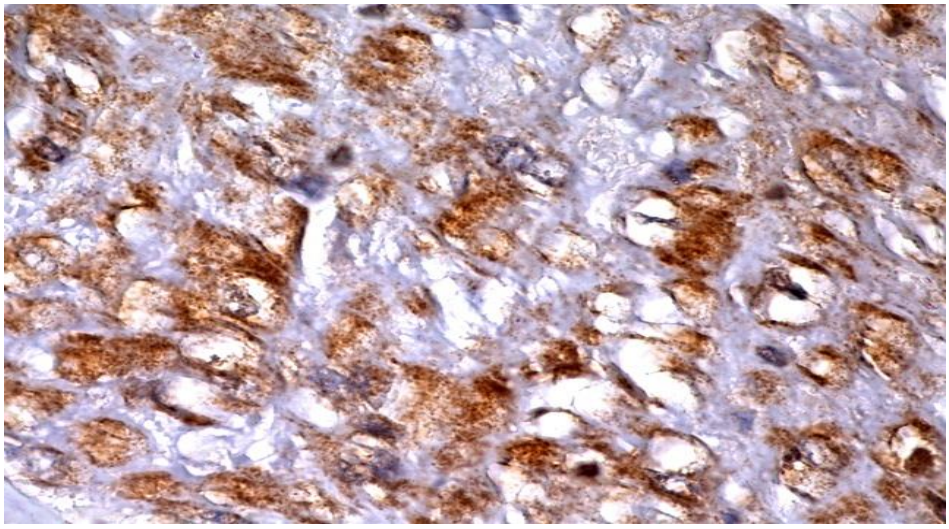

Fig. 7. Intense expression of the marker CD68 in the artery wall of a patient with ischemic stroke ×400

In the initial stage of atherogenesis, blood monocytes accumulate in the damaged layer of endothelium and subendothelial space, then they are transformed into macrophages, accumulate lipids, the cytoplasm of which acquires a foamy appearance, i.e. foam cells are formed.

Monocytes-macrophages absorb and catabolize proteins, express on their surface their short peptide fragments, which with the participation of activation proteins are transmitted to T-lymphocytes (Gotsman I., Gupta R., Lichtman A. H., Gotsman I., 2007)

The immune response in AS is represented by cellular and humoral links of the response. The cellular response to atherogenesis is provided by T-helpers (CD4) and cytotoxic T-lymphocytes (CD8), and the humoral response is provided by B-lymphocytes (CD20), which produce immunoglobulins.

The expression of helper T-lymphocytes (CD4, membrane expression) in the areas of atherosclerotic lesions was 11.18±1.76 in the group with ischemic stroke (Fig. 8) and 11.46±1.24 in the group with severe atherosclerosis.

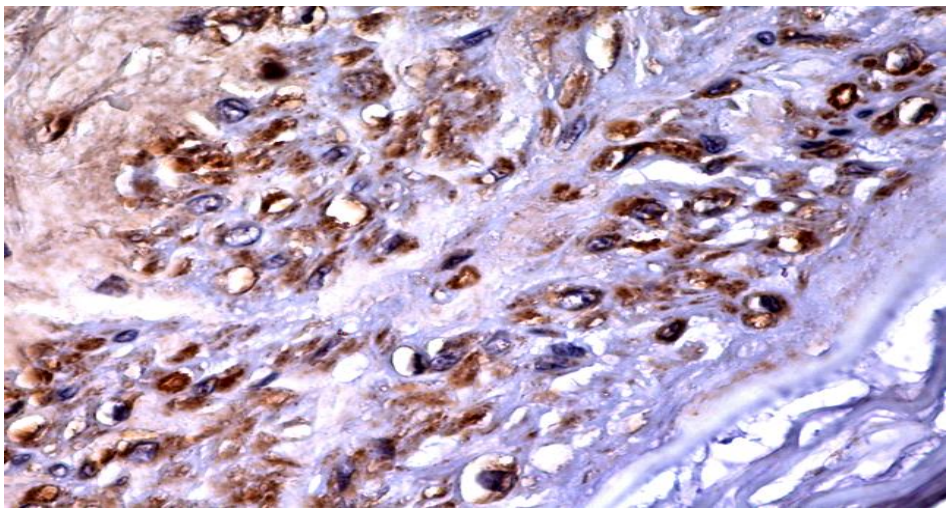

Fig. 8. Intense expression of the marker CD4 in the artery wall of a patient with severe atherosclerosis ×400

T-lymphocytes suppressors (CD8, membrane expression) (Fig. 9) were 8.56±1.16 and 9.12±1.64 (p>0.05), respectively. In the comparison group, the expression of markers in the arterial wall was absent, in some cases it was observed in the form of single cells).

B-lymphocytes (shell-cytoplasm expression) were present in a smaller number of 5.34±0.86 in the area of atherosclerotic lesions and 5.98±1.14 in ischemic stroke (p>0.05) (Fig. 10).

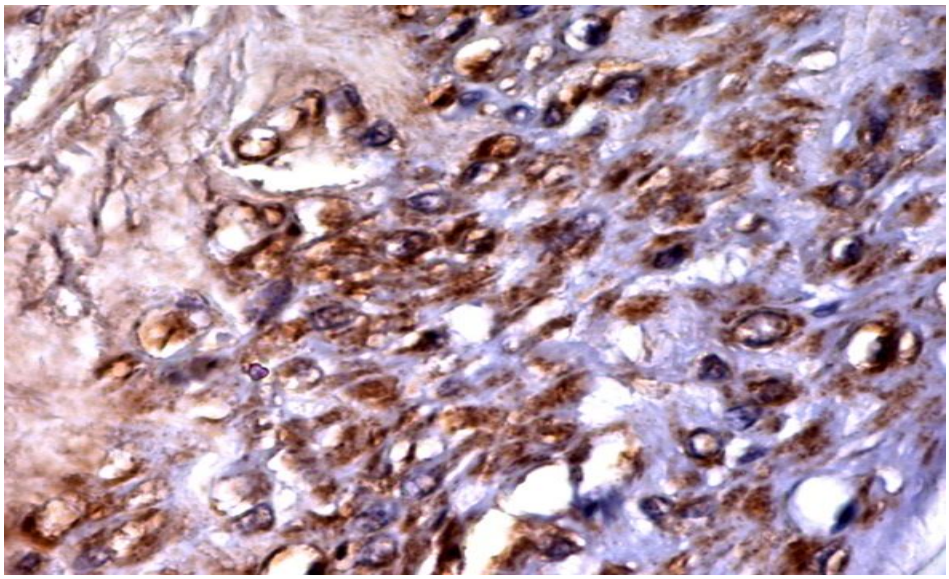

Fig. 9. Intense expression of the marker CD8 in the artery wall of a patient with severe atherosclerosis ×400

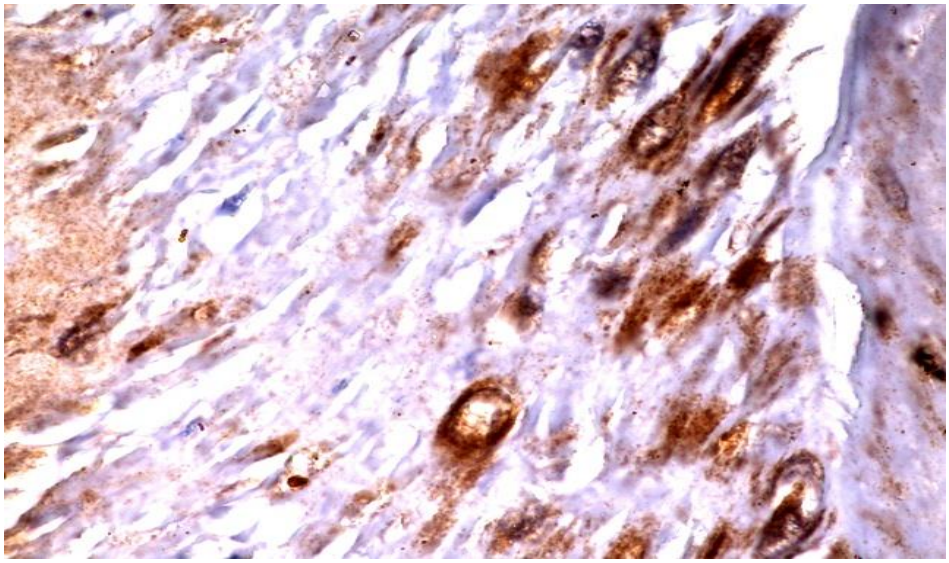

Fig. 10. Expression of the CD20 marker in the artery wall of a patient with ischemic stroke ×400

Lymphocytes and macrophages in areas of atherosclerotic lesions of the arteries are in contact with each other and produce activating antigens. Interaction within the intima occurs due to direct intercellular contacts and molecular factors, with the participation of which they exchange signals that perform para- and autocrine regulation (Ammirati E, Moroni F, Magnoni M, Camici PG., 2015).

Therefore, it should be noted that lymphocytes and macrophages form the molecular basis of many important processes, including the inflammatory response and the immune response.

4. Discussion of research results

To study the changes in the vascular endothelium of the brain and the role of immunocompetent cells in atherosclerosis, studies of the structural features of cerebral vessels in those who died of ischemic stroke on the background of atherosclerosis were done.

An important role in the pathogenesis and morphogenesis of atherosclerosis is assigned to the endothelium, which is reflected in the "theory of endothelial damage". According to this theory, the cause of atherosclerotic lesions of the arteries is the occurrence of defects in the endothelium with platelet adhesion, which is accompanied by the release of biologically active substances that activate platelet aggregation and thrombus formation [12–14]. Endothelium is currently considered as a complex metabolic system, which plays an important role in autoregulation of blood circulation, maintaining homeostasis by regulating vascular tone [11, 15].

Immunohistochemical study of the endothelial marker CD31 in the arterial wall in the group of deaths with ischemic stroke on the background of atherosclerosis revealed uneven deposition of antigen in the endothelium – 3.2±0.3 points ($p> 0.05$). In the endothelium of the cerebral arteries during stroke on the background of atherosclerosis, structural changes were observed in the form of rupture, desquamation and exfoliation, the formation of clusters of desquamated endotheliocytes. In the comparison group in the reaction to CD31 revealed a uniform deposition of antigen – 5.6±0.2 points, which was significantly higher than in the study groups ($p<0.05$). The morphological characteristics of the changes obtained during the study did not differ from the data presented in other modern studies. The obtained data indicate, and are identified with the data of foreign authors, that the endothelial barrier plays an active role in the formation of atherosclerotic plaque by regulating the penetration of endothelial cover, by local secretion of vasoactive mediators [12].

In recent years, the development of atherosclerosis is considered from the standpoint of immune inflammation, where the role of immunocompetent cells is important. In the areas of atherosclerotic lesions, a significant number of CD68-positive cells with marker expression in the cytoplasm was observed, which was in the group with ischemic stroke 16.68±1.82 ($p>0.05$). It is believed that under the influence of hemodynamic factors endothelial cells of the arteries synthesize macrophage chemoreactive factor, which leads to the accumulation in the intima of monocytes-macrophages, which play an important role in the development of atherosclerosis [10, 14–16]. In the initial stage of atherogenesis, blood monocytes accumulate in the damaged layer of endothelium and subendothelial space, then they are transformed into macrophages, accumulate lipids, the cytoplasm of which acquires a foamy appearance, i.e. foam cells are formed [9, 13]. Thus, the data from modern publications are comparable with the results of our study.

The immune response in atherosclerosis is represented by cellular and humoral links. The cellular response to atherogenesis is provided by T-helpers (CD4) and cytotoxic T-lymphocytes (CD8), and the humoral response by B-cells (CD20), which produce immunoglobulins [283, 313]. Helper T-lymphocytes (CD4) in the areas of atherosclerotic lesions were 11.18±1.76 in the group with ischemic stroke and 10.32±1.24 in the group without manifestations of cerebrovascular disease ($p>0.05$). T-suppressor lymphocytes (CD8) were 8.56±1.16 and 9.12±1.64 ($p>0.05$), respectively. B-lymphocytes were present in smaller numbers – 5.34±0.86 in the area of atherosclerotic lesions in ischemic stroke ($p>0.05$).

Thus, macrophages and lymphocytes play a significant role in the morphogenesis of changes in the vascular wall in atherosclerosis, as evidenced by their accumulation in areas of atherosclerotic lesions of the arteries. Macrophages in the vascular wall due to the accumulation of lipids are transformed into foam cells, and lymphocytes due to the cellular and humoral immune response potentiate the further formation of atheromatous plaque.

Study limitations. Limitations are related to the possibility of conducting research only on autopsy material.

Prospect for further research in this area is further study of the mechanisms of damage to the arterial wall of the brain and the mechanisms of immune processes in the morphogenesis of atherosclerotic changes in cerebral vessels.

5. Conclusions

1. For the formation of atherosclerotic changes in the arteries of the brain requires damage to the endothelium at the level of changes in endothelial cells that secrete factors that affect the adhesion and penetration of low-density lipoproteins into the intima.

2. Immunocompetent cells, macrophages and lymphocytes play a significant role in the formation of atherosclerotic plaque, as evidenced by their accumulation in areas of atherosclerotic lesions of the arteries. Macrophages in the vascular wall by the accumulation of lipids are transformed into foam cells, and lymphocytes due to the cellular and humoral response potentiate the further formation of atheromatous plaque.

3. The morphological basis of ischemic stroke is atherosclerotic lesions of cerebral vessels with lipoidosis and fibrous plaques in the vessel wall, proliferation of the intima, resulting in narrowing of the lumen of blood vessels with the development of ischemia.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Received date 12.08.2020
Accepted date 23.09.2020
Published date 30.11.2020

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