МОРФОФУНКЦИОНАЛЬНЫЕ ИЗМЕНЕНИЯ МИКРОГЛИИ У МОЛОДЫХ И СТАРЫХ КРЫС WISTAR

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Резюме. Болезнь Альцгеймера (БА) является одним из наиболее распространенных нейродегенеративных заболеваний, приводящих к деменции. На сегодняшний день не существует эффективных методов лечения этого заболевания, так же как и единого мнения относительно механизмов, лежащих в основе его патогенеза. Получение данных об этих механизмах *in vivo* возможно только путем моделирования нейродегенерации у лабораторных животных. Среди различных теорий инициации нейродегенерации активно изучается влияние микроглии, а также инфламэйджинг – хроническое системное низкоуровневое возраст-ассоциированное воспаление. Оно проявляется увеличением числа стареющих клеток с секреторным фенотипом, ассоциированным со старением (SASP). В конечном итоге это приводит к манифестации и прогрессированию возраст-зависимых заболеваний, в том числе БА. Целью исследования была оценка возрастных изменений микроглии, про- и противовоспалительных цитокинов в головном мозге, а также субпопуляций лимфоцитов в периферической крови. В работе использовали самцов крыс Wistar двух возрастных групп – старых (возраст 24 месяца) и половозрелых (возраст 3 месяца) при отсутствии какого-либо дополнительного воздействия. В гиппокампе оценивали морфологические изменения микроглии на препаратах, окрашенных антителами к Iba1. В префронтальной коре головного мозга с помощью ПЦР-РВ исследовали уровень экспрессии провоспалительных – IL-6 и TNFα, противовоспалительных – IL-10 и TGF-β, цитокинов, а также маркеров активации микроглии – iNOS и MMP-9. В периферической крови оценивали содержание основных субпопуляций лимфоцитов с помощью проточной цитофлуорометрии. Показано, что по сравнению с половозрелыми крысами старые животные характеризуются значительными изменениями морфологии микроглии, увеличением уровня экспрессии провоспалительных и снижением противовоспалительных цитокинов, повышением маркеров активации микроглии. При старении

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наблюдалось снижение процентного содержания моноцитов и В-клеток в периферической крови. Полученные данные свидетельствуют о развитии инфламэйджинга, который проявляется в виде активации микроглии, смещения баланса продукции цитокинов в сторону провоспалительных и, как следствие, активации миграции моноцитов и В-лимфоцитов из крови в ткани. Таким образом, исследование роли воспаления в развитии БА целесообразно выполнять на старых животных, физиологическое состояние которых соответствует таковому у людей. Дальнейшие исследования в этой области позволят расширить понимание механизмов инициации и прогрессирования нейродегенерации, необходимое для разработки новых и эффективных терапевтических подходов к лечению БА.

Ключевые слова: нейродегенерация, старение, микроглия, инфламэйджинг, воспаление, иммуносенесценция

MORPHOFUNCTIONAL CHANGES OF MICROGLIA IN ADULT AND OLD WISTAR RATS

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Abstract. Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases leading to dementia. There is no effective treatments for this disease so far, as well as a consensus concerning the mechanisms of its pathogenesis initiation. Obtaining data on them *in vivo* is possible only by modeling neurodegeneration in laboratory animals. Among the various theories of the initiation of neurodegeneration, the impact of microglia is vigorously studied recently, as well as inflammaging, which is a term for chronic age-related low-grade systemic inflammation. It manifests in the increasing number of senescent cells with senescence-associated secretory phenotype (SASP). Eventually, it leads to manifestation and progression of age-related diseases, such as AD. The aim of the study was to evaluate age-related changes in microglia, pro- and anti-inflammatory cytokines expression levels in the brain, as well as ones of microglial activation, and also subpopulations of lymphocytes in peripheral blood. We used male Wistar rats of two age groups, which were composed of old (age 24 months) and adult (age 3 months) rodents, without any additional exposure. In the hippocampus, morphological changes in microglia were assessed on preparations stained with antibodies to Iba1. In the prefrontal cortex, RT-qPCR was used to study the level of expression of pro-inflammatory IL-6 and TNFα, anti-inflammatory IL-10 and TGF-β cytokines, as well as microglial activation markers iNOS and MMP-9. In the peripheral blood, the relative numbers of the main subpopulations of lymphocytes and monocyte were measured by flow cytometry. It was shown that, compared with adult rats, old animals are characterized by significant changes in the morphology of microglia, an increase in the level of expression of pro-inflammatory and a decrease in anti-inflammatory cytokines, and an increase in microglia activation markers. With aging, a decrease in the percentage of monocytes and B cells in peripheral blood was observed. These data indicate the development of inflammaging, which displays itself in microglia activation, a shift in the balance of cytokine production towards pro-inflammatory ones, and, as a result, activation of the migration of monocytes and B lymphocytes from the blood into tissues. Thus, it is justified to study the role of inflammation in the development of AD in old animals whose physiological state corresponds to that in humans. Further research in this area will expand the understanding of the mechanisms of initiation and progression of neurodegeneration, which is necessary for the development of novel and effective therapeutic approaches to the treatment of AD.

Keywords: neurodegeneration, aging, microglia, inflammaging, inflammation, immunosenescence

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Introduction

Alzheimer's disease (AD) is one of the most prevalent types of neurodegenerative diseases leading to dementia. Today there are around 50 million patients with AD worldwide [10], and this number will triple by 2050 [11]. AD remains a great burden for not only patients and people who provide the care of them. It also makes an economic load on health care systems of many countries worldwide, creating an annual global cost of almost 1 trillion USD. Another challenge associated with AD is an absence of any effective treatment so far. Existing and approved drugs can only faintly relieve the symptoms of AD.

There is no consensus concerning the initial mechanisms of AD pathogenesis [1]. The impact of inflammaging, or chronic age-related low-grade systemic inflammation [3] is one of the most perspective and intensively studying hypotheses so far [5]. According to recent data microglia, which are resident immune cells in the central nervous system (CNS), might play the key role of AD development as well [8]. Cellular senescence manifesting with senescence-associated secretory phenotype (SASP) leads to microglia activation and is associated with the consistent increasing of pro-inflammatory mediators' production [9] forming a vicious circle of the disease. It is also worth noting that microglia cells, as well as other types of immune cells, undergo the process of cellular senescence themselves, which affects and probably disrupts their function.

Although neurodegenerative diseases, such as AD, belong to the group of age-related pathologies but their modeling is still being conducted only on adult rodents, whereas experiments on old animals can provide more relevant data. **The purpose of this work** was to study morphofunctional changes of microglia in adult Wistar rats in comparison with old ones to reveal age-related alterations.

Material and methods

The work was performed on adult (n = 10, age 3 months) and old (n = 10, age 24 months) male Wistar rats, euthanized by overdose (15 mg/kg) of Zoletil (Vibrac Sante Animale). The study was approved by the Bioethical Commission of the Avtsyn Research Institute of Human Morphology of Federal state budgetary scientific institution "Petrovsky National Research Centre of Surgery" (Protocol No. 36 (12) March, 28, 2022). All experimental work involving animals was carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986).

For ICH-P study, frontal histological sections of brains (6.0 mm posterior relative to bregma) were stained with antibodies Iba1 (1:100; P4C288Ra01, Cloud Clone) and secondary HRP Donkey-anti-Rabbit antibodies (1:500; 416035, Novex Life Technologies). The pictures were captured with the Leica microscope (DM 2500 Leica Microsystems) on magnification 1600.

The expression of IL-6, IL-10, TNFα, TGF-β, iNOS, and MMP-9 mRNA was assayed by real-time qPCR in tissue fragments of the prefrontal cortex. The levels of all aforementioned mRNA expression relative to GAPDH expression level as a reference were determined using a qPCRmix-HS SYBR (Eurogen, Russia) containing fluorescent intercalating dye SYBR Green I. Amplification with detection and digital analysis of fluorescence in real time was carried out on DT-96 Real-Time PCR Cycler (DNA-Technology JSC, Moscow, Russia) in a standard mode at 95 °C for 5 minutes followed by 95 °C for 15 seconds, 62 °C for 10 seconds + reading and 72 °C for 20 seconds 45. All the primers sequences were picked up by on-line soft Primer-BLAST.

Absolute and relative numbers of lymphocytes various subpopulations were counted using flow cytometry (Beckman Coulter, USA) in peripheral blood. The following antibodies (eBioscience, USA) were used for immune phenotypic analysis: anti-rat CD3-PE for total T lymphocyte population, anti-rat CD4-FITC for $CD3^+CD4^+$ for T helpers, anti-rat CD8-PE-Cy5 for $CD3^+CD8^+$ for T cytotoxic cells, anti-rat CD45R-FITC for $CD45R^+$B cells, and anti-rat CD43-PE for $CD43^+$ monocyte. Erythrocytes were lysed with the OptiLyse C solution (eBioscience, USA). The results were analyzed by Statistica 8.0 software (StatSoft, Inc.) using Mann–Whitney U test.

Results and discussion

In a morphological study, adult rats' microglia had a regular size and thin ramified processes, which are features of resting functional status (Figure 1A, see 2nd page of cover). At the same time, old rats' microglia had an increased size and spheroidal swelling, hypertrophic, beaded, and tortuous processes (Figure 1B, see 2nd page of cover).

The result of qPCR showed that IL-6 and TNFα expression levels were higher in old rats than in adult rats by 1.5-fold and 3-fold accordingly. Also iNOS, which is a marker of M1 activated microglia, was

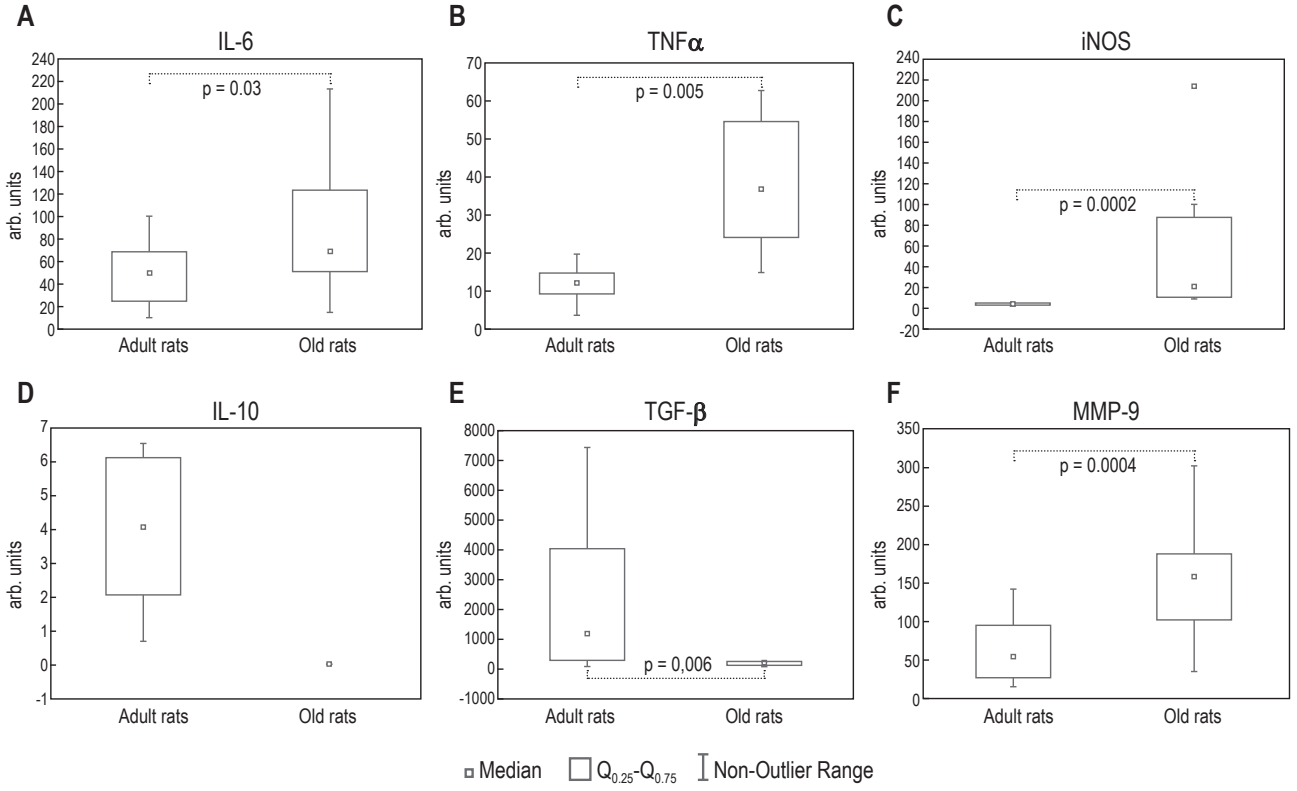

Figure 2. mRNA expression levels of IL-6 (A), TNFα (B), iNOS (C), IL-10 (D), TGF-β (E), and MMP-9 (F) in the prefrontal cortex of adult (left bar) and old (right bar) Wistar rats

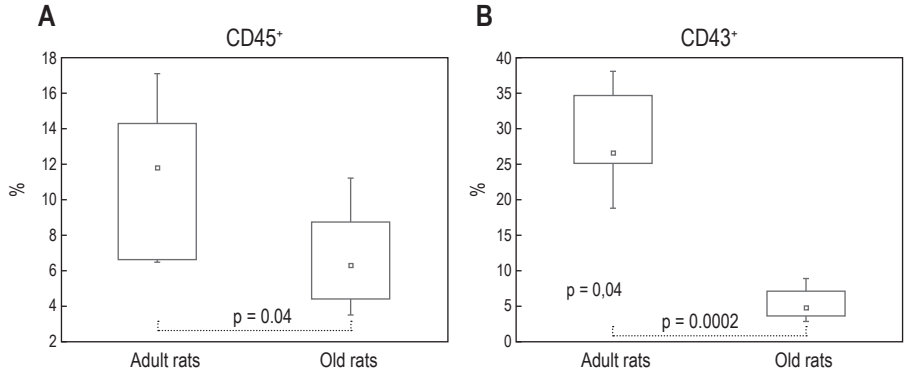

Figure 3. Percentage of CD45R+B cells (A) and CD43+ monocyte (B) in peripheral blood of adult (left bar) and old (right bar) Wistar rats

5-fold higher in old rats in comparison with adult rats. Old rats group showed no IL-10 expression at all, whereas the expression level of TGF-β was 7.25-fold higher in adult rats. At the same time *MMP9*, which is a marker of M2 activated microglia, was 3-fold higher in old rats group (Figure 2).

Additionally, the immune phenotypic analysis of monocyte and lymphocytes subpopulations was performed to estimate the impact of aging on the number of various immune cells in peripheral blood. Flow cytometry data demonstrated no statistically significant differences in the relative numbers of lymphocytes ($CD3^+$), including $CD3^+CD4^+$T helpers and $CD3^+CD8^+$T cytotoxic cells, between the groups. However, the percentage of $CD45R^+$B cells and $CD43^+$ monocyte was almost 2-fold and 6.5-fold higher in adult rats in comparison with old rats (Figure 3).

Hence, we observed morphological changes of microglial cells, the decreasing of anti-inflammatory cytokines expression levels and the increasing of pro-inflammatory ones as well as microglia activation markers, and the reduction of monocyte and B cells' numbers in old rats due to aging.

Our data are consistent with latter results obtained from humans. It was shown that identical changes of

microglia appeared in healthy human with aging [6]. Shahidehpour et al. described age-related changes of microglia morphology as hypertrophic and dystrophic and stated there were a strong evidence that dystrophic microglia are disease-associated one. It is generally accepted that the CNS resident immune cells have so called "resting" and activated states. Microglia activation, just as macrophages' one, leads cells to pro-inflammatory M1 polarization or anti-inflammatory M2 polarization. But also there is a continuum of different intermediate phenotypes between M1 and M2, and microglia can take turns from one state to another depending on microenvironment [4]. So, it is not a surprising found of presence both M1 and M2 microglia in healthy adult and old rats.

At the same time, the significant rise of iNOS and MMP9 expression levels in old rats implies there is a higher number of activated immune cells caused by aging itself due to the development of SASP. Being secretory active and likely senescent as well, these microglial cells might not only fail their surveillance and clearance functions, but also take part in aggravation of already existing neuroinflammation with unpleasant consequences. Revealing exact mechanisms to prevent them is a task yet to come.

We showed the reduction of monocyte and B cells' numbers in peripheral blood of old rats. In recent research concerning lymphopoiesis in both human and mouse aging revealed a link between the level of TNFα, consistently produced by peripheral B cells themselves during aging, and restraining of B cell lymphopoiesis in the bone marrow [2]. Meanwhile, Snodgrass et al. showed a decreasing of circulating monocyte pool due to aging in humans [7]. It could happen due to abating of monocytopoiesis in bone marrow as well as B cells' or cells destruction caused by pro-inflammatory background or other reasons; but it also could be related with an intensification of monocyte migration to different tissues, including brain parenchyma, since inflammaging is a system condition involving the whole organism.

Conclusion

Hence, this study confirms a great deal of differences between adult and old rodents' physiological state. Obviously, their reactions to the same exposure of various factors will be quite different as well. Since old rats demonstrate age-related changes in immune system's cells similar to humans', using aged animals for modeling of neurodegeneration is justified. Further investigations in this search field will provide more essential data for future inventions of novel and efficacious therapeutic approaches.

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