ИЗУЧЕНИЕ ЛОКАЛЬНОЙ ЭКСПРЕССИИ ГЕНОВ ИНФЛАММАСОМНОГО КОМПЛЕКСА ПРИ МОДЕЛИРОВАНИИ ДЕГЕНЕРАЦИИ СЕТЧАТКИ *IN VIVO*

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Резюме. Нейродегенеративная офтальмопатология является одной из основных причин необратимой слепоты и инвалидности в мире. В патогенезе заболеваний данной группы в последнее время все большее внимание уделяется роли локального воспаления, обусловленного активацией врожденного иммунитета и механизмам его генетической регуляции. В последние годы в области экспериментальной офтальмологии появились работы, которые продемонстрировали возможность сборки инфламмасомных комплексов NLRP1, NLRP3 при действии гипергликемии, влиянии кислородной депривации клеток сетчатки, а также при моделировании компрессионного стресса, подобного таковому при глаукоме, однако механизм участия инфламмасом в развитии нейродегенеративных заболеваний глаз остается невыясненным.

Целью работы явилось изучение локальной экспрессии генов, кодирующих белки инфламмасомного комплекса NLRP3 (NLRP3, CASP-1) в экспериментальной модели дегенерации сетчатки на кроликах.

Исследования выполнены в образцах тканевого комплекса (ТК) сетчатка/ретинальный пигментный эпителий (РПЭ), выделенного из глаз 14 кроликов породы новозеландских альбиносов, на которых моделировалось дегенеративное поражение сетчатки путем однократного субретинального введения 0,01 мл 0,9% раствора хлорида натрия, и 7 здоровых кроликов без поражения глаз. Оценка уровней экспрессии генов NLRP3 и CASP-1 в ТК сетчатка/РПЭ проводилась методом полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР). По результатам проведенного исследования, отмечалось статистически значимое увеличение экспрессии гена NLRP3 ($p < 0,001$) в ТК сетчатка/РПЭ глаз экспериментальных животных, что, возможно, свидетельствует об участии компонентов инфламмасомы NLRP-3 в развитии нейродегенеративного поражения сетчатки. В то же время экс-

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прессия гена, кодирующего CASP-1, обнаружена только в ТК сетчатка/РПЭ опытных глаз и, вероятно, обусловлена локальными воспалительными механизмами в ткани сетчатки.

Высокий уровень мРНК NLRP3, CASP-1, определявшийся во всех образцах ТК сетчатка/РПЭ опытных глаз на поздних сроках (3 и 6 мес.) эксперимента, позволяет предположить формирование механизмов (например, активированного глиального фенотипа), поддерживающих воспаление в ткани ретины, что должно учитываться при активно разрабатываемых в настоящее время трансплантационных методиках лечения ретинальной дегенерации.

Ключевые слова: нейродегенерация, сетчатка, компоненты инфламмасомного комплекса, NLRP3, CASP-1, экспрессия генов, ОТ-ПЦР

INVESTIGATION OF LOCAL EXPRESSION OF NLRP3 INFLAMMASOME COMPLEX GENES IN MODELING RETINAL DEGENERATION *IN VIVO*

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Abstract. Neurodegenerative ophthalmopathology is one of the main causes of irreversible blindness and disability in the world. In the pathogenesis of diseases of this group, more and more attention has recently been paid to the role of local inflammation caused by the activation of innate immunity and the mechanisms of its genetic regulation. In recent years, works have appeared in the field of experimental ophthalmology that have demonstrated the possibility of NLRP1, NLRP3 inflammasome complexes assembling when exposed to hyperglycemia, oxygen deprivation of retinal cells, as well as modeling compressive stress similar to that in glaucoma [15]. However, the mechanism of inflammasome involvement in the development of neurodegenerative eye diseases remains unclear. The aim of the study was to investigate the local expression of genes encoding proteins of the NLRP3 inflammasome complex (NLRP3, CASP-1) in an experimental model of retinal degeneration in rabbits. The studies were performed on samples of tissue complex (TC) of the retina/retinal pigment epithelium (RPE) (retina/RPE TC), isolated from the eyes of 14 New Zealand albino rabbits, in which degenerative retinal lesion was modeled by a single subretinal injection of 0.01 mL of 0.9% sodium chloride solution, and 7 healthy rabbits without eye damage. The formation of retinal degeneration was judged on the basis of changes in morphofunctional parameters obtained during specialized ophthalmological research methods (optical coherence tomography, fundus autofluorescence, electroretinography) at follow-up periods of 1, 3 and 6 months. The level of expression of NLRP3 and CASP-1 genes in the retina/RPE TC was evaluated by reverse transcription polymerase chain reaction (RT-PCR). According to the results of the study, a statistically significant increase in NLRP3 gene expression ($p < 0.001$) was noted in the retina/RPE TC of experimental animals, which may indicate the involvement of NLRP-3 inflammasome components in the development of neurodegenerative retinal lesions. At the same time, the expression of the gene encoding CASP-1 was detected only in the retina/RPE TC of experimental eyes and is probably due to local inflammatory mechanisms in the retinal tissue.

The high level of NLRP3, CASP-1 mRNA, detected in all retina/RPE TC samples of experimental eyes at late stages of the experiment (3 and 6 months), allows us to assume the formation of mechanisms (for example, activated glial phenotype) that support inflammation in retinal tissue. This should be taken into account in actively developing transplantation methods for the treatment of retinal degeneration.

Keywords: neurodegeneration, retina, components of the inflammasome complex, NLRP3, CASP-1, gene expression, RT-PCR

Introduction

Neurodegenerative eye pathology is a heterogeneous group that includes age-related macular degeneration (AMD), hereditary retinal dystrophy, primary open-angle glaucoma (POAG), diabetic retinopathy (DR), and some other forms. These diseases have different etiologies, but their common characteristic is a progressive destructive-degenerative process that affects all cellular elements of the neural retina with a violation of its integrity and leads to irreversible loss of visual functions, blindness and disability [4].

Significant visual impairment occurs at the advanced stages of neurodegenerative eye pathology: in this case, the implementation of therapeutic measures is limited, associated with certain difficulties (requires specialized invasive interventions) and does not always lead to positive functional results, and for some forms, such as, "geographic atrophy" and hereditary retinal degenerations, there is no treatment [12]. Diseases of this group are multifactorial, the pathogenesis of which remains completely unexplored: currently, in addition to the main factors of their development and progression – age (AMD, POAG), increased intraocular pressure (POAG), metabolic (AMD, DR), endocrine (DR), hemodynamic disorders (systemic and local), an increasing number of attention is paid to the role of local inflammation caused by activation of the innate immune response [8, 10].

In the retina, microglia macrophages are cells of innate immunity that recognize and form a responsive reaction to patterns associated with pathogens and damage (PAMPs and DAMPs) [1]. Being located in the inner and outer plexiform layers of a healthy retina, normally moderately activated microglia phagocytoses cellular debris and regulates neurovascular interactions [11, 14].

A number of inducing factors lead to the formation of an activated M1 phenotype of glial macrophages that implement a protective response, which is neutralized after a short-term adverse effect [5]. Prolonged activation of M1-microglia can cause neurotoxicity due to the dominance of excessive inflammatory response [2, 5, 6].

Indeed, *in vitro* experiments have shown that DAMPs released during trauma (including compression, surgical), prolonged exposure to UV light, toxic products of oxidative modification of biomolecules, etc., bind to pattern-recognition receptors (PRRs), cause activation of microglia macrophages and its transition to the M1 phenotype, secreting high levels of pro-inflammatory cytokines TNFα, IL-1β, IL-6, nitric oxide and reactive oxygen species, contributing to the maintenance of inflammation and damage to the neural retina [4, 7].

In recent years, works have appeared in the field of experimental ophthalmology that have demonstrated the possibility of assembling inflammasome complexes NLRP1, NLRP3 under the influence of hyperglycemia, the effect of oxygen deprivation of retinal cells, as well as when modeling compression stress similar to that in glaucoma [15]. However, the mechanism of inflammasome involvement in the development of neurodegenerative eye diseases remains unclear. **The purpose of the research** was to study the local expression of genes encoding NLRP3 inflammasome proteins (NLRP3, CASP-1) in an experimental model of retinal degeneration in rabbits.

Materials and methods

The study was carried out in compliance with the international principles of the Helsinki Declaration on Humane Treatment of Animals, the principles of humanity set out in the European Community Directive (86/609/EC), and the "Rules for conducting work using experimental animals". Degenerative retinal damage was modeled in 14 New Zealand albino rabbits (experimental group: age 2.5-3.0 months, weight 2.0-2.5 kg) by a single subretinal injection at a distance of 1-1.5 mm below the optic nerve disc of 0.01 mL of 0.9% sodium chloride solution with the formation of a subretinal bubble according to the methodology developed in the Department of retina and optic nerve pathology of the Helmholtz National Medical Research Center of Eye Diseases [13]. Preoperative preparation of animals included intramuscular administration of painkillers 0.3 mL of zoletil 50 and 0.55 mL of 2% xylazine; also before surgery 0.3 mL of 0.4% dexamethasone and 0.5 mL of dicinone were administered.

The formation of retinal degeneration was judged on the basis of changes in morphofunctional parameters obtained during specialized ophthalmological research methods (optical coherence tomography, fundus autofluorescence, electroretinography) at follow-up periods of 1, 3 and 6 months. At these times, animals were removed from the experiment by air embolism after anesthesia (according to the order of the Ministry of Higher Education of the USSR No. 724 of November 13, 184), and then eye enucleation was performed.

The control group included 7 somatically healthy rabbits without ocular pathology. The study protocols were approved at a meeting of the local ethics committee of the Helmholtz National Medical Research Center of Eye Diseases [13].

Twenty-eight samples of retina/RPE TC were used as a material for molecular biological study, isolated from the eyes of experimental animals according to standard protocols. The biomaterial was transferred to cryotubes and stored at a temperature of -70 °C until the research was carried out.

Using the Vector NTI program (Invitrogen), nucleotide sequences (5'-) for rabbit genes NLRP3,

CASP1 and reference GAPDH were selected and subsequently synthesized (DNA Synthesis, LLC, Russia):

CASP1-F
CTGAATGTCAACACCATCTTTGAG;
CASP1-R
CTATTCCTTGGTTTTCACCACGA;
CASP1-probe FAM-
CCCAAGGTGATCATCATCCAAGCC-BHQ1;
NLRP3-F
TGCAGATGTTGGAGTTAGACAGC;
NLRP3-R
GCAGGTCAGGATCGTGCAG;
NLRP3-Probe FAM-
CCTCACCTCGCACTGCTGCTGG-BHQ1;
GAPDH-F
GCTGGTCATCAACGGGAAGG;
GAPDH-R
GGTGAAGACGCCAGTGGATT;
GAPDH-Probe FAM-
CTTCCAGGAGCGAGATCCCGCC-BHQ1

Samples of retina/RPE TC were homogenized, and mRNA extraction was performed using the Gene JET RNA Purification Kit (Thermo Scientific, USA) in accordance with the manufacturer's instructions. Reverse transcription reaction was performed using the OT-1 kit from "Syntol" company (Russia). The obtained cDNA fragments were amplified by real-time polymerase chain reaction (RT-PCR) using a DT-96 thermocycler from DNA Technology (Russia). For PCR-RV, kits for determining the expression of the NLRP3, CASP-1, GAPDH genes were used (DNA synthesis, Russia).

To determine the relative amount of cDNA in the sample, the method of normalized expression of ΔΔCt was used. The results were expressed in relative units (rel. Units): the ratio of the threshold cycle value of amplification of the studied gene to the threshold cycle value of amplification of the reference gene (the "housekeeping" gene) GAPDH: ΔΔCt = (ΔCt of the sample) / (ΔCt GAPDH).Statistical processing of the results was carried out using the StatTech v. 3.1.1 software package (Stattech, Russia). Quantitative indicators were evaluated for compliance with a normal distribution (Shapiro–Wilk criterion). In the absence of a normal distribution, quantitative data were described using the median (Me) and the lower and upper quartiles ($Q_{0.25}$-$Q_{0.75}$); the Mann–Whitney U test was applied when comparing groups. The critical level of significance in testing statistical hypotheses was $p < 0.05$.

Results and discussion

The results of the study are presented in Table 1 and Figures 1, 2.

In the retina of 43% of healthy animals' eyes (n = 6) and in all cases of the experimental group (n = 14), expression of the gene encoding NLRP3 was detected. The level of NLRP3 mRNA in the TC of experimental animals with retinal degeneration was statistically significantly higher than that in normal tissue, with a median expression of 35.0 rel. Units (Table 1, Figure 1)

An individual analysis revealed that in the vast majority of cases (70%), the degenerative process in the retina was associated with a significant increase in the expression of the NLRP3 gene, the indicators of which exceeded the upper limit of the established normal range. In about one-third of experimental eyes, its transcriptional activity was within normal values (from 1.9 to 2.1 rel. units).

Changes in NLRP3 gene expression in the TC of experimental eyes were the same at all follow-up periods (at 1, 3 and 6 months).

The expression of the gene encoding CASP-1 was studied in the retina/RPE TC of animals in the main group and control (Table 1, Figure 2). None of the studied samples of healthy retina showed expression of the CASP-1 gene, unlike the material from experimental animals, in which the median expression activity in retina/RPE TC was 38.0 rel. units (p = 0.00).

The pattern of changes in mRNA levels of CASP-1, as well as NLRP3, in the retina/RPE TC of experimental eyes was the same at all observation periods (at 1, 3 and 6 months) in the group with retinal neurodegeneration.

NLRP3 is a member of the PRRs superfamily and belongs to the NOD-like receptor (NLR) family

TABLE 1. ANALYSIS OF GENE EXPRESSION OF COMPONENTS OF THE NLRP3 INFLAMMASOME COMPLEX (NLRP3, CASP-1) IN GROUPS OF RABBITS

Group	Expression level (rel. units)					
	NLRP3			CASP-1		
	Me	($Q_{0.25}$-$Q_{0.75}$)	p	Me	($Q_{0.25}$-$Q_{0.75}$)	p
Control group (n = 14)	0.0	0.0-23.0	< 0.001*	0.0	–	< 0.001*
Retinal degeneration (n = 14)	35.0	10.0-37.0		38.0	13.0-39.0	

Note. *, differences considered significant at p value of 0.005 or less.

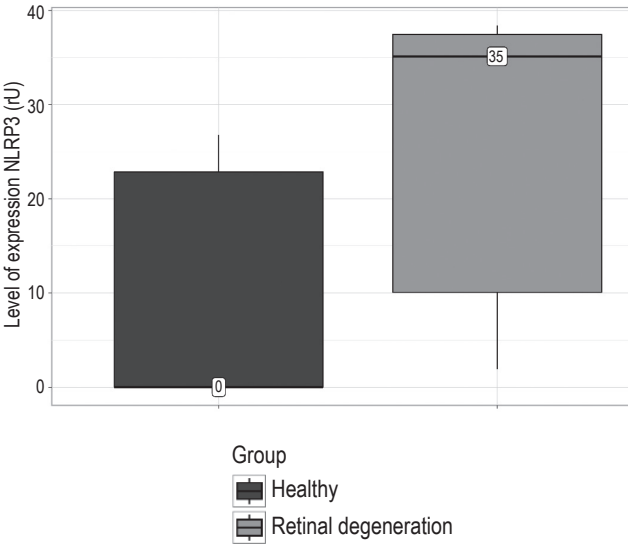

Figure 1. Comparative analysis of expression levels (rel. units) of the NLRP3 gene in the TC retina/RPE of animals without ophthalmopathology (norm) and with degenerative retinal changes

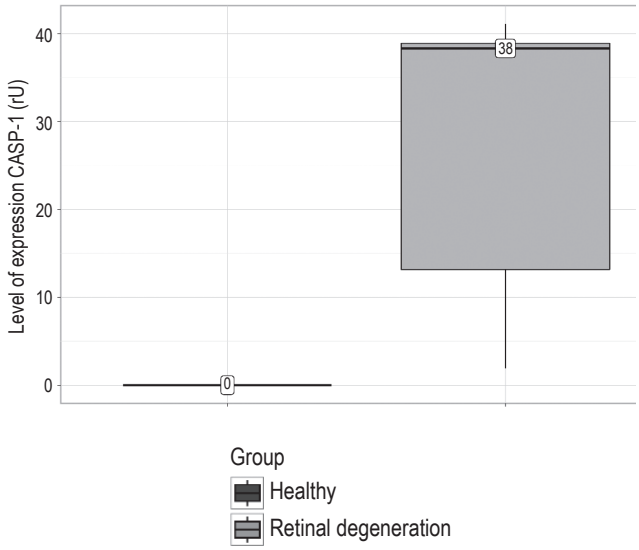

Figure 2. Comparative analysis of expression levels (rel. units) of the CASP-1 gene in the TC retina/RPE in control groups (healthy without ophthalmopathology) and with degenerative retinal changes

localized in the cell cytoplasm. After recognizing danger signals, NLRs are activated, oligomerized, and initiate the assembly of an inflammasome complex with the activation of caspases and maturation of cytokine precursor molecules pro-IL-1β and pro-IL-18 into biologically active forms. In addition, activated caspases are capable of initiating pyroptosis: a programmed proinflammatory form of cell death through the proteolytic protein gazdermine [9]. A significant increase in the level of NLRP3 mRNA ($p < 0.001$) in the retina/RPE TC of the eyes of experimental animals detected in our study indicates the involvement of this receptor in the development of retinal neurodegeneration.

The expression of the gene encoding CASP-1, found only in the retina/RPE TC of experimental eyes and absent in controls, suggests the presence of special local mechanisms regulating the activity of this protein (and therefore the inflammatory reaction) in healthy retina tissue.

Our results are consistent with data from a number of studies (mainly performed *in vitro* or in models on small rodents), which have demonstrated the ability of individual retinal cell elements (astrocytes, Muller cells, microglia) and RPE to actively produce pro-inflammatory mediators, including the IL-1 family, in response to danger signals: ischemia, photo-oxidative damage, hypertension, ATP stimulation. It has been shown that the production of these cytokines is controlled by the activation of an inflammasome complex formed in the cells of the pigment epithelium, retina and vascular membrane of the eye [3].

Conclusion

In our study, special attention was drawn to the preservation of the local response from the studied NLRP3 components: high expression activity of protein genes (NLRP3 and CASP-1) was determined in all retina/RPE TC's of experimental eyes at late stages (3 and 6 months) of the experiment. This circumstance suggests the formation of an M1 activated glial phenotype that supports inflammation in the retinal tissue, which requires further research and should be taken into account in actively developing transplantation methods for the treatment of retinal degeneration in the future.

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