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ИЗУЧЕНИЕ ЛОКАЛЬНОЙ ЭКСПРЕССИИ мРНК ГЕНОВ МЕДИАТОРОВ ВОСПАЛЕНИЯ В МОДЕЛИ АТРОФИИ РЕТИНАЛЬНОГО ПИГМЕНТНОГО ЭПИТЕЛИЯ И ДЕГЕНЕРАЦИИ СЕТЧАТКИ, ИНДУЦИРОВАННОЙ СУБРЕТИНАЛЬНЫМ ВВЕДЕНИЕМ ФИЗИОЛОГИЧЕСКОГО РАСТВОРА В ЭКСПЕРИМЕНТЕ У КРОЛИКОВ

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Резюме. Дегенеративно-дистрофические заболевания сетчатки, в частности возрастная макулярная дегенерация (ВМД), в настоящее время считаются ведущей причиной слепоты и слабовидения в развитых странах, имеют тенденцию к неуклонному росту. В публикациях последних лет представлены доказательства участия воспалительных механизмов в развитии и прогрессировании ВМД, расшифрованных благодаря успехам в области исследований врожденного и адаптивного иммунитета. Однако иммунопатогенез атрофической формы ВМД – «географической атрофии» (ГА) остается практически неизученным. Целью работы явилось исследование локальной экспрессии мРНК воспалительных цитокинов IL-1β, IL-18, CCL2/MCP-1 в модели атрофии РПЭ, индуцированной субретинальным введением 0,9%-ного раствора хлорида натрия в эксперименте у кроликов. Исследования выполнены в образцах тканевого комплекса сетчатки-РПЭ-хориоидеи (ТК), выделенного из глаз 23 кроликов породы новозеландских альбиносов, на которых моделировалась атрофия РПЭ путем субретинального введения 0,9%-ного раствора хлорида натрия, и 5 здоровых кроликов без глазных поражений. Животным опытной и контрольной групп за неделю до оперативного вмешательства, в раннем периоде, в сроки формирования устойчивой атрофии (РПЭ) проводились оптическая когерентная томография (ОКТ) и аутофлюоресценция глазного дна (АФ). Оценка уровней экспрессии генов провоспалительных цитокинов в ТК выполнялась методом ОТ-ПЦР. Показано, что субретинальное введение 0,01 мл 0,9%-ного раствора хлорида натрия, индуцирующее развитие атрофии РПЭ у кроликов в эксперименте, ассоциируется с разнонаправленными изменениями экспрессии mRNA

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генов IL-1β, IL-18, MCP-1/CCL2 относительно нормы. Охарактеризованы три типа ответа в тканевом комплексе хориоидея / РПЭ / сетчатка, формируемые во время развития атрофических изменений и определяемые величиной локальной экспрессии генов цитокинов: 1) гипо/ареактивность – снижение/отсутствие экспресии; 2) нормореактивность – умеренное повышение; 3) гиперреактивность – гиперэкспрессия. У 69,6% животных при формировании устойчивой атрофии отмечалось повышение (от умеренного до гиперответа) локальной экспрессии mRNA MCP-1/CCL2, а в трети случаев – значительное усиление экспрессии mRNA IL-1β – факторов, повреждающих гематоретинальный барьер и способствующих нарушению иммунной привилегии заднего отрезка глаза. Полученные данные могут быть полезны в изучении различных видов атрофии РПЭ и при разработке новых стратегий лечения офтальмопатологии, в доклинических исследованиях.

Ключевые слова: ВМД, атрофия ретинального пигментного эпителия, комплекс «сетчатка – хориоидея», провоспалительные цитокины, экспрессия генов, ОТ-ПЦР

EXAMINING LOCALLY EXPRESSED mRNA OF INFLAMMATORY MEDIATOR GENES IN A MODEL OF RETINAL PIGMENT EPITHELIUM ATROPHY AND RETINAL DEGENERATION INDUCED BY SUBRETINAL SALINE INJECTION IN RABBITS

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Abstract. Degenerative-dystrophic retinal diseases, particularly age-related macular degeneration (AMD), are now considered to be the lead cause of blindness and low vision in developed countries, with a steadily increasing trend. Recent publications provide evidence for the involvement of inflammatory mechanisms in TMD development and progression unveiled due to advances in innate and adaptive immunity research. However, the immunopathogenesis of atrophic AMD form, "geographic atrophy" (GA) remains largely unstudied. Objective: to investigate local mRNA expression of inflammatory cytokines IL-1β, IL-18, CCL2/MCP-1 in a model of RPE atrophy induced after subretinal injection of 0.9% sodium chloride solution in experimental rabbits. The investigation was carried out in tissue complex retina-RPE-choroid (TC) samples isolated from eyes of 23 albino New Zealand rabbits after modeling RPE atrophy by subretinal injection of 0.9% sodium chloride solution and 5 healthy rabbits lacking eye lesions. Animals in the experimental group (one week before surgical intervention, in the early period, and in the period of sustained RPE atrophy formation) and controls were subjected to optical coherence tomography (OCT) and ocular fundus autofluorescence (FAF). Evaluation of proinflammatory cytokine gene expression levels in TC was performed by RT-PCR. Results. Subretinal injection of 0.01 ml of 0.9% sodium chloride solution induced experimental RPE atrophy development in rabbits vs. control that was associated with multidirectional changes of IL-1β, IL-18, MCP-1/CCL2 gene mRNA expression. Three types of response in the TC, formed during development of atrophic changes and determined by the value of local cytokine gene expression were characterized: 1) hypo/ no response – decreased/no expression; 2) normal response – moderate increase; 3) hyper response – overexpression. 69.6% of animals with persistent atrophy had a moderate to hypertrophic increase in locally expressed mRNA MCP-1/CCL2, whereas 30% cases had significantly increased IL-1β mRNA expression – factors damaging the blood-retinal barrier and contributing to posterior segment immune privilege. It should be taken into account while developing new strategies for treatment of ophthalmic pathology, in particular the currently actively studied and tested options for RPE stem cell transplantation into subretinal space. The data obtained may be useful to investigate various types of RPE atrophy and develop new strategies of ophthalmopathology treatment in preclinical studies.

Keywords: AMD, atrophy of the retinal pigment epithelium, retina-choroid complex, proinflammatory cytokines, gene expression, RT-PCR

Introduction

Degenerative-dystrophic retinal diseases are currently considered as the lead cause of blindness and low vision in developed countries and have a steadily increasing trend. Retinal pigment epithelium damage (RPE) is the initial step in the development of degenerative changes in age-related macular degeneration, leading to photoreceptor death and irreversible loss of central vision. RPE is formed by a single layer of hexagonal polarized pigmented epithelial cells and located between the choroid and the neurosensory retina [10].

Normally, the RPE exerts many specialized functions aimed at maintaining retinal homeostasis and performing the visual cycle [4, 10]. RPE plays an important role in ocular immune privilege by forming the outer part of the haematoretinal barrier and secreting membrane-bound and soluble immunosuppressive and apoptotic factors into the subretinal space [3, 5]. PPE cells are incapable of regeneration, and their damage is irreversible [9].

An effective treatment for GA has not been developed [11]. Despite intensive research, the obstacles in development of promising new treatments for AMD are primarily due to incomplete knowledge of the disease pathogenesis.

Recent publications show that the process of RPE and central retinal photoactive zone damage is associated with the activation of identified inflammatory mechanisms owing to advances in innate and adaptive immunity research [13]

Thus, simulating unfavorable conditions such as hypoxia, hyperglycemia, oxidative and hyperosmolar stress were shown to initiate inflammatory signaling pathways in RPE cells and neuroglia, closely related to activation of the transcription factor NF-κB, assembly of the NLRP3-inflammatory complex and processing of IL-1β and pro IL-18 to mature forms via secreted multiple inflammatory mediators and chemoattractant molecules [6, 7].

With the accumulation of IL-1β and IL-18 in the intercellular space – acting in paracrine and autocrine manner can reduce the viability of RPE cells [1, 12], promote development of a new round of inflammation by stimulating intracellular proinflammatory signaling pathways, production of chemokines (IL-8, MCP-1/CCL-2) [2] and recruitment of immunocompetent cells to the lesion.

However, numerous experimental studies are currently carried out mainly on isolated RPE cells or with varying quantitative and qualitative RPE composition in combination with glial, myeloid and lymphoid cell elements *in vitro*.

It is important to consider that all closely interacting retinal cellular communities, the supplying blood vessels, the choroids, as well as leukocytes migrating to the inflammation focus are involved in the process of fundus lesions in AMD along with RPEs.

Studying the patterns of local gene expression of proinflammatory cytokines in the TC in experimentally simulated animal PPE atrophy is an important issue because it may allow determining a role and place of proinflammatory mediators in the pathological process and help developing new treatment strategies for severe degenerative retinal diseases in humans.

Purpose: to investigate the local mRNA expression for the proinflammatory cytokines IL-1β, IL-18, MCP-1/CCL2 in a model of RPE atrophy induced after a single subretinal saline injection in rabbit experiment.

Materials and methods

Modeling RPE atrophy and retinal degeneration

The international principles of the Helsinki Declaration on the Humane Treatment of Animals, the principles of humanity set out in the European Community directive (86/609/EC) "Regulations on the Handling of Test Animals" were complied in the experimental studies. 23 male New Zealand albino rabbits (age 2.5-3.0 months, weighted 2.0-2.5 kg) were injected once with 0.01 ml of 0.9% sodium chloride solution into the subretinal space below the retina 1-1.5 mm downwards from the optic nerve head to generate a subretinal bladder. To reduce a risk of exudative-hemorrhagic intra- and postoperative complications, animals were injected intramuscularly with 0.3 ml of Zoletil 50 and 0.55 ml of 2% xylazine, and 0.3 ml of 0.4% dexamethasone, 0.5 ml of dicinone) before surgery. The control group consisted of 5 somatically healthy rabbits without ocular pathology.

Specialized ophthalmological examinations

Optical coherence tomography (OCT), autofluorescence (FAF) study using ™SD-OCT (Heidelberg Engineering, Germany) were performed one week before modeling RPE atrophy, in early period (from 2 days to 1 week) and late period (from 2-4 weeks and later) after surgery by sacrificing anaesthetized animals via air embolism method (Order of the USSR Ministry of Education and Science No. 724 of 13.11.184).

RT-PCR method for determination of cytokine expression levels in eye tissues of experimental animals. Molecular biological studies were performed in 53 TC samples isolated from enucleated eyes of experimental and control rabbits according to standard protocols. Biomaterial was placed in cryovials and stored at -70°C until the study. Using the NCBI GenBank electronic database and Primer-BLAST, OligoCalc: Using the Oligonucleotide Property Calculator and BLAST Standard Nucleotide Software Package, the oligonucleotide sequences for the genes presented below were selected. IL-1β: For 5-aatctgtacctgtcctgcgtg-3 ; Rev 5-ggttggggtctacactctcc-3 ; IL-18: For 5-accagaagag-gcttgcatca-3 ; Rev 5-tccaggttctcatcgttttcagt-3 ; MCP1/CCL2: For 5-atgaaggtctctgcaacgct-3 ; Rev

5-cccttggccagtttggtcat-3 ; GAPDH: For 5-gattgtcagcaacgcatcctg-3 ; Rev 5-ctccacaatgccgaagtggt-3 .

TC samples were homogenized for 90 s at 45,000 rpm (homogenizer for operation in microvolumes Silent Crusher S Heidolph, Germany).

Isolation of mRNA from tissue samples was carried out using a sorbent-column method (RNeasy Mini Kit, Qiagen, Germany) added with 1 μl RNase inhibitor (RNase Inhibitor, Qiagen, Germany) and DNase treatment (DNase Max Kit, Qiagen, Germany) according to the manufacturer's instructions. The amount of obtained mRNA was monitored using a spectrophotometer at a 260 nm wavelength (Cytation 5 imaging reader (Biotek)).

cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad, USA): 9 μl of Nuclease-free water, 4 μl 5 iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase, 5 μl mRNA and 1 μl were mixed RNase Inhibitor (Qiagen). The reverse transcription reaction was performed according to the manufacturer's protocol.

The resulting cDNA fragment was amplified by RT-PCR in real time mode on a CFX96 Touch thermal cycler (Bio-Rad, USA).

The composition of the reaction mixture: 9.4 μl dist. water, 1.5 μl buffer, 0.5 μl dNTP, 1 μl cDNA, 0.3 μl SYBR GreenI [1: 25000] (Evrogen, Russia), 0.3 μl Taq polymerase (0.5 U/μl, Biosan, Latvia), 1 μl forward primer (F1), 1 μl reverse primer (R1). Concentrations of primers of the studied and reference genes: F1_IL-1β 1 μM (Mw 63.68 g/mol); R1_IL-1β 1 μM (Mw 60.81 g/mol); F1_IL-18 1 μM (Mw 61.16 g/mol); R1_IL-18 1 μM (Mw 69.50 g/mol); F1_CCL-2 1 μM (Mw 60.98 g/mol); R1_CCL-2 1 μM (Mw 60.56 g/mol); F1_GAPDH 1 μM (Mw 63.86 g/mol); R1_GAPDH 1 μM (Mw 60.83 g/mol).

The normalized ΔΔCt expression method was used to determine the relative sample cDNA amount. The results were expressed as relative units (relative units): the ratio of the threshold cycle of amplification of the studied gene to the value of the threshold cycle of amplification of the reference gene GAPDH: ΔΔCt= (ΔCt sample)/ (ΔCt GAPDH).

Statistical data processing

The data were analyzed by using the Statistica 6.0 software package (StatSoft Inc., USA). The normality distribution was estimated by the Kolmogorov-Smirnov method. In case the distribution of parameters differed from normal, nonparametric methods of analysis were used. Mann-Whitney U-test was used to determine the significant differences (p) in two independent samples. The critical level of significance for statistical hypothesis testing was taken as $p < 0.05$.

Results and discussion

Between 2 and 7 days after surgery, changes in the rabbit fundus were determined as neurosensory retinal detachment with hyporeflective content underneath, retinal tear with elevated edges according to the cannula injection site, disorganization of retinal layers, irregular hyperfluorescence was determined on FAF indicating RPE lesion.

Reactive changes in the fundus (from 2 days to 1 week) were accompanied by significantly increased TC IL-18 mRNA expression in experimental vs. control group lacking eye damage: the mean value was 23.35±5.43 rel. units (Figure 1).

Analysis of the early tissue response revealed no changes in IL-1β mRNA expression that was comparable to that in the control group.

It is known that IL-1β, in addition to enabling specific protective immunological mechanisms, acts as one of the main mediators of rapid response in development of nonspecific defense such as mounting local inflammatory response to damaging and pathogenic agents.

it might seem paradoxically, that the lack of changes in IL-1β gene mRNA expression in MCs in all animals with early reactive response noted in our study is consistent with the data obtained by N.M. Luyeshi et al. thoroughly investigating kinetics of the proinflammatory response in an mouse ischaemia/reperfusion brain injury model showing that IL-1α mRNA, but not IL-1β, was expressed by microglial cells in the early postischemic period: increased expression of the latter was observed at later stages [8].

The early post-operative period was also trended to increased local expression of MCP-1/CCL-1 gene mRNA: special attention was paid to its wide range of upward shifts relative to normal: from a slight/moderate increase (0.05 rel. units) to a hyperreactive response – a 4000-fold increase in expression compared to healthy eye tissue (Figure 1).

2-4 weeks after surgery, specialized ophthalmic examinations identified a circular light focus on the infra-red image; in this area, thinning of the outer retinal layers, hyperreflectivity of the choroid due to increased laser beam penetration beneath the retina, occurring in the absence of RPE with its high reflectivity was noted on the OCT. Thinning of the RPE and the choroid was identified in the lesion area. Autofluorescence examination identified an area of hypofluorescence corresponding to the atrophy zone, with irregular dissections of hyperfluorescent foci in the periphery.

In the late periods after induction of atrophy (from 2-4 weeks to 2 months), the parameters of the relative mRNA expression for IL-1β, IL-18, and MCP/CCL2 in MC exceeded the upper normal limit; at the same time, IL-1β mRNA was significantly increased (up to hyper-response) compared to that found in the early (reactive) postoperative period, while maintaining an enhanced CCL2 response and slightly lowered IL-18 mRNA expression (Figure 1).

A feature of the tissue response in the late stages after surgery – with established atrophy was described as multidirectional changes in the expression of all

cytokine mRNA genes studied in MC in a wide range relative to the norm.

An individual analysis of the IL-1β and IL-18 genes mRNA showed that overexpression was observed in approximately the same frequency of cases: in 29.4% (on average, up to 2624 rel. units) and 23.5% (on average, 59 rel.units); changes in expression within the normal range were found in 35.3% of animals (on average, 1.29 rel. units – IL-1β and 1.59 rel. units – IL-18); hypo-response and lack of expression were detected in 35.3% and 41.2% of cases, respectively. It should be noted that at later stages with established stable atrophy (from 2-4 weeks and later, up to 2 months), the level of local MCP-1/CCL2 mRNA expression also varied: hyper-response was noted in 17.6% of cases (in on average, up to 597.2 rel. units); moderately increased expression was determined in more than half of the animals in the experimental group; normal reaction and lack of expression (from 0.0 to 0.014 rel. units) were detected in 29.4% of cases.

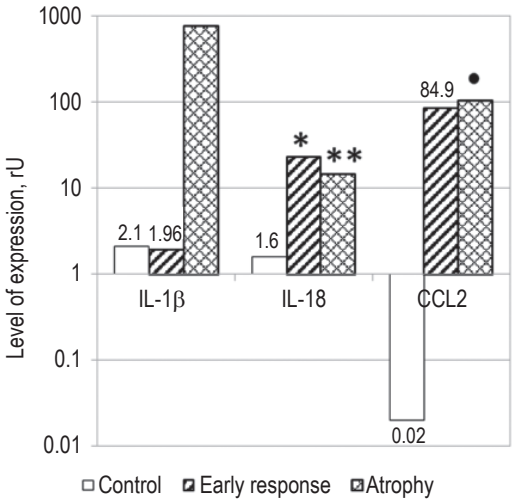

Figure 1. Level of mRNA expression of the genes IL-1β, IL-18, CCL2 / MCP-1 (using a logarithmic scale on the ordinate) in MCs of healthy rabbits (control), at early (7 days) and distant (from 14 days and later) terms after the single subretinal injection of physiological sodium chloride solution in the experiment)

Note. *, the significance of the difference in relative expression in the early response (with reactive changes in the fundus) compared to the control ($p < 0.05$); **, the significance of the difference in relative expression in the RPE atrophy group compared to the group of early (reactive) changes ($p< 0.05$); ● – a tendency to differ in the group of early (reactive) changes compared to the control ($p < 0.06$).

Conclusion

1. A single subretinal injection of 0.01 ml of 0.9% sodium chloride solution, which induced RPE atrophy in experimental rabbits was found to be associated with multidirectional changes in IL-1β, IL-18, MCP-1/CCL2 mRNA gene expression relative to normal.

2. Three types of response in the TC of the choroid / RPE / retina, formed during the development of atrophic changes and determined by the magnitude of local cytokine expression were identified:

– lack of expression – lack of response/hyporeactivity;
– normal/moderately increased response;
– overexpression – hyperreactivity.

It is possible that certain types of responses, characterized by the magnitude of the proinflammatory cytokine expression are genetically determined being associated with polymorphisms in the IL-1β, IL-18, MCP-1/CCL2 genes; however, this assumption requires further target research.

3. No relationship was found between the strength of the response (the magnitude of local mRNA expression) to atrophy-inducing stimulus and the size of atrophic retinal lesion.

The circumstances noted above as well as the data that RPE induction of atrophy in one-third of animals was accompanied by significantly increased IL-1β mRNA expression and, in 69.6% increased local MCP-1/CCL2 mRNA expression, the factors damaging haematoretinal barrier and contributing to the immune privilege disorder in the eye posterior segment , should be taken into consideration while developing new strategies of ophthalmic pathology treatment, particularly extensively studied and tested options for RPE stem cell transplantation into subretinal space.

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