Regular and ectopic photoreceptors in vivo, in vitro and under pathological conditions

Ph.D. Thesis

Szabó Klaudia

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor: Lukáts Ákos, M.D., Ph.D.

Official reviewers: Récsán Zsuzsanna, M.D., Ph.D.
Módis László, M.D., D.Sc.

Head of the final examination board: Benyó Zoltán, M.D., D.Sc.
Members of the final examination board: Madarász Emília, D.Sc.
Alpár Alán, M.D., D.Sc.

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1. Introduction

The structure of the mammalian retina is well organized and is therefore a widely used model of neuroscience researches. The neural retina includes separated layers containing cell bodies and projections. Photoreceptors (rods and cones) are special neurons that are capable of absorbing photons. They contain photosensitive pigments in their outer segments surrounded with the pigmented epithelium. Their cell bodies are located in the outer nuclear layer (ONL) and their synapses are formed in the outer plexiform layer (OPL) with bipolar and horizontal cells. The inner nuclear layer (INL) contains the perikarya of bipolar-, horizontal-, amacrine- and Müller cells. The synapsis of the bipolar and amacrine cells project to the inner plexiform layer (IPL). Ganglion cells located in the ganglion cell layer (GCL) receive information from photoreceptors via bipolar cells. The axons of ganglion cells are collected in the nerve fiber layer (NFL) and reach higher brain centers of vision as the optic nerve (ON). Two additional layers are created by the end feet of Müller glia cells. The outer limiting membrane (OLM) separate the photoreceptor inner segments from their perikarya and the inner limiting membrane (ILM) are formed under the NFL layer. Some of the retinal neurons become ectopic during retinal development. Many of these cells have been identified in the retina so far, and some of them have been shown to have a specific function in their ectopic position. For example, a population of the starburst amacrine cells responsible for transmitting direction sensitive information is localized in the GCL and makes synapses in the ON sublayer of the IPL. Photopigment expressing ectopic cells also exists, S-opsin, M/L-opsin and rhodopsin-expressing cells have been discovered in the rodent

retina. They express an incomplete repertory of phototransduction proteins. The possible functions of these populations are unknown yet. We also observed a misplaced rhodopsin positive cell (MRC) population in the inner retinal layers (INL and GCL) of in vitro organotypic retinal cultures of the rat retina. The detailed characterization of these cells were performed in the developing retina of four different rodent species.

Photoreceptors were also studied under pathological conditions. A well-known animal model of type 2 diabetes (T2D) was used to detect changes and possible degeneration in the photoreceptor cells and the pigmented epithelium. Diabetes mellitus is a civilization disease of our age, a systemic disorder that creates various complications in different tissues. Diabetic retinopathy is the ophthalmic complication due to diabetes. The diagnosis is based on the detection of the impairment of the vasculature, therefore, most of the publications available discuss vascular changes. However, it is known that functional changes like color vision defects, decreased contrast sensitivity, photopic and scotopic electrophysiological changes, may precede clinically detectable vascular degenerations. In our previous study we found signs of photoreceptor and pigmented epithelium damage in type 1 diabetic (T1D) model prior to detectable vascular alterations. As most patients suffer from type 2 diabetes, we aimed to characterize photoreceptors in Zucker Diabetic Fatty (ZDF) rat, a model of T2D and we aimed to compare the results with our previous study of T1D model.

2. Objectives

Part I. The aim of the present study was to perform a detailed characterization on the MRCs in the inner retina. The morphology and relative number of these cells were assessed in four different rodent species, and a series of double labeling experiments were performed to describe the staining characteristics of these cells. To assess the possible functions and photoreceptor differentiation, we screened for the presence of different elements of the phototransduction cascade and synapse formation. To address the question what is the fate of these cells after completion of development of the retina we aimed to examine the possibility of apoptosis and searched for the possible presence of MRCs in the adult retina.

Part II. We aimed to give a detailed qualitative and quantitative description on the early histopathological changes in the retina of ZDF rats. We aimed to measure retinal thicknesses, evaluate apoptosis and to characterize photoreceptor and pigmented epithelium degeneration pattern in the diabetic retina compared to control. We compared our results with previous studies dealing with experimentally induced T1D rat s.

3. Methods

3.1. Developmental study

Experiments were carried out on rodent species (Sprague-Dawley rat, Syrian hamster, C57bl mouse and Siberian dwarf hamster). Animals were sacrificed with ketamine narcosis, in the following ages: P4, P7, P10, P14, P18, P21, P24 and P28 in rats, P7, P10, P14 for other species (P0=date of birth). Eyes were enucleated, the cornea, lens and vitreous body removed. Eye cups were fixed and cryoprotected in 30% sucrose solution overnight. Eye cups were embedded and tangential frozen sections were prepared.

3.2. In vitro, organotypic retinal culture

Euthanasia of the animals occurred with cervical dislocation followed by decapitation. Eyes were enucleated, the sclera, choroidea, the lens and the vitreous body were removed, but the pigmented epithelium remained on the retina. The retinas were spread on a semipermeable culturing membrane. The membrane was placed on a sterile plate and the space under the membrane was filled with breeding medium. The cultures were kept in a thermostat with an atmosphere of 5% CO_2 and 100% humidity for two weeks at 37ºC. The retinas were fixed and then cryoprotected. Frozen sections from the embedded retinas of 10-20 μm thick slices were prepared.

3.3. Type 2 diabetes mellitus model

Experiments were carried out on ZDF inbred rats obtained from the Research Laboratory of the Heart and Vascular Center, Semmelweis University lead by Tamás Radovits, M.D., Ph.D.. In ZDF rats T2D and related complications develop due to a leptin receptor gene mutation and a special diet. Rats were supplied with a special diet (Purina 5008) and water ad libitum. Blood glucose levels were checked from the tail vein every five weeks. At the age of 32 weeks, anesthesia was induced and maintained with isoflurane. Animals were euthanized and decapitated. The eyes were removed and preparated. After fixation eye cups were embedded and 20 μm tangential frozen sections were made.

3.4. Immuncytochemical analysis and lectin histochemistry

The sections were first incubated with the monoclonal/polyclonal primary antibody that were detected with species-specific Alexa-conjugated secondary antibodies (Alexa-488, Alexa-594). Sections for lectin histochemistry were incubated with biotinylated lectins for 2 hours and detected with streptavidin-conjugated secondary antibodies (Alexa 594). The nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI).

3.5. Fluorescent microscopy

Sections of the MRCs and the ZDF retinas were viewed and images were recorded using confocal microscope. The final montages were created

and labels were added by Adobe Photoshop. Only minor adjustments concerning the brightness/contrast were made (in case of ZDF retinas parallel on picture pairs) to enhance the visibility of the morphologic features demonstrated.

3.6. Apoptosis

Apoptotic MRCs were recognized in the developmental experiments by the pyknotic nuclei morphology. Another detection method of apoptotic cells is the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which was also applied in the in vivo developmental experiments and on the retina of diabetic animals.

3.7. Cell counting and quantitative analysis

Statistical analysis were performed using R Statistical Program and P-values less than 0.05 were considered significant. To determine the number of MRCs on 15μm thick vertical centered sections passing through NO were manually calculated. The following parameters were studied: (1) number of MRCs/total number of rhodopsin positive cells, (2) apoptotic MRCs and (3) percentage of apoptotic MRCs/total MRC per age.

In the T2D model cell count was performed on 20 μm thick vertical sections. Cell counting was also based on cross sections passing through the ON. The M-cone cells were counted on central, midperipheral and peripheral retinal regions superiorly and inferiorly from the ON. To assess the effect of diabetes on the thickness of the retina, the distance between the outer and inner

limiting membranes (ILM-OLM), and the thickness of the outer nuclear layer (ONL) alone was measured in both superior and inferior directions. In the ONL, nuclei are typically arranged in columns on well oriented sections. To further estimate the number of photoreceptor cells, additionally we also counted the number of nuclei in the ONL in at least three columns per each location parallel with the thickness measurements.

4. Results

4.1. Developmental studies

Ectopic rhodopsin positive cells located in the INL and GCL were first identified in in vitro organotipic retinal cultures prepared from newborn rats. In cultures their presence may be a result of a degeneration or change in gene expression as consequence of the culturing conditions themselves, so in order to decide if they are present also under in vivo conditions, early postnatal rodent retinas from four different species were analyzed by immunocytochemistry.

MRCs were detectable in the retinas of all four species examined with all antibodies tried, whether directed against the C- or N-terminal part of rhodopsin, indicating that their presence is neither the consequence of culturing conditions, nor caused by non-specific binding of the antibodies. Morphologically MRCs resembled resident cell populations of the inner retina, with bipolar, amacrine and ganglion cell-like morphologic variations. Outer segment-like processes were seen only rarely. They composed approximately 2% of all rhodopsin positive elements, and their number was relatively constant between P7-P14. From P18 we observed a continuous decline in the number of MRCs that almost completely disappeared from the retina by P28. However, a small number of cells were detectable in the GCL even in adult retinas,

We could not confirm the presence of any other photopigment (S-opsin, M-opsin, melanopsin) in MRCs and they do not express any marker that normally recognize Müller-cells, microglia, rod bipolar-, horizontal-,

amacrine- or ganglion cells. From the elements of the phototransduction cascade all MRCs express recoverin and rod arrestin, while other proteins were only detectable in some of the cells.

Most MRCs were shown to be eliminated by apoptosis by the end of the 4th postnatal week. However, some of the cells in the GCL survive till adulthood. They show constant morphology (relatively small cell body, narrow dendritic tree synapsing exclusively in the ON sublayer of the IPL) and although they do not any more express detectable amounts of rhodopsin they can be easily recognized based on their special rod-like nuclear morphology and labeling pattern with recoverin and rod arrestin.

4.2. Results on ZDF rats modelling T2D

There was no significant difference in the **weight** of the animals at the time of anesthesia between the diabetic and the control group. Except for the time of euthanasia, the **blood glucose levels** of control animals remained within the normal range, while diabetic rats had elevated blood glucose levels even as early as the 7^{th} postnatal week, continued to rise till week 12 and remained steadily high thereafter.

There was no decrease in total **retinal thickness** (OLM-ILM) or in the thickness of ONL alone at any position examined. To the contrary: there was a significant increase at all positions in total retinal thickness and in all inferior retinal positions in ONL thickness, most probably due to edema formation, that is known to occur in diabetes. In case of edema formation, retinal thickness may not decrease even in case of an increased rate of apoptosis, so despite the lack of decrease in retinal thickness we estimated the

rate of apoptotic cell loss with two different methods. 1) in the ONL nuclei are arranged in columns on well oriented sections, so we calculated the number of cells in each column in the same positions where the thickness measurements were made and found no difference. 2) we calculated the number of TUNEL positive cells per section and again found no increase in diabetic specimens compared to controls. So we can conclude that the retinas were inspected prior to massive loss of cells.

Rod outer segments exhibited highly degenerated profiles in all diabetic specimens, with only some minor regional differences detectable. Uneven outer segment profiles were observed, and the border between inner- and outer segments became less well distinguishable. Unlike in T1D rats however no difference in the labeling pattern of the rod interphotoreceptor matrix was detectable. When testing the elements of the phototransduction cascade by immuncytochemistry, only minor differences were noted. Rod arrestin localized normally to the outer segments in light-adapted conditions was translocated to the inner segments and perikarya in diabetic ZDF rats.

No major difference was detectable in the labeling intensity and in the number of **M- and S-cones** in diabetic specimens compared to controls. Concerning morphology however, an evident outer segment degeneration was detectable form the majority of M-cones all over the retina. Outer segments showed incomplete fragmentation with the fragments connected by thin stalks. There was no change detectable in the staining intensity of cone arrestin, but the outer segment degeneration was evident with this staining as well. PNA labeling revealed no difference in the cone interphotoreceptor matrix.

The number of **dual cones**, coexpressing both the S- and M-opsins showed a remarkable increase in diabetic specimens. In controls, such dual

elements were detectable only in the peripheral retina in small numbers. In diabetic specimens they appeared in the central regions as well, while in the periphery almost all cones were dual in nature.

Possible degeneration of the retinal pigmented epithelium was assessed by immunocytochemistry with an antibody against RPE65. The evident reduction in staining intensity indicates the dysfunction of this layer as well.

5. Conclusions

5.1. Developmental experiments

- Ectopic rhodopsin positive cells located in the INL and GCL are detectable in the developing retina of all four rodent species studied, and may represent a subpopulation of the rod photoreceptors believed to be homogeneous so far.
- Most MRCs are likely just lost during retinal development and are eliminated from the retina after development is complete.
- A small proportion of MRCs remain with standard morphology in the adult rodent retina. The gene expression pattern of the cells changes partially: they do not express detectable amount of rhodopsin, but the expression of recoverin and rod arrestin proteins is upregulated. Their function of adult MRCs is unknown yet.
- In transplantation experiments, rod photoreceptors are selected from the retina based on their rhodopsin positivity. Our experiments have shown that rhodopsin containing cells do not only differentiate to regular rod photoreceptors but could give rise to MRC-s as well. Selecting MRC-s for transplantation could be responsible for improper integration and functioning in the recipient retina.

5.2. Experiments in Type 2 Diabetes

- There was no decrease in the thickness of the whole retina (OLM-ILM distance) and ONL thickness alone. At the same time, there was no change in the number of photoreceptors in the columns of the ONL layer and there was no increase in the number of apoptotic cells with the TUNEL assay, so our studies were performed prior to the mass destruction of the retinal cells.
- As a result of diabetes, the outer segment of rods and most M-cone photoreceptors as well as the pigmented epithelium show degeneration, without a detectable decrease in cell numbers.
- The number of dual cones expressing both S- and M-opsins increased detectably similarly to our previous study on type 1 diabetic rat model. Dual cones also appear temporarily during M-cone development, so the phenomenon may suggest regeneration or dedifferentiation. Within the cell, both S- and M-opsin activates the same signal transduction pathway, so dual cones are not able to transmit color information. The appearance of dual cones may present the anatomical basis for diabetic color vision disorders.
- When comparing our results with that of the previously studied streptozotocin-induced type 1 diabetic model we found that despite the differences in the early pathomechanism of the two diabetic disorders (different blood glucose levels, insulin levels, lipid levels…), a surprisingly similar degeneration pattern was obtained for both the photoreceptors and the pigmented epithelium. This indicates that the alterations are most likely due to high blood glucose levels while other factors, including insulin signaling seems to have a less prominent role in the development of the

degeneration.

- The changes were detectable in both models before significant apoptosis and clinically evident vascular pathology and may potentially explain photopic and scotopic ERG alterations, color vision disturbances and the decrease in contrast sensitivity reported by others in animal models and human patients.

6. Bibliography

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