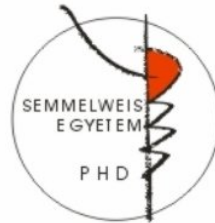


PATHOLOGICAL ALTERATIONS OF THE RAT RETINA IN EARLY STREPTOZOTOCIN- INDUCED DIABETES

PhD thesis

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Budapest, 2015

1. INTRODUCTION

Diabetic retinopathy is a common complication of diabetes mellitus. Diabetic retinopathy is among the leading causes of legal blindness in adults, therefore globally it is a great social and economical burden. Diabetic retinopathy is clinically characterized by progressive exudative, ischaemic and proliferative vascular alterations.

It is becoming clear however, that early in the course of the disease, preceding clinically detectable vasculopathy, retinal microvasculature and neural retina are already compromised. Neuroretinal functions are impaired in early diabetes, as confirmed by many electrophysiological and clinical studies from the past decades. In human patients, impaired rod and contrast sensitivity and abnormal electroretinogram recordings have been reported to feature early diabetes. Acquired color vision impairment may occur in diabetes with a prevalence of as high as 30-80%, diabetic patients even without vascular retinopathy have an increased risk for color vision impairment. Both red-green and yellow-blue chromatic sensitivities are reported to be affected by diabetes, showing a strong correlation with care of the disease. Also, according to studies on rats, visual deficits such as impaired visual acuity and contrast sensitivity, scotopic and photopic electroretinogram abnormalities are already present as early as 4 weeks after streptozotocin induction of diabetes. The underlying neuropathology of these functional alterations is still undiscovered.

Neurodegeneration as an early event of diabetic retinopathy is a widely proven issue today. Apoptotic bodies were observed independently of the vasculature by several groups assessing postmortem diabetic human retinas and different animal models as well. In streptozotocin-induced diabetes with the progression of the disease, the thickness of retinal layers (especially nuclear layers) is remarkably reduced due to apoptosis. The decreasing thickness of the inner (INL) and outer nuclear layers (ONL) is reported to occur at earliest 6

months after the induction of diabetes, while the ganglion cell layer (GCL) is reported to become affected 4 weeks – 9 months after the induction, depending on experimental setups.

In morphological studies, several types of neuroretinal elements, including glia cells, amacrine cells and ganglion cells were shown to be affected in diabetes. Gliosis is one of the earliest diabetic alterations documented. There is evidence in the literature for glia activation as early as 1 month after the induction of diabetes, although the onset and range of the changes in the labeling may vary under different experimental conditions.

The importance of early changes is underlined by literature evidence suggesting that early neurodegenerative changes and clinically detectable sight-threatening stages of diabetic retinopathy may directly be associated with each other.

2. OBJECTIVES

Based on the literature, 12 weeks after streptozotocin induction of diabetes in rats, functional alterations are already present, however, no vasculopathy is detectable clinically yet. The question arises whether or not pathological alterations will accompany functional deficits in early diabetes. Our aim was to demonstrate the effects of early streptozotocin-induced diabetes on different types of cells in the neural retina of rats, as follows.

- Is there a significant loss of cells due to apoptosis in early diabetes?
- Can glia activation be detected in the early diabetic retina?
- Do the morphology, fine structure, density and distribution of photoreceptor cells change in early diabetes?
- Are the retinal pigment epithelial cells affected by diabetes morphologically and ultrastructurally?
- Are certain types of neural cells (horizontal, bipolar, amacrine and ganglion cells) in the inner layers of the retina affected by diabetes morphologically and in number?

3. METHODS

Animals and tissue preparation

Male Wistar and Sprague-Dawley rats aged 12 weeks, were utilized in this study. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) at a dose of 70 mg/kg body weight dissolved in sodium citrate buffer (0.1 M, pH 4.5), while control rats were injected with buffer alone (n=7/group in Wistars and n=12/group in Sprague-Dawley rats). Rats were considered diabetic and enrolled in the study if fasting blood glucose concentration exceeded 20 mmol/l one day after STZ injection.

All animals were euthanized with an overdose of ketamine 12 weeks after the induction of diabetes. Eyes were oriented, enucleated, then the cornea, lens and vitreous body were carefully removed. All posterior eyecups from Wistar rats and some eyecups from Sprague-Dawley rats were fixed in 4% paraformaldehyde (PFA) diluted in phosphate buffer (PB; 0.1 M, pH 7.4), for 2 hours at room temperature. After repeated rinses in PB, retinas were detached in some cases (n=7/group in both strains) and processed further as whole mounts. Following cryoprotection posterior eyecups were embedded, then cryosectioning was performed. Sections were mounted onto gelatinized glass slides, and stored at -20 °C until use.

TUNEL assay

To evaluate the rate of apoptosis, terminal deoxynucleotidyl-transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using an in situ cell death detection kit. Total number of stained nuclei was counted on vertical cryosections (n=4/group, 4 sections/animal), counted from the optic nerve head to the ora serrata both superiorly and inferiorly.

Lectin cytochemistry and immunohistochemistry

For the selective evaluation of neuroretinal cell types and pigment epithelium, lectin cytochemistry and immunohistochemistry were performed using antibodies specific to certain cells and opsins. Analyses were carried out on whole mount retinas and on retinal cryosections.

Electron microscopy

After PFA fixation in Wistar rats (n=4/group) 1x1 mm pieces of the posterior eyecups were cut superior to the optic nerve and were postfixed with 1.5% glutaraldehyde diluted in PB (for 90 minutes, at room temperature). In Sprague-Dawley rats, posterior eyecups that were not fixed with PFA (n=4/group), were fixed with a solution containing 2.4% PFA and 1.65% glutaraldehyde diluted in PB (for 30 minutes, at 37 °C).

After fixation, samples were repeatedly rinsed with cacodylate buffer (CB; 0.1 M) at room temperature then further postfixation was carried out with 1% osmium-tetroxide diluted in CB (for 1 hour, at 4 °C). After repeated rinsing, retinas were embedded in araldite. Semithin and ultrathin sections were prepared and they were assessed using a transmission electron microscope to study the fine structure of the photoreceptor outer segments and the retinal pigment epithelium (RPE). The thickness of the pigment epithelial layer was measured on electron micrographs derived from identical locations of the diabetic and control retinas (n=4 specimens/group, 3 measurements/section).

Measurement of retinal thickness

Measurements were carried out on vertical cryosections at the level of the optic nerve, centrally at distances of 250 and 500 μm from the optic nerve in superior and inferior directions, while peripheral values were obtained at distances of 250 μm superior and inferior to the ora serrata. We measured the

distance between the outer and inner limiting membranes and the thickness of the ONL in 3 sections per retina in Wistar and Sprague-Dawley rats (n=4 diabetic and control retinas in each strain).

Analysis of the density and distribution of cones

To compare the density and distribution patterns of cone-types expressing middle (M-) and short (S-) wavelength sensitive opsins and also of dual cones (co-expressing both opsins), whole mounted retinas were immunostained with opsin-specific antibodies (n=4 for each group of Wistar rats).

On average 83.2 ± 4.7 fields of microscopic view per retina were analyzed in a systematic random manner with a NeuroLucida work station. Outer segments were counted manually, in $130 \times 130 \mu\text{m}$ squares in case of M-cones and in $250 \times 250 \mu\text{m}$ squares in case of S-cones. Based on the measured data, estimation was created using the 'Adaptive Neuro-Fuzzy Inference System' to approximate cone numbers in the uncounted regions and to construct isodensity maps.

Cell counting in the inner retinal layer

In order to study the number of parvalbumin, calbindin and recoverin positive cell types, whole mounted temporal or nasal retinal halves were immunostained and flat mounted (n=3 in each group of Wistar rats). Labeled cell types were counted manually over areas of $250 \times 250 \mu\text{m}$ at 10 evenly distributed central retinal positions.

The number of amacrine and displaced amacrine cells labeled with protein kinase C-alpha (PKC- α) were counted on vertical sections at the level of the optic nerve head (n=3/group in Wistar rats). Cells were counted over the

retinal length of 700 μm in 6 different central locations per section (1, 2 and 3 microscopic view fields superior and inferior to the optic nerve).

Statistical analysis

Blood glucose, body weight, thickness of the retina and RPE cells, as well as the number of TUNEL, parvalbumin, calbindin, recoverin and PKC- α positive elements were analyzed using Student's t-test. A *p*-value of less than 0.05 was considered statistically significant.

4. RESULTS

General clinical features of control and diabetic groups

In both strains blood glucose concentration values were all normal before the injections. In the diabetic groups, blood glucose levels were elevated (> 20 mmol/l) from day 1 throughout the observation period of 12 weeks, the values were significantly higher in diabetes compared to the vehicle-treated groups.

Analyses of retinal thickness and apoptosis

There was no significant increase in the number of TUNEL positive elements in diabetes compared to controls. In line with this, the thickness of the whole retina and the thickness of the ONL alone were also unchanged. These data clearly indicate that the retinas were inspected by us prior to significant loss of cells, which is in agreement with the literature.

Glia activation in diabetes

In our diabetic retina samples, changes in the protein expression of macroglial intermediate filaments were arranged in a patchy manner, GFAP labeling was upregulated, co-occurring with the downregulation of vimentin expression. The patches with the changes were distributed irregularly throughout the retina, with no specific localization to the central or peripheral regions, or to the superior or inferior retinal halves.

Photoreceptor cells in control and diabetic retinas

Regarding the **density and distribution of cones** expressing M-opsin, in control whole mount retinas a typical centro-peripheral gradient was present with central peak densities reaching approximately 5000 cones/mm². Density values dropped significantly towards the periphery. As for the S-cones, there

was a detectable difference between the superior and the inferior retinal halves with average values of 100-200 cones/mm² superiorly and 800-900 cones/mm² inferiorly. We detected a sharp increase in the density of S-cones at the superior peripheral rim of the retina, reaching extremely high numbers (> 1200 cones/mm²). Comparing control values with those of the diabetic retinas, similar density and distribution patterns were revealed for both cone-types. The average total number of cones counted per retina did not show any statistically significant difference. Total numbers of 5621 ± 754 vs. 5467 ± 660 M-cones and 3048 ± 776 vs. 2725 ± 259 S-cones were counted in the control and diabetic retinas respectively.

Dual cones (co-expressing both opsins) were present in a high number in the diabetic retinas, however, they rarely were observed in the adult control specimens. Dual cones reached especially high local densities in the peripheral regions of diabetic retinas, several times higher than in controls.

When the **morphology of cone outer segments** was studied, vast majority of M-cones showed clear signs of outer segment degeneration. The outer segments seemed to be fragmented, but thorough investigations showed that all pieces were connected with thin stalks. Interestingly, in diabetic rats the cone matrix sheath labeled with PNA lectin was preserved in its original form and did not seem to follow deformations of the outer segments. We also detected morphological changes in a small population of S-cones in the Wistar rat retinas. The outer segment of these cells was reduced or invisible, while the complete cell body was labeled with S-opsin specific antibodies.

Studying the **morphology of rod outer segments**, in diabetes the appearance of the rhodopsin positive outer segments was found to be characteristically blurred instead of the fine rod-like appearance seen in the control retinas of both strains. Electron microscopic investigations confirmed a remarkable disorganization of the outer and inner segments of photoreceptor

cells in the diabetic groups. In Sprague-Dawley rats, where the degeneration of rod outer segments was more prominent, PNA lectin labeling was not limited only and selectively to interphotoreceptor matrix of the cone cells, but rods also showed a discrete staining. The other type of lectin examined, the rod interphotoreceptor matrix-specific WGA labeling was diminished in these cells when compared to controls.

Retinal pigment epithelial cells in control and diabetic rats

At ultrastructural level a significant reduction of the thickness of RPE cells could be seen in the diabetic Wistar rats. In line with the altered morphology, RPE65 immunoreactivity was also drastically weaker in the diabetic retinas when compared to controls. All the above mentioned changes of the pigment epithelium were less pronounced in Sprague-Dawley rats: the reduction of the thickness of RPE cells was not statistically significant and there was only a moderate decrease in the immunoreactivity of RPE65.

Inner retinal cell types in control and diabetic rats

In the case of parvalbumin labeling (calcium-binding protein, labeling mainly AII amacrine cells) a decreased immunoreactivity was shown in all cell types: the decrease in the labeling occurred also in patches, similarly to the alterations of intermediate filaments expression. However, the number of parvalbumin positive AII amacrine cells did not decrease significantly, based on whole mounted retina samples. Apart from the patches, parvalbumin labeling appeared to be normal in diabetes.

In diabetes there were no detectable changes in the number of calbindin positive horizontal cells and recoverin positive bipolar cells according to controls, based on whole mounted retina specimens. However, at the extreme periphery the inner cell layer of the ciliary part of the retina was stained

intensely with calbindin, only in diabetes. The morphology of the axon terminals and synapses of recoverin positive cone bipolar cells are characteristically changed in diabetic retinas.

No evident change was observable when labeling with markers calretinin (calcium-binding protein, labeling amacrine and displaced amacrine cells), melanopsin and anti-Brn-3a (labeling ganglion cell types).

Labeling of rod bipolar cells with PKC- α did not change, although two other PKC- α positive populations of cells (most probably amacrine cells) in the INL were present in significantly higher numbers. In the GCL cells immunoreactive for PKC- α (most probably displaced amacrine cells) were also detected in a higher number in diabetes than in controls.

5. CONCLUSIONS

- Prior to clinically detectable vasculopathy, neurodegenerative elements are already present in the diabetic retina. We demonstrated novel pathological alterations of the neuroretina in early diabetes, which may explain functional deficits described elsewhere.
- The changes in early diabetes recorded by us are probably preceding apoptotic loss of cells.
- New aspects of glia activation in diabetes are demonstrated here: upregulation of GFAP and downregulation of vimentin expression co-occur in patches. Also the decrease in the immunoreactivity of parvalbumin, a calcium-binding protein could be observed in patches. These alterations may precede alterations affecting the whole retina.
- For the first time, remarkable degenerative signs were demonstrated affecting the outer segments of rods and cones in diabetes. However, the density and distribution of cones and probably the density of rods were shown not to decrease in this early phase of diabetes.
- We failed to prove the involution of RPE as the primary cause of outer segment pathology in experimentally induced diabetes. RPE damage and outer segment degeneration probably occur independently in the rat strains studied.
- We report different distribution patterns of diabetic alterations of different cell types in the inner retinal layers, referring to different sensitivity of cell types towards diabetes.

- We are the first to raise the idea that diabetic and developing retinas may share some common features. The following changes may represent a regenerative potential in adult diabetic rats:
 - 1) In diabetic retinas dual cones are present in a high number, especially in the peripheral regions. Dual cones co-expressing both opsins are abundant in early postnatal life of rats, when M-cones develop via transdifferentiation from originally S-opsin expressing elements, but this process had been so far an unrevealed feature of the diabetic retina.
 - 2) The staining patterns of PKC- α (a known marker for rod bipolar cells) and calbindin (a calcium-binding protein) are characteristically changed in diabetes. Both features show close resemblance to that of the retina under development.

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