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Qualitative evaluations of reactive microglial heterogeneity in cultured porcine retina

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Summary. A late stage of several retinal disorders is retinal detachment, a complication that results in rapid photoreceptor degeneration and synaptic damage. The porcine retina is a favorable in vitro model for studies of the degenerative processes that follow retinal detachment. Photoreceptor degeneration and synaptic injuries develop rapidly in the cultured porcine retina and correlate with resident microglial cell transition into a reactive phenotype. In this in vitro study, we used retinas cultured for five days and analyzed reactive CD11b and Iba1 immunoreactive microglia that localized close to/within the synaptic outer plexiform layer (OPL) and in the outer nuclear layer (ONL). A subpopulation of the CD11b and Ibalimmunoreactive microglia also expressed CD68 immunoreactivity on lysosomal membranes or as a diffuse cytoplasmic stain. Some CD68 immunoreactive microglia were juxtaposed to L/M-opsin immunoreactive cone photoreceptors in the ONL. CD11b and Iba immunoelectron microscopy further suggests the presence of a dark microglial phenotype in the degenerating cultured porcine retina. For immunoelectron microscopy, nickel-enhanced diaminobenzidine (DAB) staining resulted in clearly distinguished reaction products in the cytosol of dark microglia.

Key words: Retina, Microglia, CD11b, Iba1, CD68, Photoreceptor, Immunoelectron microscopy

Introduction

Diurnal animals use their cone photoreceptors during daylight conditions for the high resolution of vision and color perception. Cone photoreceptors are concentrated in area centralis in felines and porcine and in macula lutea in higher primates (Rapaport and Stone, 1984; Chandler et al., 1999). The physiological basis for visual

Corresponding Author: Kjell Johansson, PhD., Department of Science, Kristianstad University, 291 88 Kristianstad, Sweden. e-mail: kjell.johansson@hkr.se www.hh.um.es. DOI: 10.14670/HH-18-772 resolution and color discrimination starts at the synaptic level in the outer plexiform layer OPL (Burger et al., 2021), in which cone pedicles establish very specialized synaptic contacts with bipolar and horizontal cells. Retinal detachment diseases are accompanied by the loss of photoreceptors and synaptic damage in the OPL, which results in the transition of microglia into a reactive state (Rashid et al., 2019; Rathnasamy et al., 2019). Cone photoreceptor synaptic pedicles respond within hours to detachment, which results in impaired neural processing at the synaptic level in the OPL (Cuenca et al., 2014; Burger et al., 2021) and eventually loss of visual acuity and trichromatic color detection in humans.

Retinal detachment in vivo represents an established experimental approach for studies of photoreceptor degeneration and synaptic damage. Such studies showed that synaptic impairment occurs as early as two hours post-detachment (Wang et al., 2016; Halász et al., 2021) and photoreceptor death begins within 12 hours after the injury (Okunuki et al., 2019). Both rod and cone photoreceptors show synaptic damage after detachment: rods retract spherules and cones alter pedicle morphology within hours after detachment and show loss of intracellular ribbons (Specht et al., 2007; Townes-Anderson et al., 2021). Alteration of ribbon synapses (Striebel et al., 2021) as well as endoplasmic reticulum-stress (ER-stress)-mediated photoreceptor degeneration (Massoudi et al., 2023) also develop following protein accumulation in photoreceptors in vivo. Preparation and organotypic culture of retinal tissue explants start with an acute retinal detachment, resulting in a photoreceptor degenerative process that mimics those described in retinal detachment in vivo (Zhang et al., 2021) including microglial reactivation (Iandiev et al., 2006). Photoreceptor degeneration in cultured adult specimens progresses rapidly and involves synaptic damage, accumulation of opsins ER-stress, and the reactivation of resident microglial cells (Ferrer-Martín et al., 2014; Johansson and Mohlin, 2023).

Analyses of microglia during various retinal diseases and injuries have focused on neuroinflammation, migration, and the presence of reactive microglia in the



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ONL and phagocytosis of apoptotic photoreceptors and outer segments (Jiao et al., 2015; Zhao et al., 2015; Makabe et al., 2020; Murenu et al., 2022; Wagner et al., 2022). Experimental retinal detachment studies report contradictory results in that reactive microglia may reduce (Okunuki et al., 2019) or contribute to photoreceptor degeneration (Wang et al., 2021), opposite results that may relate to microglial heterogeneity (Gao et al., 2022). Similar studies of photoreceptor degeneration also demonstrate that reactive microglial processes project into the ONL and among neural elements in the outer plexiform layer (OPL) (Santos et al., 2010; Ferrer-Martin et al., 2014; Jiao et al., 2015; Zhao et al., 2015; Zabel et al., 2016; Johansson et al., 2020). Thus, reactive microglia may at least be divided into two morphological subtypes: one associated with synaptic structures and one closely localized to photoreceptor cell bodies. According to the localization, the latter may be considered microglial satellites described in CNS areas (Stratoulias et al., 2019).

By electron microscopy, a presumptive dark microglial subtype with cell bodies and processes at/within the OPL has recently been suggested in cultured porcine retina exposed to ambient light for eight hours/day (Johansson et al., 2020). Like the dark microglia found in synaptic areas in the degenerative brain parenchyma (Bisht et al., 2016), the retinal subtype associates with synaptic structures in the OPL. In the current study, we further assess cultured porcine retina and the suggested presence of dark microglia in proximity to photoreceptor synaptic terminals by a preembedding, immunoelectron microscopic approach with nickel-enhanced diaminobenzidine (DAB) reaction. Reactive microglia in the degenerating outer retina were also studied using antibodies directed against the lysosomal marker CD68. These microglia were observed at the OPL as well as close to degenerating photoreceptor cell bodies in the ONL.

Materials and methods

Animals and culture paradigm

Adult porcine retinas were processed and cultured for five days in vitro (5 DIV) as described previously (Johansson et al., 2020). Briefly, whole eyes were collected from a local slaughterhouse and transported in cold CO₂-independent media (Gibco, Life Technologies, Carlsbad, CA). Dissection began with the removal of the anterior segment, which was removed as the lens and vitreous body. The neural retina was gently detached from the underlying pigment epithelium and retinal explants of about 10 mm² were punched out close to the area centralis. Retinal explants (~10 mm²) were divided into four equal sections and explanted on Millicell®-PCF 0.4 µm culture plate inserts (Millipore, Bedford, USA) with the vitreal side up. One or two retinal sections were explanted onto each insert, which was placed in 6-well culture dishes containing 1.2 ml of culture medium per well. The culture medium consisted of Dulbecco's

Modified Eagle Medium/F12 (DMEM/F12; Gibco, Paisley, UK) supplemented with 2% B27 supplement (Gibco), 1% N2 supplement (Gibco), 0.2% Heparin 5000 IU/mL (Leo Pharma, Malmö, Sweden), 1% penicillin/streptomycin and 2 mM glutamine (Sigma-Aldrich, St Louis, USA), and was exchanged every second day. Retinas were cultured in light/dark cycles (8h illumination of warm white light) for 5 days at 37°C with 95% humidity and 5% CO₂. The experimental procedures were approved by the Swedish Board of Agriculture (6.7.18-16476/2022, Jönköping, Sweden).

Light and confocal microscopy

For light and confocal microscopy, cultured retinas (n=5) were fixed by immersion in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M Sorensen's phosphate buffer (Sigma-Aldrich) for 2h at 4°C. Fixed specimens were washed, cryoprotected in 20% sucrose and sectioned at 10-20 µm. Antigen-retrieval before immunohistochemistry was performed by using 0.01 M sodium citrate pH 6.0 for 3x15 sec in a microwave oven at 450 W, and thereafter washed for 2h with phosphate-buffered saline (PBS) (Gibco) at 4°C. The sections were incubated overnight at 4°C either with a rabbit CD11b antibody (1:2000; Abcam, Cambridge, UK) or a rabbit Ibal antibody (1:1000; Proteintech Group, Chicago, USA), diluted in PBS (Gibco) containing 0.25% Triton-X 100 (Sigma-Aldrich). For light microscopy rinsed sections were incubated with an HRP-conjugated antirabbit IgG (Cell Signaling, Beverly, USA) for 45 min at room temperature and developed using diaminobenzidine, H₂O₂ and with/without nickel enhancement according to the manufacturer's instructions (Vector Laboratories, Burlingame, USA). Some sections were counterstained with Mayers hematoxylin (HistoLab, Göteborg, Sweden). Labeled sections were viewed using an Olympus BX60 (Olympus-Europe, Hamburg, Germany), and images were captured at 40 or 60X using a digital acquisition system (Olympus DP74 camera). Adobe Photoshop (Adobe CC 2017, Adobe Systems, San Francisco, CA) was used to adjust contrast and brightness.

For confocal microscopy, the primary antibodies were visualized using the VectaFluorTM horse antirabbit DyLight[®]488 Antibody kit R.T.U (Vector Laboratories), according to instructions given by the manufacturer. The sections were mounted using Vectashield containing DAPI (Vector Laboratories) to visualize nuclei. Confocal stacks were captured and processed using a Leica SP8 (Leica Microsystems, Wetzlar, Germany) confocal microscope with appropriate filter settings and LAS X software (Leica Microsystems, Wetzlar, Germany). Adobe Photoshop (Adobe CC 2017, Adobe Systems, San Francisco, CA) was used to adjust contrast and brightness.

Immunoelectron microscopy

Specimens destined for immunoelectron microscopy

were fixed as above but 0.1% glutaraldehyde (Agar Scientific, Stansted, UK) was used with 4% paraformaldehyde. Fixed and rinsed retinas (n=2-3) were cut into small, triangular pieces using a surgical blade under a dissection microscope. Antigen retrieval was performed using 0.01 M sodium citrate pH 6.0 for 2x15 sec in a microwave oven at 450 W and washed for 1h with Sorensen's phosphate buffer followed by PBS (Gibco) at 4°C. Free-floating retinal pieces were incubated with an antibody raised against CD11b (1:2000; Abcam) or Iba1 (1:1000; Proteintech Group) diluted in PBS (Gibco) for 48h at 4°C. Some pieces were incubated with an anti-CD11b antibody diluted as above in PBS containing 0.02% saponin to improve antibody penetration and staining.

The pieces were rinsed and incubated with an HRPconjugated anti-rabbit IgG (Cell Signaling, Beverly, USA) for 2h at room temperature. Rinsed pieces were developed using diaminobenzidine (DAB), H₂O₂, and nickel enhancement according to the manufacturer's instructions (Vector Laboratories). Post-fixation of specimens was carried out for 1h in 0.15% osmium tetroxide (Agar Scientific), followed by dehydration and embedding in Epon resin (Sigma-Aldrich). Counterstaining of ultra-thin sections was performed with lead nitrate (BDH, Dubai, UAE) and uranyl acetate (Ted Pella, Reading, USA). The primary antibodies were omitted in control specimens. The sections were examined using a JEOL 1400 Plus Transmission electron microscope (JEOL, Tokyo, Japan), and images were captured with a Matakataki CMOS digital camera (JEOL).

Results

Light and confocal microscopy

In the normal porcine retina CD11b immunoreactive microglial cell bodies were observed in the inner nuclear layer and exhibited thin processes that terminated in the OPL (Fig. 1A). The usage of nickel in the developing solution rendered a bluish-black nickel-enhanced DAB precipitate in immunoreactive microglia, which were easily distinguished in sections counterstained with Mayers hematoxylin (for visualization of the cellular and plexiform (synaptic) layers) (Fig. 1B). Nickel enhancement of the immunoreactive microglia was also clear against a very low background in non-counterstained sections (compare Fig. 1B and C). In 5 DIV retinas, distinguished CD11b and Iba1 immunoreactive microglia were observed close to/within the OPL, in the ONL, and among photoreceptor inner segments (IS) (Fig. 1B-E) as described previously (Johansson et al., 2020). Thick CD11b immunolabeled microglial cell processes as well as small dark ones (Fig. 1C-E) were observed within the synaptic layer. Judging from confocal stacks (Fig. 1D), CD11b immunoreactivity appeared unevenly distributed and fluorescent dense aggregates were evident in cell bodies and processes.

Small CD11b immunoreactive fluorescent profiles were also observed within the OPL and in the ONL (Fig. 1D). On the other hand, Iba1 immunoreactivity showed an even distribution within the labeled microglia (Fig. 1E).

CD68 immunoreactivity

Antibodies directed against CD68 were used in combination with CD11b or Iba1 to identify lysosomes in reactive, phagocytic microglia (Ferrer-Martín et al., 2014; Jurga et al., 2020) in the OPL and ONL at 5 DIV (Fig 2A). Reactive CD68 immunoreactive microglia associated with the synaptic integrity of the OPL and phagocytosis of degenerating cone photoreceptors were studied using antibodies directed against L/M-opsin. As demonstrated in Fig. 2C,D, the intensities of CD68 and Iba1 immunoreactivities differed within single sections. Microglia without CD68 immunoreactivity (asterisk in Fig. 2B) usually displayed intense Iba1 immunoreactivity, whereas microglia with intense cytosolic CD68 immunoreactivity expressed weak Iba1 immunoreactivity (insert in Fig. 2B and asterisk in Fig. 2C). Microglia with a mixture of the two immunoreactivities resulted in a blended, less intense hue, occasionally evident in lysosomal membranes (Fig. 2B).

A similar variation in labeling pattern was also observed in retinas labeled for CD68 and L/M-opsin immunoreactivities (Fig. 2D-F). Cones labeled with L/M-opsin antibodies usually only displayed intense immunolabeling (arrows in Fig. 2D-F). It was possible to observe L/M-opsin and CD68 immunoreactive cones with different staining patterns and morphologies (Fig. 2D). One of the cones showed a rather intact morphology and weak L/M-opsin immunoreactivity but less pronounced CD68 immunoreactivity. The other immunoreactive cone was heavily condensed with cytosolic L/M-opsin and CD68 immunoreactivities, resulting in a yellowish hue. Cones with similar staining characteristics and morphologies as compared with relatively normal cones (asterisk and arrow, respectively, in Fig. 2E) were also evident. Clusters of juxtaposed nuclei (arrow in Fig. 2F) and mixed L/M-opsin and CD68 immunoreactivities were evident and may indicate closely associated microglial cells and a cone.

Immunoelectron microscopy

The electron-dense nucleus and cytoplasm are features used to identify dark microglia by conventional electron microscopy (Bisht et al., 2016; Johansson et al., 2020). During the immunoelectron microscopic analyses of Cd11b immunolabeling in 5 DIV retinas, electrondense cells were evident close to the OPL. Even though the electron density varied from dark to grey, the cells were clearly distinguished from the surrounding profiles by their dark nucleus (if present) and cytoplasm (Fig. 3A,C). It was also possible to distinguish differently sized vacuoles in the cytoplasm (Figs. 3C, 4D) and dilated/denuded ER (Fig. 3A), morphological features Reactive microglial heterogeneity in cultured porcine retina











Fig. 1. Micrograph images of CD11b and Iba1-immunolabeled microglial cell bodies and processes in normal and 5 DIV cultured retinas. A. Fluorescent micrograph of CD11b-immunolabeled microglial cell bodies (arrows) in normal retina. Note the thin processes (small arrows) terminating in the OPL. B-E. 5 DIV cultured retina. B. Light microscopy of CD11b immunoreactive microglia (arrows) with nickel-enhanced DAB reaction product. The retina is counterstained with Mayers hematoxylin to show the nuclear and plexiform (synaptic) layers. B, C. CD11bimmunolabeled microglial cell bodies and processes (arrows) close to the OPL visualized by the blueish-black nickel-enhanced DAB reaction product. Insert in C shows a microglial cell at higher magnification. D. Confocal Z-stack merged image showing CD11b-immunolabeled microglial cells (asterisk) and processes (large arrows) close to/within the OPL. CD11b immunoreactivity distributes within the cytosol of cell bodies and processes, with some fluorescent cytosolic aggregates (small arrows). E. Confocal Z-stack merged image showing Iba1 immunoreactive microglia (arrows) close to the OPL and in the ONL. The immunoreactivity distributes rather evenly within the cytosol. Nuclei are DAPI-stained (blue) in A and D-F. Abbreviations: GCL ganglion cell layer; INL inner nuclear layer; ONL outer nuclear layer; IPL inner plexiform layer; OPL outer plexiform layer; IS inner segments. Scale bars: 10 μ m.

Reactive microglial heterogeneity in cultured porcine retina





Fig. 2. Merged confocal stacks of 5 DIV retinas. **A.** Low magnification confocal Z-stack merged stack of the ONL showing CD11b immunoreactive microglia (arrows). Note also the co-expression of CD68 immunoreactivity in the microglial cell bodies in the ONL. **B-F.** Visualization of CD68 immunoreactivity (red) simultaneously with either Iba1 (green in **B, C**) or L/M-opsin (green in **E-F**). **B.** Microglia lacking CD68 immunoreactivity display intense Iba1 immunoreactivity (asterisk in **B**), whereas microglia with very faint Iba1 immunoreactivity display intense CD68 immunoreactivity (asterisk in **C**). Double immunoreactive microglia (arrows and insert in **B**) were also observed to display a weak hue of red and green fluorescence. **D-F.** Several L/M-opsin immunoreactive cones in the ONL were also immunoreactive for CD68 (asterisks in **D**, **E**). The intensity of the immunoreactivity in the co-labeled cones varied and differed from single L/M-opsin immunolabeled cones (arrows in **E, F**). **F.** Cell cluster (small arrow) in the ONL containing distinct and separated CD68 and L/M-opsin immunoreactivities. Nuclei are DAPI-stained (blue). Abbreviations as in Fig. 1. Scale bars: 10 µm.

indicating a dark microglial phenotype. The dark cells expressed a cytoplasmic distribution of nickel-enhanced DAB-reaction products that varied in shape from small irregular to spherical grains: both had sharp edges and were clearly distinguished from the background (Fig. 3A,C,E). The density of the grains was usually low and scattered in the cytoplasm, which appears consistent with immunoreaction products evident in confocal stacks.

Via Iba1 immunoelectron microscopy, it was possible to identify labeled cells in the ONL (Fig. 3B,D), corroborating the confocal observations. The analyzed cells contained cytosolic Iba1 nickel-enhanced DAB reaction products with a similar appearance as described above. In general, the cytoplasm of the labeled cells appeared grayish with certain granularity and structures resembling vacuoles (Fig. 3D).

Omission of the primary antibody abolished the deposition of nickel-enhanced DAB reaction products in the dark cells (Fig. 4A,B,D). The addition of a detergent to the primary antibody solution to increase antibody penetration and improve CD11b immunolabeling was not successful. Even though the specimens were exposed to a detergent-supplemented antibody solution, it was not possible to detect any obvious increase in immunoreaction products in the labeled cells (Fig. 4C). In general, the use of a detergent resulted in poorly preserved ultrastructure and destroyed membranes of profiles in the OPL (Fig. 4C,D).

Discussion

Using light and confocal microscopy as well as immunoelectron microscopy, the present study focused on an attempt to correlate CD11b and Iba1 immunolabeling to the recently described presumptive dark microglia in degenerating porcine retina. The current study also focused on the distribution of reactive and phagocyting CD68 immunoreactive microglia. After 5 DIV, reactive CD11b and Iba1-immunolabeled microglia localized close to and arborized within the OPL and in proximity to photoreceptor cell bodies in the ONL. Immunoelectron microscopy made it possible to detect nickel-enhanced DAB reaction products in cells with a dark appearance that varied in electron density. A suggested CD11b-immunolabeled dark microglial phenotype was found close to the OPL, whereas a nondark phenotype expressing Iba1 nickel-enhanced DAB precipitate was observed in the ONL. Confocal microscopy demonstrated CD68-immunolabeled microglia at the OPL and within the ONL, close to synaptic elements and occasionally in proximity to cone cell bodies, respectively. In general, CD68 immunoreactivity varied and was observed as an even cytoplasmic distribution or localization to lysosomal membranes. It is assumed that the reactive CD68 immunoreactive microglia represent two subpopulations with one subtype associated with the synaptic structures in the OPL. The other subtype was closely associated with photoreceptor cell bodies and may be characterized as satellite microglia (Stratoulias et al., 2019). It could not be determined with immunoelectron microscopy whether the presumptive dark microglia also expressed CD68 immunoreactivity.

Microglia in degenerating retina

The impact of reactive microglia on synaptic damages *in vivo* and *in vitro* is not well-known, however, increased densities of microglia at the OPL and ONL in the degenerating retinoschisis mouse retina (Vijayasarathy et al., 2021) as well as following lightinduced retinal injuries (Xu et al., 2021), are known. Different actions following microglia depletion include degeneration of photoreceptor synapses and impaired light response (Wang et al., 2016), as well as inhibition of photoreceptor death by regulating neuroinflammatory processes (Ôkunuki et al., 2019). Recent data suggest that photoreceptor degeneration occurs independently of microglial responses following low light exposure (Bruera et al., 2022), while other studies show a synchronized appearance of reactive microglia and photoreceptor degeneration (Santos et al., 2010; Zhao et al., 2015). Because of the rapidity of degenerative events *in vitro* (Johansson et al., 2020), the temporal correlation between microglia reactivation and degenerating photoreceptors is not clearly distinguished. Collectively, reactive microglia exhibiting CD68 immunoreactivity localize close to the cell body as microglial satellites and to synaptic structures of degenerating photoreceptors, supporting the presence of microglial heterogeneity in different microenvironments of the injured retina (O'Koren et al., 2019; Gao et al., 2022).

Iba1 immunoreactive microglial satellites forming soma-soma contacts with different types of neurons are known not only in CNS areas but also in the nuclear layer of the inner retina (Bakina et al., 2022). It is here demonstrated that this subtype of microglia also inhabits the ONL and localizes juxtaposed to photoreceptor cell bodies. Generally, conventional markers such as CD11b and Iba1 can be used to identify microglial satellites in the retina. Because the ONL in the normal retina is devoid of microglia, the microglial satellites described in the current study are correlated with an injury and can be regarded as reactive judging from their expression of CD68 immunoreactivity.

Dark microglia and synaptic integrity in the OPL

Microglial cells are closely associated with the final shaping of synaptic contacts during the development and maturation of the retina (Dixon et al., 2021). Photoreceptor synaptic damage mechanism(s) and the reactivation of microglia are not well understood, but electron microscopy and immunohistochemical data on synaptic alterations/damage are increasing (Khodair et al., 2003; Fontainhas and Townes-Anderson, 2011; Mohlin et al., 2014), and engulfment of synaptic



the ONL. Abbreviations as in Fig. 1. Scale bars: 1 μ m.

structures has been demonstrated (Xu et al., 2021). This and our previous study (Johansson et al., 2020) indicate that CD11b and Iba1-immunolabeled microglia are closely associated with the OPL in the degenerating porcine retina. Based on immunohistochemical and electron microscopy data, presumptive dark microglia in porcine retina may represent a microglial phenotype with morphological similarities to that observed in pathologic CNS regions (Bisht et al., 2016). Apart from the ultrastructural and immunohistochemical criteria, the retinal dark microglial phenotype appears to be associated with damaged synaptic structures in the OPL but not vasculature. The presumptive dark microglia in the retina may be a specific phenotype that also seems to express CD68 immunoreactivity, which strengthens the electron microscopy observations of lysosomes/vacuoles



Fig. 4. Immunoelectron micrographs of control specimens and CD11b nickel-enhanced DAB immunoreactivity in 5 DIV specimens processed with antibody solution containing detergent. **A, B.** Control specimens (omitted primary antibody) with cell bodies with dark cytoplasm lacking DAB precipitate. **C, D.** Specimens processed with antibody solution containing detergent. **C.** Dark microglial cell body with scattered cytosolic nickel-enhanced DAB precipitate (arrows) and vacuole (asterisk). **D.** Dark microglia (no nucleus in this section) in the control specimen (omitted primary antibody). Cytosol with several vacuoles (asterisk). Note the poorly preserved ultrastructure of the OPL neuropil in **C** and **D**. Abbreviations as in Fig. 1. Scale bars: 1 μ m.

indicating phagocytic activity.

CD68 immunoreactivity and reactive microglia

This and previous studies (Johansson and Mohlin, 2023) demonstrate that reactive microglia localized at the OPL and in proximity to photoreceptor cell bodies express CD68 immunoreactivity. The CD68 immunoreactivity is distributed to different cellular compartments, lysosomes, and cytosol, and may be of functional importance. According to Santos and colleagues (Santos et al., 2010), an even cytosolic distribution is correlated with increased reactivity, whereas the distribution of CD68 immunoreactivity to lysosomes characterizes less reactive microglia. It was possible to observe multi-nucleated cones with heavily condensed L/M-opsin immunoreactivity together with cytosolic CD68 immunoreactivity, which may indicate a degenerating and partly phagocyted photoreceptor. Whether the declining L/M-opsin immunoreactivities observed with CD68 immunoreactivity correlate with the lysis of an afflicted cone cell has yet to be determined. However, degenerating cone photoreceptors are known to redistribute the L/M-opsin to the cell body and the synaptic pedicles in cultured retinas (Mollick et al., 2016).

Methodological considerations

CD11b and Iba1 nickel-enhanced DAB reaction products, usually identified as heavily electron-dense grains with sharp borders, were clearly distinguished in the cytosol after 48h incubation. This type of grain was not observed in control specimens processed without primary antibodies. The ultrastructure of the parenchyma was relatively well-preserved, and cell bodies and profiles in the synaptic layer were clearly distinguished. However, stained microglia displayed a less prominent electron-dense cytoplasm compared with dark microglia observed by conventional electron microscopy (Bisht et al., 2016; Johansson et al., 2020). One possibility for this discrepancy may be that antigen retrieval resulted in less stable fixation which, combined with the use of H_2O_2 in the DAB reaction, bleached the cytoplasm/nucleus of the labeled dark microglia. The use of a detergent to enhance antibody penetration and improve the amount of staining resulted in poor ultrastructure and no improved DAB staining.

Conclusion

The degenerative photoreceptive responses and accompanying presence of reactive microglia that develop after detachment *in vivo*, are most likely mimicked in the cultured retina. After five days of culture, the OPL and the ONL contained CD11b/Iba immunoreactive microglia of which some also displayed CD68 immunoreactivity. The reactive microglia showed similar immunoreactivities, however, their localization in proximity to the synaptic area or photoreceptor cell bodies indicates the presence of heterogeneity that may be of functional importance. Also, the immunoelectron microscopy data indicated the presence of microglial cell heterogeneity. Retinal explants can be used to further investigate the role of microglia in degenerating retina *per se*, as well as to unravel the role of the different microglial subtypes in different cellular compartments of degenerating photoreceptor cells.

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Contributions. KJ and CM designed the study, conducted the experiments, and wrote the manuscript.

Declaration of interest. KJ (none), CM (none).

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