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Hyperoxia causes reduced density of retinal astrocytes in the central avascular zone in the mouse model of oxygen-induced retinopathy

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ABSTRACT

The mouse model of oxygen-induced retinopathy (OIR) is commonly used to investigate various aspects of the pathogenesis of the retinopathy of prematurity (ROP) as well as angiogenesis in general. Retinal astrocytes were suggested to be involved in retinal angiogenesis. This study aimed to describe their localization and cell density during the course of physiological vascularization and pathological revascularization.

Mice expressing H2B-GFP (green fluorescent protein fused to histone 2B) from the endogenous Pdgfra promoter were kept in 75% oxygen from P7 (post natal day 7) to P12 (mouse model of OIR). Retinal flatmounts or cryosections were immunostained for glial fibrillary acidic protein (Gfap), glutamine synthetase (Glul), collagen IV (Col IV), desmin (Des), caspase 3 (Casp3), paired box 2 (Pax2), or Ki67. Astrocytic nuclei were counted with the ImageJ macro AuTOCellQuant. The hypoxic state of the retina was investigated by Hypoxyprobe.

The GFP signal of the Pdgfra reporter mice co-localized with Pax2, a nuclear marker for retinal astrocytes. This bright label was much easier to quantify than Gfap or Pax2 staining. Quantification of the cell density of astrocytes during physiological development specified the spreading of astrocytes in a concentrical wave from the optic nerve head towards the periphery. Astrocyte density was reduced during the remodelling of the primary vascular plexus into a hierarchical vascular tree (maximal astrocyte density at P1: 2800 astrocytes/mm2, final astrocyte density: 800 astrocytes/mm2). In the OIR model, cell density of astrocytes was elevated in the peripheral vascularized zone. In contrast, astrocyte density dropped to a half (400 astrocytes/mm2) of the normal value in the central avascular zone during the hyperoxic phase between P8 and P10 by apoptosis and rose only after P17 as the retinal network normalized. An additional drop of astrocyte density was observed within the angles between the large vessels of the central avascular zone during hypoxia between P12 and P17. Astrocyte density was not altered at vascular tufts.

The hyperoxia effect on astrocytes including the reduced astrocyte density is not the reason for vascular tuft formation. Hypoxia-affected astrocytes in combination with a reduced astrocytic network in the central avascular zone during the hypoxic phase are important determinants in the formation of pathological features during retinal revascularization.

Introduction

While Müller glia is derived from retinal progenitor cells developing in the retina, retinal astroglial cells originate from the brain and penetrate the retina via the optic nerve. In the mouse eye, they arrive at the optic nerve head (ONH) at embryonic day 17 and spread radially towards the periphery along the inner surface of the retina (Huxlin et al., 1992). They form a scaffold on which the endothelial cells, coming from the ONH as well, are spreading forming the inner retinal vascular network (Fruttiger, 2007). The formation of the retinal vasculature starts at birth and is completed at post natal day 7 (P7). During the late stage of the physiological vascularization, astrocytes are redistributed until they are closely associated with retinal vessels.

In humans, prematurely newborns often need a therapy with an elevated oxygen concentration to support breathing. A side-effect of this treatment is the disturbance of the development of the retinal vasculature that is normally formed within the last weeks of gestation and is completed at birth. To simulate this process in the mouse model of oxygen-induced retinopathy (OIR), young mice are treated with 75% oxygen from P7 to P12 (Smith et al., 1994). The development of the inner retinal vascular network is not yet finished at P7, and hyperoxia results in the disappearance of the central capillary network within one day resulting in a large central avascular zone (Lange et al., 2009). Cells within this area suffer from hypoxia after the mice are removed from hyperoxia back to room air (21% oxygen) at P12. This results in rapid revascularization of the central avascular zone including a pathological component called vascular tuft formation (Stahl et al., 2010). The OIR model is generally useful for studying (pathological) revascularization of a tissue that already has been vascularized before vascular obliteration. It was reported that astrocytes were reduced in

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the central avascular zone during the hypoxic phase, and that addition of fresh astrocytes or VEGF (vascular endothelial growth factor A) or FGF2 (basic fibroblast growth factor) avoids pathological tuft formation (Dorrell et al., 2010). The intention of the present study was to investigate astrocytes in the OIR mouse model in greater detail.

In most previous studies, astrocytes were labeled with antibodies against the intermediate filament Gfap showing the star-like shape of astrocytes. Unfortunately, this cell shape makes counting of astrocytes very difficult while counting dots would be much easier. Therefore, transgenic reporter mice expressing nuclear GFP in retinal astrocytes (Hamilton et al., 2003) were used to identify and count astrocytes in the present study. Using this tool, it was found that astrocyte density was already reduced in the hyperoxic phase between P8 and P10 and was normal (neither accumulation nor thinning) at vascular tufts.

Results

Specificity of GFP expression in Pdgfra-GFP mice

Traditionally, astrocytes were labeled with antibodies raised against Gfap. However, the starlike pattern of Gfap expression and the intensive interconnection of the cells complicate cell counting. Therefore, in the present study, a mouse line with GFP expression driven by the Pdgfra promoter was used to label astrocytes. To show that GFP is expressed in astrocytes, the GFP label was compared to Gfap expression (Fig. 2). In retinal flatmounts, both labels matched well as there was no cell showing either only GFP or only Gfap expression. The same was true for cryosections where the astrocytes were found in the ganglion cell layer. In this case, Gfap showed a much broader staining because the GFP signal was confined to the nucleus while Gfap was found within the whole cell including the large ramified extensions. The nuclear GFP signal also matched that of Pax2 (Fig. 2) which is another astrocyte specific marker found in the nucleus. The Pax2 label was much weaker than that of Pdgfra, especially in adult mice, and could not be used for automated cell counting. Some additional GFP signal was detected in the outer plexiform layer that was suspected to be derived from expression in Müller cells (stained by glutamine synthetase, Glul) or bipolar cells (Mudhar et al., 1993). In retinal flatmounts, this layer could be clearly separated from the ganglion cell layer in the microscope by focussing. Additional GFP expression was found outside the retina in choroid and sclera. GFP expression could be clearly distinguished from vessels (Col IV staining) and pericytes (Desmin staining, Fig. 2) that did not express GFP.

Astrocyte density in relation to vascular development

Retinal astrocytes are derived from brain astrocytes that penetrate the retina via the optic nerve shortly before birth. Therefore, they are first found in the central zone, migrating to the intermediate zone and finally to the peripheral zone (Fig. 3). During normal development, their cell density is highest between P1 and P3 with 2800 cells/mm^2 and decreases afterwards. Eventually, from P12 onwards, astrocytes are equally distributed with a final density of 800 cells/mm^2. The high density at the migration front during centrifugal spread is reminiscent of the situation found for endothelial cells. The migration front of endothelial cells, however, slightly lags behind that of astrocytes.

The shape of the astrocytes at the migration front was bipolar with elongated nuclei which is typical for migrating astrocytes (Figs. 3A and 4A). Correspondingly, only little cell proliferation was observed by immunohistochemical staining for Ki67 (Fig. 4B) indicating that the increase of astrocytes in the retina was mainly attributable to cell migration from the optic nerve.

In the central parts of the retina where the development of the vascular network was more advanced, astrocytes had a star-like shape and round nuclei (Fig. 4A). All astrocytes were found to be in close association to vessels and capillaries while those in between disappeared resulting in a reduced overall astrocyte density (Fig. 5A). Reduction in astrocyte density was accompanied by apoptosis of astrocytes (Fig. 5B). Apoptotic astrocytes showed degraded nuclei, condensed cytoplasm and Casp3 expression.

Drop of astrocyte density in the avascular zone during the hyperoxic phase

When tested in the OIR model, the distribution of retinal astrocytes showed marked differences from the physiological development. Reduced regression of the astrocyte density in the peripheral zone was observed between P10 and P21 of the OIR model compared to the physiological development which showed a stronger drop of cell density (800 cells/mm^2 at P14). This resulted in a higher density of peripheral astrocytes (1200 cells/mm^2 at P14, Fig. 6) in the OIR model.

In the central avascular zone, astrocyte density was equal at P7 and P8 but then dropped from 1200 cells/mm^2 at P8 to 400 cells/mm^2 at P10 (Fig. 6). Thereafter, cell density remained almost constant for 7 days and then returned to the normal density of 800 cells/mm^2. As no nuclei with weak GFP expression were found, the drop of cell density was not a simple reduction of GFP expression by downregulation of Pdgfra but a reduction of the number of bright nuclei. This was similarly observed

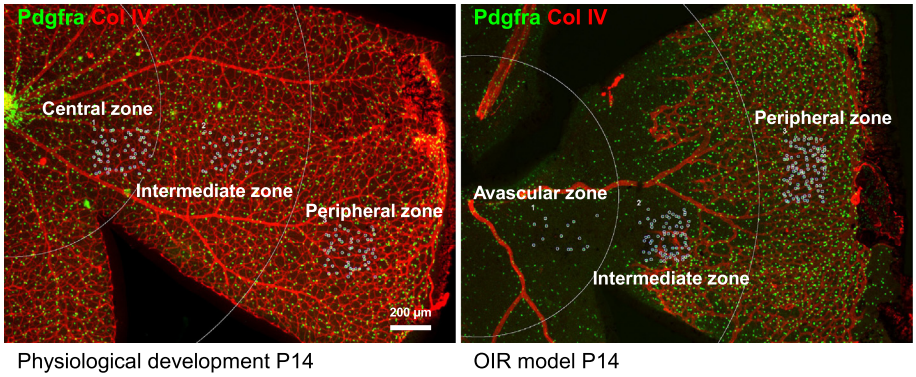

Fig. 1. Astrocyte counting method. Each quarter of each flatmount was photographed in the red channel (Col IV, vessels) and the green channel (GFP, astrocytes). Using the ImageJ macro AuTOCellQuant, the ONH and the edge of the retinal were marked in the red channel picture and the distance was divided into three equal circles defining the central, intermediate, and peripheral zones. One rectangle was selected in each zone for counting astrocytes which was performed at the selected positions in the green channel picture. The output of AuTOCellQuant is a table with the values of the counted astrocytes as well as a composite image indicating the positions of the counted astrocytes (white circles). Two examples are presented.