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Research article

Lymphatic and vascular markers in an optic nerve crush model in rat

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ABSTRACT

Only few tissues lack lymphatic supply, such as the CNS or the inner eye. However, if the scleral border is compromised due to trauma or tumor, lymphatics are detected in the eye. Since the situation in the optic nerve (ON), part of the CNS, is not clear, the aim of this study is to screen for the presence of lymphatic markers in the healthy and lesioned ON. Brown Norway rats received an unilateral optic nerve crush (ONC) with defined force, leaving the dura intact. Lesioned ONs and unlesioned contralateral controls were analyzed 7 days (n = 5) and 14 days (n = 5) after ONC, with the following markers: PDGFRb (pericyte), Iba1 (microglia), CD68 (macrophages), RECA (endothelial cell), GFAP (astrocyte) as well as LYVE-1 and podoplanin (PDPN; lymphatic markers). Rat skin sections served as positive controls and confocal microscopy in single optical section mode was used for documentation. In healthy ONs, PDGFRb is detected in vessel-like structures, which are associated to RECA positive structures. Some of these $PDGFRb^+/RECA^+$ structures are closely associated with $LYVE\text{-}1^+$ cells. Homogenous PDPN-immunoreactivity (IR) was detected in healthy ON without vascular appearance, showing no co-localization with LYVE-1 or PDGFRb but co-localization with GFAP. However, in rat skin controls PDPN-IR was co-localized with LYVE-1 and further with RECA in vessel-like structures. In lesioned ONs, numerous $PDGFRb^+$ cells were detected with network-like appearance in the lesion core. The majority of these $PDGFRb^+$ cells were not associated with RECA-IR, but were immunopositive for Iba1 and CD68. Further, single $LYVE\text{-}1^+$ cells were detected here. These $LYVE\text{-}1^+$ cells were Iba1-positive but PDPN-negative. PDPN-IR was also clearly absent within the lesion site, while $LYVE\text{-}1^+$ and $PDPN^+$ structures were both unaltered outside the lesion. In the lesioned area, $PDGFRb^+/Iba1^+/CD68^+$ network-like cells without vascular association might represent a subtype of microglia/macrophages, potentially involved in repair and phagocytosis. PDPN was detected in non-lymphatic structures in the healthy ON, co-localizing with GFAP but lacking LYVE-1, therefore most likely representing astrocytes. Both, PDPN and GFAP positive structures are absent in the lesion core. At both time points investigated, no lymphatic structures can be identified in the lesioned ON. However, single markers used to identify lymphatics, detected non-lymphatic structures, highlighting the importance of using a panel of markers to properly identify lymphatic structures.

1. Introduction

Lymphatic vessels play an important role in the maintenance of tissue fluid homeostasis and the transport of immune cells. Under physiological conditions the central nervous system (CNS) and the eye, at least at its inner parts, are considered as alymphatic. However, in healthy human eyes lymphatic vessels have been described in the conjunctiva during developmental and adult conditions (Cursiefen et al., 2006; Herwig et al., 2014) and a secondary invasion due to inflammation, trauma or uveal melanoma (Heindl et al., 2009; Maruyama et al., 2005; Wessel et al., 2012).

The normal rat ON represents a highly structured entity, with longitudinally arranged columns of nerve fibers, associated astro-

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and oligodendrocytes and concomitant vascular supply. Since the ON is considered as a part of the CNS, it is assumed that it also represents an alymphatic tissue. Although expression of the lymphatic marker podoplanin (PDPN) was detected in the lamina cribrosa and in the dura mater surrounding ON in human fetal eyes (Cho et al., 2012; Herwig et al., 2014), reports of lymphatic structures in the adult ON are lacking.

In the present study a combination of PDPN and LYVE-1 is used to identify lymphatics in the healthy and lesioned ON. PDPN, a mucin-type transmembrane glycoprotein, is considered as a lymphatic endothelial cell marker, first described in 1996 (Wetterwald et al., 1996). However, PDPN expression is also described in a variety of different other cells, e.g., osteocytes and osteoblasts (Wetterwald et al., 1996), ciliary epithelia (Williams et al., 1996) or epidermal basal layer cells (Schacht et al., 2003, 2005). In pathological conditions, PDPN was further detected in lymphatic and tumor cells, e.g. in squamous cell carcinomas and germ cell tumors (Astarita et al., 2012; Schacht et al., 2005; Sgaramella et al., 2016). In addition, the essential role of PDPN during development was detected by studying mice deficient for the PDPN gene, showing defects in the cardiac (Mahtab et al., 2008) and respiratory system (Ramirez et al., 2003), as well as defects in lymphatic vasculature formation (Schacht et al., 2003). As PDPN expression is also found in non-lymphatic, non-vascular cells and in cells undergoing epithelial-mesenchymal transition (Martin-Villar et al., 2006), identification of lymphendothelial cells requires the combination with additional lymphatic markers.

The transmembrane glycoprotein receptor, LYVE-1, was identified based on its structural and functional similarity with the hyaluronan receptor CD44 (Banerji et al., 1999) and initially thought to be exclusively restricted to lymphatic vascular endothelium. However, besides LYVE-1 expression in lymphatic endothelial cells, its expression has been reported in non-endothelial lymphatic cells: macrophages around human sclera vessels (Schlereth et al., 2014) and in the choroid (Schroedl et al., 2008), in conjunctival mouse cells (Chen et al., 2005) or dendriform cells and macrophages in the anterior eye segment (Birke et al., 2010; Kaser-Eichberger et al., 2015). Further, LYVE-1 expression on blood vessels during development has been demonstrated in mouse (Gordon et al., 2008).

Although several lymphatic markers exist, an exclusive marker is still lacking. Therefore, the unequivocal identification of lymphatic vessels requires a combination of several different markers including podoplanin (PDPN), lymphatic vessel endothelial hyaluronan receptor (LYVE-1), vascular endothelial growth factor receptor 3 (VEGFR-3) or prospero-related homeobox transcription factor (Prox1) (Baluk and McDonald, 2008; Schroedl et al., 2014).

As the formation of lymphatic structures is reported following trauma or tumor in tissues lacking lymphatics under physiological conditions, the aim of the present study was to detect potential lymphangiogenesis after ON lesion. Therefore, the lymphatic markers PDPN and LYVE-1, the vascular marker PDGFRb and RECA-1 as well as the microglial/macrophagic markers Iba1 and CD68 were analyzed in healthy and lesioned rat ON. The optic nerve crush (ONC) model was used to study CNS trauma, mimicking injury following blunt trauma.

2. Material and methods

2.1. Optic nerve crush (ONC)

Male and female Brown Norway rats (4–7 month, n = 10) were subjected to an unilateral optic nerve crush of the right eye, leaving the dura intact. For that, animals were anesthetized with a combination of ketamine/xylazine (100/5 mg/kg i. p. Sigma-Aldrich, Vienna Austria). The treated eye received topical anesthesia with 4.0 mg oxybuprocainhydrochloride (0.4% Novain, Agepha) and was disinfected with Povidon-Iod-complex solution (5% Betaisodona solution, Mundipharma). For the ONC, the ON was dissected by making a small incision in the temporal/superior conjunctiva using a surgical microscope (Universal S3B + OPMIMD Zeiss West Germany). Following this first incision, blunt dissection was used to avoid tissue damage and intraorbital trauma. The exposed ON was grasped 1–3 mm behind the ON head with a self-clamping cross-action forceps (model 35-513-10, Martin Instruments, Germany) for 20 s, ensuring a constant and consistent force in all experiments. After the ONC, retinal blood circulation was controlled by applying a cover slip on the cornea and observing retinal blood circulation with the surgical microscope. Prior to the application of the cover slip the eye was moisturized (Hylo-COMOD, sodium hyaluronate 1 mg/ml, Ursapharm, Germany). During the whole procedure (5–10 min) the animals were placed on a heat blanket to maintain normal body temperature (37–38 °C, Homeothermic Blanket Control Unit, Harvard Apparatus). To prevent possible infections, dexagenta-POS eye ointment (0,3 mg Dexamethasone, 5,0 mg Gentamicin Sulfate, Chroma Pharma, Vienna, Austria) was applied on the treated eye; the contralateral untreated eye received Vita-POS eye ointment (250 I.E./g Retinol palmitate, Croma Pharma) to prevent desiccation during the wake-up period. All animal procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee.

2.2. Tissue preparation

Animals were sacrificed 7 d (n = 5) and 14 d (n = 5) after ONC by an overdose of pentobarbital i. p. (Release, 300 mg/ml; 600 mg/kg, WDT, Germany). Eye cups and optic nerves were removed and fixed in 4% paraformaldehyde for 1 h at RT, rinsed in 0.1 M phosphate buffer (PO4, pH7.4) over-night (4 °C) and transferred into PO4 containing 15% sucrose (24 h at 4 °C). Eyes were embedded in tissue embedding medium (NEG50, Fisher Scientific) and frozen at −80 °C using liquid nitrogen-cooled iso-pentane (VWR, Austria) and stored

Table 1
Primary antibodies used in the study.

Species	Antigen		Company	Dilution
rb	LYVE-1	Lymphatic vessel endothelial hyaluronan receptor	Acris, DP3500PS	1:50
gt	PDPN	Podoplanin	R&D, AF3244	1:50
ms	RECA-1	Rat endothelial cell antibody	Serotec, MCA970GA	1:500
gp	GFAP	Glial fibrillary acid protein	Progen, GP52	1:500
rb	Iba1	Ionized calcium binding adapter molecule 1	Wako, 019-19741	1:500
gt	Iba1		Abcam, ab107159	1:500
gt	PDGFRb	Platelet-derived growth factor receptor beta	R&D, AF1042	1:150
rb	NG2	Chondroitin Sulfate Proteoglycan	Millipore, AB5320	1:300