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Absence of lymphatic vessels in the developing human sclera

Simona L. Schlereth [a, *], Barbara Neuser [a], Martina C. Herwig [b], Annette M. Müller [c], Konrad R. Koch [a], Herbert A. Reitsamer [d], Falk Schrödl [d], Claus Cursiefen [a], Ludwig M. Heindl [a]

[a] Department of Ophthalmology, University of Cologne, Kerpenerstr. 62, 50924 Cologne, Germany
[b] Department of Ophthalmology, University of Bonn, Ernst-Abbe-Str. 2, 53127 Bonn, Germany
[c] Center of Pediatric Pathology and Pathology, MVZ Venusberg, University of Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany
[d] Department of Ophthalmology and Anatomy, Paracelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria

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ABSTRACT

The adult sclera is free of lymphatic vessels, but contains a net of blood vessels. Whether and when this selectively lymphangiogenic privilege is achieved during embryologic development is not known yet. Therefore, we investigated the developing human sclera for blood- and lymphatic vessels in 34 abortions/stillborns (12–38 weeks of gestation). The probes were subdivided into three groups (group 1: 12–18 weeks of gestation, $n = 10$; group 2: 19–23 weeks of gestation, $n = 13$; group 3: 24–38 weeks of gestation, $n = 11$), and prepared for paraffin sections followed by immunohistochemistry against CD31 to detect blood vessels, and against lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1)/podoplanin to detect lymphatic vessels. We could show, that in the human episclera distinct CD31 + blood vessels are present as early as week of gestation 13. Their amount increased during pregnancy, whereas stromal CD31 + blood vessels were elevated in early pregnancy and regressed with ongoing pregnancy. In the lamina fusca CD31 + blood vessels were absent at any time point investigated. Single LYVE1 + cells were identified primarily in the episclera; their amount decreased significantly with increasing gestational ages (group 1 compared to group 3: $p < 0.01$). However, LYVE1+/podoplanin + lymphatic vessels were not detectable in the sclera at any gestational ages analyzed. In contrast to the conjunctiva where LYVE1+/podoplanin + lymphatic vessels were detectable as early as week 17, the amount of LYVE1 + cells in the sclera was highest in early pregnancy (group 1), with a significant decrease during continuing pregnancy ($p < 0.001$). These findings are the first evidence for a fetal lymphangiogenic privilege of the sclera and show, that the fetal human sclera contains CD31 + blood vessels, but is primarily alymphatic. Our findings suggest a strong expression of selectively antilymphangiogenic factors, making the developing sclera a potential model to discern antilymphangiogenic mechanisms.

1. Introduction

The human sclera is the rigid collagen cover of the eye with a specialized vascular architecture. Besides the episcleral vessel net and some perforating blood vessels, this tissue is relatively avascular. In adults, most of the scleral blood vessels are surrounded by lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1)+/CD68 + macrophages (Schlereth et al., 2014). In contrast to blood vessels, the healthy adult human sclera is free of lymphatic vessels, as our group could show recently (Schlereth et al., 2014). During embryogenesis blood vessel development within the choroid and retina is well described (Saint-Geniez and D'Amore, 2004), but only little attention has been paid to scleral vasculogenesis, which is therefore nearly unknown. This is not only the case for scleral blood vessels, but also for lymphatic vessels. Whether or when the lymphangiogenic privilege, which has been shown for adults, is achieved during development is not known yet.

Other structures in the eye, for example the lens or the hyaloid body contain blood vessels during development, and become avascular by regression of these vessels later in development. For example, in mice the vasa hyaloidea propria disappears between

* Corresponding author. Tel.: +49 221 478 4313; fax: +49 221 478 5094.
E-mail addresses: Simona.Schlereth@uk-koeln.de (S.L. Schlereth), b.neuser-erftstadt@t-online.de (B. Neuser), Martina.Herwig@ukb.uni-bonn.de (M.C. Herwig), Annette.Mueller@ukb.uni-bonn.de (A.M. Müller), Konrad.Koch@uk-koeln.de (K.R. Koch), h.reitsamer@salk.at (H.A. Reitsamer), f.schroedl@salk.at (F. Schrödl), Claus.Cursiefen@uk-koeln.de (C. Cursiefen), Ludwig.Heindl@uk-koeln.de (L.M. Heindl).

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day 12 and 16 (Ito and Yoshioka, 1999) and lately it has been shown, that this regression is regulated by a melanopsin-dependent light response (Rao et al., 2013). In contrast, other structures of the eye are primarily alymphatic, e.g. the cornea (Cursiefen et al., 2006b). For the developing fetus, it is not known, whether the fetal sclera is primarily alymphatic or if there is a regression of lymphatic vessels during embryogenesis. Time point and mode of development, as well as possible topography of lymphatic vessels are unknown. This question has important implications, as the adult sclera is selectively alymphatic, but not avascular. During pathologic conditions such as trauma (Wessel et al., 2012) or tumor (Heindl et al., 2009), lymphatic ingrowth can occur, which in case of ocular melanoma is associated with higher recurrence rate, higher metastasis rate and lower survival rate (Heindl et al., 2011b, 2010c).

Therefore, we wanted to study the fetal human sclera and investigate blood and lymphatic vessel existence and location as a basis for future experiments, investigating molecular mechanisms, which are selectively inhibiting lymphangiogenesis. The identification of responsible factors might have clinical implications for other diseases involving lymphangiogenesis.

2. Material and methods

2.1. Human fetal bulbus donors

Human eyes from stillborn fetuses and abortions between 12 and 38 weeks of gestation (WoG) ($n = 34$; mean: 21.7 weeks, of both sex: 19 male, 15 female) were obtained from the Division of Ophthalmic Pathology, University Eye Clinic Bonn, according to the Declaration of Helsinki, with informed consent. Both, the University of Bonn and the University of Cologne had approval of the local Ethics committees.

2.2. Formalin fixated paraffin embedded scleral probes

Eyes were fixed in phosphate buffered saline (PBS) containing 4% formaldehyde and prepared for paraffin embedding. Subsequently pupil-optic disc sections of 4 μm thickness were obtained and analyzed at anterior, equatorial, and posterior location. Following deparaffinization via series of graded alcohols, immunohistochemistry was performed as described previously (Schlereth et al., 2014). Briefly, sections were incubated with CD31 (monoclonal mouse anti human antibody, Dako, Hamburg, Germany, diluted 1:50 in PBS), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1 – polyclonal rabbit anti human antibody, Zytomed, Berlin, Germany, diluted 1:50 in PBS), podoplanin (monoclonal mouse anti human D2-40 antibody, Dako, ready-to-use) or CD68 (monoclonal mouse anti human antibody, Dako, diluted 1:100 in PBS) for 60 min for LYVE1 and 30 min for CD31, podoplanin and CD 68 at 20 °C. For CD31, LYVE1, and podoplanin labeling, antigen retrieval was required (20 min at 98 °C). CD68 was pretreated with Fast Enzyme (DCS diagnostics, Hamburg, Germany) for 10 min. DCS Detection line (DCS diagnostics) was performed according to manufacturer's instructions. After incubation with the primary antibody, the secondary antibody was added on the sections for 10 min at 20 °C and was marked with a peroxidase-Label (HRP). AEC (3-Amino-9-ethylcarbazole) was used as a chromogen. The sections were counterstained with hemalaun. To confirm the normal anatomy of the eye, the sections were stained with hematoxylin & eosin (H&E).

To exclude an unspecific secondary antibody binding, controls were performed by omission of the primary antibodies and resulted in no staining. Conjunctiva and placenta (for CD68) specimens were used as positive controls and showed appropriate results (i.e. immunoreactivity in conjunctival and placental macrophages (Vinnars et al., 2010); not shown).

Our definition of lymphatic vessels required the co-localization of the two established lympathic markers LYVE1 and podoplanin and a visible vessel lumen (Bock et al., 2013). Our definition of blood vessels required immunoreactivity for the endothelial marker CD31 and a visible vessel lumen.

2.3. Morphometric and statistical analyses

The fetal human sclera and conjunctiva were analyzed immunhistochemically for the existence of blood (CD31+) and lymphatic vessels (LYVE1+/podoplanin+) at anterior, equatorial, and posterior location in a blinded fashion by two independent persons. The probes were subdivided into three groups: 1) 12–18 WoG, $n = 10$; 2) 19–23 WoG, $n = 13$; and 3) 24–38 WoG, $n = 11$.

Sections were analyzed using a Leica DM2500 microscope (Leica GmbH, Wetzlar, Germany) and documented digitally using a JVC digital camera KY-F75U (JVC, Yokohama, Japan). In all gestational groups, CD31 + vessels and LYVE1 + immunoreactive cells were quantified at the different locations (episclera-anterior, -equatorial, -posterior and scleral stroma-anterior, -equatorial and posterior and conjunctiva). Therefore, pictures of 0.8 mm^2 size were taken from each localization. The amount of cells or vessels detected in the pictures was graded into four levels. The grading score applied as follows: 0 for negative result (no LYVE1 + cells or CD31 + vessel in the picture), 1 for 1–2 LYVE1 + cells/1–2 CD31 + vessels, 2 for 3–5 LYVE1 + cells/3–5 CD31 + vessels and 3 for more than 5 LYVE1 + cells/>5 CD31 + vessels) by two independent persons (Table 1). The number of CD31 + blood vessels or LYVE1 + cells was evaluated in relation to the gestational age.

Statistical analysis was performed using the Mann-Whitney-U-Test and the Wilcoxon Test in SPSS software (IBM Corporation, Armonk, NY, USA). *P*-values< 0.05 were considered statistically significant.

3. Results

3.1. The human scleral stroma contains CD31 + blood vessels as early as week of gestation 13

To study fetal hemangiogenesis, formalin embedded paraffin-fixated eyes of 34 human fetuses were investigated for age-dependent and location-specific differences in CD31 + blood vessels. In general, we detected blood vessels as early as week 13 in the episclera, the limbus and the conjuctiva. At the same time, the stroma displayed a reduced amount of blood vessels and the lamina fusca did not show any blood vessels at all time points. The number of episcleral blood vessels increased during development in all locations investigated from group 1 (anterior episclera score: 2.1 ± 1 equatorial episclera score: 0.5 ± 0.9 and posterior episclera score: 1.3 ± 1) to a higher score in group 3 (anterior episclera score:

Table 1
Scoring system used for analysis of LYVE1 + cells and CD31 + blood vessels within sclera or conjunctiva.

	Definition	Score
No LYVE1 + cells or no CD31 + vessels	Absent	0
Low amount of LYVE1 + cells or CD31 + vessels	1–2 cells/1–2 vessels	1
Moderate amount of LYVE1 + cells or CD31 + vessels	3–5 cells/3–5 vessels	2
High amounts of LYVE1 + cells or CD31 + vessels	>5 cells/>5 vessels	3