Transient Expression of Lymphatic Markers in Retrobulbar Intraconal Orbital Vasculature During Fetal Development

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PURPOSE. The aim of this study is to investigate the presence of orbital lymphatic vessels during fetal and neonatal development and in adults using a panel of lymphatic markers.

METHODS. This was a retrospective observational case series. For analyzing lymphatic vessels, we used formalin-fixed paraffin-embedded enucleated eyes from 25 human fetuses between 13 and 24 weeks of gestation and postnatal eyes from 15 children and 5 adults. Immunohistochemical analysis of lymphatic vessels was performed for the markers: lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1), podoplanin (D2-40), Prospero-related homeobox gene-1 (Prox-1), pan-endothelial marker CD31, and blood vessel endothelium specific CD34.

RESULTS. Vasculature showing endothelial expression of LYVE-1, D2-40, Prox-1, and CD31 in combination with absence or weak expression of CD34, as would be expected for lymphatic vessels, was seen in 11 of 25 fetuses in an age range from 14 weeks to 23 weeks of gestation (44%). This lymphatic vascular staining pattern was also observed in 4 of 15 liveborn children (27%), all within 1 month of age, of which two were born prematurely at 32 and 34 weeks of gestation. Interestingly, an incomplete lymphatic staining pattern was observed in another 4 fetuses and two liveborn children of 4 months and 7 years old. No expression of lymphatic markers was observed in adult orbital vasculature.

CONCLUSIONS. No retrobulbar intraorbital lymphatic vessels were observed in adults, however, we did observe transient expression of lymphatic markers in retrobulbar intraconal orbital vasculature during fetal and early neonatal development. The orbit may, therefore, be proposed to possess a full range of lymphatic plasticity.

Keywords: lymphatics, transient expression, fetal development
Transient Expression of Lymphatic Markers

METHODS

Sample Selection

Human eyes were obtained by enucleation from termination of pregnancy fetuses between gestational weeks 13 and 24 \((n = 25)\), deceased children between the ages of 0 and 15 years of age \((n = 15)\), and adults \((n = 5)\) as part of routine diagnostic procedures. All studies complied with the regulations of the local ethics committee. Clinical information and gestational age of fetuses and ages of the children and adults are provided in Table. Because prenatal ultrasound examination showed congenital malformations, pregnancy was terminated. The fetal eyes were harvested in case of brain malformations or with a differential diagnosis that involved syndromes that may be associated with ocular malformations. None of the diagnoses involved syndromes associated with (lymphatic) vascular malformations, like Turner-, Proteus-, Sturge-Weber-, or Klippel-Trenaunay-Weber syndrome. None of the eyes showed developmental anomalies upon macroscopic and microscopic evaluation. None of the liveborn children or adults suffered orbital infectious or inflammatory disease.

Eyes were fixated in buffered 10% formaldehyde, and pupil-optic nerve sections of 4 mm thickness were obtained after paraffin embedding. Pupil-optic nerve sections were stained for hematoxylin & eosin (H&E), and the presence of sufficient retrobulbar orbital fat for evaluation was confirmed. In addition, immunohistochemistry was performed on serial pupil-optic nerve sections.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) sections were analyzed for the presence of lymphatic vessels. Four-micrometer thick sections were stained for Podoplanin (Clone D2-40, Ref.: 760-4395; Cell Marque, Rocklin, CA, USA), Prospero Homebox-1 (Prox-1, Clone D2J6F, dilution 1:1500; Cell Signaling, Leiden, The Netherlands), Cluster Differentiation 31 (CD31, Clone JC70, Ref.: 760-4378; Cell Marque, Rocklin, CA, USA), and Cluster Differentiation 34 (CD34, Clone QBEnd/10, Ref.: 790-2927; Ventana Medical Systems, Tucson, AZ, USA) with the Ventana Benchmark Ultra automated staining system (Ventana Medical Systems). Briefly, after deparaffination the sections were processed for 32 to 64-minute antigen retrieval using Cell Conditioning Solution 1 (CC1 Ventana Ref.: 950-124). Following 32-minute incubation (16-minute for CD31) with the primary antibody, detection was performed using the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ref.: 760-501; Ventana Medical Systems) in combination with the Amplification Kit (Ref.: 760-080; Ventana Medical Systems). Sections were counterstained with hematoxylin II (Ref.: 790-2208; Ventana Medical Systems). Due to technical limitation of the Ventana automated staining system, detection of LYVE-1 required manual interference in the protocol. For anti-LYVE-1 (Clone AF2089, dilution 1:1000; R&D Systems, Minneapolis, MN, USA) primary antibody staining was executed using the Ventana Discovery Benchmark automated staining system (Ventana Medical Systems). The following adaptations from the protocol were required: endogenous peroxidase was blocked in order to prevent unspecific signal using Inhibitor CM from the DISCOVERY ChromoMap DAB Kit (RUO) (Ref.: 760-159; Ventana Medical Systems) for 4 minutes. The secondary antibody incubation was performed with anti-Goat-HRP (Ref.:760-159; Ventana Medical Systems) for 32 minutes. Detection was executed manually with 3-amino-9-ethylcarbazoleolue (AEC) diluted in 0.2M Sodium Acetate with H2O2. The slides were counterstained with Mayer's Hematoxylin (Cat. 4085.9005; Klinipath, Duiven, The Netherlands).

Scoring of Immunohistochemistry

Three independent reviewers scored the slides to reach consensus: a pathologist, an ophthalmologist, and a research technician with ample experience in ophthalmic pathology. Based on the presence or absence of specific signals in the orbital vasculature, lymphatic vessels were identified using a panel of immunohistochemical markers on serial sections. As described in an earlier study, a vascular structure was identified as a lymph vessel when it showed combined endothelial expression of D2-40, Prox-1, LYVE-1, and CD31 and absence or weak expression of CD34. These requirements are in accordance with the first international consensus on the methodology of lymphangiogenesis quantification in solid human tumors and the consensus statement on the immunohistochemical detection of ocular lymphatic...
vessels.\textsuperscript{14,16} Lymphatic vessels of the perlimbal conjunctiva served as internal controls, external control tissue (lymph node) was applied in case of absence of conjunctival tissue.

\section*{RESULTS}

\textbf{Immunohistochemistry for Endothelial Lymphatic Markers}

Positive endothelial staining for any of the lymphatic vascular markers was observed in the retrobulbar perioptic orbital fat of fetuses and young children. None of the adult cases showed positive endothelial staining for lymphatic markers, which is in concurrence with earlier unreported observations in enucleation specimen for uveal melanoma.\textsuperscript{17} Positive staining for all lymphatic markers tested in combination with weak or absence of staining for CD34 was seen in 11 of 25 fetuses in an gestational age ranging from 13 to 24 weeks (44\%) (Table, Fig. 1). In another three fetuses, CD34 staining could not be evaluated with certainty because of high positive staining of perivascular orbital connective tissue. This could potentially have resulted in an underestima-
FIGURE 1. Overview of identification of lymphatic vessels with Prox-1, D2-40, LYVE-1, CD34, and CD31 in second trimester fetuses. Lymphatic phenotype staining pattern of a 14-week-old fetus (Table, case 2) is shown by a positive endothelial staining for Prox-1, D2-40, and LYVE-1 (A, B, C, respectively) combined with a weaker staining for CD34 compared to the surrounding blood vessel endothelium. (D) Positive staining for CD31. (E) Lymphatic staining pattern of an 18-week-old fetus (Table, case 8) showed positive endothelial phenotype staining for Prox-1, D2-40, and LYVE-1 (F, G, H) combined with a negative staining for CD34 (I) and a positive staining for CD31. (J) Lymphatic phenotype staining pattern of a 21-week-old fetus (Table, case 11) showed positive endothelial staining for Prox-1, D2-40, and LYVE-1 (K, L, M, respectively) combined with a weak staining for CD34 (N) and a positive staining for CD31. Note the asterisk (*) showing strong positive staining in blood vessel endothelium for CD34 as reference. (O) Staining pattern of vasculature in a 24-week-old fetus (Table, case 25) showed negative staining for Prox-1, D2-40, and LYVE-1 (P, Q, R, respectively) combined with a positive staining for CD34 (S) and a positive staining for CD31. (T) No lymphatic phenotype staining pattern was observed in this case.

Transient Expression of Lymphatic Markers in Relation to Age

Three cases, one fetus of 13 weeks of gestation, one term born, one 4-month-old child, and one 7-year-old child, showed a vascular structure that exhibited incomplete lymphatic endothelial marker staining (Table). The 13 weeks of gestation fetus with a vascular structure staining positive for LYVE1, Prox1, and CD31 was a termination of pregnancy because of the combination of omphalocele and encephalocele. CD34 could not be evaluated in this case. The 4-month-old child with a vascular structure positive for LYVE-1, D2-40, CD31, and CD34 died of hemophagocytic lymphohistiocytosis, which was not present in the orbital tissues. The 7-year-old child had a vascular structure that stained positive for LYVE-1 in combination with CD31 and CD34 positive staining. This implies a transient expression of lymphatic markers and that Prox-1 may the first marker to be lost followed by D2-40 and LYVE 1. Moreover, for second trimester fetuses, a lymphatic vascular pattern was observed in a maximum of 14 of 25 cases (56%, including...
FIGURE 2. Overview of identification of lymphatic vessels with Prox-1, D2-40, LYVE-1, CD34, and CD31 in third trimester and older children
and adult. Lymphatic phenotype staining pattern of a 14-week-old premature born child at 34 weeks of gestation (Table, case 29) is
shown by a positive staining for Prox-1, D2-40, and LYVE-1 (A, B, C, respectively) combined with a negative staining for CD34 (D) and
a positive staining for CD31. (E) Incomplete lymphatic staining pattern of a 4-month-old child showed positive staining for LYVE-1 and
D2-40 (G, H), but a negative staining for Prox-1. (F) Whereas CD34 and CD31 both show positive staining, (I, J) Incomplete lymphatic
staining pattern of a 7-year-old child showed positive staining for LYVE-1 (M), but negative staining for Prox-1 and D2-40. (K, L) CD34
and CD31 both showed positive staining. (N, O) Vascular staining pattern of an adult showed no positive staining of Prox-1, D2-40, and
LYVE-1 (P, Q, R), but did show positive staining for CD34 and CD31. (S, T) No lymphatic phenotype staining pattern was observed in adult
cases.

those cases where CD34 could not be evaluated), whereas
in third trimester premature and term born children, such a
complete pattern was only observed in 4 of 15 (27%) cases.
No lymphatic vascular patter was found in adults, also indi-
cating a progressive loss of expression of lymphatic markers
with age.

DISCUSSION
This study examined the presence of retrobulbar intraorbital
lymphatic vessels during fetal and neonatal development
and in adults by using a panel of lymphatic markers on
enucleation specimen. Our panel of markers was designed
to distinguish lymphatic vessels from blood vessels. Our
criteria to identify lymphatic vessels also relies on the
absence or weak expression of CD34, which should be
carefully interpreted in early development and adulthood.
During early development, CD34 is expressed in mesenchy-
mal cells, 26 hematopoietic progenitor cells, 28 and developing
vasculature. 29 Furthermore, endothelial cell fate during early
development is much less understood, which makes identifi-
cation of lymphatic vessels challenging in embryonic tissue.
Whereas in adulthood, CD34 can be occasionally and irreg-
ularly expressed in lymphatic vessels depending on the type
of vessel, localization, and tissue, albeit in a much lower
staining intensity when compared to blood vessels. 30,31
When identifying lymphatics in neural-rich tissue, such
as the eye, one needs to be aware of positive staining of
D2-40 32 and CD34 33 in nerve sheath cells. Especially in an
immunofluorescent approach, this can mimic a vascular

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structure, and, thus, may appear as a lymphatic vessel. With the use of a broader lymphatic marker panel, these structures can be identified as nonlymphatic structures by lack of LYVE-1, Prox-1, and CD31 (data not shown). We could not detect retrobulbar intraorbital lymphatic vasculature in adult samples using five specific markers. This is in concordance with earlier unreported observations, when 16 adult eyes were examined for lymphatic vessel recruitment in uveal melanoma.17 However, we did find transient expression of lymphatic markers in retrobulbar orbital vasculature during fetal and early neonatal period. Others did not observe intraorbital lymph vessels in four 10 to 12 weeks of gestation old fetuses that were serially sectioned.25 Although, in that study, only podoplanin was used as an immunohistochemical marker and our cases are of more advanced gestational age. The use of multiple markers in the current study may explain the increased likelihood for identification of lymphatic vasculature. The fact that not all cases from a similar gestational age proved to be positive may be explained by a potential for under detection because a limited amount of orbital fat was available in the enucleation specimen. Complete removal, embedding, and sectioning of the orbital contents may be expected to increase the likelihood for identification of such vessels in future. Because such a procedure would not be justified as part of a diagnostic procedure, a separate specific parental consent would be required. The observation that, in the current study, expression of lymphatic markers decreases with (gestational) age, made us hypothesize that this is most likely a transient developmental phenomenon that does not signify consistent orbital lymphangiogenesis. These findings are similar to what has been reported for the murine cornea.13 That study demonstrated that the mouse cornea was endowed with a significant number of lymphatic vessels that underwent spontaneous formation and regression during a critical period after birth, which was not observed for blood vessels. Lymphatic growth can be reactivated in the adult cornea and orbit after inflammatory stimulation. The transient expression of lymphatic vessel markers in retrobulbar orbital vessels during fetal and early neonatal development may, therefore, share overlapping features with lymphatic vessel growth and regression during postnatal development in the mouse model. In line with what was described for the murine cornea, it could be speculated that the lymphatic status of the orbit is orchestrated and maintained by a similar combination of pro- and antilymphatic factors already known or yet to be discovered. Certain physiologic or pathologic stimulations will tip the balance in favor of lymphatic formation or regression.13 This may also explain previous studies of adult orbital soft tissues that have reported that orbital fat lacks lymphatic vessels, but that inflammation can induce both the growth of new blood vessels and lymphangiogenesis in orbits that are inflamed or in orbital infection.9 16

In conclusion, this study describes that transient developmental expression of lymphatic markers is a feature observed in retrobulbar intracranial vasculature during the fetal and early neonatal period. The orbit may, therefore, be proposed to possess a full range of lymphatic plasticity.

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