Experimental Eye Research 172 (2018) 1–9

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Histochemical characteristics of regressing vessels in the hyaloid vascular system of neonatal mice: Novel implication for vascular atrophy

Ayuko Kishimoto[a], Shunsuke Kimura[a], Junko Nio-Kobayashi[a], Hiromi Takahashi-Iwanaga[a], Ah-Mee Park[b], Toshihiko Iwanaga[a,*]

[a] Laboratory of Histology and Cytology, Department of Anatomy, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan
[b] Department of Microbiology, Kindai University Faculty of Medicine, Osaka 589-8511, Japan

ARTICLE INFO

Keywords:
Galectin-3
LYVE-1
Integrin
Hyaloid vessels
Regression
Immunohistochemistry

ABSTRACT

The hyaloid vasculature constitutes a transitory system nourishing the internal structures of the developing eye, but the mechanism of vascular regression and its cell biological characteristics are not fully understood. The present study aimed to reveal the specificity of the hyaloid vessels by a systematic immunohistochemical approach for marker substances of myeloid cells and the extracellular matrix (ECM) in neonatal mice. Macrophages immunoreactive for F4/80, cathepsin D, and LYVE-1 gathered around the vasa hyaloidea propria (VHP), while small round cells in vascular lumen of VHP were selectively immunoreactive for galectin-3; their segmented nuclei and immunoreactivities for Ly-6G, CD11b, and myeloperoxidase indicated their neutrophilic origin. VHP possessed thick ECM and a dense pericyte envelope as demonstrated by immunostaining for laminin, type IV collagen, integrin β1, and NG2. The galectin-3$^+$ cells loosely aggregated with numerous erythrocytes in the lumen of hyaloid vessels in a manner reminiscent of vascular congestion. Galectin-3 is known to polymerize and form a complex with ECM and NG2 as well as recruit leukocytes on the endothelium. Observation of galectin-3 KO mice implicated the involvement of galectin-3 in the regression of hyaloid vasculature. Since macrophages may play central roles including blocking of the blood flow and the induction of apoptosis in the regression, galectin-3$^+$ neutrophils may play a supportive role in the macrophage-mediated involution of the hyaloid vascular system.

1. Introduction

The hyaloid vascular system, consisting of the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL), is a transiently existing vessel network important for nourishment of the developing lens and maintenance of the primary vitreous. This vascular system in mice starts to form at embryonic day 10.5 (E10.5) and becomes fully developed by E13.5 (Smith et al., 2002). It regresses and then replaces developing permanent retinal vessels; in the mouse, VHP and TVL initiate the regression after birth and largely disappear around postnatal day 16 (Ito and Yoshioka, 1999). The regression is associated with apoptosis of endothelial cells/pericytes and phagocytosis of their apoptotic bodies by macrophages (Jack, 1972; Mitchell et al., 1998; Taniguchi et al., 1999). The exact regulatory mechanism responsible for the involution of hyaloid vessels is poorly understood; there are, however, some contributing factors: an increased expression of apoptosis factors, the down-regulation of angiogenic growth factors such as VEGF, and cessation of the blood flow (for review, Saint-Geniez and D'Amore, 2004; Hegde and Srivastava, 2017).

Macrophages are known to gather around regressing blood vessels of VHP (Ito and Yoshioka, 1999; Taniguchi et al., 1999). Electron microscopic studies have demonstrated the phagocytosis of vessel-derived debris by macrophages (Taniguchi et al., 1999). Using macrophage-deficient mice, it has been shown that macrophages play an essential role in the regression of the hyaloid vascular system (Lang and Bishop, 1993). Histochemically, Zhang et al. (2010) detected a topographical association of macrophages with VHP using an antibody against LYVE-1, which was originally identified as a receptor molecule for hyaluronan. LYVE-1 is structurally homologous to another hyaluronan receptor, CD44, which is widely expressed on leukocytes, dendritic cells, and tumor cells (Lesley et al., 1994). For further characterization of the macrophages in regressing hyaloid vessels, we tried to use another unique marker of macrophages, galectin-3 (Sato and Hughes, 1994; Liu et al., 1995; Sano et al., 2003), in the immunostaining of whole mount preparations of developing murine eyes.

Galectin is one of the animal lectins which recognize β-galactoside

* Corresponding author. Kita 15-Nishi-7, Kita-ku, Sapporo 060-8638, Japan.
E-mail address: tiwanaga@med.hokudai.ac.jp (T. Iwanaga).

https://doi.org/10.1016/j.exer.2018.03.024
Received 7 December 2017; Received in revised form 7 March 2018; Accepted 23 March 2018
Available online 27 March 2018
0014-4835/

of glycoconjugates and contribute to various biological functions, such as cell differentiation, migration, and apoptosis (Yang et al., 2008). Among fifteen galectin subtypes, galectin-3 is the only one classified as a chimera-type and possesses a unique ability for self-assembly. In addition to monocytes/macrophages, this endogenous lectin-like protein is contained in several blood cells including neutrophils, eosinophils, and mast cells (Liu, 2000, 2005; Dumic et al., 2006). Although galectin-3 is present predominantly in the cytoplasm, it is released from cells by a non-classical mechanism, due to a lack of the signal sequence or transmembrane domain in the protein (Hughes, 1999; Dumic et al., 2006). Extracellular galectin-3 binds to cell surface oligosaccharides, thereby exerting various functions represented by apoptosis (Suzuki et al., 2008; Zhuo et al., 2008) and cell adhesion (Sato and Hughes, 1992; Inohara et al., 1996; Kuwabara and Liu, 1996). Notably, Galectin-3 forms a molecular complex with NG2 chondroitin sulfate proteoglycan and extracellular matrixes (ECM) such as integrin (Ochieng et al., 1998; Fukushi et al., 2004) and laminin (Kuwabara and Liu, 1996; Hughes, 2001) to promote cell-ECM attachment. Support for the involvement of galectin-3 in physiologic and pathologic consequences of blood vessels has been attributed to thrombogenesis and neovasculogenesis. Leukocytes expressing galectin-3 attached to the vein wall in a thrombosis model of mice and the expression levels of monomers to trimmers were up-regulated in loco (DeRoo et al., 2017).

When we stained the hyaloid vessels of neonatal mice using several anti-galectin-3 antibodies, the LYVE-1-immunoreactive macrophages were found to be negative, but cells accumulating in the lumen of hyaloid vessels were selectively immunoreactive for galectin-3. In contrast, the developing blood vessels in the retina contained few galectin-3-expressing cells in the vascular lumen. The present study examined the detailed characteristics of the galectin-3-expressing cells in the hyaloid vessels and suggests their possible involvement in the regression of the hyaloid vasculature.

2. Methods

2.1. Animals and tissue sampling

Pregnant ddY mice and CD1 mice were supplied by Japan SLC (Shizuoka, Japan) and Charles River Laboratories Japan (Yokohama, Japan), respectively. The eyeballs of E17.5 embryos and neonates at postnatal days 1, 3, 5, 7, 9, 15, and 20 (ddY strain) were mainly used in the present study. The generation of galectin-3-deficient mice of the CD1 strain was described previously (Hsu et al., 2000). Samples from the galectin-3 KO mice were obtained at postnatal days 9, 15, and 20. Mice were sacrificed by the intraperitoneal injection of an overdose of pentobarbital sodium (Schering Plough Animal Health, the Netherlands). The eyeballs were enucleated and fixed for 2 h in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4. The retina with vitreous was spread by cutting into fours, and the lens were isolated from eyeballs under a dissecting microscope.

All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

2.2. Immunohistochemistry

After immersion in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton-X100, the whole mount preparations in glass bottles were pre-incubated with a normal donkey serum. For double or triple immunostaining, they were incubated for 3 days with a mixture containing two or three antibodies from the following list: goat anti-mouse galectin-3 antibody (AF1197; R&D Systems, Minneapolis, MN), rabbit anti-mouse LYVE-1 antibody (11–034; AngioBio, Del Mar, CA), rabbit anti-myeloperoxidase antibody (PA5-16672; ThermoFisher Scientific, Rockford, IL), goat anti-mouse cathepsin D antibody (AF1029; R&D Systems), rat anti-mouse F4/80 antibody directly labeled with or without Alexa Fluor 647 (BM8; BioLegend, San Diego, CA), rat anti-mouse integrin β1(CD29) antibody (MAB2405; R&D Systems), rabbit anti-laminin antibody (ab11575; abcam, Cambridge, UK), rabbit anti-NG2 chondroitin sulfate proteoglycan antibody (AB5320; Millipore/Chemicon International, Temecula, CA), rabbit anti-type IV collagen antibody (ab19808; abcam), rat anti-mouse/human CD44 antibody labeled with PE (IM7; affymetrix/ThermoFisher Scientific), rat anti-mouse Ly-6G/Ly-6C antibody (RB6-8C5; Novus Biologicals, Littleton, CO), rat anti-mouse CD68 antibody labeled with or without Alexa fluor 488 (FA-11; affymetrix and BioLegend), rat anti-mouse/human CD11b antibody labeled with or without Alexa Fluor 488 (M1/70; BioLegend), rat anti-mouse CD31 antibody (MEC 13.3; BD Pharmingen, Franklin Lakes, NJ), rabbit anti-MCT1 antibody (MCT1-Rb-Af900; Frontier Institute, Ishikari, Japan), and guinea pig anti-GLUT1 antibody (GLUT1-GP-Af610; Frontier Institute). The antigen-antibody reactions were detected by incubation of 6 h with Cy3-conjugated donkey anti-goat, anti-rat, or anti-rabbit IgG antibody; Cy5-conjugated donkey anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA); Alexa Fluor 488-conjugated donkey anti-rat, anti-rabbit, or anti-guinea pig IgG antibodies (Invitrogen/ThermoFisher Scientific). Stained samples were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview FV300; Olympus, Tokyo, Japan). The specificity of immunoreactions on sections was confirmed according to a conventional procedure, including absorption tests.

For statistic evaluation, galectin-3$^+$ cells and LYVE-1$^+$ macrophages were counted in five independent areas of VHP, 600 × 800 μm in size, randomly chosen from 5 eyes at each stage and the numbers of galectin-3$^+$ cells and LYVE-1$^+$ cells were counted. Data are expressed as mean ± standard errors of the mean (SEM). Statistical comparisons between stages were evaluated by one-way analysis of variance (ANOVA) with Sidak's multiple comparisons test using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Values in the graphs represent the mean ± SEM and $P < 0.05$ was regarded as significant.

2.3. Silver-intensified immunogold method for electron microscopy

Some of the fixed tissues containing the VHP were dipped in 30% sucrose solution overnight at 4 °C, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections 12 μm in thickness were mounted on poly-L-lysine-coated glass slides. They were pretreated with normal donkey serum for 30 min, incubated with the goat anti-galectin-3 antibody (1 μg/mL) overnight, and subsequently reacted with rabbit anti-goat IgG covalently linked with 1-nm gold particles (Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon. Ultrathin sections were prepared and stained with an aqueous solution of uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

3. Results

3.1. Galectin-3-expressing cells gather in the hyaloid vessels

As reported by Zhang et al. (2010), LYVE-1-immunoreactive cells of round or irregular shapes gathered around the blood vessels of the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL) in fetuses and neonates. They were immunostained positively with F4/80 antibody (Fig. 1a), a reliable marker antibody of murine macrophages, and also immunoreactive for cathepsin D (Fig. 1b), one of the lysosomal degradation enzymes, thus strongly indicating their macrophagic origin. They were distributed only along the VHP/TVL but completely absent in other regions without hyaloid vessels (Fig. 1c). They appeared to attach or be juxtaposed to the blood vessels of VHP with short