Bush-like integrin filament networks associated with hyaloid vasculature in murine neonate eyes

Toshihiko IWANAGA, Junko NIO-KOBAYASHI, and Hiromi TAKAHASHI-IWANAGA

Laboratory of Histology and Cytology, Department of Anatomy, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

(Received 4 January 2019; and accepted 2 February 2019)

ABSTRACT

The vitreous of perinatal mice temporarily develops a unique vascular system, called the vasa hyaloidea propria (VHP). Observations showed the vessels possessed an extracellular matrix including the basement membrane in their entire length. Immunostaining of whole mount preparations of VHP with integrin β_1 antibody displayed a bush-like network consisting of long and straight fibers which were associated with the VHP but extended apart from the blood vessels. Electron microscopically, each fiber was composed of a bundle of thin filaments different from collagen fibrils. Macrophages associated with the VHP appeared to be arrested by the integrin bushes. The integrin bushes fragmented and disappeared by postnatal day 10, just before the regression of the VHP. Macrophages were involved in the digestion and clearance of integrin bushes. The vitreous integrin bushes appear to provide a scaffold for architectural maintenance of the hyaloid vessels and macrophages.

The hyaloid vascular system is a transiently existing vessel network nourishing the developing lens and maintaining the primary vitreous. It consists of the vasa hyaloidea propria (VHP) and the tunica vasculosa lentis (TVL). After full development in fetuses or neonates, it regresses to be replaced by developing permanent retinal vessels; in the mouse, the VHP and TVL initiate the regression several days after birth and largely disappear around postnatal day 16 (4, 9). The regression is mainly due to the programmed cell death of endothelial cells and associated pericytes (10, 16, 19). Numerous macrophages gather around developing and regressing blood vessels of the VHP/TVL and phagocytose their apoptotic bodies (4, 19). Electron microscopic studies have demonstrated the active phagocytosis of vessel-derived debris by macrophages (19). Using

macrophage-deficient mice, it has been shown that macrophages play an essential role in the regression of the hyaloid vascular system (12). Recently, we reported the involvement of galectin-3-expressing granulocytes in the regression process (11).

Another characteristic of the VHP is to float in the vitreous, which lacks any firm structural components. Although the vitreous contains collagen fibrils, they are neither a concrete nor well-organized architecture. It is reasonable that the “floating vessels” and wandering macrophages require some scaffolds in the vitreous.

Integrins comprise a large family of heterodimeric cell surface receptors that link the extracellular matrix and the intracellular filaments, namely the cytoskeleton (8). In a previous study of the regressing hyaloid vasculature, we noticed a unique morphology of β_1 integrin-immunoreactive fiber networks closely associated with hyaloid vessels. We considered that these integrin networks can serve as a scaffold of hyaloid vessels. Notably, the integrin networks disappeared just before the entire regression of hyaloid vessels. It is known that endothelial cells during angiogenesis use integrin receptors to

Address correspondence to: Dr. T. Iwanaga
Department of Anatomy, Hokkaido University Graduate
School of Medicine, N15W7, Kita-ku, Sapporo 060-
8638, Japan
Tel: +81-11-706-5033, Fax: +81-11-706-7151
E-mail: tiwanaga@med.hokudai.ac.jp

adhere to and migrate into the extracellular matrix (ECM). Integrins not only promote the adhesion of endothelial cells but also regulate cell growth and differentiation. Among many integrin forms, β_1 and β_3 integrins may be especially important in the angiogenic processes (3, 6, 8). Furthermore, Carnevale *et al.* (5) indicated that β_1 but not β_3 integrins are required for postangiogenic neovessel survival, by the aortic ring assay of angiogenesis. Failure of the hyaloid vasculature to undergo programmed involution results in persistent hyperplastic primary vitreous (PHPV), also known as persistent fetal vasculature (PFV). On the analysis of different gene knockout/transgenic mouse models manifesting PHPV, Hegde and Srivastava proposed that integrins play a significant role in the regression of hyaloid vasculature (7). In this study, we reveal unusual features of integrin fibers in the developing and regressing hyaloid vasculature in the murine eye.

MATERIALS AND METHODS

Animals and tissue sampling. Pregnant ddY mice were supplied by Japan SLC (Shizuoka, Japan). The eyeballs of E17.5/E18.5 embryos and neonates at postnatal days 1, 3, 5, 7, 9, 15, and 20 were used in the present study. Mice were sacrificed by an 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 partially cutting into fourths, 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.

Immunohistochemistry. After immersion in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton-X 100, the whole mount preparations in glass bottles were pre-incubated with a normal donkey serum. For double immunostaining, they were incubated for 3 days with a mixture containing two antibodies from the following list: rabbit anti-mouse LYVE-1 antibody (11-034; AngioBio, Del Mar, CA), rat anti-mouse integrin β_1 (CD29) antibody (MAB2405; R&D Systems), rat anti-mouse CD31 antibody (MEC 13.3; BD Pharmingen, Franklin Lakes, NJ), rabbit anti-laminin antibody (ab11575; abcam, Cambridge, UK), rabbit anti-type IV collagen

antibody (ab19808; abcam), rabbit anti-MCT1 antibody (MCT1-Rb-Af900; Frontier Institute, Ishikari, Japan), guinea pig anti-GLUT1 antibody (GLUT1-GP-Af610; Frontier Institute), and goat anti-mouse galectin-3 antibody (AF1197; R&D Systems, Minneapolis, MN). The antigen-antibody reactions were detected by an incubation of 6 h with Cy3-conjugated donkey anti-rat, anti-rabbit IgG antibody, or donkey anti-goat IgG (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.

Silver-intensified immunogold method for electron microscopy. Some of the fixed tissues 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 μ m in thickness were mounted on poly-L-lysine-coated glass slides. The sections were pretreated with normal donkey serum for 30 min, incubated with the rat anti-integrin β_1 antibody (1 μ g/mL) overnight, and subsequently reacted with goat anti-rat IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; 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).

Scanning electron microscopy (SEM). Under pentobarbital anesthesia, mice at postnatal day 3 were transcardially perfused with Lock's solution saturated with O₂ and subsequently with a mixture containing 2.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Posterior halves of the eyeballs and portions of the cerebral cortex were removed, cut into fine pieces about 1.5 mm in size, and immersed in the same fixative overnight. The fixed tissue pieces were macerated with 6N NaOH for about 18 min at 60°C (18), rinsed, and mildly disrupted by suction and ejection with a glass pipette in 0.02 M phosphate buffer (pH 7.3). After disruption the specimens were immersed in 1% tan-

nic acid buffered with 0.1 M phosphate (pH 7.3), followed by 1% OsO₄ buffered with phosphate (0.1 M, pH 7.2) for 1 h. The osmicated specimens were dehydrated through a graded series of ethanol, transferred to isoamyl acetate, and critical-point-dried with liquid CO₂. The dried specimens were coated with osmium in a plasma osmium coater (Nippon Laser and Electronics Laboratory, Nagoya, Japan), and examined in a Hitachi SU8010 scanning electron microscope (Hitachi) at an acceleration voltage of 5 kV.

RESULTS

When whole mount preparations of developing eyes from E17.5 fetuses were double-stained for β_1 integrin and laminin, only small ring-like membranous structures were immunolabeled for integrin on the outer surface of the VHP vessels (Fig. 1). The immunoreactivity was restricted to the periphery of the ring-like structures, and their sizes (less than 5 μ m) were smaller than erythrocytes. Blood vessels of the VHP had not yet developed an entire sheath of laminin-immunoreactive basement membrane at this stage. At E18.5, integrin fibers apart from blood vessels first appeared in some regions of the vitreous and were associated with round cell bodies with integrin immunoreactivity. However, the integrin-immunoreactive round cells almost disappeared after birth, while the networks of integrin fibers developed more extensively.

Immunostaining of the neonatal ocular tissues at postnatal days 1–3 using CD31 antibody and laminin antibody revealed the entire images of the VHP (11). The VHP possessed a thick basement membrane consisting of extracellular matrix elements including laminin and β_1 integrin. Integrin-immunoreactive linear structures of varying thickness were extended in association with the VHP to form a dense filamentous network (Fig. 2a). Noteworthy, no networks were formed in any areas of the vitreous without the VHP. The dense distribution of integrin fibers was most developed in neonates of days 1–5 and was reminiscent of a tangled web or bush. The integrin bushes were complicated and interlaced, though they were projecting in a certain direction in some places (Fig. 2b). The fibers displayed various thicknesses and frequently dotted images. Interestingly, the β_1 integrin fibers arrested LYVE-1⁺ macrophages and loosely bound them with vessels of the VHP (Fig. 3a). The integrin bushes were not immunoreactive for laminin, in contrast to blood vessels immunoreactive for both laminin and integrin β_1 . The

Fig. 1 The first appearance of integrin β_1 immunoreactivity in the vasa hyaloidea propria (VHP) of mouse fetuses. Double staining for integrin and laminin in a fetus at E17.5 displays small round structures with integrin immunoreactivity on the laminin-immunoreactive VHP vessels. In a fetus of E18.5, integrin-immunoreactive fibers and associated round cells (arrows) appear close to VHP. VHP shows no immunoreactivity for integrin β_1 in these fetuses. Bar 10 μ m (a), 20 μ m (b).

blood vessels were equipped with fine cytoplasmic processes in running courses, which were identified easily under the scanning electron microscope, as mentioned below. These cytoplasmic processes were immunoreactive for laminin but not for integrin β_1 .

After postnatal day 5, the integrin bushes started to regress or be more fragmented, and almost disappeared by days 7–10. In the same conditions, macrophages around the VHP displayed a positive immunoreactivity for integrin in the cytoplasm with a granular appearance, suggesting the phagocytosis of integrin fibers (Fig. 3b).

Electron microscopically, integrin immunoreactivity was found between the plasma membrane and basal lamina in the inner limiting layer of the retina,

Fig. 2 Integrin bushes in the vitreous of neonates. **a.** Double immunostaining of integrin β_1 (green) and laminin (red) intensely label blood vessels of VHP, being yellow in color due to double immunoreactivities in postnatal day 1. Fibers of various thickness, immunoreactive only for integrin, extend straightly and interlace to form bushes. **b.** The integrin fibers project in a given direction in some places of vitreous at postnatal day 5. Round cells (red), possibly macrophages, are immunoreactive for galectin-3. Bar 20 μ m (a), 50 μ m (b)

which was used for the positive control (Fig. 4). This image by transmission electron microscopy indicates that the immunoreactive localization is plausible based on the functional significance of integrin. Immunoreaction products for integrin in the vitreous were precipitated along fine extracellular filaments of an intermediate electron density (Fig. 5). The integrin-positive filaments lacked any striations and coursed solitarily or in small bundles. They extended in various directions to form networks in accord with light microscopic observations.

Scanning electron microscopy observations of the day 3 vitreous demonstrated fine filaments which well corresponded in diameter to the integrin-posi-

Fig. 3 Relationship of integrin bush and macrophages in the vitreous. In Fig. 3a (Day 3), integrin fibers contact with LYVE-1-positive macrophages, suggesting that macrophages are trapped by the integrin fibers. In Fig. 3b (Day 7), the integrin bushes disappear, and an integrin immunoreactivity is recognizable in the cytoplasm of macrophages. Bar 20 μ m

tive fibers, and thicker, cord-like cell processes arising from the VHP endothelium (Fig. 6). The former, extracellular fibers coursed quite independently from the latter endothelial cell processes. The fibers frequently repeated joining with and separating from one another to constitute an extraordinarily fine meshwork in the vitreous. On the other hand, the endothelial processes, about 150 nm thick, usually originated from the basal cell-surfaces in groups, to display a tuft-like appearance as demonstrated by immunostaining for laminin (inset in Fig. 6).

DISCUSSION

The present study revealed the morphological characteristics of filamentous networks immunolabeled by the integrin β_1 antibody; their unique distribution

Fig. 4 Immunogold method for detection of integrin under electron microscope (silver-intensified immunogold method). In the inner limiting layer of the retina used as a positive control, gold particles showing the existence of integrin are localized between the basal lamina and plasma membrane of Müller cells in a day 3 neonate. Bar 1 μm

Fig. 5 Electron microscopy of integrin-immunoreactive fibers at day 3 neonate. Gold particles label a bundle of filaments. Bar 1 μm

in the vitreous suggests that they may function as a scaffold for vessels of the VHP and associated macrophages. The integrin bushes may correspond to filamentous remnants observed under a scanning electron microscope in the late postnatal period of rats (13). We can see a similar figure in an *in vitro* study where the integrin coats of neovessels accompanied fuzzy extracellular filamentous structures with β_1 integrin immunoreactivity (Fig. 1 in Ref. 5).

The integrin bushes in the vitreous contacted with hyaloid vessels and macrophages, but also extended for long distances apart from the hyaloid vessels. The hyaloid vasculature is a temporary vasculature and regresses in the early stages of postnatal development in rodents. It is worth noting that the integrin bush disappeared before the regression of the hyaloid vessels, suggesting an involvement of the integrin bush in maintaining the hyaloid vessels and

Fig. 6 Scanning electron microscopy in a neonate at day 3. Two types of fibers/filaments are associated with VHP vessels in the vitreous. Thick ones are cytoplasmic processes with a consistent diameter of about 150 nm, while thin fibers are very fine and interlace to form networks. The cytoplasmic processes are light-microscopically identified in immunostained sections for laminin (arrows in inset).

macrophages.

It is generally accepted that integrin is a membranous protein anchoring the cytoskeleton to the extracellular matrix such as laminin and fibronectin. The integrin bushes observed in the present study are unexpected phenomena, since they run in the free space of the vitreous apart from blood vessels, which are the only cellular architecture present in the vitreous. Immunoelectron microscopy revealed that immunoreaction products for integrin β_1 were associated with a bundle of fine filaments resembling actin filaments. What is the essential constitution of the filamentous structures? In adult eyes, the vitreous with a gel-like viscosity contains filamentous structures. The major constituents are collagen fibrils which are very long, of uniform diameter (10–20 nm), and unbranched (14). The collagen fibrils are randomly spaced filaments making up a three-dimensional network in the vitreous gel. As a minor component, fibrillin-containing microfibrils are present in the adult vitreous, although they represent the major structural elements of the zonules. The present immunohistochemical study failed to detect the existence of actin (in phalloidin positivity) or fibrillin-1 (in immunoreactivity) in the neona-

tal vitreous of mice. Further study is needed for identification of the filamentous core proteins.

The vitreous humour is essentially composed of the extracellular matrix, rich in hyaluronan. Macrophages, densely distributed along the VHP, expressed the LYVE-1 hyaluronan receptor (11, 20). This is helpful for the residing of macrophages in the hyaluronan-rich vitreous. The present immunostaining for integrin β_1 demonstrated the positive labeling of macrophages with dotted appearances in the cytoplasm, parallel to the fragmentation and disappearance of integrin bushes. This image suggests the simple uptaking and degradation of integrin by associated macrophages with a higher phagocytotic activity than shown in our previous study (11). However, there is a possibility that endothelial cells themselves uptake and degrade integrin with the aid of macrophages. Macrophage-derived exosomes, which are released extracellularly, induced the internalization and degradation of integrin β_1 by cultured endothelial cells (HUVECs) (15). The exosome treatment in this study did not affect other angiogenic regulators, such as integrin β_3 and VE-cadherin, on expressing on HUVECs.

Numerous studies have reported the involvement

of integrins in neovascularization. An *in vitro* study for angiogenesis demonstrated the existence of an integrin coat immunoreactive for integrins β_1 and β_3 around neovessels, indicating their functional roles in angiogenesis (5). Integrins of the β_1 family drive the synthesis, secretion, and insolubilization of laminin-1, resulting in the initiation of basement membrane formation in embryoid bodies (1). Although we did not examine the expression of other integrin families, many studies have pointed to a more central role for β_1 integrins in vascular development (6). Stimulation with vascular endothelial growth factor (VEGF) markedly increased the expression of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ at the cell surface, and antibody α_1 or β_1 but not α_2 antibody inhibited the attachment of VEGF-stimulated cells to collagen IV and laminin-1 (17). Furthermore, treatment with integrin-specific neutralizing antibodies induced a marked inhibition of angiogenesis only by the anti- β_1 integrin antibody, indicating a rapid regression associated with the fragmentation of neovessels and apoptotic changes in both endothelial cells and pericytes (4). Embryonic stem cells with β_1 integrin-deficiency can differentiate into endothelial cells but fail to produce a well-organized vascular network (2).

The integrin-immunoreactive elements appearing before birth remain to be identified. It is not likely that the immunoreactive small ring-like structures originate from any cellular elements. The round cell bodies associated with the integrin-immunoreactive fibers may play a role in the initial formation of the integrin bush, though the cell types and mechanism forming the integrin bush are unknown. A complete understanding of these unsolved problems will require observation by immunoelectron microscopy assisted by the use of extensive marker substances. In conclusion, the unique integrin bushes demonstrated in the present study may prove important as scaffolds of hyaloid vessels and macrophages, and function in maintaining the floating vessels in the vitreous.

REFERENCES

1. Aumailley M, Pesch M, Tunggal L, Gaill F and Fassler R (2000) Altered synthesis of laminin 1 and absence of basement membrane component deposition in β_1 integrin-deficient embryoid bodies. *J Cell Sci* **113**, 259–268.
2. Bloch W, Forsberg E, Lentini S, Brakebusch C, Martin K, Krell HW, Weidle UH, Addicks K and Fassler R (1997) β_1 integrin is essential for teratoma growth and angiogenesis. *J Cell Biol* **139**, 265–278.
3. Brooks PC, Clark RA and Cheresch DA (1994) Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* **264**, 569–571.
4. Brown AS, Leamen L, Cucevic V and Foster FS (2005) Quantitation of hemodynamic function during developmental vascular regression in the mouse Eye. *Invest Ophthalmol Vis Sci* **46**, 2231–2237.
5. Carnevale E, Fogel E, Aplin AC, Gelati M, Howson KM, Zhu W-H and Nicosia RF (2007) Regulation of postangiogenic neovessel survival by β_1 and β_3 integrins in collagen and fibrin matrices. *J Vas Res* **44**, 40–50.
6. Francis SE, Goh KL, Hoidalva-Dilke K, Bader BL, Stark M, Davidson D and Hynes RO (2002) Central roles of $\alpha_v\beta_1$ integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol* **22**, 927–933.
7. Hegde S and Srivastava O (2017) Different gene knockout/transgenic mouse models manifesting persistent fetal vasculature: are integrins to blame for this pathological condition? *Life Sci* **171**, 30–38.
8. Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11–25.
9. Ito M and Yoshioka M (1999) Regression of the hyaloid vessels and pupillary membrane of the mouse. *Anat Embryol* **200**, 403–411.
10. Jack RL (1972) Ultrastructure of the hyaloid vascular system. *Arch Ophthalmol* **87**, 555–567.
11. Kishimoto A, Kimura S, Nio-Kobayashi J, Takahashi-Iwanaga H, Park A-M and Iwanaga T (2018) Histochemical characteristics of regressing vessels in the hyaloid vascular system of neonatal mice: Novel implication for vascular atrophy. *Exp Eye Res* **172**, 1–9.
12. Lang RA and Bishop JM (1993) Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell* **74**, 453–462.
13. Latker CH and Kuwabara T (1981) Regression of the tunica vasculosa lentis in the postnatal rat. *Invest Ophthalmol Vis Sci* **21**, 689–699.
14. Le Goff MM and Bishop PN (2008) Adult vitreous structure and postnatal changes. *Eye* **22**, 1214–1222.
15. Lee HD, Kim YH and Kim D-S (2014) Exosomes derived from human macrophages suppress endothelial cell migration by controlling integrin trafficking. *Eur J Immunol* **44**, 1156–1169.
16. Mitchell CA, Risau W and Drexler HC (1998) Regression of vessels in the tunica vasculosa lentis is initiated by coordinated endothelial apoptosis: a role for vascular endothelial growth factor as a survival factor for endothelium. *Dev Dyn* **213**, 322–333.
17. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP and Detmar M (1997) Angiogenesis promoted by vascular endothelial growth factor: Regulation through $\alpha_v\beta_1$ and $\alpha_3\beta_1$ integrins. *Proc Natl Acad Sci USA* **94**, 13612–13617.
18. Takahashi-Iwanaga H and Fujita T (1986) Application of an NaOH maceration method to a scanning electron microscopic observation of Ito cells in the rat liver. *Arch Histol Jpn* **49**, 349–357.
19. Taniguchi H, Kitaoka T, Gong H and Amemiya T (1999) Apoptosis of the hyaloid artery in the rat eye. *Ann Anat* **181**, 555–560.
20. Zhang H, Tse J, Hu X, Witte M, Bernas M, Kang J, Tilabun F, Hong Y-K, Qiu M and Chen L (2010) Novel discovery of LYVE-1 expression in the hyaloid vascular system. *Invest Ophthalmol Vis Sci* **51**, 6157–6161.