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REGULAR ARTICLE

Integrin αvβ5 in endothelial cells of rat splenic sinus: an immunohistochemical and ultrastructural study

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Abstract Localization of integrins β1-8, α1, α2, α3, α5, α6 and αv in sinus endothelial cells of the rat spleen was examined by immunofluorescence microscopy. Labeling for anti-integrin β5 and integrin αv was detected and colocalized in the entire circumference of endothelial cells. Labeling for integrin β5, vinculin and actin filaments demonstrated that they lay close to each other in the basal part of the endothelial cells. Although the other integrin βs, including integrin β1 and integrins α1, α2, α3, α5 and α6 in combination with integrin β1, were localized in leukocytes, slightly large cells, megakaryocytes and/or platelets in the sinus lumen and splenic cords, they were not detected in endothelial cells. Labeling for vitronectin, a component of the extracellular-matrix-binding integrin αvβ5, was strongly stained in the periphery of the wall of sinuses, as was collagen IV and, in addition, was localized in the cytoplasm of endothelial cells. Ultrastructural localization of integrin β5, vitronectin and clathrin was examined by immunogold electron microscopy to elucidate the involvement of integrin αvβ5 in the endocytosis of vitronectin in sinus endothelial cells. Electron microscopy with detergent extraction revealed abundant coated pits and coated vesicles in endothelial cells. Immunogold labeling for vitronectin was present in pits, vesicles and the stacked endoplasmic reticulum. Double-labeling for integrin β5 or integrin αv and clathrin revealed that they were colocalized in some vesicles in close proximity to the apical and lateral plasma membrane of the endothelial cells. The possible functional roles of integrin αvβ5 in endothelial cells of the splenic sinus are discussed.

Keywords Integrin · Vitronectin · Endocytosis · Sinus endothelial cells · Spleen · Rat (Wistar)

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Introduction

The endothelial cells that line sinus capillaries in the red pulp in the mammalian spleen are structurally different from other vascular endothelial cells. They are spindle-shaped and run longitudinally in sinus capillaries. In particular, the structural difference in their basement membrane is marked, as it does not fully sheathe the basal plasma membranes of the sinus endothelial cells but is transformed into ring-shaped bands, called ring fibers, hooping the spindle-shaped endothelial cells. Splenic sinus endothelial cells are only attached to ring fibers by focal adhesion and their basal plasma membranes, except for focal adhesions, are exposed to the splenic cord, whereas the focal adhesions of sinus endothelial cells are associated with a highly ordered network of distinctive stress fibers (Drenckhahn and Wagner 1986; Uehara and Miyoshi 1999a). These structures are believed to be formed for the passage of blood cells, i.e., the intercellular spaces between sinus endothelial cells are sometimes open, playing a crucial role in controlling blood-cell passage through the splenic cord. Although the ultrastructure of these sinus endothelial cells has been investigated, the way that the passage of blood cells is controlled in the endothelium remains unclear. Recently, the cell-cell junctions of splenic sinus endothelial cells have been examined by laser-scanning confocal microscopy and electron microcopy, which revealed that poorly developed tight junctions (TJs) and predominant adherens junction (AJs) are located throughout the lateral plasma membrane of sinus endothelial cells. In addition, the colocalization of vascular

endothelial (VE)-cadherin, β-catenin and p120-catenin in sinus endothelial cells was assessed by immunofluorescent microscopy and immunogold electron microscopy. AJs mediated by the VE-cadherin complex have been suggested to regulate the passage of blood cells through the sinus endothelium (Uehara and Miyoshi 1997, 1999b; Uehara 2006). Furthermore, ZO-1 not only acts as a molecule scaffold bringing together many proteins, such as claudins and actin, in the cytoplasmic face of TJs but also works as a cross-linker between the cadherin/catenin complex and actin-based cytoskeleton in that of AJs (Itoh et al. 1997). ZO-1 has been revealed to be colocalized with VE-cadherin and α-catenin and with claudin-5 along the junctional membranes of adjacent sinus endothelial cells, suggesting that ZO-1 plays an important role in regulating the cell-cell junctions of sinus endothelial cells for blood-cell passage (Uehara and Uehara 2008). In contrast to the cell-cell junctions of splenic sinus endothelial cells, little information exists about cell-extracellular matrix (ECM) junctions, i.e., focal adhesions, of splenic sinus endothelial cells, in spite of them being a crucial site in the regulation of microvascular barrier function (Wu 2005).

Focal adhesions are composed of integrins providing an important structural basis for anchoring the endothelial lining to its surrounding ECMs, such as fibronectin, laminin, collagen and vitronectin, in the vascular wall. Integrins are receptors for ECM in the plasma membrane of cells and are composed of a heterodimetric molecule, with α and β subunits, mediating cell anchorage and migration. Mammalian genomes contain 18 α subunit and 8 β subunit genes and to date, 24 different α−β combinations have been identified at the protein level. Integrins share characteristics with a dependence receptor. Although integrins do not conform in all characteristics to the established definitions of dependence receptors, alternations in the expressions of integrins and their ligands during physiological and pathological events regulate cell fate in a ligand-dependent manner (Stupack 2005). Vascular endothelial cells have been reported to express integrins α1β1, α2β1, α3β1, α5β1, α6β1, α6β4, αvβ3 and αvβ5 (Stupack and Cheresh 2002; Wu 2005).

We examined the immunohistochemical localization of integrins β1-8, α1, α2, α3, α5, α6 and αv by confocal lase-scanning microscopy and labeling for anti-integrin β5 and integrin αv was detected in splenic sinus endothelial cells. Integrin αvβ5 binds vitronectin, a major component of ECM and integrin β5 was demonstrated to be colocalized with vitronectin during the endocytic process in cultured fibroblasts by indirect immunofluorescence microscopy; this colocalization is inhibited by the inhibition of clathrin (Memmo and McKeown-Longo 1998). In addition, sinus endothelial cells have abundant clathrin-coated vesicles in the cytoplasm and clathrin-coated pits originating in the apical, lateral and basal plasma membranes (Uehara and Uehara 2010). Therefore, the ultrastructural localization of integrins β5 and αv, vitronectin and clathrin was examined by immunogold electron microscopy in order to clarify one of the functions of integrin αvβ5 in sinus endothelial cells in the spleen. Western blotting was carried out to examine the specificity of antibodies and to investigate the molecular weight of the examined integrins α and β in the rat spleen.

Materials and methods

Immunohistochemistry for confocal laser-scanning microscopy

Three 8-week-old male Wistar rats were anesthetized prior to thoracic aorta cannulation for perfusion with Ringer's solution, followed by 3 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The spleen was removed and the red pulp was cut into small blocks and then immersed in the same fixative for 1 h. The specimens were rinsed in buffer, infused with 20 % polyvinyl pyrolidone and 2.3 M sucrose in buffer and rapidly frozen in liquid nitrogen. Some specimens were used for immunohistochemistry for confocal laser-scanning microscopy and the others were used for immunogold labeling for electron microscopy. The animals were treated according to the animal welfare regulations of Japan, with permission from the ethics commission of Fukuoka University.

Semi-thin cryosections (about 0.5 μm in thickness) of the samples were mounted on glass slides and treated with 3 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 10 min. The slides were subsequently incubated with the primary antibody (Table 1) in PBS containing 1 % BSA at 4 °C overnight. After being rinsed with PBS, the sections were incubated with secondary antibody conjugated with Alexa-488 (Molecular Probes, Eugene, USA) containing Alexa-Fluor-546-phalloidin in PBS to demonstrate distinctive stress fibers in the basal part of sinus endothelial cells. In addition, sinus endothelial cells were identified by immunostaining with anti-CD141 antibody (Steiniger et al. 2007). To examine the more detailed localization of integrins and stress fibers in sinus endothelial cells, some specimens were observed by using triple fluorescence immunostaining (Uehara and Uehara 2011). In addition, in order to examine the relationships of integrins β5 and αv with stress fibers and of integrin β5 and vinculin with stress fibers, some specimens were observed by using triple fluorescence immunostaining. Semi-thin cryosections of the aorta with the vasa vasorum, small intestine and cardiac muscle of the rat were prepared by the same method as positive controls; leukocytes, epithelial cells, or endothelial cells in these tissues showed positive reactions. Negative controls were also performed. All samples were examined by using a laser-scanning confocal microscope (LSM710, Zeiss).

Table 1 Primary antibodies (*IHC* immunohistochemistry, *ImG* immunogold electron microscopy, *WB* Western blotting)

Antigen	Host	Clonal type	Source	Catalog number	Application dilution	
					IHC, ImG	WB
Integrin β1	Goat	Poly	Santa Cruz Biotechnology	sc-6622	1:100	1:2000
Integrin β2	Mouse	Mono	Santa Cruz Biotechnology	sc-80850	1:200	1:1000
Integrin β3	Mouse	Mono	Santa Cruz Biotechnology	sc-7311	1:100	1:2000
Integrin β4	Rabbit	Poly	Santa Cruz Biotechnology	sc-9090	1:50	1:1000
Integrin β5	Rabbit	Poly	AnaSpec Inc	ANA-53588	1:50	1:1000
Integrin β6	Mouse	Mono	Milipore	MAB20762	1:100	–
Integrin β6	Goat	Poly	Santa Cruz Biotechnology	sc-6632	–	1:3000
Integrin β7	Rabbit	Poly	Santa Cruz Biotechnology	sc-15330	1:100	1:4000
Integrin β8	Rabbit	Poly	Santa Cruz Biotechnology	sc-25714	1:100	1:4000
Integrin α1	Rabbit	Poly	Santa Cruz Biotechnology	sc-10728	1:200	1:3000
Integrin α2	Rabbit	Poly	Santa Cruz Biotechnology	sc-9089	1:200	1:8000
Integrin α3	Goat	Poly	Santa Cruz Biotechnology	sc-6587	1:100	1:4000
Integrin α5	Mouse	Poly	Santa Cruz Biotechnology	sc-10729	1:100	1:3000
Integrin α6	Goat	Poly	Santa Cruz Biotechnology	sc-6597	1:100	1:2000
Integrin αv	Rabbit	Poly	Santa Cruz Biotechnology	sc-6617-R	1:100	1:4000
CD141	Rabbit	Poly	Santa Cruz Biotechnology	sc-13164	1:100	–
Clathrin	Mouse	Mono	Sigma	c-1860	1:100	–
Collagen IV	Rabbit	Poly	Cosmo Bio, LSL	LB-1403	1:1000	–
Vinculin	Mouse	Mono	Sigma	V9264	1:500	–
Vitronectin	Rabbit	Poly	Cosmo Bio, LSL	LB-2096	1:1000	–

Electron microscopy

Saponin extraction

The spleens of adult male Wistar rats were cut into pieces and immersed in 0.5 % saponin for 20 min in HEPES buffer (pH 7.3). The specimens were fixed for 1 h in 2.5 % glutaraldehyde containing 0.2 % tannic acid, post-fixed for 1 h in 1 % osmium tetroxide in the same buffer, dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunogold labeling

Ultrathin cryosections of the frozen specimens (about 80 nm in thickness) were collected on grids and pretreated with 3 % BSA. Samples were incubated for 1 h at room temperature with anti-vitronectin antibody in PBS containing 1 % BSA and then incubated with 15-nm colloidal gold-conjugated secondary antibody. Some specimens were double-stained with anti-integrin β5 and anti-clathrin, anti-integrin αv and anti-clathrin antibodies and incubated with 15-nm and 5-nm colloidal gold-conjugated secondary antibodies, respectively. The specimens were fixed in 2 % glutaraldehyde in 0.1 M phosphate buffer and subsequently incubated in 0.5 % uranyl acetate and 1.8 % methylcellulose in distilled water. Excess fluid was removed and the grids were air-dried. Appropriate controls included the omission of primary or secondary antibody. All preparations were observed under a Hitachi 7000 electron microscope.

Western blots

Spleens were taken from three 8-week-old male Wistar rats and homogenized in an extraction reagent including protease inhibitors (Sigma-Aldrich, Mo., USA). The rat spleen contains a large amount of lymphoid tissues, hematopoietic tissues, blood vessels, nerves and a network of tortuous sinus endothelium. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Calif., USA). Each sample (15 μg/lane) was loaded onto 7.5 % SDS-polyacrylamide gels by using a Bio-Rad Mini-Protean 3 cell (Bio-Rad) and then transferred to polyvinyl-difluoride membranes (Millipore, Mass., USA). The membranes were blocked with 5 % nonfat milk powder and 0.05 % Tween 20 in TRIS-buffered saline (TBS) and incubated overnight at 4 °C with primary antibody (Table 1) in blocking solution. The membranes were washed with TBS containing 0.05 % Tween 20, incubated with secondary antibody conjugated with horseradish peroxidase at room temperature for 60 min and washed with TBS-Tween 20. Western blotting luminal reagent for enhanced chemiluminescence and Hyperfilm (Amersham, UK) were used to visualize peroxidase

activity. Control for nonspecific binding was determined by the omission of the primary antibody.

Results

Western blots

Western blotting analysis was performed using crude extracts from the whole spleen. Strong signals for integrins β and α were detected. Integrin β1 was detected at about 110 kDa, integrins β2, β3, β4 and β7 at about 40 kDa and integrins β5 and β6 at about 90 kDa. Two bands for integrin β8 were noted at about 90 and 40 kDa. Integrin αv was found at about 60 kDa, integrins α1 and α3 at about 70 kDa, integrin α2 at about 70 and 50 kDa and integrins α5 and α6 at about 110 and 100 kDa, respectively (Fig. 1).

Immunohistochemistry for confocal laser-scanning microscopy

Triple immunofluorescent staining for a combination of integrin β, CD141 and actin filaments showed that integrin β5 was localized in the entire circumference of sinus endothelial cells, leukocytes and slightly large cells in the sinus lumens and splenic cords and fibroblasts in the splenic trabeculae in the red pulp. However, integrins β1, β2, β3, β4, β6, β7 and β8 were not detected in sinus endothelial cells, although integrin β1 was localized in leukocytes and platelets, integrins β2 and β6 were located in leukocytes and integrin β3 was present in platelets, megakaryocytes, and slightly large cells in sinus lumens and splenic cords (Fig. 2). Labeling for integrins β4, β7 and β8 was not detected in any cells in the red pulp.

Labeling for a combination of integrin α, CD141 and actin filaments demonstrated that integrins α1, α2, α3, α5 and α6 were not localized in sinus endothelial cells. However, integrins α1 and α6 were detected in leukocytes and integrin α5 was noted in leukocytes and slightly large cells in the sinus lumen and splenic cords. Integrin α2 was detected in leukocytes and megakaryocytes, whereas integrin α3 was present in leukocytes, megakaryocytes and platelets (Fig. 3). Labeling for a combination of integrin αv, CD141 and actin filaments showed that integrin αv was localized in the entire circumference of sinus endothelial cells and, in addition, in leukocytes, slightly large cells in the sinus lumen and splenic cords and fibroblasts in splenic trabeculae. Labeling for a combination of integrin β5, integrin αv and actin filaments revealed that integrin β5 and αv were colocalized in the entire circumference of sinus endothelial cells. Vinculin is a cytoplasmic actin-binding protein in focal adhesions and plays an important role in governing cell-matrix adhesion. Labeling for a combination of integrin β5, vinculin and actin filaments demonstrated that they lie close to each other in the basal part of sine endothelial cells (Fig. 4).

Labeling for collagen IV and actin filaments showed that collagen IV was distinctly localized in the periphery of the wall of sinuses and surrounded the wall of the sinus with an appearance of broken lines. Collagen IV lie in the vicinity of stress fibers in the basal part of sinus endothelial cells but these fibers were not colocalized in the wall of sinuses. Labeling for vitronectin and actin filaments revealed that vitronectin was localized in the cytoplasm of sinus endothelial cells with distinctive stress fibers. Vitronectin was strongly stained in the periphery of the wall of sinuses (Fig. 5).

Electron microscopy

Saponin extraction

After saponin extraction, sinus endothelial cells were electron-lucent and clathrin-coated vesicles including the cytoskeletal elements and other organelles were easily visible. In particular, a coat of clathrin was distinctly visualized as spines

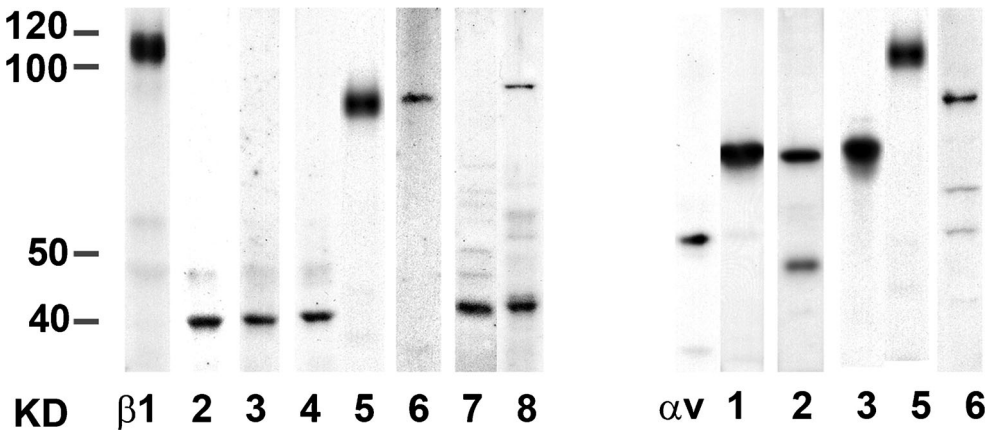

Fig. 1 Western blotting analysis of integrin β and of integrin α in crude extract from the rat spleen. Integrins β1-7 were detected as a single band and integrin β8 as two bands. Integrins αv, α1, α3, α5 and α6 appeared as a single band and integrin α2 as two bands. Molecular weight markers are given in kilo Daltons (*KD*) *left*

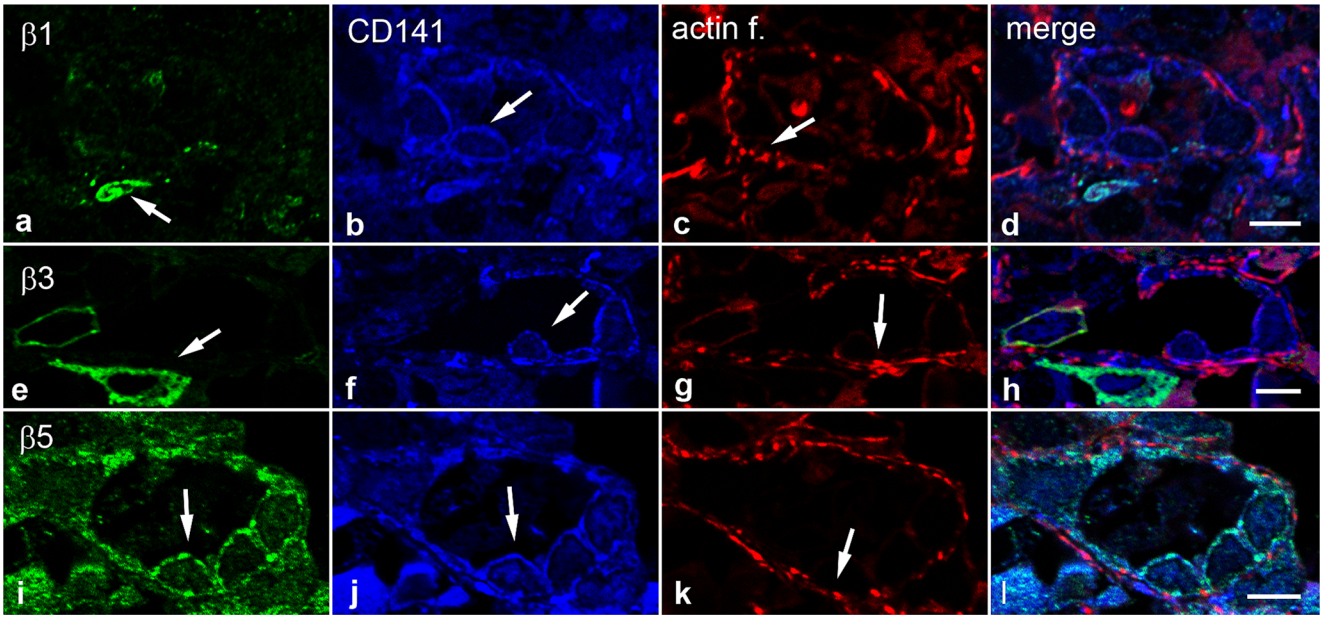

Fig. 2 Immunofluorescent localization of integrin β (*green*) and CD141 (*blue*) in semi-thin sections of the sinus endothelium in the red pulp by triple immunofluorescent staining with phalloidin-conjugated fluorescence (*red*) for actin filaments (*actin f.*). **a–d** Immunostaining for integrin β1 (*green*), CD141 (*blue*) and actin filaments (*red*). **a** Integrin β1 (*arrow*) is detected in a cell in the sinus cord (*arrow*). **b**, **f**, **j** CD141 is detected in the entire circumference of the sinus endothelial cells (*arrow*) and leukocytes in the sinus lumen and the splenic cord. **c**, **g**, **k** Actin filaments demonstrating an apical cortical layer of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells (*arrow*). **d** The merged image indicates that integrin β1 is not localized in the sinus endothelial cells. **e–h** Immunostaining for integrin β3 (*green*), CD141 and actin filaments. **e** Integrin β3 is detected in the circumference of a cell in the sinus lumen (*arrowhead*) and in the cytoplasm of a cell in the splenic cord (*arrow*). **h** The merged image indicates that integrin β3 is not localized in the sinus endothelial cells. **i** Integrin β5 is localized in the entire circumference of the sinus endothelial cells (*arrow*). **l** The merged image indicates that integrins β5 and CD141 are colocalized in the entire circumference of the sinus endothelial cells. *Bars* 5 μm

associated with vesicles or polygonal networks. Clathrin-coated vesicles were abundant in sinus endothelial cells and clathrin-coated pits, the cavities of which contained electron-dense material, originating in the apical, lateral and basal plasma membranes. Clathrin-coated vesicles were associated with the endoplasmic reticulum (Fig. 6).

Immunogold labeling

Single labeling for vitronectin was present collectively in vesicles in the apical and lateral part of sinus endothelial cells and in the stacked endoplasmic reticulum and vesicles in the basal part of sinus endothelial cells. It was also present in the ring fibers beneath the sinus endothelial cells (Fig. 7). Following double-labeling for integrin β5 or integrin αv and clathrin, labeling for integrin β5 and integrin αv was present in the plasma membrane and in vesicles in close proximity to the apical and lateral plasma membrane of sinus endothelial cells. Labeling for clathrin was present in the vesicles closely adjacent to the apical, lateral and basal plasma membrane and gold particles sporadically assembled in the cytoplasm of sinus endothelial cells. Two kinds of labeling for integrin β5 or integrin αv and clathrin were colocalized in some pits and vesicles in close proximity to the apical and lateral plasma membrane of sinus endothelial cells (Fig. 8).

Discussion

In this study, we demonstrated, using immunofluorescence microscopy, that integrins β1-4 and β6–8 and the previously reported integrins α that bind integrin β1 in endothelial cells are not detectable in splenic sinus endothelial cells but that integrin αvβ5 is localized in the entire circumference of sinus endothelial cells. Integrin αvβ5 lies close to stress fibers, vinculin, collagen IV and vitronectin in the basal part of sinus endothelial cells. Furthermore, integrin αvβ5 was demonstrated to be involved in the endocytosis of vitronectin (a regulator of cell adhesion and cellular motility through binding integrin αvβ5) from the blood plasma into sinus endothelial cells by immunogold electron microscopy. Integrin αvβ5 is reported to regulate vascular permeability and barrier function (Su et al. 2007). Moreover, integrins are presumed to play a role in mechanotransduction of endothelial cells in response to shear stress, to lead the reorganization of the cytoskeleton (Davies et al. 1994; Ingber 2002; Chien et al. 2005). In view of the key role of sinus endothelial cells in the filtering out of damaged or senescent cells from the blood, a role of integrin αvβ5 in this process is suggested and now needs to be evaluated.

The specificity of the antibodies for integrins β and integrins α used in this study was confirmed by Western

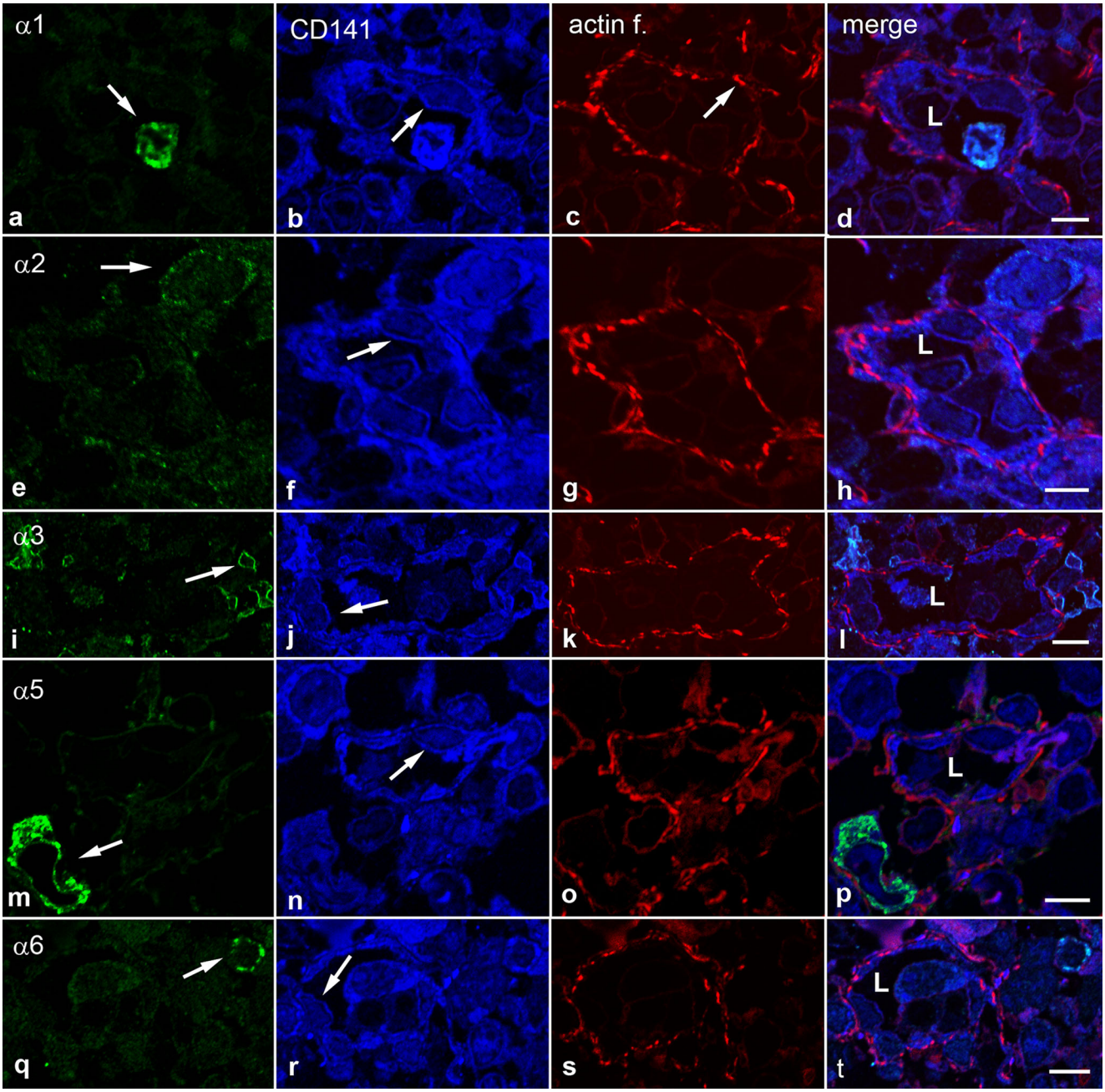

Fig. 3 Immunofluorescent localization of integrin α and CD141 in semithin sections of the sinus endothelium in red pulp by triple immunofluorescent staining with phalloidin-conjugated fluorescence. **a–d** Immunostaining for integrin α1 (*green*), CD141 (*blue*) and actin filaments (*red*). **a** Integrin α1 is only detected in a leukocyte in the sinus lumen (*arrow*). **b**, **f**, **j**, **n**, **r** CD141 is detectable in the entire circumference of the sinus endothelial cells (*arrow*) and in leukocytes in the sinus lumen and the splenic cord. **c**, **g**, **k**, **o**, **s** Actin filaments demonstrate an apical cortical layer of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells (*arrow*). **d** The merged image indicates that integrin α1 is not localized in the sinus endothelial cells. **e–h** Immunostaining for integrin α2 (*green*), CD141 (*blue*) and actin filaments (*red*). **e** Integrin α2 is detected in a slightly large cell in the splenic cord (*arrow*). **h** The merged image indicates that integrin α2 is not localized in the sinus endothelial cells. **i–l** Immunostaining for integrin α3 (*green*), CD141 (*blue*) and actin filaments (*red*). **i** Integrin α3 is detected in platelets in the splenic cord and sinus lumen (*arrow*). **l** The merged image indicates that integrin α3 is not localized in the sinus endothelial cells. **m–p** Immunostaining for integrin α5 (*green*), CD141 (*blue*) and actin filaments (*red*). **m** Integrin α5 is only detected in a slightly large cell in the splenic cord (*arrow*). **p** The merged image indicates that integrin α5 is not localized in the sinus endothelial cells. **q–t** Immunostaining for integrin α6 (*green*), CD141 (*blue*) and actin filaments (*red*). **q** Integrin α6 is only detected in a leukocyte in the sinus lumen (*arrow*). **t** The merged image indicates that integrin α6 is not localized in the sinus endothelial cells (*L* sinus lumen). *Bars* 5 μm

blotting performed with crude extracts of whole spleen. The integrin β subunit is composed of about 750 amino acids and has a molecular weight of 90–110 kDa, whereas the integrin α subunit is composed of about 1000 to 1200 amino acids and

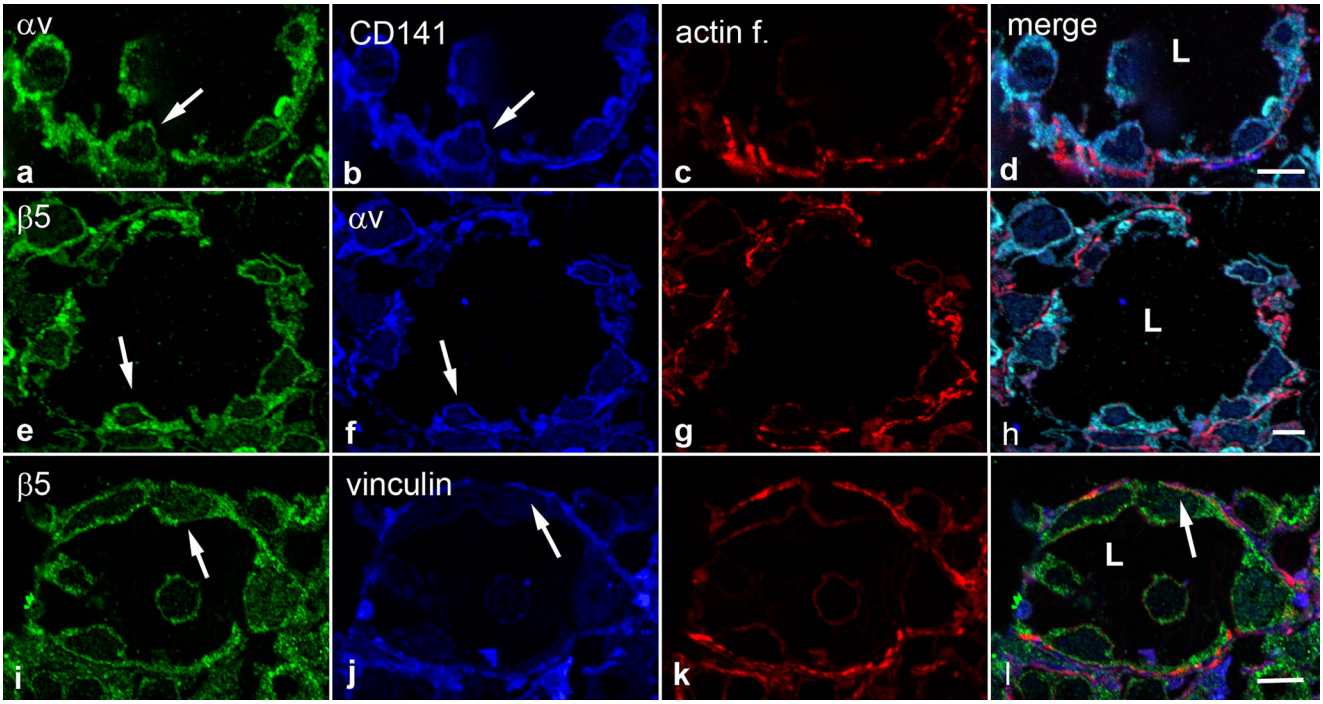

Fig. 4 Immunofluorescent localization of integrin αv, integrin β5, CD141 and vinculin in semi-thin sections of the sinus endothelium in red pulp by triple immunofluorescent staining with phalloidin-conjugated fluorescence. **a–d** Immunostaining for integrin αv (*green*), CD141 (*blue*) and actin filaments (*red*). **a** Integrin αv is localized in the entire circumference of the sinus endothelial cells (*arrow*). **b** CD141 is detected in the entire circumference of the sinus endothelial cells (*arrow*). **c** Actin filaments demonstrate an apical cortical layer of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells. **d** The merged image indicates that integrin αv and CD141 are colocalized in the entire circumference of the sinus endothelial cells. **e–h** Immunostaining for integrin β5 (*green*), integrin αv (*blue*) and actin filaments (*red*). **e** Integrin β5 is localized in the entire circumference of the sinus endothelial cells (*arrow*). **f** Integrin αv is also found in the entire circumference of the sinus endothelial cells (*arrow*). **g** Actin filaments demonstrate an apical cortical layer of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells. **h** The merged image indicates that integrin β5 and integrin αv are colocalized in the entire circumference of the sinus endothelial cells. **i–l** Immunostaining for integrin β5 (*green*), vinculin (*blue*) and actin filaments (*red*). **i** Integrin β5 is localized in the entire circumference of the sinus endothelial cells (*arrow*). **j** Vinculin is detected in the basal part of the sinus endothelial cells (*arrow*). **k** Actin filaments demonstrate an apical cortical layer of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells. **l** The merged image indicates that integrin β5, vinculin and actin filaments lie in close proximity to each other (*arrow*in **l**) in the basal part of the sinus endothelial cells (*L* sinus lumen). *Bars* 5 μm

has a molecular weight of 120–180 kDa. In this study, the molecular weights of the detected bands of the examined integrins were different from the expected molecular weights. The examined integrins might have been fragmented or undergone some processing after extraction leading to a change in molecular weight or their charges might have been changed after translation and processing in the spleen.

Vascular endothelial cells have been reported to express large amounts of integrin β1 (Stupack and Cheresh 2002; Wu 2005). Integrin β1-knockout homozygous mice die during the early stages of development; analysis of integrin-β1-deficient chimeric mice has revealed the role of integrin β1 during mouse development. In chimeric mice, no-integrin β1-deficient endothelial cells are found in the spleen, demonstrating that vasculogenesis in the spleen requires integrin β1 (Fassler and Meyer 1995). However, the expression of the integrins in endothelial cells varies during tissue growth, development and repair and aberrantly in the diseased state, including tumor growth (Stupack and Cheresh 2002). No integrin β1 has been detected in the sinus endothelial cells of the spleen of adult rats (Fig. 2). In addition, integrins α1, α2, α3, α5 and α6 combined with integrin β1 were not detected in sinus endothelial cells (Fig. 3). These results taken together suggest that the expression of integrins α1 β1, α2 β1, α3 β1, α5, β1 and α6β1 are down-regulated in sinus endothelial cells of the adult rat and are insufficiently expressed to be detected in sinus endothelial cells by immunofluorescent microscopy. Furthermore, some of the integrins might not be localized in sinus endothelial cells.

Integrin α6β4 is thought to interact with keratin filaments to form type I hemidesmosome (Stepp et al. 1990). Although endothelial cells do not express the associated proteins with type I hemidesmosome and do not form ultrastructurally the type I hemidesmosome, they express integrin α6β4 and plectin to form type II hemidesmosomes, which can be distinguished from type I hemidesmosomes (Uematsu et al. 1994; Homan et al. 2002). We have reported that plectin is localized predominantly in the basal part of sinus endothelial

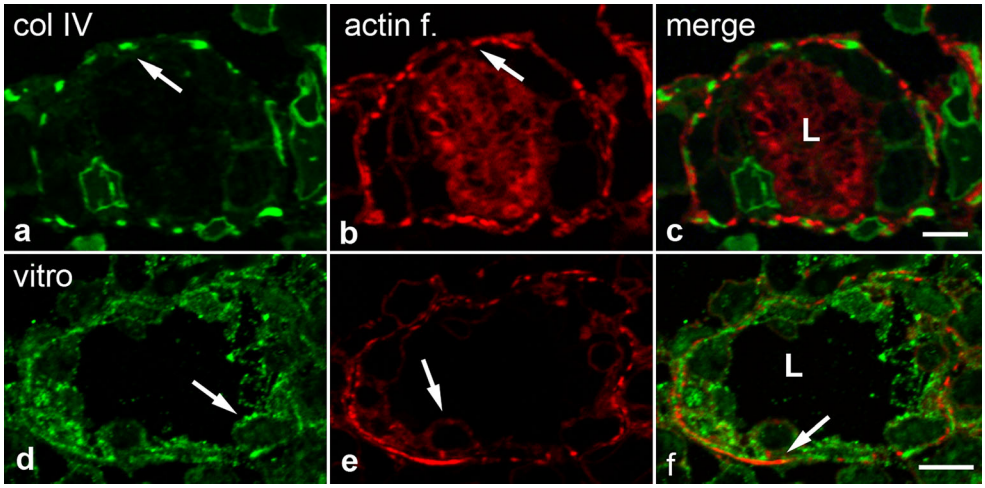

Fig. 5 Immunofluorescent localization of collagen IV (*col IV, green*) and vitronectin (*vitro, green*) and in semi-thin sections of the sinus endothelium in red pulp by double immunofluorescent staining with phalloidin-conjugated fluorescence (*red*). **a–c** Localization of collagen IV. **a** Collagen IV (*arrow*) is distinctly localized in the periphery of the wall of the sinus in which it appears as *broken lines*. **b** Stress fibers in the basal part of the endothelial cells are observed as conspicuous thick broken-lines (*arrow*). A large number of erythrocytes stained with phalloidin fill the sinus lumen. **c** The merged image indicates that collagen IV is precisely localized in the vicinity of stress fibers but collagen IV and stress fibers are separately localized in the periphery of the wall of sinuses. **d–f** Localization of vitronectin. **d** Vitronectin is localized in the cytoplasm of the endothelial cells (*arrow*). Strong labeling for vitronectin occurs in the periphery of the wall of the sinus. **e** Actin filaments demonstrate an apical cortical layer (*arrow*) of the endothelial cells and stress fibers are localized in the basal part of the sinus endothelial cells. **f** The merged image indicates that vitronectin sporadically overlies stress fibers (*arrow*) in the sinus endothelial cells (*L* sinus lumen). *Bars* 5 μm

cells but rarely in the vicinity of focal adhesions and, in addition, the type II hemidesmosome is not observed ultrastructurally (Uehara and Uehara 2010). The lack of detection of integrins α6 and β4 in the sinus endothelial cells in this study is considered to agree with our previous report.

Integrin β5 combines with integrin αv. Since integrins β5 and αv have been colocalized in sinus endothelial cells in this study, integrin αvβ5 is considered to be localized in the entire circumference of sinus endothelial cells. Endothelial cells express integrins αvβ3 and αvβ5, which mediate cell adhesion to vitronectin. Integrin αvβ3 is minimally expressed on resting or normal blood vessels but is significantly upregulated on endothelial cells in response to certain growth factors, such as basic fibroblast growth factor (Friedlander et al. 1995). In contrast to integrin αvβ3, αvβ5 is widely expressed and detected in most normal cells, such as capillary endothelial cells in the cortex and medulla of human thymus and in glomerular endothelial cells of the human kidney (Pasqualini et al. 1993). Furthermore, integrins αvβ3 and αvβ5 contribute to cell attachment to vitronectin but are distributed differently on the cell surface (Wayner et al. 1991). Integrin αvβ5 was probably detected rather than integrin αvβ3 in sinus endothelial cells in this study. Furthermore, integrin αvβ5 was not only found in the basal part of

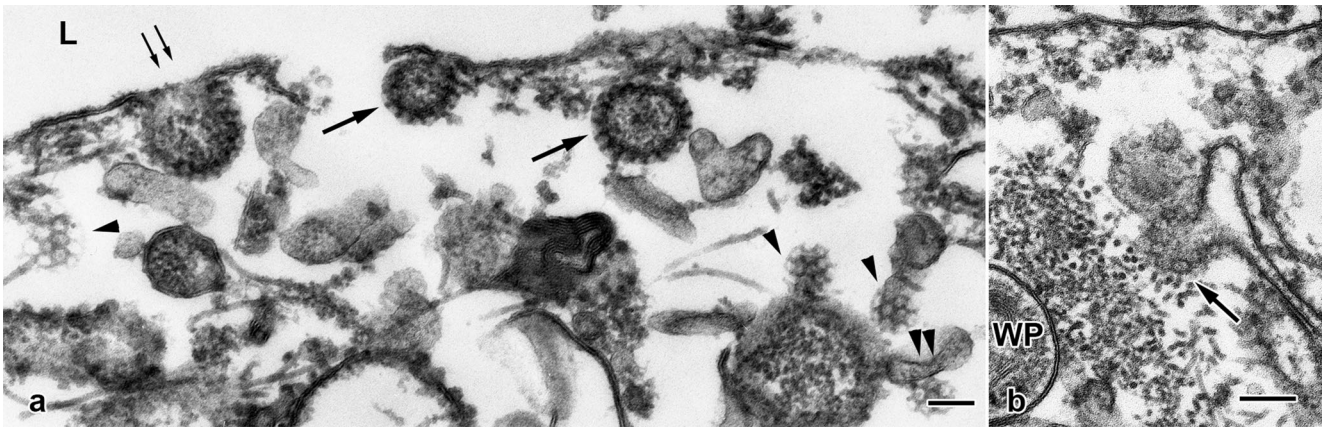

Fig. 6 Electron microscopy of sinus endothelial cells following saponin extraction. **a** In a vertical section of an endothelial cell, a coated pit (*double arrows*) containing electron-dense material is present in the luminal surface of the cell (*L* sinus lumen). Coated vesicles with spines (*arrow*) and structures of the polygonal network (*arrowhead*) are found in the cytoplasm of the cell. The polygonal network is associated with the endoplasmic reticulum (*double arrowheads*) **b** Endoplasmic reticulum sometimes has a fungiform structure with a polygonal network (*arrow*; *WP* Weibel-Palade body). *Bars* 100 mm

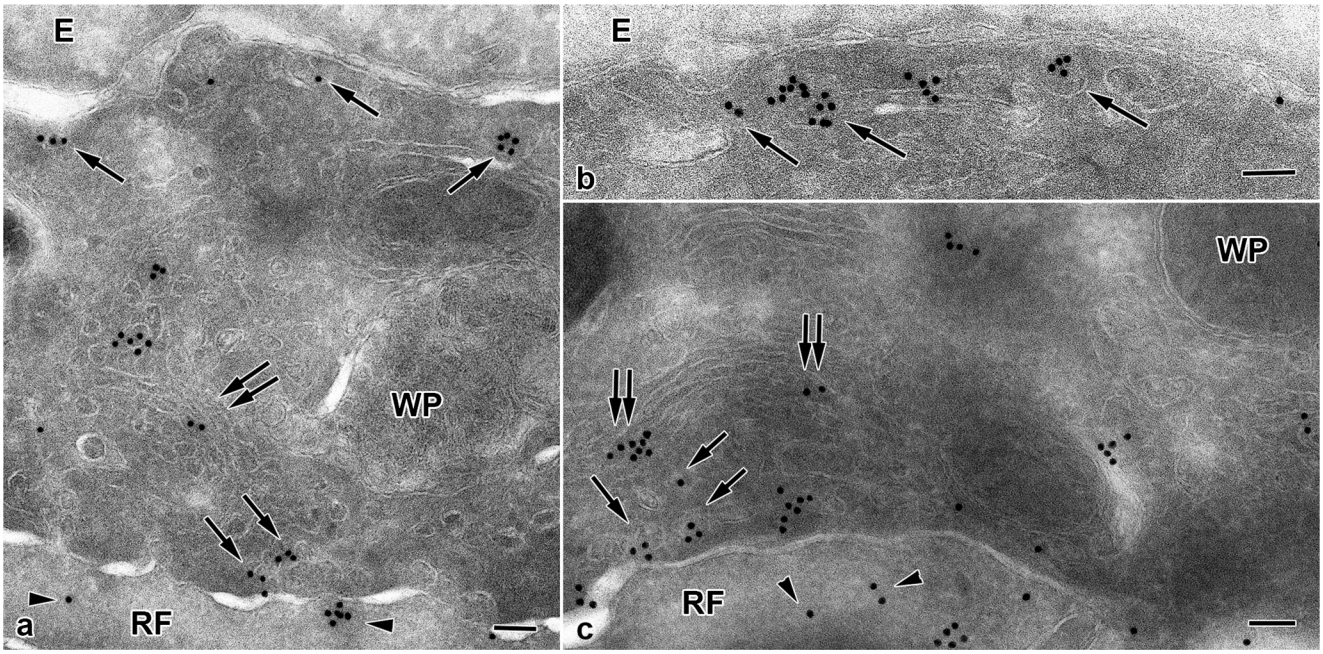

Fig. 7 Immunogold electron microscopy of vertical sections of sinus endothelial cells labeled with anti-vitronectin detected with 15-nm colloidal gold. **a** Labeling with anti-vitronectin is present in vesicles in the apical and basal part of the sinus endothelial cell (*arrow*) and in the endoplasmic reticulum of the cytoplasm (*double arrows*). Labeling is also present in ring fibers (*RF*, *arrowhead*) beneath the sinus endothelial cell (*E* erythrocyte in the sinus lumen). **b** Higher magnification of the apical part of a sinus endothelial cell (*E* erythrocyte in the sinus lumen). Labeling has accumulated in the vesicles (*arrow*). **c** Higher magnification of the basal part of a sinus endothelial cell. Labeling is present in the stacked endoplasmic reticulum (*double arrow*) and vesicles (*arrow*). Labeling is also present in the ring fibers (*RF*, *arrowhead*) beneath the sinus endothelial cell (*WP* Weibel-Palade body). *Bars* 100 nm

sinus endothelial cells but also in the entire circumference of sinus endothelial cells. Integrin αvβ5 has been reported to be the sole apical integrin receptor of retinal pigmented cells in the mammalian eye and contributes to adhesion to the outer segment of photoreceptor cells, thereby playing a key role in the cyclic phagocytosis of shed fragments of the outer segment of photoreceptor cells by retinal pigmented cells (Nandrot et al. 2008). Integrin αvβ5 in rabbit pleural mesothelial cells has been shown to be the major integrin involved in the endocytosis of asbestos fibers (Boylan et al. 1995). Moreover, integrin αvβ5 has been suggested to regulate induced pulmonary endothelial permeability by facilitating

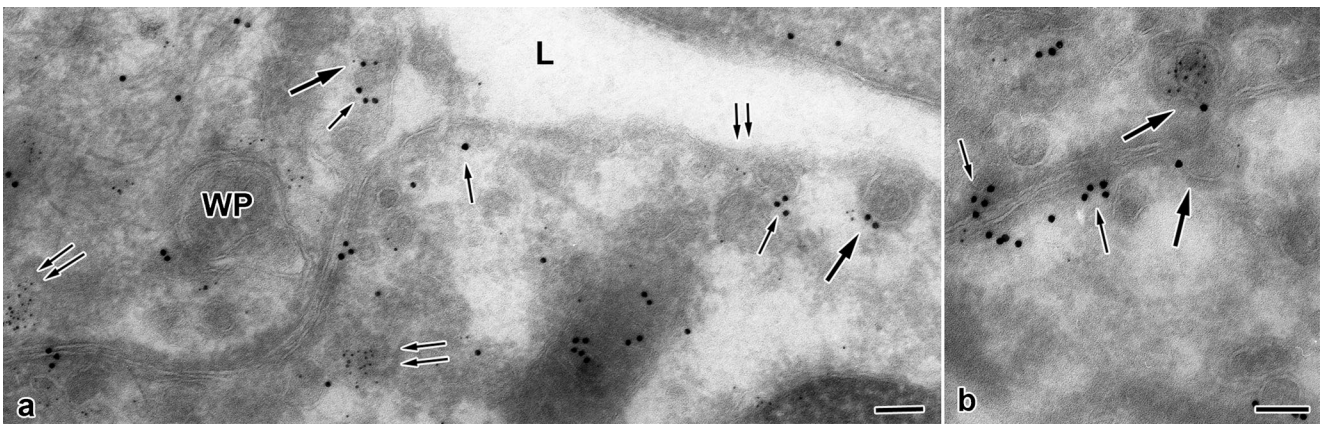

Fig. 8 Immunogold electron microscopy of vertical sections of adjacent sinus endothelial cells labeled with anti-integrin β5 or αv, each detected with 15 nm colloidal gold and anti-clathrin, detected with 5 nm colloidal gold. **a** Labeling for integrin β5 is present in the vesicles (*small arrows*) in close proximity to the apical plasma membrane (*L* sinus lumen, *WP* Weibel-Palade body). Labeling with anti-clathrin is present in the vesicles closely adjacent to the apical plasma membrane and sporadically appears in the cytoplasm of the sinus endothelial cells (*double arrows*). Labeling with anti-integrin β5 and labeling for anti-clathrin are colocalized in some vesicles in close proximity to the apical plasma membrane (*large arrows*). **b** Labeling for integrin αv is present in the vesicles in close proximity to the lateral plasma membrane (*small arrows*). Labeling with anti-integrin αv and labeling with anti-clathrin are colocalized in a pit and a vesicle in close proximity to the lateral plasma membrane (*large arrows*). *Bars* 100 nm

interactions with the actin cytoskeleton (Su et al. 2007). Therefore, integrin αvβ5 is probably involved in the endocytosis of some materials and in the regulation of permeability, including the passage of blood cells, in splenic sinus endothelial cells.

Integrins containing β1, β3 and β5 subunits interact with the microfilament system in focal adhesions (Sastry and Horwitz 1993). Vinculin is a cytoplasmic actin-binding protein enriched in focal adhesions and binds the cytoplasmic tail of integrin β via its interaction with talin. Vinculin is suggested to perform a critical function in regulating integrin clustering, force generation and the strength of adhesions (Peng et al. 2011). In this study, we found integrin β5 in close proximity to vinculin and stress fibers in the basal part of sinus endothelial cells. Therefore, integrin αvβ5 in the basal part of sinus endothelial cells probably takes part in the formation of focal adhesions and provides an important structural basis for anchoring sinus endothelial cells to ring-fiber-transforming ECMs, including collagen IV and vitronectin.

Vitronectin, a plasma protein that is primarily synthesized by hepatocytes, is found in ECMs; it binds to various biological ligands to play a crucial role in tissue remodeling by regulating cell adhesion and cellular motility through binding some members of the integrin family on the cell surface. Endothelial cells are perceived not to synthesize vitronectin and vitronectin in ECM provides a structurally and functionally distinct form from that in plasma. In vitro experiments have revealed that, following the uptake of vitronectin on the apical surface of endothelial cells, it is translocated into their lysosomal components or transcytosed to the basolateral phase in association with the ECM (Preissner and Seiffert 1998). In cultured fibroblasts, vitronectin has been demonstrated to be colocalized with integrin β5 during the endocytic process in cultured fibroblasts by indirect immunofluorescence microscopy and endocytosis is inhibited by the inhibition of clathrin endocytosis, i.e., endocytosis of vitronectin is thought to be regulated by integrin β5 (Panetti and McKeown-Longo 1993; Memmo and McKeown-Longo 1998). In this study, we revealed that sinus endothelial cells have abundant coated pits and coated vesicles and immunogold labeling for vitronectin was collectively localized in vesicles in the apical and lateral part of sinus endothelial cells. Furthermore, labeling for vitronectin is present in the stacked endoplasmic reticulum, in vesicles in the basal part of sinus endothelial cells and in ring fibers, the transformed basement membrane. This suggests that vitronectin is taken up through coated pits on the apical surface of splenic sinus endothelial cells, translocated to the stacked endoplasmic reticulum and transcytosed to ring fibers of a transformed basement membrane. Moreover, our immunogold electron microscopy revealed that labeling for integrin β5 and integrin αv is localized in the pits and vesicles in the apical and lateral part of sinus endothelial cells and that labeling for clathrin is sporadically colocalized with labeling for integrins β5 and αv. These results indicate that integrin αvβ5 regulates the endocytosis of vitronectin in splenic sinus endothelial cells and in cultured fibroblasts.

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