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Ontogeny of reactivity to endothelial cell markers during development of the embryonic and fetal rat lung

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Summary. The reactivity of endothelial cells to putative endothelial cell-specific markers varies with species,with vessel size and with the organ studied. To determine their value in studies of fetal rat lung, and whether organ immaturity would also influence reactivity, we studied endothelial cell immunoreactivity to antibodies against Factor VIII/von Willebrand factor (VIII/vWF), and binding reactivity to *Bandeiraea (Griffonia) simplicifolia* 1 lectin (BSL 1) during rat fetal lung development. Using an indirect immunofluorescent technique to detect Factor VIII/von Willebrand factor (VIII/vWF), endothelial cells lining the aortic arches were identified as early as day 11 of gestation (term= 22 days), prior to lung development. Immunoreactivity to VIII/vWF was subsequently localized to intrapulmonary endothelial cells and was not dependent on vessel size. In contrast, binding reactivity of FITC-conjugated BSL 1 was observed to both endothelial cells and to the basement membrane of developing airways, thus limiting its value as endothelial cell marker. During very early lung development solitary angioblasts could not be identified by reactivity to either VIII/vWF antibodies or to BSL 1, and neither marker appears to be of value for studies of early angiogenic events.

Key words: Endothelial cell, Factor VIII/von Willebrand factor related antigen, Immunohistochemistry, Lectin, Lung development

Introduction

We have recently reported (Buch et al., 1991) gestation-dependent changes in the transcription and translation of genes for homodimers of the growth factor platelet-derived growth factor (PDGF) and PDGF receptors during lung development, and that isolated endothelial cells from the canalicular stage of fetal lung development increase DNA synthesis in response to PDGF (Tanswell et al., 1991). Further immunohistochemical and autoradiographic studies of the temporal relationship between the expression of PDGF receptors and endothelial cell division during lung development require a reliable marker to identify endothelial cells in the embryonic and fetal rat lung.

Immunocytochemical studies of the pulmonary vasculature have most frequently been performed using antibodies to general endothelial cell (EC) markers (Hoyer et al., 1973; McComb et al., 1982; Darnule et al., 1983), such as factor VIII/vWF. Factor VIII/vWF synthesis and localization is restricted to EC and megakaryocytes (Piovella et al., 1978; McComb et al., 1982), allowing its use as a specific marker for some immunocytochemical studies (Piovella et al., 1978; Mukai et al., 1980; Burgdorf et al., 1981; McComb et al., 1982). However, because there are known to be organ-dependent variations in the reactivity of microvascular EC to such antibodies (Auerbach et al., 1985), and because we did not know whether extreme organ immaturity would also influence immunoreactivity, we elected to use an additional EC marker in these studies. Reactivity of BSL 1 lectin conjugates has been demonstrated in blood vessel EC of various rat organs (Laitinen, 1987; Hansen-Smith and Nine, 1987), and has been reported to demonstrate more capillaries than can be observed with enzyme methods (Christie and Thomson, 1989). Again, the effect of organ immaturity on EC reactivity to BSL 1 lectin is unknown.

We had also hoped that one of these markers would be able to detect primitive angioblasts during very early stages of lung development. Studies with avian lung have shown that an intrinsic vascular plexus develops from endothelial cell precursors within the pulmonary mesenchyme of the very early lung bud prior to invasion by exogenous pulmonary vessels (Labastic et al., 1986; Lance-Jones and Lagenaur, 1987; Pardanaud et al., 1987; Coffin and Poole, 1988; Poole and Coffin, 1988). The fully formed pulmonary vasculature is derived from

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the fusion of the extrinsic vascular plexuses, which follow the airways, with the intrinsic vascular plexuses derived from endothelial cell precursors in the pulmonary mesenchyme. It remains uncertain whether the mammalian lung vascular bed develops in the same fashion.

Materials and methods

Reagents

Rabbit anti-human von Willebrand factor and swine FITC-labelled anti-rabbit IgG were from Dakopatts (Glostrup, Denmark). FITC-conjugated BSL 1 lectin was from E.Y. Labs. (San Mateo, CA), and polyester wax from BDH Chemicals (Poole, England). Embedding medium (O.C.T. compound) was from Miles (Elkhart, IN). Paraformaldehyde was from BDH Chemicals (Toronto, Ontario). Neutral buffered formalin was from Fisher Scientific (Silver Spring, MO). Glutaraldehyde and sodium cacodylate were from JBS-CHEM (Pointe Claire-Dorval, Quebec). Tris-maleate, lead nitrate, magnesium chloride, adenosine 5'-triphosphate (ATP; grade I crystalline sodium salt), bovine serum albumin (BSA; fraction V) and ammonium sulfide were all from Sigma Chemical (St. Louis, MO). Normal goat serum (NGS) was from ICN ImmunoBiologicals (Costa Mesa, CA). Antifading mounting media (AF I solution) was from City-Fluor Ltd., (London, U.K.), and α-aminopropyltriethoxysilane from Pierce Chemical Company (Rockford, IL).

Pregnant Sprague Dawley rats of known gestation were obtained from Charles Rivers (Lachine, Quebec).

Tissue preparation

Animals were killed by exposure to an excess of ether. The uterus was immediately exposed and the fetuses delivered. Whole embryos were studied on day 11 of gestation, while the lungs were removed for study from days 12-21 of gestation. Tissues were immersion fixed in Carnoy´s fixative (Bancroft, 1975) for 2h, processed and embedded in polyester wax. Sections (5 μm) were cut and mounted on α-aminopropyltriethoxy-silane-coated slides. Tissue for haematoxylin and eosin staining were fixed in 10% buffered formalin phosphate overnight and washed in PBS pH 7.45 for 24h before processing. The tissues were then embedded in polyester wax and 5 μm sections cut.

Immunohistochemistry of VIII/vWF

An indirect immunofluorescent staining technique was used to study the immunoreactivity of VIII/vWF. Tissue sections, embedded in polyester wax, were dewaxed in three changes of 100% ethanol and rehydrated in a graded series of ethanol dilutions. After rinsing twice in PBS pH 7.45 non-specific binding to the sections was blocked by using 5% (v:v) NGS with 1% (v:v) BSA in PBS for 30 min. The excess of blocking solution was carefully blotted at the edges of the tissue sections and incubated over night at 4° C with a 1:400 dilution of rabbit anti-human VIII/vWF. The sections were washed in cold PBS with 0.01% (v:v) Tween 20 and incubated with 1:100 FITC-conjugated anti-rabbit IgG for 40 min, washed again in cold PBS with 0.01% (v:v) Tween 20, then mounted as above for examination under a fluorescent microscope (Leitz Laborlux D). All incubation steps were conducted in a humidified chamber, and all primary and secondary antibodies were diluted in 5% (v:v) NGS with 1% (v:v) BSA in PBS.

As negative controls slides were incubated as above, but with the primary antibody omitted.

Reactivity of BSL 1 lectin

Tissue sections were dewaxed and rehydrated as above, then incubated with 50-100 μg/ml FITC-conjugated BSL 1 lectin, diluted in PBS pH 7.45 containing 0.05 mM calcium chloride for 40 min. The sections were then washed in cold PBS with 0.01% (v:v) Tween 20 (10 min x 3) and mounted as above.

As negative controls slides were incubated with BSL 1 lectin in the presence of 0.2-0.4 M D-galactose.

Histochemical detection of magnesium-activated adenosine triphosphatase (ATPase)

The technique used in these studies was described by McLeod et al. (1987), for studies of rabbit eye development. Briefly, embryonic and fetal lungs were fixed in freshly-prepared Karnovsky's glutaraldehyde-paraformaldehyde (pH 7.2) at 23° C for 7 min. Following this, they were rinsed in 0.2M cacodylate buffer (pH 7.2) at 4° C, and then fixed in 10% neutral buffered formalin at 4° C for 24h. Lungs were washed in 5 changes of tap water at 4° C with gentle agitation, prior to a 15-min incubation in medium containing 0.2M Tris-maleate buffer (pH 7.2), 3mM lead nitrate, 6 mM magnesium chloride and 0.1% (w:v) ATP, which was added immediately prior to use. Subsequently, the lungs were washed x5 in tap water at 20° C, and then treated with 0.1M ammonium sulfide for 1 min. After several washes with tap water, to remove sulfide, the lungs were embedded in O.C.T. compound for preparation of 5 μm cryosections.

Results

Immunoreactivity to VIII/vWF was evident in EC lining the major embryonic vessels in sagittal section of day 11 whole embryo (Fig. 1a). Weak positive immunoreactivity was first observed on the first day of lung bud formation at day 12 of gestation (Fig. 1b). Thereafter there was a progressive increase in VIII/vWF tissue reactivity to day 21 of gestation (Fig. 1c). This was attributable to an increase in the number of small vessels present in the lung tissue, particularly between

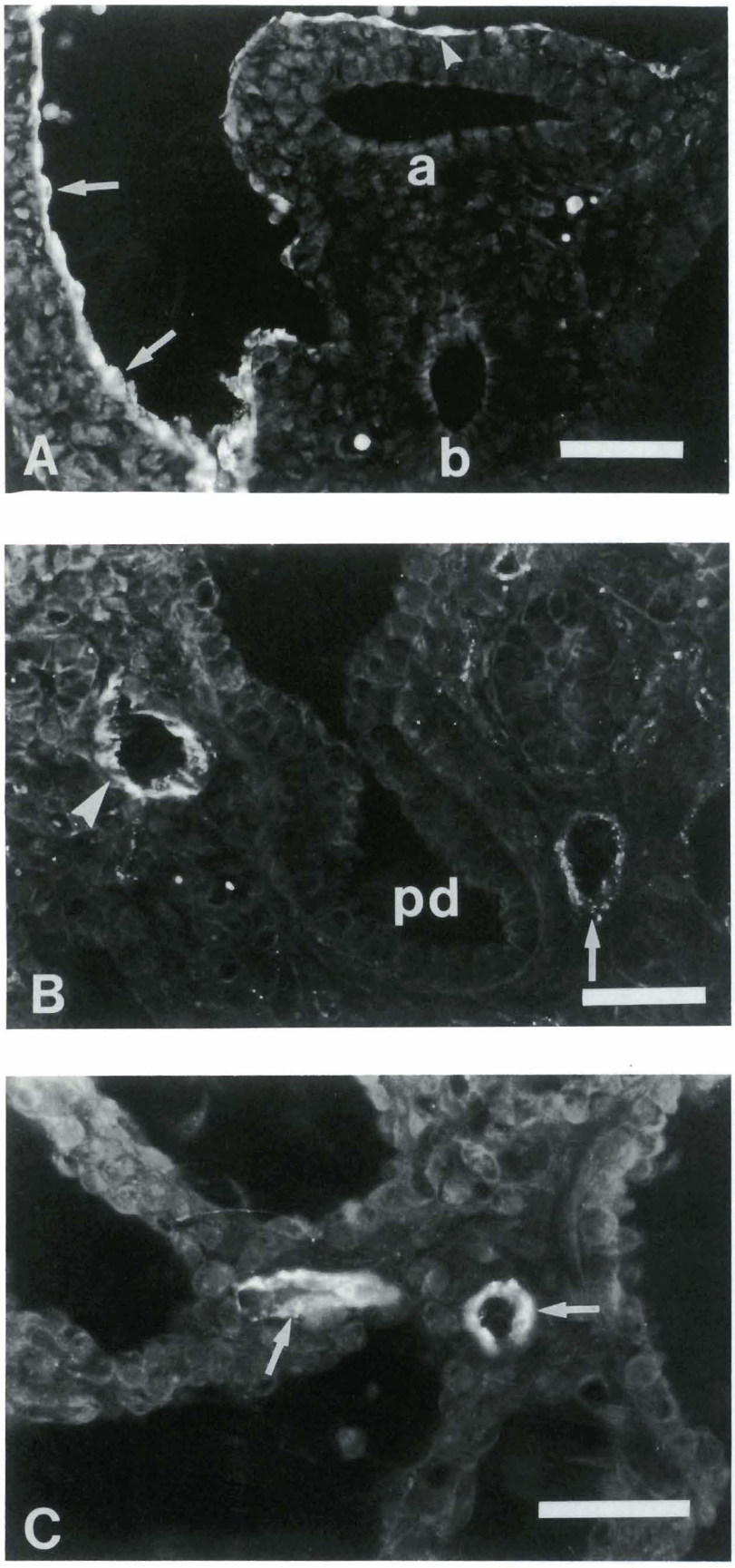
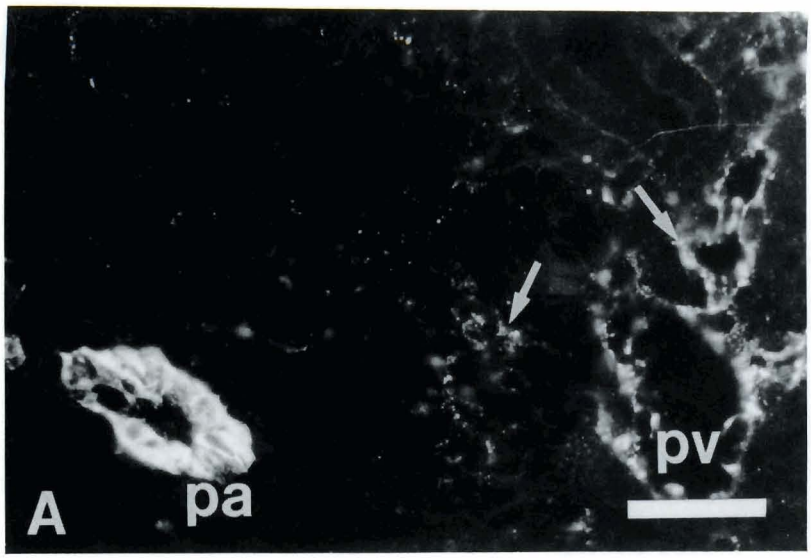


Fig. 1. (A) Sagittal section of the whole embryo at day 11 of gestation with cephalic part above, caudal below, developing heart on right and dorsum to the left. Second (a) and third (b) pharyngeal pouches are seen above the heart. Strong positive immunoreactivity to VIII/vWF is evident in endothelial cells lining the dorsal aorta (arrows) and second aortic arch (arrow head). Magnification bar = 100 μm. **(B)** Section of the lung bud at day 12 of gestation showing the pulmonary diverticulum (pd). There is a weak positive reactivity to VIII/vWF in the mesenchyme denoting the formation of intrinsic vascular plexuses (arrow). Strong positive reactivity is evident in endothelial cells lining an embryonic vessel (arrow head). Magnification bar = 50 μm. **(C)** Section of fetal lung at 21 days' gestation. Strong positive immunoreactivity to VIII/vWF is evident in endothelial cells lining microvessels (arrows). Magnification bar = 50 μm



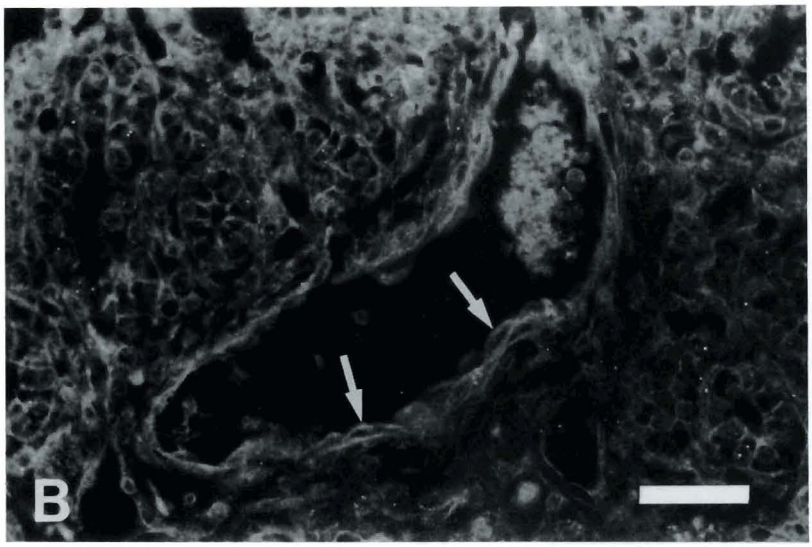


Fig. 2. (A) . The pulmonary artery (pa) and vein (pv) of the fetal lung at day 19 of gestation. The endothelial cells of both large vessels and of microvessels between the developing acini (arrows) show positive immunoreactivity to VIII/vWF. **(B).** Fetal lung at day 19 of gestation stained as above, but without the primary antibody to VIII/vWF, showing a lack of endothelial cell (arrows) immunofluorescence. Magnification bars = 50 μm

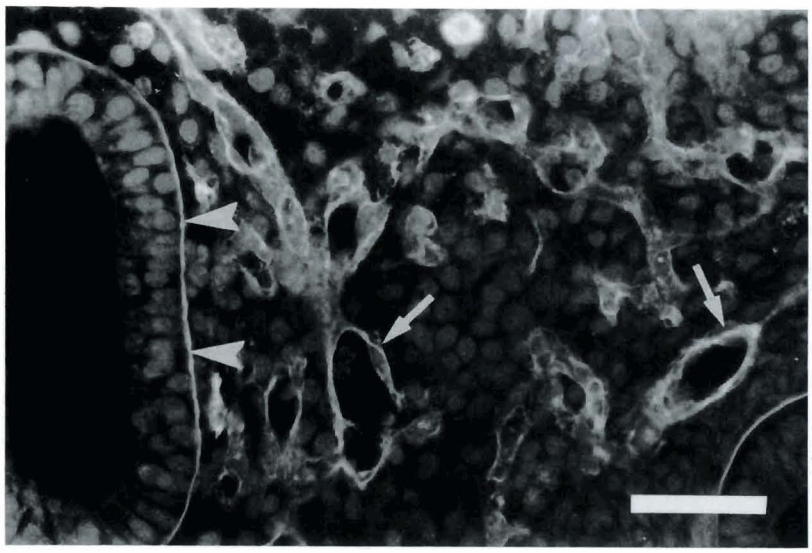


Fig. 3. Strong positive binding reactivity of BSL 1 lectin to endothelial cells (arrows) is evident in fetal lung at 16 days of gestation when numerous intrinsic vessels have formed. Basement membranes of the developing airways also show positive binding activity (arrow heads). Magnification bar = 50 μm

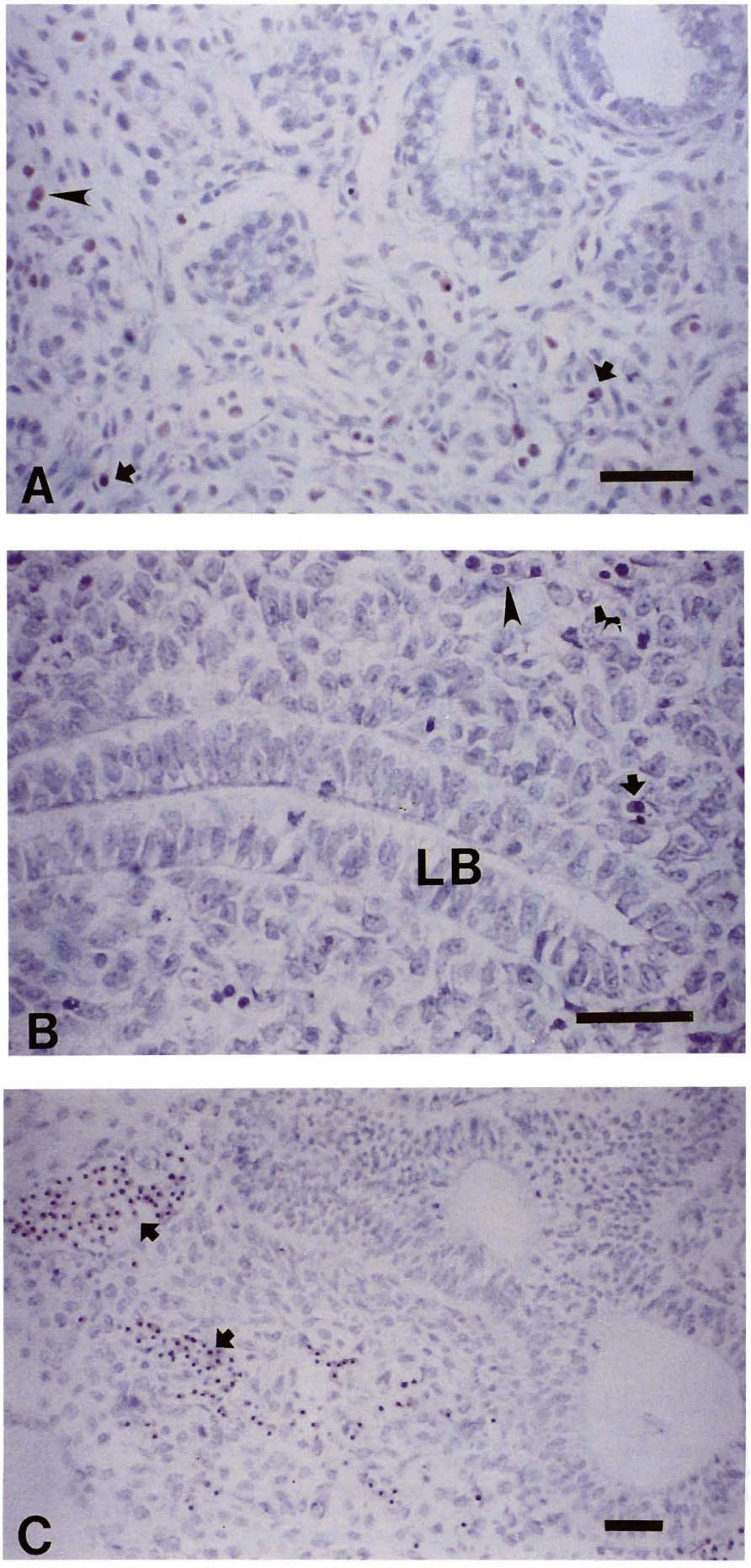


Fig. 4. Fetal rat lung of different gestational ages stained with haematoxylin and eosin **(A)** Fetal lung at day 18 of gestation with red blood cells in microvessels (arrow head) and solitary cells which could be angioblasts in stroma (arrows). **(B)** Lung bud (LB) at day 12 of gestation with solitary putative angioblasts (arrow) and angioblasts undergoing autolysis (arrow heads). **(C)** Fetal lung at day 14 of gestation containing clusters of putative angioblasts (arrows). Magnification bar = 50 μm

the acini at the periphery of the lung (Fig. 2a). No immunofluorescence was observed if the primary antibody was omitted (Fig. 2b).

Reactivity to BSL 1 lectin revealed a very similar developmental pattern to that observed with VIII/vWF immuno-reactivity. Weakly reactive intra-pulmonary EC were first observed at day 12 of gestation. The binding became more intense as gestation advanced due to an increase in the number of vessels. Reactivity to BSL 1 lectin did not detect more vessels than were evident with VIII/vWF immunoreactivity. By day 16 of gestation reactivity to BSL 1 was also evident in the basement membranes of developing airways (Fig. 3).

A summary of immunoreactivity to VIII/vWF and BSL 1 lectin reactivity to EC is given in Table 1, and a summary of pulmonary vasculature development is given in Table 2. Formation of large intrapulmonary vessels was first noted at day 17 of gestation. By day 18 of gestation, red blood cells were noted in the airway-associated vessels and in the vascular plexuses (Fig. 4a). Prior to this cells that may be angioblasts (eosinophilic cytoplasm with basophilic nucleus), as single cells or in groups, were first observed in the mesenchyme surrounding the lung bud at day 12 of gestation. Some of these cells showed features of autolysis, forming clefts between the cells (Fig. 4b). The number of these cells had markedly increased throughout the pulmonary mesenchyme by day 14 of gestation (Fig. 4c). Unfortunately neither BSL 1 lectin nor antibodies to VIII/vWF reacted to these cells or any other solitary cell type, suggesting that the relevant antigens are not expressed on endothelial cell precursors until vessel formation. Nor were we able to identify angioblasts by histochemical techniques, designed to demonstrate magnesium-activated ATPase (data not shown).

Discussion

The endothelial cell precursor, the angioblast, does not express the differentiated characteristics necessary for recognition by VIII/vWF antibodies or BSL 1 lectin. This lack of recognition could not be attributed just to fetal immaturity, since reactivity to both EC markers was evident by endothelial cells lining the embryonic vessels on day 11 of gestation, prior to lung development. It has been reported that pretreatment of paraffin embedded tissue with trypsin is required before capillaries can be identified immunohistochemically with VIII/vWF antibodies (McComb et al., 1982). We did not find trypsin-pretreatment to be necessary, perhaps due to differences in the fixative used, or to our use of low melting point (40° C) polyester wax embedding media. The use of BSL 1 lectin (Laitinen, 1987; Christie and Thomson, 1989) has been reported to enhance detection of capillaries, compared with VIII/vWF. We did not observe any quantitative differences between these EC markers, perhaps for the same reasons which made trypsin-treatment unnecessary. The intensity of basement membrane reactivity to BSL 1 in late gestation fetal lung limited its value as an EC marker..

Reactivity to VIII/vWF antibodies or BSL 1 demonstrated the presence of EC in the mesenchyme of the forming lung bud on day 12 of gestation. The number of intrinsic vessels increased progressively through to day 16 of gestation. Airway-associated vessels were first evident at day 18 of gestation, indicating that they develop at some point between 16-18 days of gestation. From day 19 of gestation onwards microvessels were evident between the acini of the fetal lung.

Our findings in the mammalian species are in keeping with the results from avian lung, which suggest that an internal vascular plexus develops from endothelial cell precursors within the pulmonary mesenchyme prior to invasion by exogenous pulmonary vessels, but are not definitive due to our inability to positively identify endothelial cell precursors. Our findings do, however confirm the value of VIII/vWF antibodies as a tool for identifying endothelial cells in the developing fetal rat lung even at very early stages of development.

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Table 1. Immunoreactivity of VIII/vWF and BSL 1 lectin binding activity of endothelial cells in developing rat fetal lung.

	E12	F14	F16	F18	F19	F21
VIII/vWF immunoreactivity						
Large vessel EC	-	-	-	+++	+++	+++
Small vessel EC	+	+	++	++	+++	+++
BSL 1 binding activity						
Large vessel EC	-	-	-	+++	+++	+++
Small vessel EC	+	+	++	++	+++	+++

Large vessel EC = Endothelial cells lining the airway-associated vessels in later gestation. Small vessel EC = Endothelial cells of intrinsic vascular plexuses and microvasculature in later gestation. + denotes weak reactivity. ++ denotes intermediate reactivity. +++ denotes strong reactivity. - denotes no large vessels present.

Table 2. Developmental profile of the pulmonary vasculature in fetal rat lung.

	E12	F14	F16	F18	F19	F21
Intrinsic vascular plexus	+	++	+++	-	-	-
Airway-associated vessels	-	-	-	++	+++	+++
Microvasculature	-	-	-	-	++	+++

+ denotes few vessels of a specific type. ++ denotes intermediate number of vessels of a specific type. +++ denotes multiple vessels of a specific type. - Represents absence of this vessel type.

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