

Endoglin (CD105) immun-expression in human foetal and neonatal lungs

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Summary. Endoglin is a 180 KDa glycoprotein mainly expressed on endothelial cells of newly formed vessels. Its expression is increased by the hypoxia inducible factor 1 (HIF-1), a potent stimulator of VEGF expression. The relative hypoxic environment in which foetal lung develops favours HIF-1 dependent gene expression, including the endoglin and VEGF ones. Herein, we analysed endoglin immunexpression in the human neonatal and foetal lung throughout gestation. Lungs from 18 foetuses (9-41 weeks), 7 preterm and 2 term infants were submitted to the immunohistochemical study. A slight immunostaining was found in some mesenchymal aggregates in the lungs of foetuses at the first trimester of pregnancy. At mid gestation, endoglin was evidenced in peri-tubular mesenchymal stem cells or in peri-canalicular vessels and in the endothelia of peri-bronchial vessels; by contrast, no immunoreaction was observed in case of Down syndrome or in a foetus with cardiac malformations. At late gestation and in preterm infants, endoglin antibody labelled endothelia of the alveolar capillaries and of peri-bronchial vessels. In case of alveolar capillary dysplasia (ACD) or macrosomy associated with maternal diabetes, endoglin expression was restricted to peri-bronchial vessels; no immunoreaction was encountered in foetuses with IUGR (intra-uterine growth restriction) or massive pulmonary haemorrhage. Lungs of term infants both displayed atelectasis; there was no evidence of endoglin immunexpression in one case, whereby only the endothelia of peri-bronchial vessels were stained in the other. Our study suggests that lung vasculogenesis endures throughout gestation. Absence of endoglin staining in some pathologic conditions may reflect lung vasculogenesis disorders; nonetheless, since each pathologic state is represented by a single case in our cohort, further studies are required to clarify this issue.

Key words: Endoglin, Lung, Foetus, Development, Vasculogenesis

Introduction

Endoglin (CD105) is a 180 KDa homodimeric transmembrane glycoprotein, which is a component of TGF- β receptor complex (Cheifetz et al., 1992). It is mainly expressed by the cycling endothelial cells of newly formed vessels in regenerating, inflamed or neoplastic tissues (Burrows et al., 1995; Miller et al., 1999; Torsney et al., 2002) and only weakly expressed or absent in normal tissues (Wang et al., 1993); thus, it is considered to be a powerful marker for neo-angiogenesis.

It is known that endoglin plays a key role in the vascular system development and morphogenesis, as documented by studies on knock out mice, whose embryos die precociously because of severe vascular and cardiac abnormalities (Li et al., 1999). Moreover, endoglin gene is the target for a dominant autosomal hereditary disease known as hereditary hemorrhagic teleangiectasia type 1, which is characterized by arteriovenous malformations in many organs, including the lung (Shovlin and Letarte, 1999).

Normal lung development takes place in a relative hypoxic environment (Lee et al., 2001) which is beneficial for both organogenesis and vascular formation (Semenza, 2005; van Tuyl et al., 2005). Indeed, the hypoxia inducible factor-1 (HIF-1), which is expressed in response to hypoxia, is able to stimulate vascular endothelial growth factor (VEGF) and endoglin expression, and, consequently, the creation of new vessels in the developing lung (Sanchez-Elsner et al., 2002; van Tuyl et al., 2005). Blood vessels have been shown to derive from two main mechanisms in the mouse lung (deMello et al., 1997). In particular, lung proximal arteries have been suggested to grow through angiogenesis, i.e. through sprouting from existing vessels, whereas the peripheral arteries appear by

vasculogenesis, which is defined by *de novo* proliferation and organization of endothelial cell precursors, which assemble into capillaries and then associate with the systemic circulation (Shannon and Detering, 1997). The pulmonary vascular development, in turn, appears to drive the airways formation (Hall et al., 2000; Schwartz et al., 2000). Indeed, epithelial branching morphogenesis is abrogated as a consequence of the inhibition of the pulmonary vascular development (van Tuyl et al., 2005).

In the present study, pulmonary lung vascular system development was investigated in normal and pathological human foetal and neonatal lung through the assessment of immuno-expression of endoglin, a marker able to specifically detect newly formed vessels.

To the best of our knowledge, this represents the first study evaluating pulmonary vascular system development through endoglin immuno-expression analysis.

Materials and methods

Human lung samples

Lung tissue samples, obtained at autopsy from 18 foetuses, 7 preterm infants and 2 term infants were collected, after written informed consent of the mother, in the Department of Human Pathology of the University of Messina, Italy. Foetuses at 9 and 10 weeks were retrieved following legal voluntary termination of pregnancy. Foetuses at 15, 16, 19, 21, 22 and 23 weeks were aborted following legal voluntary termination of pregnancy for foetal malformations, according to Italian law which allows termination of pregnancy for medical reasons up to 25 weeks of gestation. In these cases, termination was induced by prostaglandin vaginal administration.

Gestational age of the 15 foetuses ranged between 9 and 41 weeks, whereas preterm infants were born between the 25th and the 36th week of gestation.

The causes related to the foetuses and the infants death are shown in Table 1. Concerning the 9 infants enrolled in the study, 2 had died of respiratory distress syndrome (RDS) associated with hyaline membranes deposition, 5 had died of respiratory failure, 1 had died of cerebral malformations and 1 of persistent pulmonary hypertension (PPH).

Autopsies and sample collections were carried out immediately after delivery, assessing a less than 24 h interval since foetal death had occurred.

All samples were fixed in 10% formalin and embedded in paraffin for morphological diagnostic evaluation by haematoxylin and eosin staining and for immunohistochemistry. At least two samples of each lung for each foetus or infant were analyzed in three separate experiments.

The developmental stage of the analyzed lungs was assessed on the basis of histological findings accordingly to the classification proposed by Askin (1991), which

identifies three main stages: the pseudo-glandular stage, which is normally seen between 7 and 16 weeks of gestation, the canalicular stage, physiologically observed between 17 and 28 weeks and the terminal sac stage, from 28 weeks and up to the 36th week.

Then an alveolar stage, characterized by the presence of alveoli begins after 36 weeks of gestation.

Lung hypoplasia was defined on the basis of the ratio of lung weight to body weight, basing on age related standards (Page and Stocker, 1982; De Taete et al., 2005).

Immunohistochemistry

All lung specimens were fixed in 10% neutral formalin for 24 hours at room temperature, embedded in paraffin at 55°C and cut into parallel consecutive 4µm thick sections for the subsequent immunohistochemical study. Briefly, the endogenous peroxidase activity was blocked with 0.1% H₂O₂ in methanol for 20 min; then, normal sheep serum was applied for 30 min to prevent unspecific adherence of serum proteins. For the endoglin epitope retrieval, specimens were pre-treated with proteinase K (S3020, DAKO Cytomation) at room temperature for 15 min. Sections were successively incubated at 4°C overnight with the primary monoclonal antibody against endoglin (DAKO Corporation, Denmark, clone SN6h, w.d. 1:50); the bound primary antibody was visualized by avidin-biotin-peroxidase detection using the Vectastain Rabbit/Mouse Elite Kit, according to the manufacturer's instructions. To reveal the immunostaining, the sections were incubated in darkness (43) for 10 min with 3,3'-diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline solution (PBS). Nuclear counterstaining was performed by Mayer's haemalum. Specificity of the binding was assessed by omitting the primary antiserum or replacing it with normal rabbit serum or phosphate buffered saline solution (PBS, pH 7.4). Moreover, the syncytiotrophoblast present in specimens of human term placenta was tested as a positive control for endoglin immunoreaction (Gougos et al., 1992). Sections of renal cell carcinoma known to express endoglin were used as additional positive controls (Sandlund et al., 2006).

Results

At histologic examination, foetal lungs displayed the following patterns: a pseudoglandular aspect in 8 cases, a canalicular one in 5 cases, a terminal sac stage in 4 cases and an alveolar one in the fetus died at 41 weeks of gestation (Table 1). In two cases (foetuses n. 9 and n. 13 in Table 1), at 19 and 23 weeks, respectively, lung immaturity was present. Indeed, a pseudo-glandular histology was observed instead of the canalicular one expected for gestational age. In all but one preterm infants a terminal sac stage was observed in the lungs,

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whereas term infants all showed an alveolar stage of development (Table 1). In an infant (n. 25 in table 1) with persistent pulmonary hypertension (PPH), on the basis of the microscopic findings, alveolar capillary dysplasia (ACD) was diagnosed. ACD is a rare condition, characterized by a varying degrees of capillary deficiency in the alveoli (Sen et al., 2004), which cause persistent pulmonary hypertension in the

newborn.

The immunohistochemical results of our analysis are shown in Table 1. In all three separate experiments the same results were achieved.

With reference to endoglin immunoexpression in the foetal lungs, results were analyzed on the basis of gestational phase (first trimester or early pregnancy; second trimester or mid-gestation; third trimester or late

Table 1. Clinical data, histological and endoglin immunoexpression findings in the lungs of 18 fetuses, 7 pre-term and 2 term infants enrolled in the study.

N	Trimester	Gestational Age (wk)	Age at death	Cause of death	Lung	Endoglin	stage
Foetuses							
1	first	9	9wks	VT	Normal	Slight positivity in aggregates of mesenchymal cells	PG
2	first	10	10wks	VT	Normal	Slight positivity in aggregates of mesenchymal cells	PG
3	second	15	15wks	VT for trisomy 18	Normal	Positive (endotelium of peri-bronchial vessels; stem cells)	PG
4	second	16	16wks	Abruptio placentae	Normal	Positive (endotelium of peri-bronchial vessels; stem cells)	PG
5	second	16	16wks	VT for trisomy 18	Normal	Positive (endotelium of peri-bronchial vessels; stem cells)	PG
6	second	16	16wks	Oligohydramnios	Hypoplasia	Positive (endotelium of peri-bronchial vessels; stem cells)	PG
7	second	19	19wks	CVM infection	Normal	Positive (endotelium of peri-bronchial vessels; peri-canalicular stem cells)	C
8	second	19	19wks	VT for trisomy 21	Normal	Negative	C
9	second	19	19wks	VT for trisomy 18	Immaturity; hypoplasia	Positive (endotelium of peri-bronchial vessels; stem cells)	PG
10	second	21	21wks	VT for spina bifida	Normal	Positive (endotelium of peri-bronchial vessels; peri-canalicular stem cells)	C
11	second	22	22wks	VT for cardiac malformation	Normal	Positive (endotelium of peri-bronchial vessels; peri-canalicular stem cells)	C
12	second	22	11wks	VT for cardiac malformation	Normal	Negative	C
13	second	23	23wks	VT for trisomy 21	Immaturity; hypoplasia	Positive (endotelium of peri-bronchial vessels)	PG
14	third	28	28wks	Abruptio placentae	Asphyxic changes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
15	third	30	30wks	Abruptio placentae	Normal	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
16	third	32	32wks	Placental growth retardation	Hypoplasia	Negative	TS
17	third	33	33wks	Abruptio placentae	Asphyxic changes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
18	third	41	41wks	Intrapartum asphyxia	Massive pulmonary haemorrhage	Negative	A
Preterm infants							
19	/	25	1d	RDS	Hyaline membranes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
20	/	32	4d	RDS	Hyaline membranes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
21	/	33	2d	Respiratory failure	Asphyxic changes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
22	/	33	3d	cerebral malformation	Meconium aspiration	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
23	/	34	40min	Respiratory failure and macrosomy	Asphyxic changes	Positive (endotelium of peri-bronchial vessels)	TS
24	/	36	1d	Respiratory failure	Asphyxic changes	Positive (endotelium of peri-bronchial vessels; primitive alveolar capillaries)	TS
25	/	36	10d	PPHN	Changes typical for ACD	Positive (endotelium of peri-bronchial vessels)	TS
Term infants							
26	/	39	2d	Respiratory failure	Atelectasis	Negative	A
27	/	39	20min	Respiratory failure	Asphyxic changes-atelectasis	Positive (endotelium of peri-bronchial vessels)	A

wk: week; wks:weeks; d: day; min: minutes; VT: voluntary termination; RDS: respiratory distress syndrome; PPH: Persistent Pulmonary Hypertension; A-C: alveolar capillary; PG: pseudo-glandular; C: canalicular; TS: terminal sac; A: alveolar.

gestation) and on that of histologic aspect.

At the first trimester of gestation, a slight endoglin immuno-staining was found in some aggregates of mesenchymal cells within the lungs with a pseudoglandular histology of the two analyzed foetuses (9 and 10 weeks of gestation) (foetuses n. 1 and 2) (Fig. 1).

At mid- pregnancy, a positive endoglin immunoreaction was evidenced in 6/6 lungs at a pseudo-glandular stage and in 3/5 lungs at a canalicular stage of development.

Considering the 6 lungs at a pseudoglandular stage,

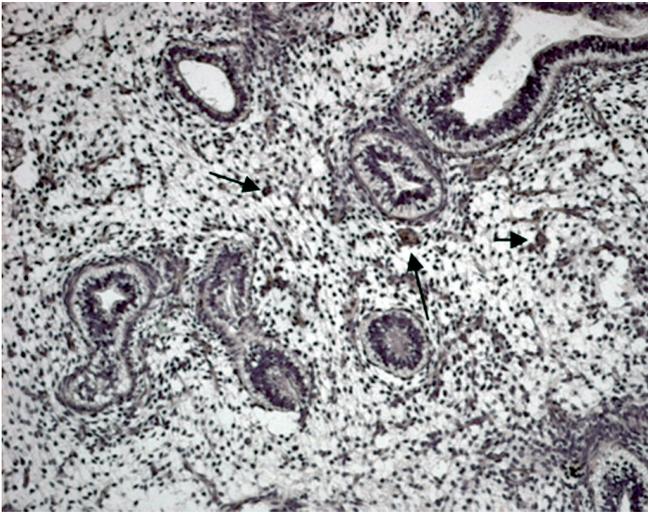


Fig. 1. Lung from a foetus at 9 weeks of gestation with a pseudo-glandular histology (foetus n. 1 in table 1). A slight immunostaining for endoglin was present in some aggregates of mesenchymal cells (arrows) (endoglin stain; x 100).

in 5/6 cases endoglin staining was present in the peritubular mesenchymal stem cells (Fig. 2a) and in the endothelia of peri-bronchial vessels (Fig. 2b). In 1/6 case (foetus n. 13), lungs were retrieved from a fetus with Down syndrome and endoglin antibody labelled endothelial cells in the peri-bronchial vessels, but no mesenchymal stem cells were stained around the tubules. Concerning the 5 fetal lungs with a canalicular histologic pattern, at the 19th week (foetus n. 7), endoglin immunostaining was present in the peri-bronchial vessels and in peri-canalicular stem cells, in analogy with the immunohistochemical pattern observed in the lungs at a pseudo-glandular stage. In the lungs at the 21st and at the 22nd week (foetuses n. 10 and 11), anti-endoglin antibody labelled peri-bronchial vessels and the newly formed peri-canalicular vessels (Fig. 3a,b). About the lungs with a canalicular histology and negative for endoglin, they had been retrieved from a foetus with Down syndrome (foetus n. 8) and from one with severe cardiac malformations (foetus n. 12) (Fig. 4), respectively.

In the third trimester of gestation, lungs displayed a terminal sac stage of development in all cases but one, which showed an alveolar stage (foetus n. 18). Endoglin immunoreaction localized in the endothelium of peri-bronchial vessels and of primitive alveolar capillaries (Fig. 5). Absence of staining was found out in a foetus (n. 16) displaying intrauterine growth restriction (IUGR) and in a case of massive pulmonary haemorrhage (foetus n. 18).

With reference to preterm infants, endoglin immun-expression was observed in 7/7 cases. Reaction was present in the endothelium of primitive alveolar capillaries and of peri-bronchial vessels in 5/7 cases, whereas it was confined to the latter in the lungs of a macrosomic infant (n. 23) born of a diabetic mother (insulin dependent diabetes mellitus, not metabolically

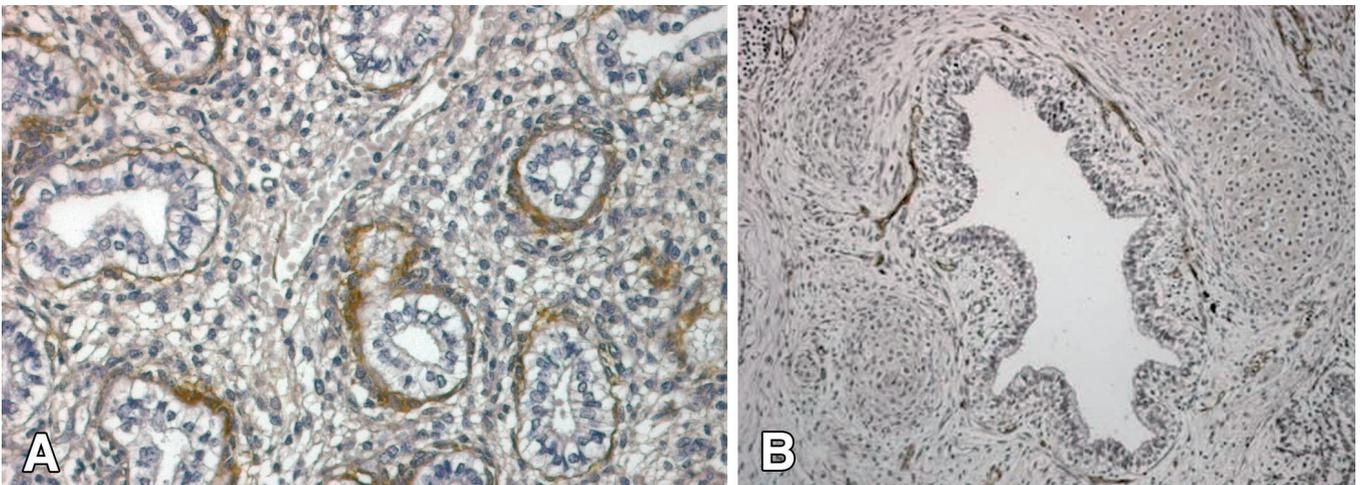


Fig. 2. Lung from a foetus at the 16th week (foetus n. 4 in table 1), showing a pseudo-glandular histology. Endoglin immuno-expression was present in (a) peri-tubular mesenchymal stem cells (endoglin stain, x 200) and in (b) peri-bronchial vessels (endoglin stain; x 200).

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well controlled) and of one with ACD (n. 25).

Lungs of both term infants showed atelectasis; no endoglin immunoreaction was found in one case (n. 26), whereby reaction localized only in the endothelium of peri-bronchial vessels in the other (n. 27).

Discussion

In the present study, endoglin immunohistochemical expression was evaluated in the lungs of foetuses at

different gestational ages, as well as in those of preterm and term infants. In the light of its role in the vascular system normal formation, endoglin immuno-expression was also analyzed in the lungs of foetuses with genetic abnormalities, such as trisomy 21 or 13, or with growth defects or pulmonary haemorrhage as well as in those of a neonate with alveolar capillary dysplasia (ACD), in the aim of verifying whether any abnormalities in lung vascular development was present in these cases. Hypoplastic lungs were also included in the present study

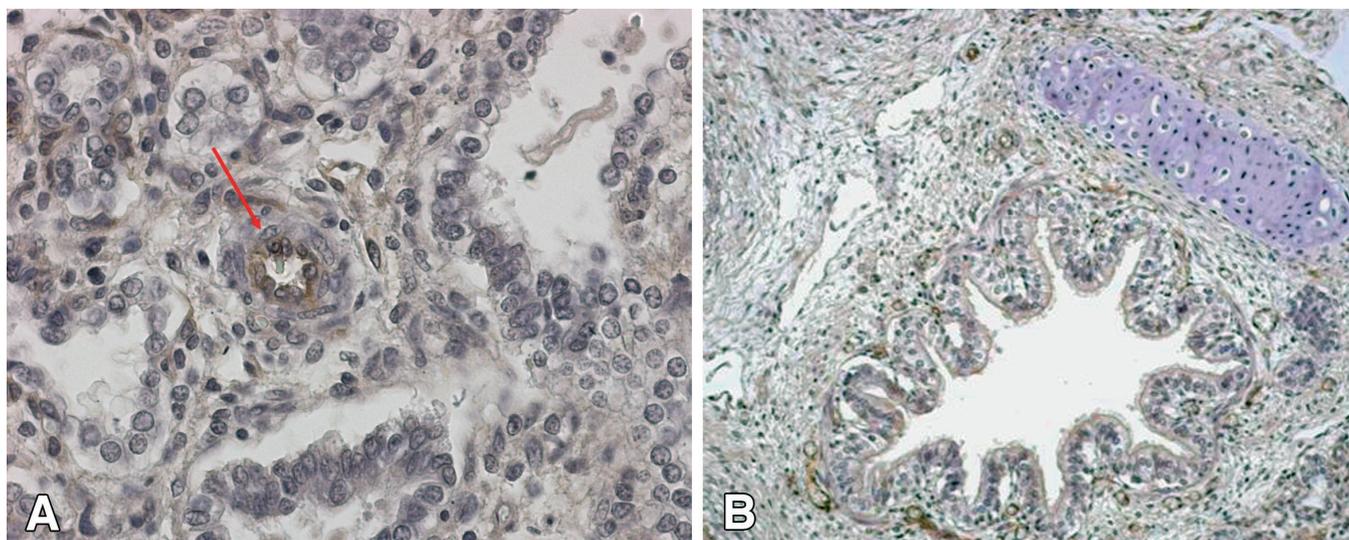


Fig. 3. Lung from a foetus at the 22nd week of gestation (foetus n. 11), displaying a canalicular histology. Endoglin antibody stained (a) peri-canalicular vessels (endoglin stain, x 400) and (b) peri-bronchial vessels (endoglin stain, x 100).

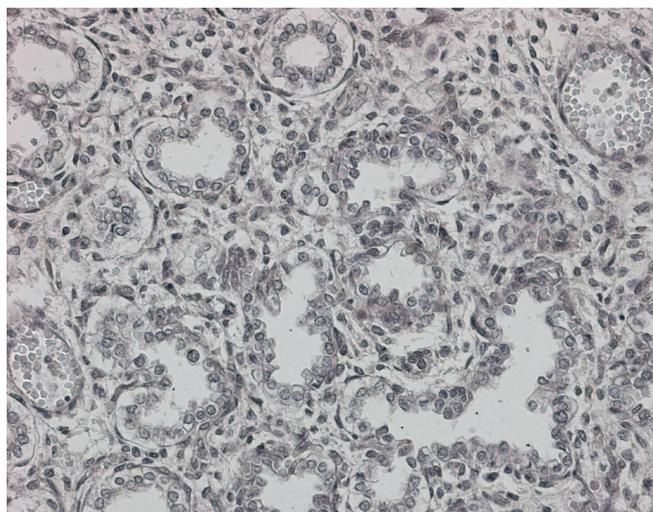


Fig. 4. Lung from a foetus, aborted at 22 weeks (foetus n. 12) because of severe cardiac malformations. A canalicular histological pattern was observed. No endoglin immuno-reaction was evident (endoglin stain, x 400).

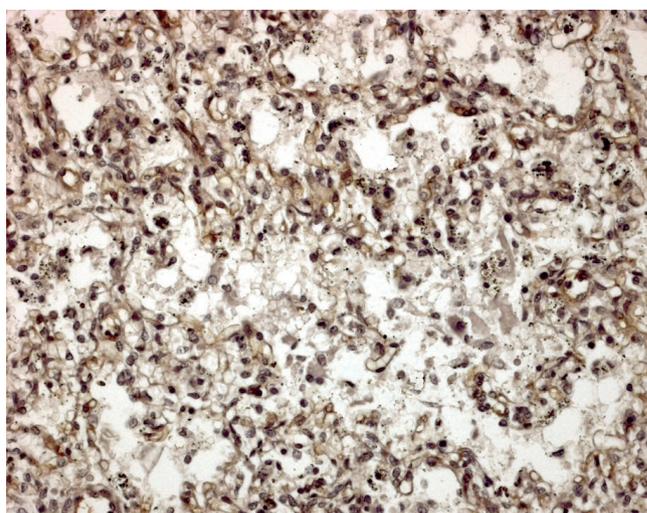


Fig. 5. Lung from a preterm infant (33 weeks) (n. 22) died after 3 days because of cerebral malformations. A terminal sac stage was evident and endoglin antibody labelled primitive alveolar capillaries (endoglin stain, x 200).

to evaluate whether impairment of organogenesis was associated with alterations in vasculogenesis.

Only a slight endoglin immunostaining was evidenced in sporadic aggregates of mesenchymal cells in the lungs of foetuses at the first trimester of gestation. At mid pregnancy, normal lungs and all but one hypoplastic lungs with a pseudoglandular histology were characterized by a positive endoglin immunoreaction in the endothelium of peri-bronchial vessels and in the peritubular mesenchymal stem cells, which are supposed to give rise to pulmonary capillaries through a process of vasculogenesis (Shannon and Deterding, 1997). Endoglin expression in stem cells is not a novel finding, since it was already reported in mesenchymal stem cells of the human lung (Sabatini et al., 2005). Groenman and colleagues (2007) documented HIF-1 and VEGFR2 immunohistochemical expression in these cells and reported VEGF positivity in the epithelium of the tubular structures. Moreover, other authors (Acarregui et al., 1999) found VEGF translocation from the epithelial cells of the pseudo-glandular lung to the basal membrane. Thus, it is suitable that mechanisms of paracrine stimulation occur between peri-tubular mesenchymal stem cells and tubular epithelial cells. HIF-1 secreted by the former cells may stimulate endoglin expression in an autocrine way and may induce VEGF synthesis in the latter through a paracrine mechanism. Correspondingly, VEGF synthesized by tubular cells may determine differentiation of the stem cells towards an endothelial phenotype and the subsequent vessels formation.

In one of the cases with a pseudoglandular pattern, absence of endoglin staining was recorded in the stem cells. Lungs from this foetus, who was aborted at the 23rd week because of Down syndrome, displayed hypoplasia and immaturity. Since endoglin is normally expressed by cycling endothelial cells (Miller et al., 1999), we can assume that its absence in the stem cells indicates that they are not in cycle so as to give rise to vascular structures in the lungs of this foetus. Then, it is tempting to speculate that, in this case, disruption of the vasculogenic process might have led to an alteration of air-ways branching which normally follows the formation of terminal vessels, thus determining lung immaturity and hypoplasia.

At canalicular stage, endoglin immuno-expression was found in the endothelial cells of peri-bronchial vessels; moreover, a positive immuno-reaction was identified in peri-canalicular stem cells at the 19th week and in peri-canalicular vessels at the 21st and at the 22nd week. The presence of endoglin positive vessels around canalicules suggests their derivation from the peritubular/peri-canalicular stem cells.

Lungs from two foetuses at canalicular stage did not display any staining for endoglin. One of these foetuses was affected by Down syndrome, while the other presented severe cardiac malformations. Thus, both the foetuses with Down syndrome included in the present study, showed a pattern of endoglin distribution differing

from that detected in normal lungs at the same stage of development. The absence of endoglin expression in the lungs of these foetuses may be related to an arrest of normal vasculogenesis and angiogenesis related to the syndrome. Besides, the association between Down syndrome and ACD has been reported (McGaughan et al., 2001; Shehata and Abramowsky, 2005; Galambos, 2006); thus we may hypothesize that if gestation had continued, pathologies like ACD might have occurred in these foetuses.

As previously stated, at canalicular stage, absence of endoglin expression was also recorded in the lungs of a foetus with cardiac malformations. It is known that mutations in endoglin gene cause hereditary haemorrhagic teleangiectasia, which is characterized by vascular and cardiac malformations (Shovlin and Letarte, 1999). As recently reviewed by Abdalla and Letarte (2006), most endoglin gene mutations result in reduced endoglin expression. Hence we may speculate that, in this foetus, mutations in the endoglin gene there exist and that they may account for both the absence of endoglin staining in the lung vessels and the cardiac malformations.

In the lungs of foetuses at the third trimester of gestation, endoglin antibody labelled peri-bronchial vessels and alveolar capillaries. No staining was evidenced in one foetus with lung hypoplasia associated with IUGR and in a case of pulmonary haemorrhage. Endoglin absence may indicate a deficiency in vasculogenesis and angiogenesis in these lungs. Even if an increase in vascularity could be expected in association with chronic hypoxia related to IUGR due to placental restriction, a recent study has demonstrated that neither HIF-1 nor VEGF increase in the placenta from IUGR normotensive pregnancies (Rajakumar et al., 2007). Thus chronic hypoxia might have led to a defective lung vasculogenesis and organogenesis instead of stimulating the formation of new vessels.

When considering the preterm newborns, the same pattern of endoglin distribution as that observed at the third trimester of pregnancy was seen in all cases except for an infant with ACD and for a macrosomic one.

In the case of ACD, endoglin immunoexpression was limited to the peri-bronchial vessels. The lack of endoglin immuno-reactivity in the few present alveolar capillaries may be related to a defect of vasculogenesis which is at the basis of the disease. Indeed, absence of endoglin, which is a marker expressed by cycling endothelial cells, indicates the arrest of vascular system growth.

The same pattern of endoglin immunoexpression was recorded in the lungs retrieved from the macrosomic pre-term newborn, died of respiratory failure. Hypoxic changes were seen at the microscopic evaluation and endoglin expression was restricted to the peri-bronchial vessels. This infant's mother was suffering from insulin dependent diabetes mellitus, which was not metabolically well controlled. Since it has been demonstrated that insulin stimulates VEGF expression

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through HIF-1 activation (Treins et al., 2002), we can assume that insufficient insulin treatment during gestation might have caused an alteration of vasculogenesis in the lung, which could be responsible for the respiratory distress arisen at birth.

In summary, in the present paper, lung vascular system development was studied for the first time by evaluating immunexpression of endoglin, a protein which is specifically expressed by endothelial cells of newly formed vessels. Our data document that endoglin is present in the precursor of endothelial cells and in the lung vessels throughout gestation, thus indicating that, in human lung, vasculogenic and angiogenic processes are not limited to the early pregnancies, but they also endure during mid- and late gestation. Moreover, endoglin expression in the lung vessels of the newborn demonstrates that lung vascular development continues even after birth, as it was reported for the air-way component by other authors (Haworth and Hislop, 2003).

Absence of endoglin immunostaining in the lung vessels of fetuses with ACD, pulmonary haemorrhage, Down syndrome or cardiac malformations may suggest an arrest of normal vascular development in these conditions; nevertheless, seen the rarity of these cases in our cohort, further studies are needed to more deeply investigate this matter.

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