

Endoglin (CD105) Up-regulation in Pulmonary Microvasculature of Ventilated Preterm Infants

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Rationale: Preterm infants exposed to mechanical ventilation and oxygen are at risk for bronchopulmonary dysplasia (BPD), a multifactorial chronic lung disorder characterized by arrested alveolar development. Studies have described disruption of microvascular development in BPD, characterized by primitive angioarchitectural patterns reminiscent of the canalicular/saccular stages of lung development. The molecular regulation of this BPD-associated dysangiogenesis remains undetermined.

Objectives: Endoglin (CD105), a hypoxia-inducible transforming growth factor- β coreceptor, has been implicated as an important regulator of angiogenesis in various neoplastic and nonneoplastic conditions. The aim of this study was to investigate the expression of endoglin and other angiogenesis-related factors in ventilated preterm human lungs.

Methods: We have studied endoglin protein and mRNA expression in postmortem lungs of short-term and long-term ventilated preterm infants. Control subjects were age-matched infants who had lived for less than 1 hour.

Measurements and Main Results: Lungs of short-term ventilated preterm infants showed significant upregulation of endoglin mRNA and protein levels, immunolocalized to the microvasculature. Similar but more variable endoglin upregulation was noted in lungs of long-term ventilated infants with BPD. The mRNA levels of vascular endothelial growth factor, angiopoietin-1, and their respective receptors were significantly lower in ventilated lungs than in age-matched nonventilated control lungs.

Conclusions: BPD is associated with a shift from traditional angiogenic growth factors (vascular endothelial growth factor, angiopoietin-1) to alternative regulators such as endoglin, which may contribute to BPD-associated microvascular dysangiogenesis.

Keywords: chronic lung disease of prematurity; bronchopulmonary dysplasia; neonatal lung disease; angiogenesis

Preterm newborns who require mechanical ventilation and supplemental oxygen are at risk for bronchopulmonary dysplasia (BPD), a chronic lung disease of newborn infants associated with significant mortality and morbidity (1). BPD in the post-surfactant era is seen mainly in very low birthweight infants and affects 30% of infants born between 24 and 28 weeks of gestation, many of whom will require long-term respiratory support (2, 3).

The dominant pathological finding in postsurfactant BPD is an arrest in alveolar development, resulting in large and simplified airspaces that show varying degrees of interstitial fibrosis (2, 4–8). Studies have shown that, in addition to impaired alveolar development, there is also a disruption of pulmonary microvascular development in infants with BPD (8–10) or in

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Microvascular development is disrupted in premature infants with bronchopulmonary dysplasia (BPD), resulting in abnormalities of lung structure that occur during the late canalicular and early saccular stages of lung development. Mechanisms that impair angiogenesis in BPD are incompletely understood.

What This Study Adds to the Field

This study suggests that endoglin, an important regulator of angiogenesis in various neoplastic and nonneoplastic conditions, contributes to BPD-associated dysangiogenesis.

BPD-like animal models such as chronically ventilated premature baboons (11, 12). In view of the intimate relation between alveolar and microvascular development during pulmonary morphogenesis (13–16), disruption of microvascular development in premature lungs has been implicated as a critical factor in the arrest of alveolar development that is characteristic of BPD (17, 18).

We have studied the growth kinetics of the pulmonary microvasculature in ventilated preterm infants (10). Using stereologic morphometric techniques and analysis of endothelial cell proliferation, we determined that the pulmonary microvasculature of ventilated preterm lungs undergoes significant expansion, rather than growth arrest, which is at least in part attributable to brisk endothelial cell proliferation during the early postnatal period (10).

Although the microvasculature in ventilated preterm lungs is quantitatively nearly normal, we (10) and others (8, 9) have described striking angioarchitectural abnormalities compared with age-matched nonventilated control lungs. Normal human lungs at term (36–40 wk of gestation) display thin alveolar septa with abundant secondary crest formation, characteristic of the early alveolar stage of lung development. Within the complex alveolar septa of normal lungs, the microvasculature forms a delicate network, characterized by extensive capillary sprouting. In contrast, we determined that the pulmonary microvasculature of long-term ventilated preterm infants at the same corrected postmenstrual age (36–40 wk) retains the vascular pattern of canalicular/saccular lungs, characterized by a persistent dual capillary pattern and primitive, nonbranching vessels.

The molecular regulation of this ventilation-induced dysangiogenesis remains undetermined. Previous studies of angiogenesis in BPD or BPD-like animal models have focused on the major factors traditionally linked with developmental angiogenesis, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), and their respective receptors. In a fetal baboon model of BPD, ventilation was associated with significantly decreased levels of VEGF and its receptor Fms-like

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tyrosine kinase-1/VEGF receptor-1 (Flt-1) (12). Studies in human preterm infants have shown variable results, in part attributable to small sample sizes, high clinical variability, and possible detection of differing (VEGF) protein isoforms. Bhatt and coworkers (9) reported decreased levels of VEGF, Flt-1, and Ang-1 receptor TIE receptor tyrosine kinase-2 (TIE-2) mRNAs as well as decreased VEGF immunostaining in infants with BPD compared with infants without BPD. Similarly, Lassus and coworkers (19) described lower VEGF in tracheal aspirates of preterm infants with severe respiratory distress/BPD compared with control subjects. In contrast, studies by Ambalavanan and Novak (20), D'Angio and coworkers (21), and Currie and coworkers (22) found no correlation between levels of VEGF in tracheal aspirates of preterm infants and risk for development of BPD.

In this study, we focused on endoglin (CD105) as potential candidate regulator of ventilation-induced angiogenesis. Endoglin is an accessory receptor for transforming growth factor (TGF)- β that is emerging as a pivotal regulatory component of the gateway for TGF- β signaling in vascular endothelial cells (23, 24). Endoglin is expressed predominantly in proliferating endothelial cells in culture and in angiogenic blood vessels *in vivo* (25). High levels of endoglin expression have been described in vascular endothelial cells in tissues undergoing active angiogenesis, such as regenerating and inflamed tissues or tumors (26–30). In addition, endoglin is weakly expressed in selected nonendothelial cell types including syncytiotrophoblast of full-term placenta (31), stromal cells (32, 33), mesenchymal cells (fibroblasts and vascular smooth muscle cells) (34, 35), and some hematopoietic cells (36, 37). Several lines of evidence implicate endoglin as an important regulator of cardiovascular development, angiogenesis, and vascular remodeling. Endoglin null mice are embryonally lethal because of vascular and cardiac abnormalities (38–40). Moreover, the gene encoding endoglin is the target for a hereditary vascular disorder known as hereditary hemorrhagic telangiectasia type I (24, 41).

The aim of the present study was to investigate endoglin expression in ventilated preterm human lungs. We report that the levels of pulmonary endoglin, immunolocalized to the microvasculature, are markedly increased in short-term ventilated preterm lungs compared with nonventilated age-matched control subjects. In contrast, mRNA levels of the more traditional angiogenic factors VEGF, Ang-1, and their receptors were significantly decreased in ventilated lungs. We speculate that this shift in angiogenic regulators may contribute to ventilation-induced dysangiogenesis in infants at risk for BPD.

METHODS

Patients

Lung samples from ventilated and control infants were obtained from the Women and Infants Hospital (Providence, RI) perinatal autopsy files between 2001 and 2005. Informed consent was obtained in compliance with institutional guidelines. The study protocol was approved by the institutional review board. The patients' medical and autopsy records were reviewed. Infants with congenital, chromosomal, or cardiac anomalies or with other conditions potentially predisposing to pulmonary anomalies (42) were excluded. In addition, cases with documented lung hypoplasia, defined as a lung-to-body weight ratio below the 10th percentile for age (43) were excluded. Records were reviewed for postmenstrual age (PMA) at birth, postnatal age, and corrected postmenstrual age at death (gestational age at birth plus postnatal age). In all patients, the postmortem interval (i.e., time between death and autopsy) was recorded.

To study the early and late effects of ventilation on pulmonary expression of angiogenic factors, patients were divided in four groups: "short-term ventilated," "long-term ventilated," "early control," and

"late control." The group of short-term ventilated infants was composed of very preterm infants (23–29 wk PMA at the time of death), who had lived for at least 4 days and had been ventilation dependent throughout life. The long-term ventilated group consisted of near-term or term infants (35–39 wk PMA at death) who had lived for prolonged periods of time (at least 6 wk), and had been ventilated for most of their life (at least 75% of their total life span). Pulmonary angiogenic expression of short- and long-term ventilated infants was compared with that of early and late control infants, respectively. Control patients consisted of age-matched nonmacerated fetuses whose intrauterine demise occurred immediately before delivery, or live-born infants who lived for less than 12 hours. Control subjects were age matched with the ventilated infants with respect to the time of death: early control infants or fetuses had died between 23 and 29 weeks PMA, late control subjects between 35 and 39 weeks PMA.

Lung Processing

Autopsies were performed at Women and Infants Hospital according to standard methods. After thorough *in situ* examination, the lungs were dissected and weighed. Biopsies taken from the right upper lobe were treated with RNAlater (Ambion, Inc., Austin, TX) for molecular analyses, as described previously (44). The remainder of the right lung was immersed in formalin. The left lung was inflation-fixed with formalin at a standardized pressure of 20 cm H₂O.

Analysis of Angiogenic Factor Expression by RNase Protection Assay

The RNase protection assay (RPA) was used to detect and quantify angiogenesis-related mRNAs in lung tissue. Total cellular RNA was isolated from lung tissue according to the method of Chomczynski and Sacchi (45). Subsequently, RPA was performed with a RiboQuant RNase protection assay kit with the hAngio-1 human angiogenesis multiprobe template set (BD Biosciences, San Jose, CA) containing DNA templates for Flt-1, Flt-4, TIE, thrombin receptor, TIE-2, CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), endoglin, Ang-1, VEGF, and VEGF-C and the housekeeping gene product, L32. Specific antisense cRNA probes were synthesized with [α -³²P]UTP in an *in vitro* transcription reaction, and 10- μ g samples of total RNA were hybridized for 16 hours at 56°C. After RNase and proteinase K treatment, the RNA:RNA duplexes were heat denatured and resolved on a 5% denaturing polyacrylamide gel. Dried gels were exposed to X-AR film (Kodak, Rochester, NY) at -70°C. The resulting bands were scanned and quantified with PhotoShop (Adobe Systems, San Jose, CA) and Image NIH software (National Institutes of Health, Bethesda, MD). Band intensities were normalized to that of L32 in the same reaction.

Western Blot Analysis of Endoglin Expression

Pulmonary endoglin protein levels were evaluated by Western blot analysis of whole lung lysates, as described in detail elsewhere (44, 46), using monoclonal mouse anti-human endoglin antibody (clone SN6h; DakoCytomation, Inc., Carpinteria, CA). Human umbilical vein endothelial cell (VEC Technologies, Inc., Rensselaer, NY) lysates were used as positive control.

Immunohistochemical Analysis

Sections of left lung were processed for avidin-biotin-immunoperoxidase staining, using anti-endoglin antibody and anti-PECAM-1 antibody (goat polyclonal anti-PECAM-1, M-20, sc-1506; Santa Cruz Biotechnology, Santa Cruz, CA). Binding was detected with 3,3'-diaminobenzidine tetrachloride (DAB). Sections were lightly counterstained with Mayer's hematoxylin, cleared, and mounted. Controls for specificity consisted of omission of the primary antibody, which abolished all immunoreactivity.

All lung sections were prepared and stained in a single session for immunohistochemical analysis. Semiquantitative analysis of the endoglin-immunostained sections was performed by a single observer blinded with respect to the nature of the study group. At least 25 randomly selected fields (magnification, $\times 200$) were scored for intensity and distribution of staining (Table 1). The intensity and distribution scores were combined to obtain a cumulative immunoreactivity score for each case.

TABLE 1. SYSTEM FOR SCORING IMMUNOREACTIVITY

	0	1	2	3
Intensity	Negative	Weak	Moderate	Strong
Distribution (% of ×20 power fields)	<10	10–25	26–50	>50

Data Analysis

Values are expressed as means ± SD. The significance of differences between ventilated and control groups was determined by unpaired Student's *t* test or Mann-Whitney U test, where applicable. The significance level was set at *P* < 0.05. StatView software (Abacus, Berkeley, CA) was used for all statistical work.

RESULTS

Patients

The age and sex distribution, relevant clinical data, and general autopsy findings of control and ventilated infants analyzed by RPA are summarized in Table 2. The mean postmortem interval (time interval between death and autopsy) ranged from 8 to 63 hours and was not significantly different between the various study groups.

RNase Protection Assay of Pulmonary Angiogenesis-related Gene Expression

The mRNA levels of angiogenesis-related genes were assayed by multiprobe RPA. As shown in Figure 1, endoglin mRNA levels, normalized to the constitutively expressed housekeeping gene L32, were significantly more than threefold higher in short-term ventilated lungs ("early") compared with control subjects (*P* < 0.05). In sharp contrast to the endoglin up-regulation, the levels of VEGF and its receptor, Flt-1, were markedly lower in short-term ventilated lungs compared with nonventilated control subjects (Table 3). Levels of Ang-1 were low in both control and short-term ventilated groups, although densitometric analysis of a longer exposed radiograph revealed significantly decreased levels in ventilated lungs compared with control subjects. Similarly, the mRNA level of the Ang-1 receptor, TIE-2, were lower in ventilated lungs (Table 3).

Endoglin mRNA levels in long-term ventilated lungs ("late") were overall higher than in lungs of age-matched late control subjects (Figure 1). However, greater variability between individual cases precluded statistical significance with the

sample sizes available for analysis. As in short-term ventilated lungs, the VEGF mRNA levels were consistently lower in long-term ventilated lungs with established BPD compared with late control subjects (Figure 1).

Western Blot Analysis of Pulmonary Endoglin Expression

Endoglin protein expression in lungs of short-term ventilated and early control infants was assayed by Western blot analysis of whole lung homogenates, according to methods described in detail elsewhere (44). Samples (*n* = 5 in each group) were obtained from the patients described in Table 2, based on the availability of good-quality protein. Representative results are shown in Figure 2. Appropriately sized (~170 and ~160 kD) endoglin-immunoreactive protein bands were detected, consistent with the L- and S-endoglin isoforms, respectively. The pulmonary L-endoglin levels of ventilated infants were more almost 10-fold higher than those of age-matched control subjects (*P* < 0.01). A smaller increase was noted for the S-endoglin isoform. Results of densitometric quantitation of L-endoglin band intensities, normalized to actin, are shown in Figure 2B.

Endoglin and PECAM-1 Immunohistochemistry

Morphologic and immunohistochemical analysis was performed in a slightly different patient group, as dictated by the availability of high-quality paraffin-embedded material. Table 4 shows the relevant clinical data of patients included for immunohistochemical analysis. Lungs of early control and short-term ventilated infants (23 to 29 wk of gestational age) showed histologic features characteristic of the early saccular stage of lung development: the peripheral lung parenchyma was composed of large-sized primitive acini, separated by relatively wide septa, showing focal septation of the acinar units by vascularized ridges ("secondary crests") (Figure 3A). Compared with control lungs, the lungs of short-term ventilated infants showed varying degrees of septal widening and hypercellularity, often associated with focal pulmonary hemorrhage and interstitial emphysema (Figure 3B).

PECAM-1 (CD31) immunostaining of early control lungs at 23–29 weeks (late canalicular/saccular stage) revealed a double capillary network within the septa that was frequently localized immediately beneath the alveolar epithelial lining (Figure 3C). Lungs of short-term ventilated infants of the same age displayed abundant microvascular structures within the septa, both sub-epithelially and centrally (Figure 3D). Although the size of the

TABLE 2. CLINICAL DATA OF PATIENTS STUDIED BY RNase PROTECTION ASSAY

	Early (23–29 wk)		Late (32–39 wk)	
	Control (<i>n</i> = 10)	Ventilated (<i>n</i> = 7)	Control (<i>n</i> = 5)	Ventilated (<i>n</i> = 5)
Age at birth*, wk	25.7 ± 2.3	24.6 ± 2.0	34.5 ± 3.7	27.0 ± 4.2
Postnatal age, d	<0.5	8.9 ± 4.7	<0.5	58.3 ± 26.2
Corrected age at death*, wk	25.8 ± 2.4	26.1 ± 2.3	35.0 ± 3.8	36.8 ± 4.2
Sex	5 M/5 F	5 M/2 F	3 M/2 F	3 M/2 F
Body weight at autopsy, g	725 ± 184	782 ± 265	2,616 ± 753	3,053 ± 862
Postmortem interval, h	27.3 ± 13.2 (8–48)	35.8 ± 20.9 (16–63)	31.8 ± 13.3 (22–51)	21.8 ± 9.5 (10–32)
Clinical/autopsy diagnosis	PROM (3); chorioamnionitis ± sepsis (4); placental insufficiency (1); COD undetermined (2)	Early BPD with complications of prematurity (3); early BPD with sepsis (4)	Sepsis (2); COD undetermined (2); abruption (1)	BPD (1); BPD with sepsis/pneumonia (1); BPD with NEC (1); BPD with viral infection (2)

Definition of abbreviations: BPD = bronchopulmonary dysplasia; COD = cause of death; F = female; M = male; NEC = necrotizing enterocolitis; PROM = premature rupture of membranes.

Values represent means ± SD of (*n*) patients.

* Age and corrected age reflect postmenstrual age.

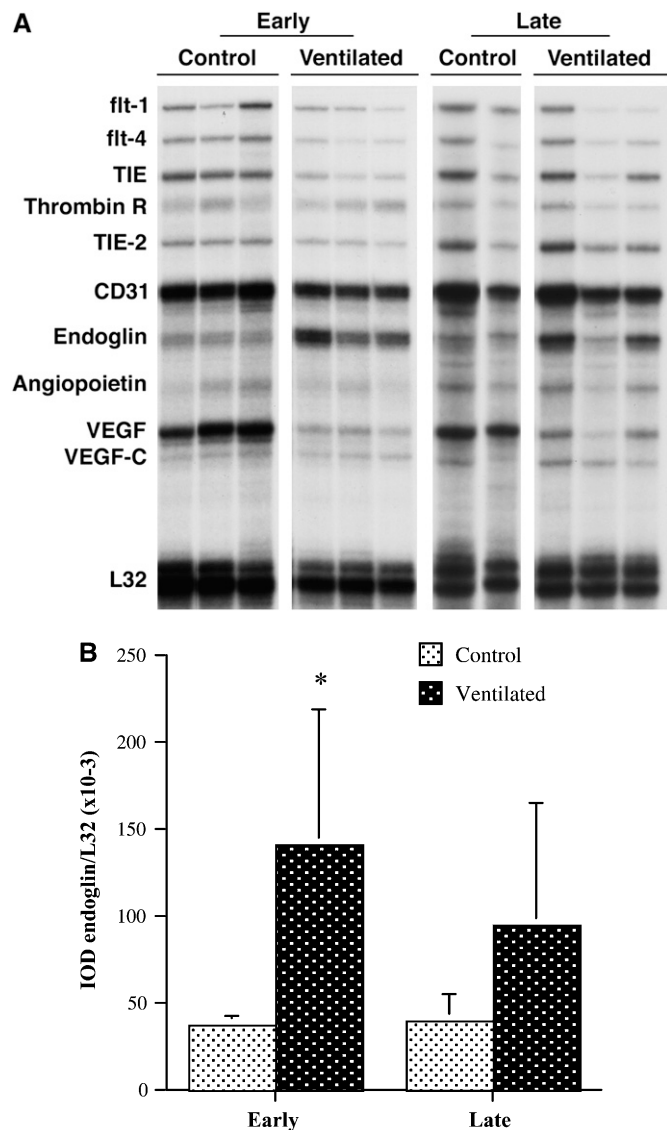


Figure 1. RNase protection assay of angiogenesis-related gene expression. (A) Representative RNase protection assay (RPA) of angiogenesis-related gene expression in whole lung homogenates of control and ventilated preterm infants. (B) Densitometry of endoglin RPA analysis. Values represent means \pm SD. * $P < 0.05$ versus control.

septal microvessels varied greatly, they were mostly of small caliber and were haphazardly arranged within the interstitium.

The cellular distribution of endoglin was determined by immunohistochemical analysis, using a monoclonal anti-human endoglin antibody (Figures 3E and 3F). Endoglin immunoreactivity was associated with microvascular structures within the peripheral lung parenchyma, as well as larger sized central vascular structures. Considerable variability in endoglin immunostaining was noted between patients of the same study group, which may, at least in part, be due to differences in protein degradation and length of fixation inherent in autopsy material. Overall, however, the cumulative immunoreactivity score, which takes into account both intensity and distribution of staining, was higher in the lungs of short-term ventilated patients compared with control patients (see Figure 5).

Lungs of late control patients showed thin septa with abundant secondary crests and focal evidence of early alveolariza-

TABLE 3. DENSITOMETRY OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOPOIETIN RNase PROTECTION ASSAY

	Early Control (n = 10)	Short-Term Ventilated (n = 7)
VEGF	283.9 \pm 95.4	22.3 \pm 4.7*
Flt-1	22.2 \pm 18.1	4.8 \pm 4.2†
Ang-1	24.0 \pm 15.8	10.7 \pm 4.7†
TIE-2	15.3 \pm 2.0	5.6 \pm 2.9*

Definition of abbreviations: Ang-1 = angiopoietin-1; Flt-1 = Fms-like tyrosine kinase-1/VEGF receptor-1; TIE-2 = TIE receptor tyrosine kinase-2 (angiopoietin-1 receptor); VEGF = vascular endothelial growth factor.

Values represent mean integrated optical density (IOD)/IOD L32 \pm SD ($\times 10^{-3}$) of (n) samples.

* $P < 0.01$ versus control.

† $P < 0.05$ versus control.

tion, consistent with the late saccular/early alveolar stage of development (Figure 4A). The lungs of age-matched long-term ventilated patients showed large and simple airspaces, separated by wide and hypercellular septa, characteristic of fully established BPD (Figure 4B). PECAM-1 immunostaining of late control lungs revealed a delicate single capillary network within the thin septa, displaying abundant branching points into secondary crests (Figure 4C). The PECAM-1-immunoreactive microvascular network of long-term ventilated BPD lungs exhibited the double “tram-track” appearance typical of more primitive stages, associated with a striking paucity of sprouting (Figure 4D). Endoglin immunoreactivity in late control lungs was weak and

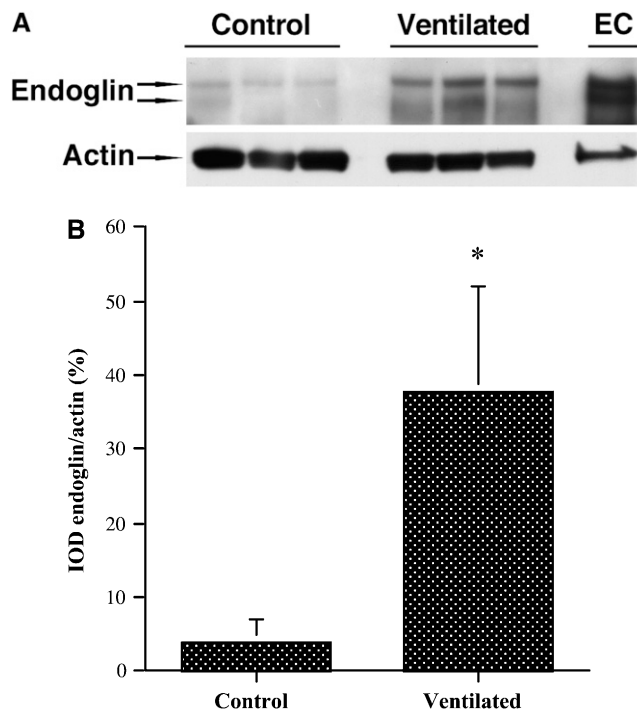


Figure 2. Western blot analysis of pulmonary endoglin protein levels in early control subjects and short-term ventilated patients. (A) Representative Western blot analysis of endoglin protein expression in whole lung homogenates. Appropriately sized bands were detected for endoglin (approximately 170 and 160 kD). Actin (42 kD) served as internal loading control. Human umbilical vein endothelial cells (EC) served as positive control. (B) Densitometry of 170-kD L-endoglin Western blot analysis. Values represent means \pm SD of five samples in each group. * $P < 0.01$ versus control. IOD = integrated optical density.

TABLE 4. CLINICAL DATA OF PATIENTS STUDIED BY IMMUNOHISTOCHEMISTRY

	Early (23–29 wk)		Late (32–39 wk)	
	Control (n = 6)	Ventilated (n = 5)	Control (n = 4)	Ventilated (n = 5)
Age at birth*, wk	25.3 ± 1.9	25.8 ± 2.4	37.0 ± 2.7	27.2 ± 2.7
Postnatal age, d	<0.5	11.6 ± 5.4	<0.5	54.0 ± 6.5
Corrected age at death*, wk	24.8 ± 2.0	27.2 ± 3.4	37.4 ± 2.9	35.8 ± 3.1
Sex	2 M/4 F	2 M/3 F	2 M/2 F	3 M/2 F
Body weight at autopsy, g	692 ± 180	853 ± 235	2,808 ± 484	3,492 ± 967
Clinical/autopsy diagnosis	PROM/extreme prematurity (3); chorioamnionitis ± sepsis (1); COD undetermined (2)	Early BPD with complications of prematurity (3); early BPD with sepsis (2)	Sepsis (1); intrapartum death/placental insufficiency (1); maternal fatty liver of infancy (1); abruption (1)	BPD (1); BPD with sepsis/pneumonia (1); BPD with NEC (1); BPD with viral infection (2)

For definition of abbreviations, see Table 2.

Values represent means ± SD of (n) patients.

* Age and corrected age reflect postmenstrual age.

limited to scattered centrally located microvascular structures (Figure 4E). Endoglin staining in long-term ventilated BPD lungs was highly variable. Overall, the immunoreactivity score tended to be higher in patients with BPD than in late control subjects (Figures 4E and 5). However, this difference did not reach significance, which may be due, at least in part, to the relatively small sample size available for analysis.

DISCUSSION

We have previously demonstrated that the pulmonary microvasculature of ventilated preterm infants undergoes marked growth, nearly proportionate to the expansion of the distal air-exchanging lung parenchyma (10). Although quantitatively almost normal, the microvasculature of ventilated lungs displays a primitive, nonbranching architecture, suggestive of a failure of angiogenesis (10). Angiogenesis, here defined as the sprouting of new vessels from preexisting ones, is a complex and highly coordinated process that requires endothelial cell activation, proliferation and directional migration, extracellular matrix generation and remodeling, recruitment of mural cells (pericytes and smooth muscle cells), and specialization of the vessel wall for regulation of vessel function (47–49). Orchestration of these complex functions is regulated by various pro- and antiangiogenic growth factors, angiogenic chemokines/cytokines, matrix-degrading proteases, and cell–extracellular matrix interactions (47–50).

Previous studies of the molecular regulation of angiogenesis in BPD lungs have focused mainly on the major angiogenic growth factors traditionally associated with vascular morphogenesis, such as VEGF and Ang-1 (9, 12, 19–22). In view of the important role of the accessory TGF- β receptor endoglin (CD105) in a variety of neoplastic and nonneoplastic angiogenic conditions (23–30), we speculated that endoglin may be implicated in ventilation-induced angiogenesis in preterm lungs as well.

Endoglin expression was studied in postmortem lung samples from short- and long-term ventilated preterm infants. We determined that endoglin mRNA and protein levels were significantly higher in short-term ventilated lungs than in age-matched control lungs; endoglin was immunolocalized to the peripheral microvasculature. A similar tendency to increased endoglin mRNA and protein levels was noted in long-term ventilated lungs with fully established BPD. In this group, however, a considerably wider range of endoglin levels was noted, which may be related to the greater clinical variability associated with a more protracted stay in neonatal intensive care. In concordance with previous reports in humans and BPD-like animal models, we determined that pulmonary levels of

VEGF and Ang-1 and of their respective receptors, Flt-1 and TIE-2, were significantly lower in ventilated infants (9, 12, 19).

The significant up-regulation of pulmonary endoglin in short-term ventilated lungs, especially in the context of down-regulated VEGF and Ang-1, suggests that endoglin may be an important angiogenic factor in ventilation-induced vascular remodeling. Similarly, endoglin up-regulation has been described in a rapidly increasing number of neoplastic and non-neoplastic conditions characterized by prominent angiogenesis (26–30). Endoglin is an accessory receptor for TGF- β (51), a pleiotropic multifunctional cytokine involved in cellular proliferation, differentiation, and migration (52, 53). Endoglin is believed to stimulate endothelial proliferation by interference with inhibitory TGF- β signaling, possible by modulating the balance between the TGF- β /activin receptor-like kinase-1 (ALK1) and TGF- β /ALK5 signaling pathways (23, 24, 54–56).

The functional involvement of endoglin in angiogenesis is based mainly on evidence derived from loss-of-function clinical or experimental conditions. Mutations in the endoglin gene have been implicated in the autosomal dominant vascular disorder termed hereditary hemorrhagic telangiectasia type I (41). Endoglin null mice (Eng^{-/-}) are embryonally lethal, dying *in utero* around midgestation because of defects in cardiovascular development and angiogenesis, leading to dilated vessels prone to rupture and hemorrhages (38–40). Mice heterozygous at the endoglin locus (Eng^{+/-}) mature to adulthood and can develop hereditary hemorrhagic telangiectasia (38).

The *in vivo* effects of endoglin overexpression on capillary growth patterns are, to our knowledge, unknown. Endoglin has been shown to regulate the expression of various components of the extracellular matrix including fibronectin, collagen, plasminogen activator inhibitor-1, and lumican (57–60), suggesting a crucial role of endoglin in cellular transmigration. In mouse fibroblasts in culture, endoglin overexpression has been shown to alter cellular morphology, migration, and intercellular cluster formation and to modulate extracellular matrix production (58). Although these studies suggest that endoglin may play a role in cellular migration and extracellular matrix remodeling essential for capillary sprouting, the functional contribution of endoglin upregulation to the primitive, nonbranching angioarchitecture of BPD lungs remains to be determined.

Endoglin is a disulfide-linked homodimeric membrane glycoprotein. Molecular cloning of the human endoglin cDNA has demonstrated the existence of two protein isoforms, arising by alternative splicing. L-endoglin, the predominant isoform, has a cytoplasmic domain of 47 residues, whereas the minor isoform, S-endoglin, contains a cytoplasmic tail of only 14 amino acids (61, 62). Both endoglin isoforms can bind ligand (62), but

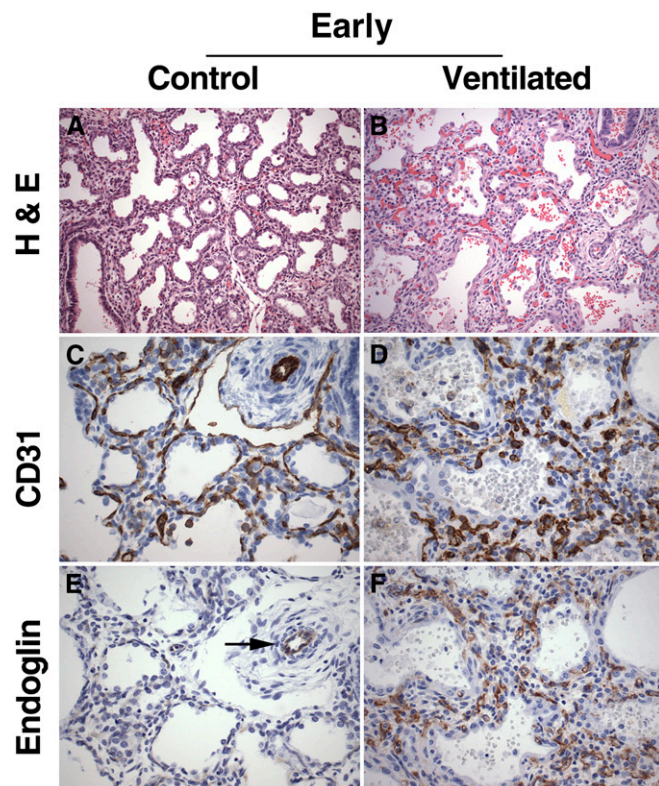


Figure 3. Lung histology and platelet endothelial cell adhesion molecule (PECAM)-1/endoglin immunohistochemistry of early control subjects and short-term ventilated patients. (A) Early control lung showing large-sized, simple airspaces, relatively wide septa, and focal early secondary crest formation, characteristic of late canalicular/early saccular stage of lung development (infant born at 24.5 wk of gestation, lived for 30 min). (B) Short-term ventilated lung showing widening and increased cellularity of the septa, associated with focal hemorrhages within the air spaces (infant born at 23 wk, lived for 5 d, ventilated). (C) PECAM-1 (CD31) immunostaining of early control lung, highlighting the double subepithelial capillary network characteristic of the canalicular/saccular stage of lung development. (D) PECAM-1 (CD31) immunostaining of short-term ventilated lung, showing abundant, disarrayed capillary structures within the expanded septa. (E) Endoglin immunostaining of early control lung corresponding to (C), showing endoglin immunoreactivity in association with a peripheral arteriole (*arrow*). Endoglin staining is virtually absent in capillary structures within the septa. (F) Endoglin immunostaining of short-term ventilated lung corresponding to (D), showing abundant endoglin-positive capillary structures within the widened septa. (A and B) Hematoxylin–eosin staining (H&E); (C and D) PECAM-1 (CD31) immunohistochemistry, DAB (3,3'-diaminobenzidine tetrachloride) with hematoxylin counterstain; (E and F) endoglin (CD105) immunohistochemistry, DAB with hematoxylin counterstain. Original magnification: (A and B) $\times 200$; (C–F) $\times 400$.

differ in their capacity to regulate TGF- β -dependent responses (63). Similar L- and S-endoglin variants have been identified in murine tissues (64). In mice, S-endoglin is believed to have antiangiogenic functions, in contrast to the proangiogenic influence of L-endoglin (64). In our study, Western blot analysis identified two endoglin-immunoreactive bands of approximately 170 and 160 kD, consistent with the L-endoglin and S-endoglin dimers, respectively. In accordance with previous reports, L-endoglin was the dominant form in human lung homogenates. Although both isoforms appeared to be upregulated in short-term ventilated lungs, the increase was more

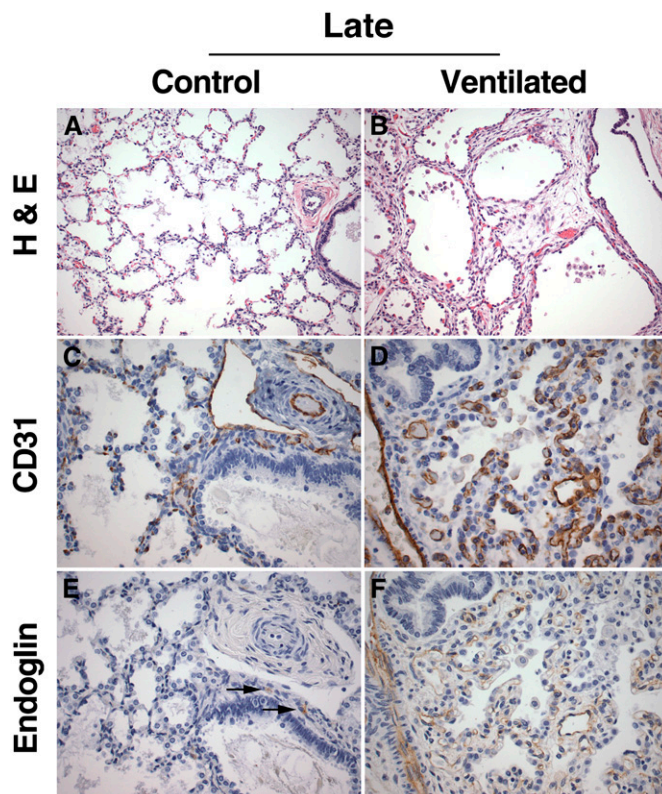


Figure 4. Lung histology and platelet endothelial cell adhesion molecule (PECAM)-1/endoglin immunohistochemistry of late control subjects and long-term ventilated patients. (A) Late control lung showing late saccular/early alveolar architecture characterized by thin alveolar septa and abundant secondary crests (nonmacerated stillborn delivered at 36 weeks gestation, cause of death unknown). (B) Long-term ventilated lung showing simple, large-sized airspaces with wide and hypercellular septa (infant born at 24 weeks, lived for 56 days, ventilated). (C) PECAM-1 (CD31) immunostaining of late control lung, showing branching capillary structures within the alveolar septa and immunoreactive endothelium in central arterial and lymphatic structures. (D) PECAM-1 (CD31) immunostaining of long-term ventilated lung, showing abundant, primitive-appearing and predominantly non-branching capillary structures within the expanded septa. (E) Endoglin immunostaining of late control lung corresponding to (C), showing weak endoglin immunoreactivity limited to rare central capillary structures (*arrows*). Endoglin staining is absent in the interstitial microvasculature. (F) Endoglin immunostaining of long-term ventilated lung corresponding to (D), showing diffuse endoglin immunoreactivity in association with capillary structures in the widened septa. (A and B) Hematoxylin–eosin staining (H&E); (C and D) PECAM-1 (CD31) immunohistochemistry, DAB (3,3'-diaminobenzidine tetrachloride) with hematoxylin counterstain; (E and F) endoglin (CD105) immunohistochemistry, DAB with hematoxylin counterstain. Original magnification: (A and B) $\times 200$; (C–F) $\times 400$.

pronounced for the L-isoform. Whether this implies that the net effect of pulmonary endoglin upregulation in ventilated preterm lungs is proangiogenic remains undetermined.

In addition to its known role in cardiovascular development and remodeling (38–41), endoglin has other functions that may potentially contribute to the pathogenesis of BPD in ventilated preterm lungs. Endoglin has been shown to play a role in actin cytoskeletal organization (65). In endothelial cells, where endoglin is highly expressed, actin filaments play a crucial role in angiogenesis and vascular permeability. It is possible that upregulated pulmonary endoglin may modulate vascular permeability

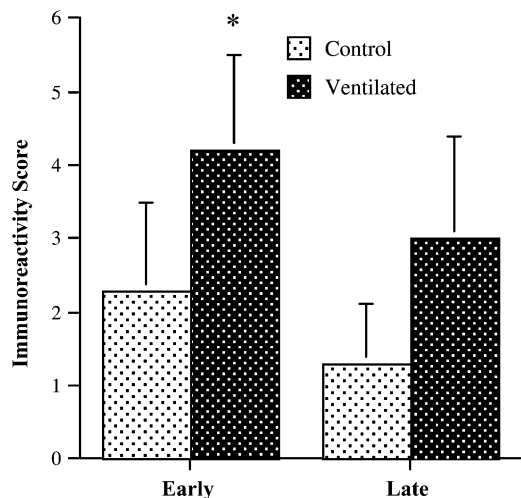


Figure 5. Endoglin immunoreactivity score. Cumulative immunoreactivity score, obtained by adding distribution and intensity scores. Values represent means \pm SD. * $P < 0.05$ (Mann-Whitney U test).

in ventilated preterm lungs, as interstitial edema is a morphologic hallmark of early BPD (10).

The regulation of endoglin overexpression in ventilated preterm lungs remains unclear. The levels of endoglin protein, mRNA, and promoter activity are known to be up-regulated by hypoxia (66) and by TGF- β_1 (63), which cooperate to induce the expression of endoglin at the transcriptional level (66). Although hypoxia may play a role in BPD-associated endoglin upregulation, the importance of other factors implicated in BPD, such as oxygen toxicity, infection/inflammation, glucocorticoids, and mechanical stretch/strain² remains to be determined. In this context, it needs to be emphasized that the terms “short-term ventilated” and “long-term ventilated,” used in this study to describe preterm infants at risk for BPD and older post-premature infants with established BPD, respectively, are mainly terms of convenience and not meant to imply that ventilation is deemed the sole or major cause of endoglin up-regulation or BPD.

Of note, this study was entirely based on postmortem human lung tissues, traditionally regarded as unsuitable for molecular analyses. For this study, no attempt was made to expedite the postmortem examination, which resulted in postmortem intervals (time between death and autopsy) often longer than 24 hours. Of interest, several samples obtained more than 24 hours after death still yielded high-quality protein and RNA, adequate for Western blot analysis and RPA.

In summary, we have documented upregulation of endoglin in the pulmonary microvasculature of ventilated preterm infants at risk for BPD. This study, performed with postmortem lung tissues, addresses the most severe (fatal) end of lung injury produced by ventilation and preterm birth. However, it is likely that similar events may occur in less severe nonlethal preterm lung injury. Although we focused on endoglin as a potential alternative angiogenic factor in BPD, it is conceivable that other factors such as angiogenic growth factors, proinflammatory chemokines, and extracellular matrix components may contribute to BPD-associated dysangiogenesis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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