

Prenatal administration of vitamin A alters pulmonary and plasma levels of vascular endothelial growth factor in the developing mouse

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Summary

Vitamin A and the retinoids play a unique role in mammalian embryonic and foetal development and are essential for both cellular differentiation and the establishment of normal morphogenesis. Vascular endothelial growth factor (VEGF) is a known potent mitogenic factor that plays a key role in lung development and function maintenance. In order to contribute to a better knowledge of the modulating effects of vitamin A in lung development, we investigated the effects of the antenatal administration of vitamin A on VEGF expression in lungs and plasma from foetuses and neonates. Pregnant mice were subjected to subcutaneous administration of vitamin A on the 12th gestational day. The lungs and plasma from foetuses and neonates were collected daily from the 15th gestational day till the day of birth. Our results show that vitamin A modulates VEGF concentrations both in lungs and plasma. Statistically significant differences were observed at gestational days 15 ($P = 0.004$ for lungs; $P < 0.0001$ for plasma), 16 ($P < 0.0001$ for lungs and plasma) and 18 ($P < 0.0001$ for lungs; $P < 0.05$ for plasma). Vitamin A tends to increase the expression of this factor in the lung, particularly during the critical period of perinatal adaptation to postnatal life. These effects seem to be spatial and temporally regulated, and point out to the important role of vitamin A during lung development.

Keywords

foetal development, lung, plasma, VEGF, vitamin A

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Vitamin A and its related compounds, the retinoids, play a unique role in mammalian embryonic and foetal development and are essential for both cellular differentiation and the establishment of normal morphogenesis (Rodrigues *et al.* 2004).

Vessel development in the early lung determines lung structure maturation, and both undisturbed angiogenesis and vasculogenesis are necessary for the successful building of the organ (Healy *et al.* 2000; Voelkel *et al.* 2006). Several signalling pathways including the vascular endothelial

growth factor (VEGF), glucocorticoid and retinoic acid (RA) have been implicated in regulating lung maturation and vascular development (Morrisey & Savani 2003).

It has been demonstrated that RA is required for the normal alveolar development in the mouse, and exogenous administration of RA restores alveolar architecture to the lungs of dexamethasone-treated mice (Hind & Maden 2004; Maden & Hind 2004; Maden 2006). Retinoic acid maintains alveolar development in mice treated with an angiogenesis inhibitor (Cho *et al.* 2005), and is required to control endothelial cell maturation and cell cycle progression during vasculogenesis and angiogenesis (Lai *et al.* 2003).

Vascular endothelial growth factor is a potent mediator of angiogenesis, which has multiple effects in lung development and physiology. It has been reported that lung presents the highest level of VEGF gene expression among normal tissues (Kaner *et al.* 2000; Voelkel *et al.* 2006). Vascular endothelial growth factor is also involved in the pathophysiology of some of the most common respiratory disorders (Papaioannou *et al.* 2006). One of these conditions is bronchopulmonary dysplasia (BPD). Infants dying from BPD have reduced pulmonary levels of VEGF (Bhatt *et al.* 2001), and intrauterine or postnatal intratracheal instillation of VEGF protects preterm mice against respiratory distress syndrome (RDS) (Compernelle *et al.* 2002). It was verified that VEGF plays a central role in the epithelial–endothelial interactions that are critical for normal lung development (Acarregui *et al.* 1999; Brown *et al.* 2001; Compernelle *et al.* 2002; Zhao *et al.* 2005). Vascular endothelial growth factor seems to interfere in a different manner, depending on its amount, the location and the underlying physiological or pathological process in lung tissue (Papaioannou *et al.* 2006). Recent studies have correlated RA and VEGF during lung development, hypothesizing that inhibition of alveolar development is associated with a block in angiogenesis due to the down-regulation of VEGF and VEGFR2, which is prevented by treatment with RA (Cho *et al.* 2005). It has been shown that RA regulates the expression of several signalling molecules including VEGF in tissues such as skin and brain development (Maden *et al.* 1996; Lachgar *et al.* 1999; Diaz *et al.* 2000). At this time, however, it is not known whether these RA functions are applicable during foetal lung development. On the other hand, clinical trials showed that vitamin A supplementation given largely to infants <1 kg resulted in a decrease in death and/or BPD without increasing mortality or neurodevelopment impairment at 18 to 22 months (Ambalavanan *et al.* 2005). Thus, interventions with vitamin A to treat infants with RDS and prevent its complications are promising, but further research must be conducted (Carlo

et al. 2004). In order to contribute to a better knowledge of vitamin A effects and its influence in the lung maturation process we investigated if the antenatal administration of vitamin A could alter the lung and plasma concentrations of VEGF in foetuses and neonates.

Materials and methods

Animals

Mice, *Mus musculus*, strain ICR (CD-1[®]; Harlan, Spain) were housed at 22 °C, fed *ad libitum*, and exposed to day–night cycles alternatively every 12 h throughout the study period. Mating was performed according to a polygamist regimen (Broustail 1967). Timed-pregnant mice (visualization of vaginal plug at day 0) were obtained on days 15–18. Pregnant mice received a single subcutaneous injection of vitamin A (150 µl, 45,000 IU of retinyl palmitate; G. Streuli & Co., Uznach, Switzerland) on gestational day 12. At the same gestational day, pregnant mice of control groups received an equivalent volume of diluent alone (saturated median chain triglyceraldehydes, Dub MCT 55/45 EP ex Dubois, Georges Walther AG, Switzerland). Pregnant mice and pups delivered naturally at term gestation were anaesthetized in the same manner. For anaesthesia induction, each animal was placed alone in an induction chamber with gas input and exhaust output lines. Anaesthesia was induced with 5% isoflurane (Isoflo, Esteve Farma Lda., Carnaxide, Portugal) in 100% oxygen with a delivery rate of 5 l/min until loss of righting reflex. The mothers were sacrificed by neck dislocation and the foetuses were delivered by hysterectomy. Pups were sacrificed by cutting large thoracic vessels. The day of birth was designated day 0. Foetuses and neonates were randomized to study groups (treated *vs.* control) similar in number at each time point. All procedures and protocols were approved by the Animal Care and Use Committee of the 'Direção Geral de Veterinária, Portugal'.

Sample collection

Blood was collected from foetuses and neonates by cutting large vessels in the thorax with a hand-modified 20G needle (Sterican 100, B. Braun, Germany) applied to a 1 ml/100 IU syringe (Ominifix 100 solo, B. Braun, Germany) (Pinto *et al.* 2007, personal communication). The needle and syringe were previously passed through a solution of ethylenediaminetetraacetic acid tripotassium salt dihydrate (Sigma, Steinheim, Germany) in NaCl 0.9% (B. Braun, Germany) at 25 mg/ml. Whole blood was centrifuged at 2500 g for 2 min (Minispin, Eppendorf, Hamburg, Germany). Plasma

was collected and stored at -80°C for further analysis. Lungs were collected, frozen and stored at -80°C or processed for paraffin embedding.

ELISA for VEGF

Frozen lungs were placed in 5 ml ice-cold phosphate-buffered saline (PBS) and homogenized using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 5000 g for 5 min at $+4^{\circ}\text{C}$ (Sigma 3K30, Steinheim, Germany) and the supernatant was used to assay VEGF concentration in lungs. ELISAs were performed with a commercial VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions. Briefly, standards and samples were incubated in duplicate wells of a microplate coated with anti-VEGF polyclonal antibody. Unbound substances were washed from the plate, and horseradish peroxidase-linked anti-VEGF polyclonal antibody was added. After incubation and further washing, H_2O_2 and tetramethylbenzidine were added. The reaction was stopped with HCl, and optical densities were read at 450 nm (correction wavelength at 540 nm) on a multiskan plate reader (Labsystems[®] Multiskan MCC/340; Vantaa, Finland). Vascular endothelial growth factor concentration was calculated by comparison with a standard curve generated using a four-parameter logistic curve-fit and on-board software. Vascular endothelial growth factor concentrations were normalized to total protein concentrations determined by the bicinchoninic acid method. Each sample value is the result of three lungs, and 36 lungs (18 treated *vs.* 18 controls) were used at each time point. To assay the VEGF concentrations in plasma the same procedure was applied, but the samples were diluted (50 μl sample + 200 μl of calibrator diluent), and 16 individual values (8 treated *vs.* 8 controls) were obtained at each time point.

VEGF Immunostaining

Paraffin-embedded lung tissue sections (3 μm) were deparaffinized with xylene, and rehydrated through an ethanol-water series. Antigen retrieval was performed by microwaving sections in Tris-HCl (10 mM Tris-HCl, pH 10.0). Endogenous peroxidases activity was quenched using a 3% solution of hydrogen peroxide. Sections were blocked with 'Large volume ultra V block' (Labvision Corporation, Fremont, CA, USA). The slides were then incubated with primary antibody (rabbit polyclonal anti-VEGF Ab-1, 5 $\mu\text{g}/\text{ml}$, Labvision-Neomarkers, Fremont, CA, USA) overnight at $+4^{\circ}\text{C}$. Tissue sections were incubated with 'Biotinylated goat anti-polyvalent secondary antibody', washed, and

incubated with 'Large volume streptavidin peroxidase reagent'. After being rinsed, the sections were incubated with diaminobenzidine (Novocastra, UK) and counterstained with haematoxylin. A negative control, using non-immune goat serum instead of the primary antibody was used. Lungs were viewed under a Nikon FX Photomicroscope (Nikon, Japan) with a $40\times$ lens for immunohistochemical scoring. The degree of positive staining was evaluated by semi-quantitative scoring on a scale of 0 (negative cases) to 3 for intensity (I) and distribution (D) adapted from Ozer *et al.* (2000). Tissues with $I \times D \leq 3$ were considered weakly positive, and those with $I \times D > 3$ were designated strongly positive.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was performed using SPSS 13.0 (Chicago, IL, USA). After checking both assumptions of normality and homogeneity of variances the one-way ANOVA was used to analyse the different groups. Immunohistochemical scores were assessed by the χ^2 test. Statistical significance was set at $P < 0.05$.

Results

Quantitative observations

VEGF pulmonary levels. The ELISA values obtained for VEGF in the lungs of control and vitamin A treated fetuses and neonates are displayed in Figure 1. At gestational day 15, VEGF levels in the lung homogenates from control fetuses are higher (76.5 ± 15.1 ng VEGF/mg total lung protein; $P = 0.004$) than the ones observed in vitamin A treated fetuses (48.05 ± 10.55 ng/mg). From this day onwards, VEGF levels in the lungs from vitamin A treated fetuses increase gradually and reach a maximum of 192.7 ± 42.8 ng/mg at the 18th gestational day. On the opposite, VEGF levels in the lungs from control fetuses suffer a nearly three-fold decrease (26.8 ± 5.4 ng/mg) at the 16th gestational day. A statistically significant difference ($P < 0.0001$) between control and treated fetuses is observed at this day. Pulmonary VEGF levels increase to 70.2 ± 10.2 ng/mg in control fetuses of 17 gestational days, but decrease again to 48.4 ± 11.3 ng/mg by the 18th gestational day. A statistically significant difference ($P < 0.0001$) between control and treated fetuses is also observed at the 18th gestational day. However, at the day of birth, VEGF pulmonary levels in control and vitamin A treated neonates are similar (213.8 ± 37.8 for control and 188.7 ± 48.3 for treated). It is important to point out that

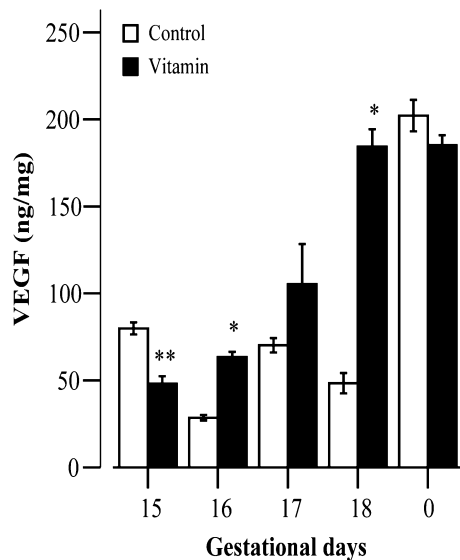


Figure 1 Vascular endothelial growth factor (VEGF) pulmonary levels during foetal development expressed in ng of VEGF/mg of protein (mean \pm SD). Sustained increase of VEGF in the lungs from vitamin A treated foetuses. In the last gestational day, VEGF levels are already similar to the ones observed in the neonate lung. On the opposite, control foetuses display reduced VEGF levels during the perinatal period. ** $P = 0.04$ (*vs.* control); * $P < 0.0001$ (*vs.* control).

pulmonary VEGF levels in vitamin A treated foetuses at the 18th gestational day are similar to those observed at the day of birth, a tendency that is not observed in control subjects (Table 1, Figure 1).

VEGF plasma levels. The results of VEGF quantification in plasma are presented in Figure 2. Vascular endothelial growth factor plasma levels in control foetuses and neonates show a gradual and sustained increase throughout develop-

ment, ranging from 13.7 ± 3.2 pg/ml at the 15th gestational day to 52.6 ± 14.9 ng/ml at the day of birth. A significant increase takes place between the 15th and the 16th gestational days, in which VEGF plasma levels nearly triple (Figure 2). At the 15th gestational day, VEGF plasma levels in treated foetuses (28.1 ± 7.5 pg/ml) more than double the values observed in control foetuses, reaching a statistically significant difference ($P < 0.0001$). A similar difference at gestational day 16 is also observed (Table 2, Figure 2). A prominent decrease in VEGF plasma levels from treated foetuses occurs at the 17th gestational day. From this gestational day and until the day of birth, VEGF levels in treated foetuses increase gradually, despite being inferior to the ones observed in control (Table 2, Figure 2). The difference between control and treated groups is statistically significant ($P < 0.05$) at the 18th gestational day, the day before birth.

Qualitative and semi-quantitative observations

Immunostaining for VEGF protein revealed that in the foetal and neonate lung VEGF is observed both in epithelial and mesenchymal cells. Lungs from control foetuses with 15 gestational days display a strong immunoexpression for VEGF regarding both intensity and distribution patterns (Table 1, $P < 0.05$ *vs.* treated foetuses). Positive cells include columnar and cuboidal cells in the tubular epithelium and the surrounding mesenchymal cells (Figure 3a). At the same age, VEGF immunoexpression in the lungs of treated foetuses is weak, but it is more pronounced in the mesenchyme and in the areas where the interlobar septa will develop (Figure 3b). At the late canalicular stage (16 gestational days) VEGF pulmonary immunoexpression is weak in control subjects, but lungs from treated foetuses display a strong immunoexpression for VEGF, in particular regarding the distribution pattern (Table 1, $P < 0.05$ *vs.* control foetuses). Immunoexpression in the vascular endothelium was also

Gestational days	VEGF immunostaining scores							
	Control				Vitamin A			
	None	Weak	Moderate	Strong	None	Weak	Moderate	Strong
15	0	0	2	2	0	4*	0	0
16	1	3	0	0	0	0	3*	1*
17	1	2	1	0	0	2	2	0
18	0	0	4	0	0	0	4	0
0†	0	0	2	2	0	0	4	0

* $P < 0.05$ (*vs.* control).

†Day of birth.

Table 1 VEGF immunostaining scores (intensity \times distribution)

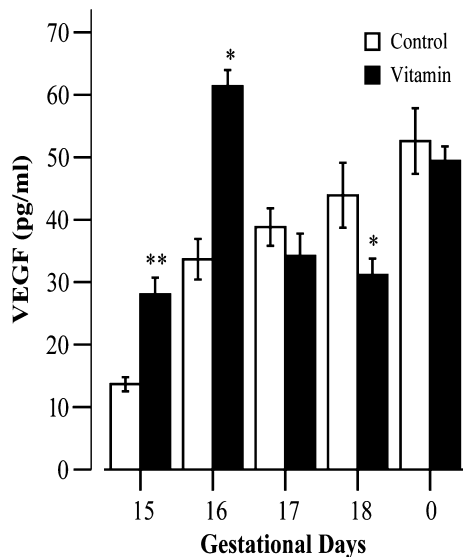


Figure 2 Vascular endothelial growth factor (VEGF) plasma levels during foetal development expressed in pg of VEGF/ml (mean \pm SD). Gradual increase of VEGF plasma levels throughout development in control subjects. A prominent increase at the 15th gestational day is observed, both in control and in vitamin A treated fetuses. In the last gestational day, VEGF plasma levels are higher in control fetuses, reaching a significant statistical difference. At the day of birth, however, control and treated animals display similar VEGF plasma levels. ** $P < 0.05$ (vs. control); * $P < 0.0001$ (vs. control).

observed. At the 17th gestational day, both control and treated groups exhibit strong pulmonary VEGF immunopositive cells throughout lung parenchyma is not homogeneous. The central areas have fewer VEGF expressing cells when compared with the peripheral lung areas, a finding specially pronounced in the control group (Figure 3d). In the lungs of treated fetuses at the late sacular period (18 gestational days) VEGF immunopositive intensity in the sacular parenchyma reaches a peak (Figure 3f), but again the distribution pattern is not homogeneous, and there are areas where the expression is scarce or even absent (Figure 3e,f). At this day, VEGF immunopositive intensity is high in the muscle cells of the vascular walls, but it is weak or even absent in the cuboidal epithelium of the distal airways, both in control and treated fetuses. At the day of birth, VEGF pulmonary immunopositive expression is very similar to that observed on the last prenatal day, but there is a trend towards a higher intensity in control animals, and a more homogeneous distribution pattern in treated ones. At this day, VEGF immunopositive expression is observed in the epithelial

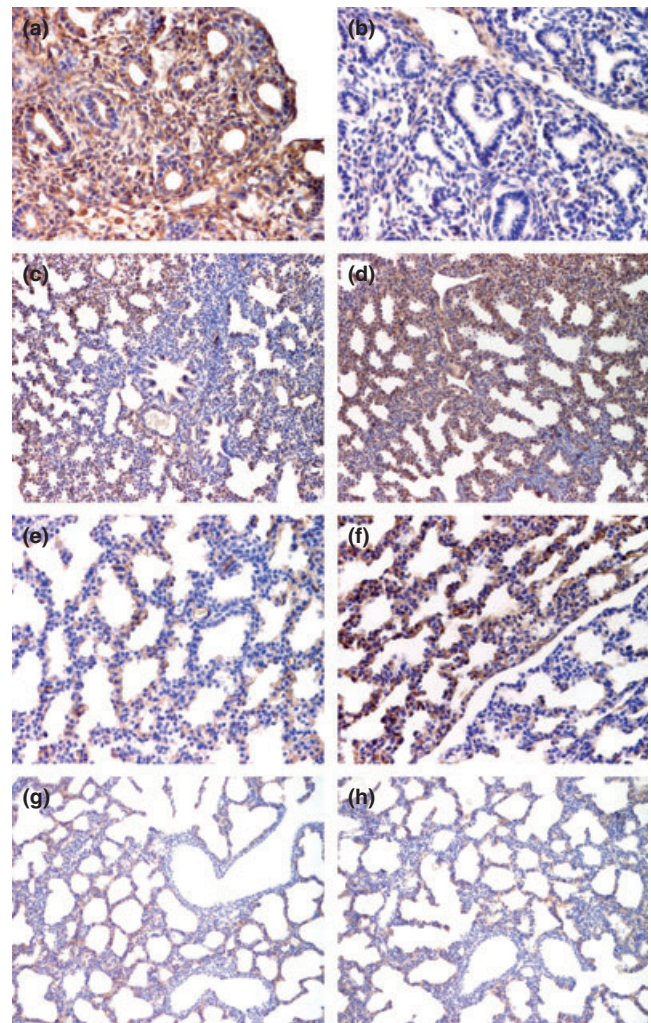


Figure 3 Immunohistochemistry for vascular endothelial growth factor (VEGF) in the lungs of control (a, c, e, g) and vitamin A treated (b, d, f, h) fetuses and neonates. Higher intensity and distribution of VEGF immunopositive patterns in control fetuses at the 15th gestational (a, b). Strong VEGF immunopositive, both for control and for treated fetuses at the 17th gestational day (c, d). The central area depicted in (c) displays fewer immunopositive cells. Higher immunopositive expression in treated fetuses at the 18th gestational day (f). The intensity and distribution expression patterns are different in contiguous areas of the sacular parenchyma. Similar immunopositive for VEGF in control and treated neonates (g, h). Notice the weak or absent immunopositive expression in the cuboidal epithelium of the distal airways. Original magnification $\times 400$ (a, b and e, f). Original magnification $\times 200$ (c, d and g, h).

and endothelial cells of lung parenchyma (Figure 3g,h), as well as in muscle cells of the walls of vascular and large airways.

Discussion

Lung-branching morphogenesis and epithelial development have been the subject of intense investigation over the past 50 years (Warburton *et al.* 2000; Cardoso 2001; Demayo *et al.* 2002). Emerging data indicate that the formation of pulmonary vascular units directs epithelial morphogenesis but the precise signalling pathways involved in this process are still to be determined (Warburton *et al.* 2000; Demayo *et al.* 2002; Cardoso & Lu 2006). A biologically active derivative of vitamin A, RA, plays an important role in both the biochemical (surfactant) and structural maturation (alveolarization) of the developing lung (Torday & Rehan 2004). A large number of physiological effects of VEGF pertain particularly to the lung, which is one of the organs with the highest expression of VEGF in animal systems (Voelkel *et al.* 2006).

This study was conducted to investigate if the antenatal administration of vitamin A, in the period where its teratogenic effects are no longer present, could alter VEGF expression in lungs and plasma from fetuses and neonates.

Our results show that vitamin A modulates VEGF expression in plasma and lungs of fetuses and neonates. Vitamin A treated fetuses present higher VEGF pulmonary levels at all but the 15th gestational day. A particularly critical and delicate period, the antenatal day (18th gestational day), presents the most striking difference in VEGF pulmonary concentrations between control and treated fetuses. In fact, VEGF pulmonary levels in treated fetuses are nearly four times higher than the levels observed in control subjects. In control fetuses, there is a peak in pulmonary VEGF concentrations at gestational day 17. This finding differs from the reports made by Ng *et al.* (2001) and Chang *et al.* (2004) who suggest that there is an increase in VEGF expression throughout lung development, peaking at the 16th gestational day. This discrepancy may be explained by the fact that the commercial ELISA kit used in our study quantifies 120 and 164 mouse VEGF protein isoforms, leaving out the VEGF188 isoform, and that we have quantified proteins and not VEGF mRNAs isoforms, as in the previous studies.

The precise regulation of VEGF pulmonary levels is of critical importance since lung VEGF overexpression causes lung dysmorphogenesis (Akeson *et al.* 2003). Decrease in lung VEGF as a consequence of VEGF neutralization or gene deletion results in poor septal formation and an emphysematous pattern (Gerber *et al.* 1997). Disruption of the VEGF/-VEGF receptor signalling not only reduces pulmonary arterial density, but also leads to impaired alveolarization, emphysema and pulmonary hypertension (Jakkula *et al.*

2000; Kasahara *et al.* 2000; Lassus *et al.* 2001). Furthermore, exogenous RA increases alveolar septal formation in adult tight skin mice, a genetic model associated with a failure in alveolar septation resulting in emphysema (Massaro & Massaro 2000).

In our study, the morphologic observations revealed that the lungs of treated animals did not display any abnormal features. Previous studies made by our group (data not published) have already indicated that the elastic fibres of the lung's interstitial compartment significantly increased in fetuses and neonate mice from vitamin A treated mothers, which is in accordance with the recent studies published by Pierce *et al.* (2007).

The results of our study suggest that the beneficial effects of vitamin A administration seem to work in a synergetic manner with the modulating action of VEGF in lung tissue. This is not surprising taking into account that RA indirectly regulates visceral endoderm survival and production of soluble signals, including VEGF (Bohnsack *et al.* 2004). Moreover, the presence of all-*trans* RA in rat lung cultured endothelial cells increases their nitric oxide production, which is one of the mediators of the VEGF effects (Uruno *et al.* 2005). Additionally, it was also shown recently that all-*trans* RA induces VEGF secretion in umbilical vein endothelial cells culture (Saito *et al.* 2007).

Recent studies also demonstrate that RA attenuates the mild hyperoxic lung injury of newborn mice, with effects on VEGF mRNA expression (Zimová *et al.* 2007). Vascular endothelial growth factor acts as a morphogen, inducing a significant increase in both epithelial- and endothelial-branching morphogenesis (Del Moral *et al.* 2006), and accelerating lung growth in rat foetal lung explants (Shinkai *et al.* 2006).

Our immunohistochemical results, regarding the intensity scores from our study, are in accordance with the results from the studies made by Zhao *et al.* (2005). However, there is a slight discrepancy regarding the type of cells displaying VEGF expression that may be explained by the different techniques used. In the study by Zhao *et al.* (2005) it is mentioned that at the 18.5th gestational day VEGF expression is restricted to a subpopulation of cells that line the sacular walls, and there is no mention of VEGF expression by the muscle cells of the vascular walls, as seen in our study. Even so, they mention that they cannot rule out the expression by other cells. The other cells would include endothelial cells, as we already observed at the 16th gestational day, despite the close proximity of epithelial and endothelial cells. Immunostaining of the vascular endothelium in the lung of fetuses and preterm infants was also observed by Lassus *et al.* (2001). It is interesting to notice that in term infants this was

not apparent, and that VEGF increased rapidly in the tracheal aspirates from preterm infants during the first days of life (Lassus *et al.* 1999). Due to these findings the authors suggested that VEGF may be indicative of pulmonary maturity. Our observations revealed VEGF immunostaining of the vascular endothelium also in term mice (day 0 of life), which can be a normal feature of the mice lung.

To the best of our knowledge, this is the first report that presents results on plasma VEGF levels in foetuses and neonates. Vascular endothelial growth factor plasma levels were previously determined by Lassus *et al.* (1999) in infants with clinically diagnosed RDS. These authors demonstrated that they remained constant during the first postnatal week. Our results show that VEGF plasma levels suffer variations during foetal development, and are modified by the maternal administration of vitamin A. It is interesting to notice that during our study, the days were significantly statistical differences were observed regarding both VEGF plasma and pulmonary levels, where precisely the same (15, 16 and 18 gestational days). Regarding this particular aspect, it is interesting to consider the hypothesis raised by Kaner and Crystal (2001), that high levels of VEGF protein on the respiratory epithelial surface may function as a physiological reservoir. If this is correct, we could then assume that the variations of plasma VEGF levels during foetal development would reflect VEGF variations in the developing lung, the physiological reservoir. The same authors suggested that in healthy humans VEGF protein levels in alveoli are 500 times higher than in plasma. In the present study we cannot directly compare VEGF plasma levels with the ones obtained in the lung homogenates, since these last were normalized to total protein concentrations. Nevertheless, there is a tendency for the plasma and pulmonary VEGF levels to change inversely, which could point out to the role of reservoir displayed by the lung, but further studies need to be made to confirm this.

Blood VEGF data is affected by several confounding circumstances, including circadian rhythm and hypoxia. Hypoxia is one of the major regulators of VEGF production (Papaioannou *et al.* 2006; Voelkel *et al.* 2006). However, Oltmanns *et al.* (2006) found that in contrast with *in vitro* studies (Ferrara *et al.* 1992; Shweiki *et al.* 1992; Namiki *et al.* 1995; Fredriksson *et al.* 2000; Mick *et al.* 2002), hypoxia decreases plasma concentrations in adult human healthy subjects. Therefore, systemic VEGF concentration may be differently regulated than the expression on a cellular basis. Whether this is also true for foetal development, and if the physiologic 'hypoxic environment' of the developing foetus decreases VEGF in plasma is still to be addressed. Circadian rhythm can also affect VEGF blood levels, but in order to avoid variations

related to this factor the sacrifice and sample collection were consistently made at the same hour of the day.

Despite very recent studies having demonstrated that the repression of VEGF expression by premature birth and mechanical ventilation was not altered by vitamin A supplementation (Pierce *et al.* 2007), we demonstrated that maternal vitamin A administration influences foetal and neonate VEGF pulmonary and plasma levels, and that VEGF/RA is one of the signalling pathways involved in this process. Furthermore, our data support the hypothesis that the maternal antenatal vitamin A administration can enhance foetal lung development. We may therefore speculate that, despite the differences between lung alveolar development in mice and humans, vitamin A antenatal administration could be beneficial for the human foetus. However, further studies and clinical trials must be performed to address this question. Bearing in mind this perspective, the present results could make an important contribution towards future appropriately designed strategies to prevent the risk of RDS and BPD in premature neonates.

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