Summary. Vascular endothelial growth factor (VEGF) is an endothelial cell growth factor expressed in normal lung tissue. The aim of the study was to investigate the expression of VEGF and its repercussions as regards alveolarization in the developing rat lung. We studied pulmonary VEGF expression at 0 and 14 days of life in Wistar rats. Rat pups were exposed to hypoxia for two hours during the first hours of life and recovered under conditions of hyperoxia or normoxia for a further two hours, or not recovered. The animals of the control group were only exposed to conditions of normoxia. Our results showed that VEGF was increased in the lungs of the animals that were exposed to hypoxia but we did not find any correlation with the septation. The VEGF was decreased in the lungs of animals exposed to hyperoxia after neonatal hypoxia. We observed this at 0 and 14 days of life, and it was correlated with a lower degree of alveolarization at 14 days of life. Our data suggest that hyperoxia after neonatal hypoxia at birth may give rise to a decrease in the expression of VEGF, possibly permanently, together with a reduction in alveolar development.

Key words: VEGF, Hypoxia, Hyperoxia, Neonatal rats, Lung development, Alveolarization

Introduction

Preterm delivery is a major health care problem, affecting 10% of all births and accounting for more than 85% of all perinatal complications and deaths (Goldenberg et al., 2000). In this context, we studied the lungs of newborn rats because they provide a model similar to the preterm human lung (Burri, 1974; Thurlbeck, 1993). It should be noted that normal lung development takes place in the relative hypoxic environment of the uterus (Lee et al., 2001).

The transition from foetal to ambient (21%) O₂ concentrations creates a relative oxidative stress for all newborns, but especially so in preterm neonates, whose lung development has been interrupted. Hypoxia is most commonly seen in babies born prematurely. Premature babies born during the late canalicular or early alveolar stage of lung development have a reduced area for gas exchange and an immature surfactant system. Supraphysiological concentrations of O₂ (hyperoxia) are often used to treat preterm newborns during the first hours of life (Haworth and Hislop, 2003). In previous works, we found morphometric changes in the lungs of neonatal rats after hypoxic or hyperoxic exposure (Pedraz et al., 2003; San Feliciano et al., 2005).

The mechanisms that regulate normal alveolar development are not yet well established. Interactions between airways and blood vessels are critical for normal lung development, suggesting that a coordinated and timely release of vascular specific growth factors would promote alveolar development.

Vascular endothelial growth factor (VEGF) is crucial for blood vessel formation. It is a potent mitogen for endothelial cells, influencing angiogenesis and vasculogenesis (Ingo et al., 1995; Lassus et al., 1999; Warburton et al., 2000; Raoul et al., 2004; Norbert et al., 2005).

Abbreviations: VEGF, Vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor; HLF, hypoxia-inducible factor -1α-like factor; IgG, immunoglobulin G; Lm, mean length; Nv, number of alveoli; ISA, internal surface area; Sv, surface density of alveolar walls; EGF, epidermal growth factor; TGF-β, transforming growth factorβ; KGF, keratinocyte growth factor; IGF, insulin-like growth factor.
VEGF in alveolarization in newborn rat lung

2006; Shibuya, 2006). VEGF expression is regulated by hypoxia-inducible factor (HIF)-1α in response to hypoxia (Moromisato et al., 1996; Gerber et al., 1997) and by HLF (HIF-1α-like factor) (Masatsugu et al., 1997). Several studies have shown that prolonged exposure of neonatal mammals to hyperoxia decreases VEGF expression (Maniscalco et al., 1997, 2001, 2005; Hosford and Olson, 2003; Tuyl et al., 2005; Zhang et al., 2005).

In light of the foregoing, the objective of the present study was to investigate the pulmonary expression of VEGF and its repercussions in alveolarization during rat lung development in an experimental model similar to the clinical situation of premature children: i.e., the acute hypoxic situation at birth and the administration of oxygen (a situation of acute hyperoxia) or air at that time.

Materials and methods

We evaluated pulmonary VEGF expression in Wistar rats at 0 and 14 days of life. All procedures were approved by the Animal Health Care Committee of the University of Salamanca. The rats were born on 21st-22nd days of gestation by natural delivery and they were mixed and distributed at random in a litter size that was adjusted to 8 pups in order to control for the effects of this on nutrition and growth (Cricic and Chase, 1978). Rat pups were exposed for two hours to hypoxia (10% O₂) at 4-8 hours of life in a sealed chamber with continuous O₂ monitoring (SERVOMEX 1440 gas analyser) and then recovered for a further 2 hours under hyperoxia (>95% O₂) (hyperoxia after hypoxia group) or normoxia (21% O₂) (normoxia after hypoxia group) or not recovered (hypoxia group). The rat pups studied at 14 days were later supported in room air. The animals of the control group were under normoxia conditions throughout the study. This experimental model has been reported by our group previously (Pedraz et al., 2003; San Feliciano et al., 2005).

Rat pups were killed at 0 and 14 days of life by intraperitoneal administration of pentobarbital, and their lungs were processed depending on the experiments performed.

Tissue preparations

For VEGF immunohistochemistry and the study of alveolarization, lungs were fixed with a 4% paraformaldehyde solution through the trachea under a constant pressure of 20 cm H₂O. The trachea was then ligated, and the lungs were immersed in 4% paraformaldehyde. The respective lungs were then processed and embedded in paraffin before 24 hours had elapsed.

Tissue microarray construction

For the study two “tissue microarrays” were built, with the incorporation of pulmonary samples from 5 animals per group and experimental situation. All samples were embedded in triplicate (blocks of the experimental groups of 14 days of life) or quintuplate (blocks of the experimental groups of 0 days of life).

Tissue cylinders with a diameter of 0.6 mm (blocks of the experimental groups of 0 days of life) or 2 mm (blocks of the experimental groups of 14 days of life) were then punched from lung areas of each “donor” tissue block and embedded in a recipient paraffin block.

Different cylinder sizes were used owing to the difference in size of the pulmonary tissue obtained between two days studied: 0 and 14. Four-µm sections of the tissue microarray block were transferred to glass slides.

Immunohistochemistry

Immunohistochemistry was performed in an automatic way using the OPTIMAX PLUS processor, and employing a 1:100 dilution of rabbit anti-human VEGF-A (sc-152 Santa Cruz Biotech, Santa Cruz). The immunostaining was carried out simultaneously to reduce to a minimum the inter-assay variability. Slides were treated with 0.5% H₂O₂ in methanol to remove endogenous peroxidase activity, blocked with 3% normal goat serum, and incubated with a 1:100 dilution of rabbit anti-human VEGF. Non-immune rabbit IgG was used as a negative control. The slides were rinsed and incubated with biotinylated goat anti-rabbit IgG. Vectastain ABC elite reagent (Vector Laboratories, Burlingame, CA) was added for 30 min. The slides were then incubated with diaminobenzidine tetrahydrochloride solution. For the densitometry analyses, no haematoxylin-eosin contrasts were carried out.

Densitometry analysis

Image processing techniques were employed to perform an optical density (OD) analysis of the immunostained tissue from 5 animals from each experimental group. Eight-bit greyscale digital images (Fig. 1) of the tissue microarrays were obtained by means of a Zeiss Axioskop 40 microscope equipped with a digital camera (Axiocam MRC5, Zeiss). The images were analyzed using ImageJ software (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008). Pixel values of 0 and 255 correspond to black and white colours, respectively. In an attempt to preserve identical illumination conditions within the dynamic range of the digital camera, between the different image-capturing sessions the settings of all components were kept unchanged and the illumination of the tissue preparation was kept constant by fixing the intensity of the microscope lamp, using neutral density filters. Thus, similar mean grey-level distributions were obtained at the beginning of each session for the digital image of an empty slide (H240). Despite the precautions taken, the illumination conditions may have varied slightly across the sessions. To minimize the risk of the results being...
influenced by fluctuations in illumination, before each session digital images were captured from a stepped-OD filter (NT32-599, Edmund Optics, York, UK) and the grey levels were calibrated in OD values. Accordingly, OD were used instead of direct grey-level measures. Two rectangular digital images (36780.65 µm² - 1292x968 pixels) were captured for each tissue core in the tissue microarray using a dry 20x objective (PL FluoTar, N.A.: 0.50). For each image, the pixels corresponding to the VEGF-like immunoreactive area were segmented by visual threshold and the measurements were limited to the selected area. The mean OD, the selected area, and the integrated OD values (mean OD x number of selected pixels) were measured. Negative controls for the antibody were performed using non-immune serum. The measurements of the negative controls did not reveal significant differences among the groups.

Morphometrics

Quantitative morphometric assessment was carried out by superimposing a sample over a square grid pattern (model CPLW 1018, Zeiss Optical, Hannover Md) for the conventional Haematoxylin-Eosin lung preparations (Blanco and Frank, 1994) and the mathematical model of Weibel (Weibel, 1979) was applied, measuring Lm (mean air space size), Nv (number of alveoli) and ISA (mean internal surface area).

Statistical Analyses

Results are expressed as means ± SD. The data were statistically studied with one-way ANOVA and the Bonferroni or Tamhane post-hoc tests as appropriate.

Results

Immunohistochemistry

VEGF immunostaining was mainly observed in distal airway epithelial cells, pneumocytes, and endothelial cells (Fig. 2).

The 14-day group had decreased VEGF immunostaining as compared with the 0-day group in all experimental groups (the control group, the hyperoxia after hypoxia group, and the normoxia after hypoxia group) with statistical significance (p<0.001).

The group exposed to hypoxia had increased VEGF immunostaining with respect to the control group, with statistical significance (p<0.01) (Fig. 3).

The group that was recovered under hyperoxia (>95% O₂) after neonatal hypoxia showed decreased VEGF immunostaining as compared with the group recovered in air (21% O₂) after neonatal hypoxia on day 0 (p<0.001) (Fig. 3). This was also observed in the 14-day samples, with statistical significance (p<0.005) (Fig. 4). The group exposed to hypoxia and recovered under conditions of hyperoxia had decreased VEGF immunostaining with respect to the control group on day 14, with statistical significance (p<0.001) (Fig. 4).

Alveolarization

In the morphometric assessment performed on 14,

![Image](image-scale-of-grey-of-histological-section-of-lung-with-vegf-immunostaining-x-200.png)
the lungs of the animals exposed to normoxia after neonatal hypoxia or hyperoxia after neonatal hypoxia had fewer alveoli (Nv), these having larger diameters (Lm) and a smaller alveolar surface for gas exchange (ISA) with respect to the control group, with statistical significance (Table 1). In the animals of 0 days of life there were no statistically significant differences between the animals exposed to normoxia after neonatal

Fig. 2. VEGF Immunohistochemistry. Lung sections for each experimental group. A. Normoxia day 0. B. Hypoxia + Normoxia day 0. C. Hypoxia + Hyperoxia day 0. D. Normoxia day 14. E. Hypoxia + Normoxia day 14. F. Hypoxia + Hyperoxia day 14. Bars: 50 μm.
hypoxia and the animals exposed to hyperoxia after neonatal hypoxia.

Discussion

We observed that VEGF expression was affected by a short period of hypoxia or hyperoxia at birth in developing rat lungs. We detected increased VEGF immunostaining in the animals subjected to conditions of acute hypoxia. In this sense, many studies have confirmed that hypoxia is an inducer of VEGF (Gerber et al., 1997; Masatsugu et al., 1997; Hosford and Olson, 2003; Asikainen et al., 2005, 2006; Groenman et al., 2007). Nevertheless, we did not find any correlation with increased alveolarization. Ferrara et al. (2003) reported that the paracrine or autocrine effects of such factors, including epidermal growth factor (EGF), transforming growth factor β (TGFβ), keratinocyte growth factor (KGF) and insulin-like growth factor (IGF), cooperate with local hypoxia in regulating VEGF release in the microenvironment. TGF-β is a negative regulator in foetal lung development, a lack of septation of the alveoli after hypoxic exposure in newborn rats being associated with an increase in the level of TGF-β (Vicencio et al., 2002).

VEGF is an important growth factor that regulates alveolarization and it plays a central role in epithelial-endothelial interactions. In this sense, Voelkel et al. (2006) and Thébaud et al. (2005) have shown that the inhibition of VEGF impairs alveolarization during lung development in rats. In our study, the rat pups that were exposed to hyperoxia after hypoxia had decreased VEGF immunostaining, and this was correlated with less alveolar septation.

Maniscalco et al. (1997) and Watkins et al. (1999) reported that pulmonary VEGF production returned to normal in New Zealand rabbits exposed to hyperoxia after they had been allowed to recover for 3 days at a reduced FiO₂ level. In our study, carried out in rats, we observed that VEGF immunostaining continued to be decreased at 14 days after recovery with oxygen at birth (Remesal et al., 2006). This suggests the persistence of a blockade in VEGF translation and hence production. In a mouse model, Yee et al. (2006) demonstrated that perinatal hyperoxia adversely affects alveolar development by disrupting the proper timing of type II cell proliferation. The recovered mice had 70% fewer type II cells when exposure to hyperoxia occurred in the postnatal period, while in adult mice those authors found type II cell recovery at 7 days.

An important question is to know the mechanisms involved in decreased alveolar and capillary formation (observed in premature infants with bronchopulmonary dysplasia) for the possible prevention of this important pathology (Bancalari, 2006). The greatest decrease in alveolarization was found in the group exposed to hyperoxia after hypoxia and the decrease in VEGF may be related to this. Since VEGF is known to be a fundamental component in normal alveolar development (Lassus et al., 1999; Warburton et al., 2000; Raoul et al., 2004; Ingo et al., 2005) hyperoxia after neonatal hypoxia induces changes in VEGF and the correlation of such changes with the lower number of alveoli may be an important element in the pathology of broncho-pulmonary dysplasia.

Acknowledgements. The authors thank Dr. Horta-Júnior for his suggestions in the densitometry analyses; Dr. Benito Zaballos for her unconditional help, and Angustias Pérez Sánchez and Estefanía Moyano for the immunostaining techniques.

References


Accepted October 14, 2008