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Effects of Oxidative Stress and Antenatal Corticosteroids on the Pulmonary Expression of Vascular Endothelial Growth Factor (VEGF) and Alveolarization

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1. Introduction

The lung is the human organ most susceptible to oxidative damage. The transition from fetal, a low-oxygen environment, to FiO_2: 0.21, induces a relative degree of oxidative stress for all newborns, but especially in the case of preterm neonates whose lung development has been interrupted. A supplementary supply of oxygen (hyperoxia) is often used to treat preterm newborns and these infants may not yet be prepared to protect their lungs from oxidative injury.

One of the most important advances in neonatal care has been the introduction of antenatal corticosteroid therapy for preventing respiratory distress syndrome and improving the survival of preterm infants (Liggins et al., 1972; 1995). Foetal and postnatal lung development is regulated by glucocorticoids (Speirs et al., 2004).

During lung development, vascular endothelial growth factor (VEGF) is an important growth factor in vasculogenesis and angiogenesis (Voelkel et al., 2006), and it plays a central role in epithelial-endothelial interactions, which are critical for normal lung development (Zhao et al., 2005). VEGF is also involved in alveolarization (Thebaud et al., 2005), and it has been reported that the inhibition of VEGF impairs this process (Zhao et al., 2005; Van Tuyl et al. 2005).

Situations such as hypoxia, hyperoxia, and the administration of antenatal corticosteroids may regulate VEGF expression and may interfere with the alveolarization process (Remesal et al. 2009, 2010; San Feliciano et al., 2011).

Among the mechanisms implicated in lung damage due to hyperoxia, alterations in the expression of pulmonary VEGF have been described (Roberts et al., 1983; Maniscalco et al., 2005; Remesal et al., 2009). In our previous experimental studies in rats, we have also observed that dexamethasone and hyperoxia have an additive effect on the inhibition of VEGF, with a decrease in alveolarization (Remesal et al., 2010).

In this chapter we shall discuss the importance of vasculogenesis and angiogenesis in lung development, the role of VEGF in lung development, the effect of oxidative stress on the pulmonary expression of VEGF and alveolarization, the effects of antenatal glucocorticoids added to oxidative stress on the pulmonary expression of VEGF and alveolarization and, finally, the role of VEGF in the pathogenesis of bronchopulmonary dysplasia (BPD).

2. Angiogenesis and vasculogenesis in lung development

Vascular development occurs during all stages of lung development. The formation of the pulmonary vasculature includes three processes: angiogenesis, which leads to central vessels by the budding of new vessels from previous ones; vasculogenesis, which results in peripheral vessels by in situ differentiation of mesenchymal cells into hemangioblasts, and the fusion between the central and peripheral systems, creating the pulmonary circulation (Papaioannou et al., 2006; Akeson et al., 2000).

Vascular development in the lung has been shown to be a determinant of the maturation of lung structure, and angiogenesis and vasculogenesis are necessary for the successful development of the organ (Voelkel et al., 2006).

Interactions between the airways and blood vessels are critical for normal lung development, suggesting that a coordinated and timely release of vascular growth factors would promote alveolar development (Thebaud et al., 2005).

Jakkula et al. (Jakkula et al., 2000) administered antiangiogenic agents, SU-5416, thalidomide and fumagillin, to neonatal rats and observed a decrease in alveolarization and lung growth, with a histological pattern similar to BPD.

Van Tuyl et al.(Van Tuyl et al., 2005) found that the inhibition of vascularization in vitro resulted in a significant decrease in the morphogenesis of the airways, suggesting that pulmonary vascular development would be a factor in lung morphogenesis.

Schwarz et al.(Schwarz et al., 2000) reported that the inhibition of neovascularization with endothelial monocyte-activating polypeptide II (EMAP II) resulted in an arrest in the morphogenesis of the airways. It has also been reported that pulmonary vascular development is dependent on reciprocal interactions with the lung epithelium. Gebb and Shannon (Gebb et al., 2000) showed that mesenchymal cells cultured in the absence of epithelial cells degenerate, and they also observed significantly fewer cells positive for VEGFR-2 (VEGF receptor 2). In contrast, lung mesenchyme recombined with lung epithelial cells contained abundant cells positive for VEGFR-2, and their spatial distribution was similar to that observed in vivo in the lungs of foetal and neonatal rats. In an in vivo study of the lung, they found, as from foetal day 11, precursor cells with a positive expression of VEGFR-2 in the mesenchyme in the developing epithelium.

Studies carried out by Burri (Burri, 2006) have shown that in the human lung microvascular maturation begins very early and partly takes place during alveolarization, ending at an age of 2-3 years.

Genetic analyses have shown that cell-cell interactions and cell-extracellular matrix, growth factors and transcription factors are involved in vascular development (Roth-Kleiner et al., 2003).

One of the most important factors related to the processes described above is clearly VEGF (Thebaud et al., 2005).

3. The role of VEGF in lung development

Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells (Ferrara et al., 1997; Lassus et al., 1999) as well as being chemotactic for these cells, and it is one of the most potent mediators of vascular regulation (Bhatt et al., 2000). Regarding water and proteins, VEGF exerts a potent effect on vascular permeability (Dvorak et al., 1995). VEGF is also necessary for the survival of endothelial cells, especially in hyperoxic environments (Watkins et al., 1999; Maniscalco et al., 2002).

In 1996, the studies of Ferrara et al (Ferrara et al., 1996) and Carmeliet el al. (Carmeliet et al., 1996) revealed the critical role of VEGF in embryonic vasculogenesis and angiogenesis. Those authors observed the death of mouse embryos, with the inactivation of a single VEGF allele.

The VEGF signalling pathway is important in vasculogenesis, particularly during foetal lung development (Gebb et al., 2000; Voelkel et al., 2006) . The expression of VEGF and VEGFR-2 has been demonstrated in airway tube and vascular mesenchymal cells during foetal development and also in vitro in cultures containing epithelial and mesenchymal elements of foetal lung (Gebb et al., 2000).

In baboons, Maniscalco et al. (Maniscalco et al., 2002) found an association between increased PECAM-1 (platelet endothelial cell adhesion molecule) and increased VEGF levels, suggesting a role of VEGF in pulmonary vasculogenesis.

Lassus et al (Lassus et al., 2001) found a higher concentration of VEGF in tracheal aspirates of preterm infants than in those from term infants. Levy et al (Levy et al., 2005) reported that in foetal lungs VEGF expression was higher in the canalicular and saccular stages.

In a murine model, Bhatt et al. (Bhatt et al., 2000) observed that VEGF mRNA levels increased three-fold during the first two weeks of postnatal age: i.e., the alveolarization phase and expansion of the microvasculature; VEGFR-2 mRNA increased in parallel. In those studies, performed by in situ hybridization, the authors demonstrated that VEGF mRNA was mainly located in the epithelial cells of the distal air spaces.

The mRNA expression of VEGFR-1 and VEGFR-2 also increases during normal lung development in mice (Costa et al., 2001; Gebb et al., 2000), and these receptors are located on the endothelial cells of pulmonary vessels in proximity to the epithelium in development. This spatial relationship suggests that VEGF would play an important role in the development of the alveolar capillary bed.

Del Moral et al. (Del Moral et al., 2006) described that exogenous $VEGF_{164}$ induces bronchial morphogenesis in cultured embryonic mouse lung. The effect of VEGF on the epithelium would be indirect, through an interaction with the mesenchyme, because these authors only found VEGFR-2 to be expressed in mesenchymal cells.

The expression of VEGF mRNA and VEGF protein is localized to distal epithelial cells in human foetal lung during the second half of pregnancy and its levels increase with time (Brown et al., 2001).

More recently, in humans Groenman et al. (Groenman et al., 2007) observed the expression of VEGF in the epithelium during the first trimester, as well as the expression of VEGFR-2 in mesenchymal cells adjacent to the epithelium and mesenchyme in vascular structures.

In a mouse model with lung renal capsule grafts, Zhao el al (Zhao et al., 2005) identified the role of VEGF in lung development. By inhibiting VEGF they observed the inhibition of vascular development and a significant alteration in epithelial development.

The absence of $VEGF_{164}$ and $VEGF_{188}$ isoforms results in a decrease in peripheral vascular development and a delayed formation of air spaces in mouse lungs (Van Tuyl et al., 2005).

It has been shown that the inhibition of VEGF results in the inhibition of angiogenesis and alveolarization in lung development in rats. The inhibition of VEGF for even short periods of time may also reduce the number of blood islands and endothelial cells expressing VEGFR-2 (Van Tuyl et al., 2005).

Thebaud B et al. (Thebaud et al., 2005) performed in vitro and in vivo studies in rat lung, reporting that the inhibition of the VEGF signal stopped alveolarization, offering a model similar to BPD and emphysema.

Use of SU-5416, which blocks the VEGF receptor, before or after birth results in a reduction in pulmonary vascularization and alveolarization (Jakkula et al., 2000).

Reports have been made of the existence of a regulatory loop in which epithelium-derived VEGF induces vascular development and endothelial relayed signals directly or indirectly, via the mesenchymal compartment, and stimulates epithelial differentiation and branching (Van Tuyl et al., 2005).
According to the studies carried out by Yamamoto et al. (Yamamoto et al., 2007) primary septum formation depends on interactions between the respiratory epithelium and the underlying vessels, suggesting a dependence of the development of pulmonary capillaries on the VEGF-A derived from the epithelium.

In human foetal lung cell cultures in the second half of gestation, Brown et al (Brown et al., 2001) found the VEGFR-2 receptor and neurofilin I in epithelial cells, suggesting a possible autocrine role of VEGF in the proliferation and differentiation of human alveolar epithelial cells. They also observed that exogenously administered VEGF increased tissue differentiation parameters and that this led to a proliferation of epithelial cells in the distal airways with the morphology of type II pneumocytes and, additionally, it increased the production of several components of surfactant.

VEGF stimulates the production of pulmonary surfactant by type II pneumocytes (Voelkel et al., 2006; Papaioannou et al., 2006; Ferrara et al., 2003), leading to lung maturation and preventing the development of respiratory distress syndrome in newborns.

Compernolle et al (Compernolle et al., 2002) performed a study in premature mice in which they observed an increase in the production of surfactant proteins B and C after treatment with VEGF, showing that type II cells express VEGFR-2. Although the precise mechanisms by which VEGF stimulates the synthesis of surfactant have not been clearly defined, it appears that VEGF stimulates the synthesis of platelet-activating factor (PAF), a potent inducer of glycogenolysis in the foetal lung, and activates kinase C protein, a central regulator of surfactant secretion and glycogen metabolism.

It has been reported that the inhibition of VEGF results in endothelial cell death, suggesting that VEGF could also be a survival factor for endothelial cells (Maniscalco et al., 2005).

4. Effects of oxidative stress on pulmonary VEGF expression and alveolarization

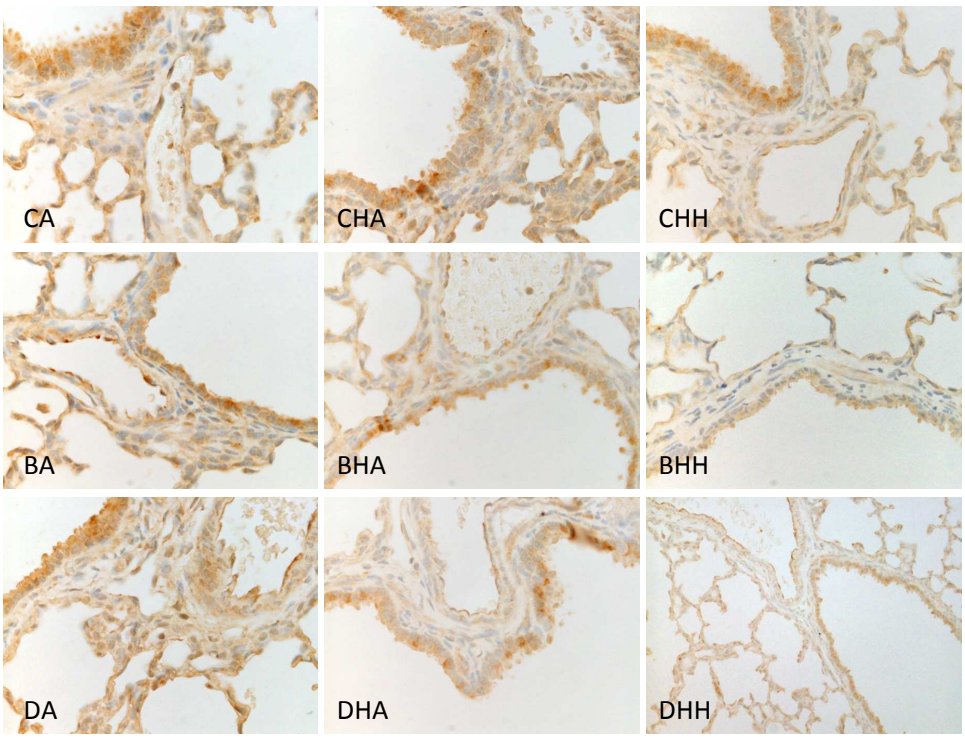
Some studies have shown the involvement of reactive oxygen species (ROS) in the mitogenic cascade initiated by tyrosine kinase receptors of many growth factors. Colavitti et al (Colavitti et al., 2002) identified reactive oxygen species as mediators involved in the signal transduction of VEGFR-2; these findings were paradoxical, however, because it is well known that angiogenesis occurs at low oxygen concentrations. Nevertheless, situations of complete anoxia are very rare under physiological conditions. Other studies have shown that low oxygen concentrations give rise to oxygen free radicals through a mechanism involving an abnormal flow of electrons in the respiratory mitochondrial chain (Chandel et al., 1998).

Preterm birth in ambient air, before the full development of antioxidant defences, elicits a relative degree of hyperoxia, which blocks normal lung vascular development and alveologenesis (Massaro et al., 2004; Thebaud, 2007).

The perinatal period is critical for proper adaptation to postnatal lung life. Lung damage during the perinatal period could interfere with lung growth, leading to abnormalities in lung structure and function that persist in children and adults. Premature infants with immature lungs often require high inspired oxygen levels, which are toxic to alveolar and endothelial cells (Crapo, 1986, 2003a, 2003b). Studies carried out by our group and other researchers have shown that hyperoxia alters alveolar growth by interfering with essential growth factors such as VEGF (Maniscalco et al., 1997; Remesal et al., 2009, 2010).

In our studies (Remesal et al., 2009, 2010) we evaluated pulmonary VEGF expression by RT-PCR and immunohistochemistry with densitometry analysis in Wistar rats at 0, 4 and 14 days of life. In order to analyse alveolarization, quantitative morphometric assessment was carried out by superimposing a sample over a square grid pattern (model CPLW 1018, Zeiss Optical, Hannover Md) for conventional haematoxylin-eosin lung preparations (Blanco et al., 1994), and the mathematical model of Weibel (Weibel et al., 1966) was applied, measuring Lm (mean air space size), Nv (number of alveoli) and ISA (mean internal surface area). All procedures were approved by the Animal Health Care Committee of the University of Salamanca. The experiments were performed following the regulations of the Directive of the Council of the European Community (DOCE L 222; 24/08/1999). The rats were born at 21-22 days of gestation by natural delivery and were mixed and randomly distributed in a litter size adjusted to 8 pups in order to control for the effects of this on nutrition and growth (Crnic, L. S. et al., 1978). Rat pups were exposed to hypoxia for two hours (0.10 FiO2) at 4-8 hours of life in a sealed chamber with continuous O_2 monitoring (SERVOMEX 1440 gas analyser) and then recovered for a further 2 hours under hyperoxia (>0.95 FiO_2) (hyperoxia after hypoxia group) or normoxia (room air, 0.21 FiO_2) (normoxia after hypoxia group) or not recovered (hypoxia group). The rat pups studied at 4 and 14 days were later maintained in room air. The animals of the control group were under normoxic conditions throughout the study.

In our studies (Remesal et al., 2009, 2010), we observed a decrease in pulmonary VEGF expression in rats that were exposed to hyperoxia after hypoxia and this finding correlated with less alveolar septation. We observed that pulmonary VEGF expression remained decreased at 14 days after recovery with oxygen at birth (Fig.1).



C: Control. B: Betamethasone. D: Dexamethasone. A: Air. HA: Hypoxia + Air. HH: Hypoxia + Hyperoxia

Fig. 1. Immunohistochemistry. Lung sections for each group.

The effect of hyperoxia on the postnatal lung varied, depending on the length of exposure, the degree of hyperoxia, and the age and animal species used in the model. In mice, McGrath-Morrow et al. (Grath-Morrow et al., 2005) found differences between the lung damage caused by hyperoxia and damage due to blocking VEGFR-2. In lungs exposed to hyperoxia the damage was more persistent; the animals had a more reduced mitotic index and showed increased apoptosis.

Roberts et al.(Roberts et al., 1983) reported that newborn rats exposed to an FiO_2 of 1 exhibited a decrease in the number of capillaries due to the toxic effect of oxygen on endothelial cells. Kunig et al. (Kunig et al., 2005) found that lungs subjected to hyperoxia had a decreased number of alveoli and showed reduced vascular growth.

Although hyperoxia could inhibit lung growth through several mechanisms, clinical and experimental studies have suggested that damage to VEGF signalling plays an important

role in the pathogenesis of BPD (Thebaud et al., 2005,; Kunig et al., 2005; Maniscalco et al., 2002;Lin et al., 2005; Thebaud, 2007). In many studies it has been shown that hyperoxia decreases VEGF levels.

Thebaud B et al.(Thebaud et al., 2005) described irreversible hypoalveolarization induced by oxygen in rat lung development, and this was associated with decreased VEGF expression and reduced vascular growth.

Wageenaar et al (Wagenaar et al., 2004) investigated gene expression by analysing "microarray" DNA in the lungs of preterm rats exposed to prolonged hyperoxia, observing a decrease in VEGF levels and those of its receptor VEGFR-2.

Endothelial cells are particularly sensitive to oxidative stress and decreases in VEGF levels due to hyperoxia further increase the susceptibility of the epithelium to hyperoxic injury and a greater loss of these cells after acute hyperoxia (Kunig et al., 2005; Lin et al., 2005). During exposure to hyperoxia, a destruction of the lung microvascular system was observed, accompanied by a cessation of endothelial regeneration, which is essential for the repair of lung injury induced by oxygen (Watkins et al., 1999).

Klekamp et al. (Klekamp et al., 1999) reported a reduction in VEGF in the lungs of rats exposed to hyperoxia (FiO_2> 0.95 between postnatal days 6 and 14) associated with alveolar cell apoptosis and a reduced expression of VEGFR-2 and VEGFR- 1.

Maniscalco et al. (Maniscalco et al., 2002) found that preterm baboons treated with oxygen had a 70% decrease in PECAM-1 protein and a 27% decrease in capillary density in the presence of dysmorphic capillaries in comparison with a control group. They observed a decrease in VEGF mRNA, supporting the hypothesis that the development of BPD could be the result of a disruption of the genetic program of angiogenic factors and receptor expression in endothelial cells.

Watkins et al. (Watkins et al., 1999) found that in damage due to hyperoxia in newborn and adult rabbits the proportion of $VEGF_{189}$ decreased, normal values being re-established during recovery under normoxic conditions.

In a model of hyperoxia exposure in mice, Zimova et al (Zimova-Herknerova et al., 2008) observed a decrease in VEGF mRNA levels that was lower when the animals were given retinoic acid.

The decrease in VEGF expression in hyperoxia could be related to the suppression of the expression of hypoxia-induced factor 2-α (HIF2-α or HLF) (Maniscalco et al., 2002). Moreover, hypoxia-induced factor (HIF) is inhibited by increasing levels of oxygen (Thebaud, 2007).

In a study carried out on newborn rats, Hosford et al (Hosford et al., 2003) showed that the expression of VEGF, VEGFR-1 and VEGFR-2 was decreased at 12 and 14 days after exposure to a hyperoxic environment during the critical period of alveolar development (4 to 14 days). They observed that under conditions of normoxia there was a strong correlation between VEGF and HLF. This correlation disappeared after hyperoxia, suggesting that low levels of HLF, after exposure to high concentrations of O_2, would not stimulate the expression of VEGF.

Roper et al. (Roper et al., 2004) found that alveolar type II epithelial cells exposed to hyperoxia showed DNA damage (broken strands, modifications of bases, changes in sister chromatids and the oxidation of guanine to 8-oxoG), even though they were morphologically intact. Hyperoxic injury was produced by the interaction of ROS with macromolecules such as DNA, lipids and proteins.

Oxidative damage to tissues could be exacerbated by damage to VEGF signalling. It has been observed that VEGF induces manganese superoxide dismutase (MnSOD) and nitric oxide (NO), both of which are scavengers of reactive oxygen species (Maniscalco et al., 2005). In VEGF-overexpressing transgenic mice, Siner et al (Siner et al., 2007) have shown that VEGF induces cytoprotection via the induction of heme oxygenase-1 (an antioxidant) and also that VEGF has the capacity to decrease levels of apoptosis markers.

In addition VEGF conferred cytoprotection through an A1-dependent mechanism, a critical regulator in lung injury and cell death induced by hyperoxia. He et al. (He et al., 2005) showed that transgenic $VEGF_{165}$-overexpressing mice had increased A1 protein levels and A1 mRNA and that they survived longer under conditions of hyperoxia with a FiO_2 of 1.

In a study of preterm baboons subjected to hyperoxia for 6 to 10 days, Maniscalco et al. (Maniscalco et al., 2005) found an increase in the levels of p53, a transcription factor that represses VEGF transcription, in distal epithelial cells. They also found oxidant DNA damage with increased 8-oxoG levels, which would be the mechanism responsible for the increase in p53 level.

In a study performed with mice to detect alveolar type II cells, Yee et al. (Yee et al., 2006) observed a loss of these cells after exposure to hyperoxia; this would be related to the decrease in VEGF levels in hyperoxia.

In studies by Maniscalco et al. (Maniscalco et al., 1995, 2002) it has been reported that VEGF is expressed in type II cells with low surfactant protein C levels. Thus, the change in the distal epithelial cell phenotype was seen in situations of hyperoxia, with more cells with high amounts of surfactant protein C, resulting in a decrease in the production of VEGF.

It has also been shown that oxidative stress inactivates the survival signal of VEGF in endothelial cells through the action of peroxynitrite (Acarregui et al., 1999) .

Furthermore, in vitro and in vivo studies have described the induction of VEGF by reactive oxygen species. In ferrets, Becker et al (Becker et al., 2000) reported an increase in VEGF in a model of lung ischemia, regardless of exposure to oxygen, and in a mouse model Corne et al. (Corne et al., 2000) observed an increase in VEGF levels in bronchoalveolar lavage fluid after 72 hours of exposure to an FiO_2 concentration of 1.

In our studies (Remesal et al., 2009, 2010) the animals were previously exposed to acute hypoxia. It has been described that early exposure to hypoxia predisposes the animals to an increased response to any further damage (Haworth et al., 2003). Saugstad (Saugstad, 1988) reported that reoxygenation following hypoxia generated an increase in oxygen free radicals levels that could not be neutralized by antioxidant defences and that resulted in injury to cellular structures. Ekekezie et al. (Ekekezie et al., 2003) and Lin et al. (Lin et al., 2005) reported that hyperoxia negatively regulates the expression of VEGF, despite recovery in air. These findings are similar to our own.

5. Antenatal glucocorticoids

One of the most important advances in neonatal care has been the introduction of antenatal glucocorticoid therapy for preventing respiratory distress syndrome and improving the survival of preterm infants (1995). Foetal and postnatal lung development is regulated by glucocorticoids (Speirs, H. J. et al., 2004). There are many reports showing that corticoids can alter the normal stages of lung development during the period of alveolarization. Corticoids are known to trigger the structural maturation of mesenchymal cells and the functional maturation of the surfactant system (Jobe, A. H., 2001).

Dexamethasone and Betamethasone are the only corticosteroids recommended for antenatal therapy (National Institute of Health NIH, 1995), and as yet there are no indications favouring the use of one preparation over the other (Crowley, 2007), although there are some reports giving preference to bethametasone (Lee et al., 2006; Miracle et al., 2008; Remesal, et al., 2010; San Feliciano et al., 2011). The dosage and number of treatment cycles in gestating women at risk of preterm delivery are still under debate because of the long-term effects of these drugs on growth (Sweet et al., 2010).

5.1 Effects of betamethasone vs dexamethasone on the expression of VEGF and alveolarization

Massaro et al. (Massaro et al., 2004) reported that in all species septation, whether antenatal or postnatal, occurs during a period in which the plasma concentration of glucocorticoids is low and that it ends when the concentration of glucocorticoids increases. Also, in studies performed by Liu et al. (Liu et al., 2004) the authors showed that dexamethasone inhibits IGF-I, this having an impact on alveolarization.

Studies by Massaro (Massaro et al., 2004) and Clerch (Clerch et al., 2004) have shown that glucocorticoid administration to rats or mice during the period of septation, when the plasma concentration of steroids is usually low, damages spontaneous septation and the development of the pulmonary vasculature. There was no further spontaneous septation and vasculogenesis when steroid treatment was withdrawn (tested in rats up to 95 days). This observation suggests that there would be a critical period for the development of these events. In those studies, using microarray analysis of pulmonary gene expression the authors identified a negative regulation of VEGFR-2 by dexamethasone, resulting in the inhibition of septation.

Furthermore, in a study in newborn rats treated with steroids during the first 4 days of life Tschanz et al. (Tschanz et al., 2003) have shown that the lung has the ability to recover from the damage caused by steroids when these are suspended.

In our studies (Remesal et al., 2010; San Feliciano et al., 2011), in order to evaluate possible differences between the effect of both antenatal glucocorticoids, dexamethasone, betamethasone or saline solution were administered intravenously to pregnant Wistar rats on the 20th and 21st days of gestation. The newborn rats were exposed to the different experimental situations described previously.

We observed a decreased pulmonary VEGF expression that was correlated with a decrease in alveolarization in the animals that received antenatal dexamethasone. This effect on VEGF and alveolarization was not found in the animals that received antenatal

betamethasone (Fig.1). We observed a negative effect on lung maturation and VEGF expression caused by antenatal dexamethasone lasting from the saccular phase until the end of the alveolarization period (Remesal et al., 2010; San Feliciano et al., 2011).

The only structural difference in the molecule of these two corticosteroids is the orientation of the methyl group at position 16, and this small difference seems to have important consequences. Other authors have reported the different biological effects of both corticosteroids. Rayburn et al. (Rayburn et al., 1997) observed better memory in rats treated with antenatal betamethasone and poorer memory in the group treated with antenatal dexamethasone.

In an in vitro study of thymocytes, Buttgereit et al (Buttgereit et al., 1999) observed that different glucocorticoids, including dexamethasone and betamethasone, differed in the strength of their genomic and non-genomic effects; they found that dexamethasone was five times more potent in inhibiting cellular respiration.

Other authors have reported differences in genomic and non-genomic effects between the two molecules and the expression of NMDA receptors (McGowan et al., 2000; Setiawan et al., 2007) and in the expression of the sodium channels of respiratory epithelial cells. Although we have found no reports comparing the effects of betamethasone or dexamethasone on lung VEGF, our results indicate that the molecules do have different actions (Remesal et al., 2010; San Feliciano et al., 2011).

In clinical studies, different findings have been reported with the use of either molecule (dexamethasone or betamethasone). Baud et al (Baud et al., 1999) published a multicenter study with a cohort of 883 children with gestational ages of 24 to 31 weeks over a period of 4 years. The mothers of 361 infants had received betamethasone; the mothers of 165 infants had received dexamethasone, and the mothers of 357 children had not received glucocorticoids. The authors compared the frequency of cystic periventricular leukomalacia among the three groups using a multivariate analysis adjusted for confounding factors such as sex, chorioamnionitis, infection, multiple gestation, and other relevant factors. 8.4% of children in the group that had not received antenatal steroids developed cystic periventricular leukomalacia as compared to 4.4% of the children in the antenatal betamethasone-treated group and 10.9% of those in the antenatal dexamethasone-treated group.

In the study by Lee et al. (Lee et al., 2006), which included 3600 children with birth weights of less than 1500 grams, the authors found a decrease in mortality in the children who had received antenatal betamethasone as compared with the group that had not received antenatal steroids; they failed to find this decrease in mortality in the antenatal dexamethasone-treated group. Also, dexamethasone was associated with an increase in neonatal mortality as compared with betamethasone. They found a lower incidence of severe retinopathy in children who had received antenatal betamethasone and this would be related to the inhibition of TNF-α. This factor was also involved in angiogenesis. In that study, the authors also observed a lower frequency of intraventricular hemorrhage in children who had received antenatal betamethasone. Similar results have also been published in the Cochrane meta-analysis reported by Crowley (Crowley, 2007).

Feldman et al. (Feldman et al., 2007) reported a study of 334 preterm infants with birth weights of less than 1500 grams at birth: 186 children had received antenatal betamethasone

and 148 had received antenatal dexamethasone. The most important finding observed in that study was that the children who had received antenatal betamethasone had a lower incidence of respiratory distress syndrome and BPD.

In cultured embryonic rat lung, Oshika E et al (Oshika et al., 1998) showed that treatment with dexamethasone resulted in growth retardation, abnormal branching, dilated proximal tubules, and a suppression of the proliferation of the epithelial cells of the distal tubules.

Schellenberg and Liggins (Schellenberg et al., 1987) administered dexamethasone to pregnant rats and studied its effects on lung development in the offspring during foetal and postnatal life. The results revealed an inhibition of lung growth and body growth, and a reduction in DNA contents in the animals treated with dexamethasone. Dexamethasone appeared to affect the populations of cells that produce elastin and cells that produce collagen during foetal lung development.

In in vitro studies of human lung fibroblast cultures, it was observed that dexamethasone inhibited fibroblast proliferation and chemotactic activity in a dose-dependent manner (Brenner et al., 2001).

There are different studies, such as ours, describing a relationship between steroids and VEGF. In studies carried out by Vento et al.(Vento et al., 2002) on preterm infants, the authors found that lung VEGF levels were decreased in the dexamethasone-treated group as compared with the untreated group.

Many in vitro studies (Nauck et al., 1997; Nauck et al., 1998; Harada et al., 1994; Tanabe et al., 2006) have described a strong negative regulation of the induction of VEGF expression by dexamethasone in different cell types, including alveolar epithelial cells.

Hewitt et al (Hewitt et al., 2006) showed that VEGF expression was decreased in the placentas of pregnant rats treated with dexamethasone.

In an in vitro study, Gille et al (Gille et al., 2001) found that in human keratinocyte cells treated with glucocorticoids VEGF mRNA was rapidly degraded within 2 hours. The decrease in mRNA stability could be one mechanism of glucocorticoid-induced inhibition of VEGF.

In an in vitro study of human foetal lungs, Acarregui et al. (Acarregui et al., 1999) found increased levels of VEGF mRNA when the lung tissue was maintained in dexamethasone for 4 days as from the onset of incubation in 0.20 FiO_2. However, when the epithelium was first incubated in FiO_2 0.20 and then treated with dexamethasone, mRNA levels were not increased. According to those authors, dexamethasone induced differentiation in type II cells and this was responsible for the variations in the levels of VEGF.

In studies of preterm infants, Lassus et al. (Lassuset al., 1999, 2001) found no differences in the concentration of VEGF in tracheal aspirates from children who had received antenatal steroids and those who had not. They also failed to find any differences in the concentration of VEGF in preterm infants treated early on with postnatal dexamethasone.

Moreover, D'Angio et al. (D'Angio et al., 1999) have described higher levels of VEGF in tracheal aspirates in preterm infants treated with dexamethasone.

In a study carried out in mice, Compernolle et al. (Compernolle et al., 2002) observed that antenatal dexamethasone stimulated the expression of foetal VEGF when administered at low doses (0.8 mg / kg), but suppressed the production of VEGF when administered at high doses (2.4 mg / kg). Thus, excessive amounts of glucocorticoids could neutralize the beneficial effects of VEGF in the lung.

Conversely, a study in mice conducted by Bhatt et al. (Bhatt et al., 2000), who administered dexamethasone (0.1-5 mg/ Kg/ day) from postnatal day 6 to 9, analyzed VEGF mRNA and VEGFR-2 mRNA levels in lungs, and found that VEGF and VEGFR-2 increased with increasing doses of dexamethasone. They also analyzed the effects of dexamethasone on the amount of mRNA HLF, which has been associated with an increased transcription of VEGF in normoxia and hypoxia. In the group treated with 5mg/Kg/day of dexamethasone the amount of HLF-α in the lung tripled and this could be suggested as a possible mechanism accounting for the effects of dexamethasone on lung VEGF mRNA levels. There were no differences between dexamethasone and control groups in the study of the VEGF protein. Dexamethasone treatment did not alter the pattern of VEGF-expressing cells; mainly distal alveolar epithelial cells.

The different experimental models used, or the fact that the effect of dexamethasone on the differentiation of type II cells might lead to a relative increase in VEGF mRNA (Acarregui et al., 1999) could explain the discrepancies found in the effect of dexamethasone on VEGF expression.

In our studies (Remesal et al., 2010; San Feliciano et al., 2011) we observed that dexamethasone inhibited septation in rats (with postnatal alveolarization), as reported by Massaro and Massaro (Massaro et al., 1986). We believe that the effect of dexamethasone on the decrease in VEGF may be related to the impairment in alveolarization.

Also in our studies (Remesal et al., 2010; San Feliciano et al., 2011), we found no decrease in VEGF in the group treated with antenatal betamethasone (Fig.1). We have found few studies in the literature addressing the effect of betamethasone and VEGF.

In a study by Aida et al. (Aida et al., 2004) it was reported that VEGF stimulates the expression of glucocorticoid receptors, although these might possibly have a more reduced function.

In an experimental study on sheep, Suzuki et al. (Suzuki et al., 2006) found no differences in the levels of VEGF mRNA between the group treated with antenatal betamethasone and the group that received no treatment.

Roubliova et al. (Roubliova et al., 2008) conducted a study that included 112 rabbit foetuses. The mothers received 0.05 or 0.1 mg/ Kg/ day of betamethasone at 25 and 26 days of gestation, but the mothers of the control group only received saline. The authors studied lung VEGF expression and found that VEGF was increased in endothelial cells, epithelial cells, and smooth muscle cells, these results being similar to those found in our study. They also observed that this effect was dose-dependent.

5.2 Effects of antenatal glucocorticoids added to oxidative stress damage on the pulmonary expression of VEGF and alveolarization

In our studies (Remesal et al., 2010) we have observed that dexamethasone and hyperoxia have an additive effect on the inhibition of VEGF, with a decrease in alveolarization (Fig.1).

It has been reported that both factors (dexamethasone and hyperoxia) arrest alveolarization (Frank , 1992; Veness-Meehan et al., 2000)

There are other studies that have reported that hyperoxia and dexamethasone jointly enhance decreases in the expression of VEGF (Ozaki et al., 2002; Edelman et al., 1999).

In the study performed by Ozaki et al. (Ozaki et al., 2002) the authors assessed the response of VEGF in the retinas of newborn rabbits after treatment with dexamethasone, exposure to hyperoxia with FiO_2 0.8 to 1 for 4 days, and exposure to hyperoxia with subsequent recovery in air for 5 days. They found that VEGF mRNA was decreased after hyperoxia and recovery in air in the animals that had received dexamethasone as compared to animals that had received dexamethasone and were in ambient air.

Edelman et al. (Edelman et al., 1999) used a model of corneal neovascularization induced in rat cornea by cauterization. They observed that after cauterization an increase occurred in VEGF mRNA and protein produced by leukocytes and macrophages adjacent to the lesion. This was a model in which hypoxic zones also occurred around the lesion due to the cauterization, and hypoxia could induce the expression of VEGF. They found that treatment with dexamethasone or systemic hyperoxia inhibited the increase in VEGF and that combined treatment with both dexamethasone and hyperoxia had an additive effect.

HGF (hepatocyte growth factor) is a growth factor with mitogenic activity against epithelial cells. Dexamethasone suppresses the gene expression of HGF and inhibits the growth factors responsible for the induction of HGF mRNA expression (Lassus et al., 2002). It has also been shown that glucocorticoids inhibit the inflammatory cytokine production. It has been shown that interleukin 1 and 6 induce the expression of HGF in vitro (Tamura et al., 1993; Zarnegar, 1995). Through its effect on the HGF, dexamethasone exerts an adverse influence on lung development and the repair of acute lung injury in the lungs of preterm infants (Lassus et al., 2002).

There is a balance between retinoic acid and glucocorticoids during normal lung development. Alterations in this balance produce abnormal alveolarization (Jobe, 2003). It has been observed that the survival of newborn rats exposed to oxygen improves with concurrent treatment with retinoic acid and dexamethasone, suggesting a possible complementary effect (Veness-Meehan et al., 2000). Retinoic acid could compensate the acceleration of septal maturation due to the administration of steroids, the benefit of treatment with these drugs persisting during epithelial maturation (Bourbon et al., 2005). It has also been observed that vitamin A levels in the blood of ventilated premature children whose lung function improved with glucocorticoid treatment were higher (Shenai et al., 2000).

According to our own results (Remesal et al., 2010) it would appear that betamethasone inhibits the negative action of hyperoxia on VEGF.

Chandrasekar et al. (Chandrasekar et al., 2008) found that betamethasone reduced intensity of oxidative stress and improved the response of the pulmonary arteries to vasodilators in lambs with pulmonary hypertension. They observed that betamethasone increased the levels of eNOS and MnSOD.

6. VEGF and BPD

The first definition of BPD was given by Northway in 1967 (Northway et al., 1967) as chronic lung disease in childhood as a result of therapy with mechanical ventilation and oxygen for respiratory distress syndrome after premature birth. Traditionally, BPD has been defined by the persistence of respiratory signs and symptoms, the need for supplemental oxygen to treat hypoxemia, and radiographic abnormalities at 36 weeks corrected age.

In light of the new therapeutic possibilities available today, BPD has been seen to affect very immature preterm infants. In the pathogenesis of BDP, the interruption of lung development plays a more important role than volutrauma or inflammation (Jobe, 1999). Alveolar hypoplasia and dysmorphic changes of the pulmonary microvasculature have been consistent findings in animal models of BPD and in the autopsies of children who died of BPD (Maniscalco et al., 2002; Coalson et al., 1988, 1999; Bhatt et al., 2001; De Paepe et al., 2006).

Among the mechanisms that arrest alveolar development in BPD, the following have been implicated: "oxidative stress", mechanical ventilation, proinflammatory factors, glucocorticoids, bombesin-like peptides, and poor nutrition (Jobe, 1999; Jobe et al., 2001).

Premature infants have a non-developed microvasculature and suffer from lung damage due to hyperoxia. (Crapo,1986, 2003a, 2003b ; Spyridopoulos et al., 1997). Given the critical role of endothelial cells during lung development, strategies that alter the signal of VEGF during the foetal and perinatal periods would lead to BPD (Voelkel et al., 2006; Lin et al., 2005).

The importance of VEGF in foetal lung growth has led to the vascular theory of BPD, which has considerable importance in lung injury due to prematurity, mechanical ventilation, and hyperoxia treatment during perinatal life (Abman, 2001; Voelkel et al., 2006).

The predominant lung histology at autopsy in children with bronchopulmonary dysplasia is characterized by an arrest in lung development, including alveolar development and vascular growth (Kunig et al., 2005; Lin et al., 2005; Jobe, 1999; Jobe et al., 2001; Abman, 2001; Kunig et al., 2006).

Bhatt et al. (Bhatt et al., 2001) reported a decrease in the expression of VEGF, VEGFR-1 and TIE-2 in the lungs of children who had died of BPD and had dysmorphic capillaries, suggesting an arrest of the development of the pulmonary vasculature.

Ambalavanan and Novak (Ambalavanan et al., 2003) found lower levels of VEGF in the tracheal aspirates of preterm infants upon mechanical ventilation in the first 24 hours of life, such infants developing BPD in comparison with those who did not develop BPD. The results of experimental studies carried out on rats suggest that the inhibition of VEGF receptor activity reduces alveolarization and vascular growth, leading to histological changes in the lung that resemble those found in BPD (Lin et al., 2005).

The decrease of VEGF in BPD has implications for the survival of endothelial cells. In addition, VEGF inhibits tumour necrosis factor (TNF-α) and hence endothelial cell apoptosis (Bhatt et al., 2001). It has also been reported that the inhibition of VEGF receptors in immature lungs reduces the expression of nitric oxide synthase (NOS) and the bioactivity of NO and later contributes to the development of structural damage and functional BPD (Papaioannou et al., 2006; Lin et al., 2005; Tang et al., 2004).

The baud et al. (Thebaud et al., 2005) performed intratracheal treatment with gene therapy with VEGF to rats exposed to hyperoxia during lung development and observed a longer survival and preservation and restoration of normal alveolarization, even when treatment was performed with already established BPD. VEGF gene transfer also increased the expression of NOS, suggesting that some of the beneficial effects of VEGF could be mediated by NO.

Kunig et al. (Kunig et al., 2005, 2006) treated newborn rats with human recombinant VEGF during and after exposure to hyperoxia, observing an increase in vascular growth and improved alveolarization. VEGF treatment after the damage caused by hyperoxia in neonatal rats improved alveolarization, vascular growth and lung growth, and prevented the development of BPD.

In the above studies (Thebaud et al., 2005; Kunig et al., 2005), VEGF led to highly permeable immature capillaries and pulmonary oedema. However, in a combination of gene transfer of VEGF and angiopoietin-1, the alveolarization and angiogenesis were preserved and improved, with more mature and fewer permeable capillaries.

These studies would increase the possibilities for the treatment of children with severe BPD, although caution should be exercised on attempting to extrapolate findings from animal models for the treatment of human disease.

In the case of children with BPD, De Paepe et al. (De Paepe et al., 2006) have suggested that changes in lung architecture would not simply be due to a decrease in angiogenesis, describing an increase in pulmonary capillary density in children who had been subjected to mechanical ventilation for long periods.

Akeson et al. (Akeson et al., 2003) studied transgenic $VEGF_{164}$-overexpressing mice. They noted that when the increased expression of $VEGF_{164}$ was in the proximal airways the abnormalities did not occur at the junction of the vascular network of the lung. When the expression of this molecule was higher in the distal airways, there was an alteration of the junction of the vascular network, demonstrating that normal pulmonary vascular development requires a precise spatial expression of VEGF-A for proper morphogenesis, and indicating that caution should be exercised in the use of VEGF as a therapeutic agent in neonates.

Changes in the lungs of patients with BPD are similar to those seen in our dexamethasone and hyperoxia groups, with fewer and larger alveoli (Remesal et al., 2010; San Feliciano et al., 2011). Betamethasone does not decrease VEGF expression or alveolarization and seems to inhibit the negative action of hyperoxia on VEGF (Remesal et al., 2010).

7. Conclusions

VEGF is one of the most important growth factors related to lung development and it is negatively regulated by oxidative stress.

With the limitation that we did not use an experimental model to reproduce BPD, the results of our studies suggest that repeated administration of antenatal dexamethasone, mainly associated with supplemental oxygen therapy, might worsen the morphological changes seen in BPD, with a negative effect on lung maturation and VEGF expression until the end of the alveolarization period.

Our studies also support the notion that betamethasone could be the drug of choice for treating pregnant women at risk of preterm delivery.

8. Acknowledgments

We wish to dedicate this chapter to the memory of Prof. Carmen Pedraz

9. References

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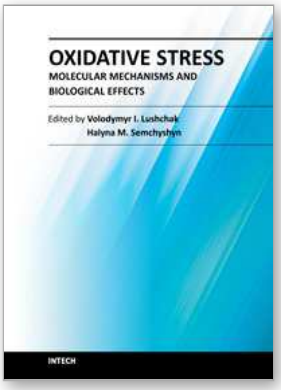

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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

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