Chapter 5

Angiogenesis and Pulmonary Hypertension

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http://dx.doi.org/10.5772/56054

1. Introduction

The aim of this chapter is to present an overview of salient findings in human beings and animal models (particularly in the chicken), as related to known participating molecules in angiogenesis within the lung as a response to induced and natural environmental hypoxia, in the framework of the pathobiology of pulmonary hypertension (PH).

Hypoxic PH is now recognized as an important disease within the PH types. More than 140 million human beings are settled in geographical zones located 2500 m above sea level (Peñaloza and Arias, 2007). Animals which provide proteins for human consumption, have different degrees of susceptibility to develop PH, especially the commercial chicken, which is particularly prone to develop PH, either from hypoxia (Gómez et al., 2007, 2008; Vásquez and Hernández, 2011; Areiza et al, 2011 and others) or low temperatures (Pakdel et al., 2005; Pan et al., 2005). The chicken has been proposed as a model to study PH in humans (Al-Ruyabe et al., 2010; Wideman and Hamal, 2011).

Exposure to hypoxia has been reported to cause different effects on the pulmonary vasculature. These include increasing the extent of pulmonary vascular network, increased vascular tone, vascular remodeling and in some cases quantitative reduction of microvessels. Neo-formation of blood vessels in the lung, takes place through a mechanism not fully understood and often controversial. Remodeling of pulmonary arterioles has been thoroughly studied. It includes thickening of the medial muscle and adventitia layers. Many tasks have been directed to discover the role of the endothelium in maintaining pulmonary vascular tone and in the PH remodeling process. Endothelial dysfunction leads to chronically impaired production of vasodilators such as nitric oxide (NO) and prostacyclin along with prolonged over expression of vasoconstrictors such as endothelin-1 (ET-1). These changes affect vascular tone and promote vascular remodeling. Given that most of these mediators affect the growth of smooth muscle cells, an alteration in their production may facilitate the development of pulmonary

vascular hypertrophy and structural remodeling, which are characteristic of PH (Budhiraja et al., 2004; Humbert et al., 2004; reviewed by Hernández and Sandino de, 2011).

Both processes, angiogenesis and remodeling of pulmonary arterioles, appear to share the participation of similar molecules. In fact, some of them, known to increase pulmonary blood vessels tone during hypoxic exposure, are also involved in generating pulmonary blood vessels. In this framework, three main research cellular targets can be identified: the endothelium, the smooth muscle cells of the wall of pulmonary arterioles and, adventitial connective tissue cells.

PH has been related with malfunction of potassium channels and over-regulation of calcium channels on the cell membrane of vascular muscle cells as well as endothelial dysfunction. The latter, as it pertains with decreasing NO and prostacyclin (vasodilators) delivering and increment of vasocontrictors, such as endothelin-1 (ET-1) Inagami *et al.*, 1995; Voelkel and Tuder, 2000)

In adult subjects, angiogenesis could be taken as a compensatory process during hypoxic exposure. This process would be in addition to the well-known mechanisms of augmenting blood perfusion in less vascularized areas in the lung, hyperventilation and erythropoiesis. Those areas might be a potentially available area for *de novo* vascular formation. Human beings living in highlands, have developed a greater lung capacity through evolution, as an adaptive mechanism to hypobaric hypoxia (Frisancho, 1970). Also, high landers show increased lung capillary density than lowlanders, which was interpreted as an effect of hypoxia on angiogenesis (Bisschop et al, 2010).

Ooi et al (2000) and Howell et al (2002, 2003) determined that hypoxic hypoxia (10% oxygen) provoked augmentation in volume and total length in pulmonary blood vessels in rats, together with an increment in endothelial superficial area and cells numbers. In another work, chronically hypoxic rats, which exhibited a greater degree of vascular development through angiogenesis, had higher cardiac mass index values than the correspondent ones in animals subjected to hypoxia and hypercapnia or normoxia (Howell et al., 2004). In mice subjected to hypoxia, Pascaud et al (2003) gave evidence that inhibition of hypoxia-induced angiogenesis, enhanced the degree of PH.

Angiogenesis is regulated by angiogenic and anti-angiogenic molecules (Pascaud et al., 2003; Maharaj and D`Amore, 2007). Genetic and epigenetic factors permit blood vessels formation (Pauling and Vu, 2004). Taraseviciene-stewart et al (2001) found that plexiform lesions in idiopathic pulmonary hypertension (iPAH) were associated with disordered angiogenesis due to exaggerated mitosis of endothelial cells. Tuder and Voelkel (2002) reported endothelial cell proliferation in cases of primary PH, and, since these cells exhibited markers of angiogenesis, the authors named this process a "disordered or misguided angiogenesis". In this context, endothelium progenitor cells are now claimed to be therapeutic targets in PH due to their possible involvement in PH pathobiology. Nitric oxide synthases (eNOs), vascular endothelial growth factor (VEGF), angiopoietins and their receptor tie-2, which are associated with vascular remodeling, were all expressed in a monocrotaline induced model of PH (Cho et al.,

http://dx.doi.org/10.5772/56054

2009). Furthermore, induced ischemia results in pronounced cell proliferation and consequent lung vascularization in mice, within a period of 20 days (Wagner et al, 2006).

If vascular growth in the lung is an adapting mechanism to hypoxia, genetically predisposed individuals will develop hypoxic PH, and have a diminished pulmonary vascular growth capacity. Hence, angiogenesis might be the expression of genetic adaptation or predisposition to PH inducers.

2. Angiogenic promoting factors

The hypoxia inducible factor (HIF) system is oxygen sensitive. It includes HIF-1 and HIF-2. They bind and activate many of the same genes, but differ in their participation during hypobaric hypoxia. For instance, HIF-2 alone is responsible for the liberation of erythropoietin through stimulation of connective tissue cells in the kidney (Paliege et al., 2010) and other hematological reactions to hypoxia (reviewed by Tissot van Patot and Gassmann, 2011). HIF-1 is a key molecule in regulating the expression of several angiogenic molecules. Among the hypoxia-inducible genes which have important HIF-1 binding sites and are believed to participate in the pathogenesis of PH and angiogenesis as well, are those encoding VEGF (Liu et al., 1995), the VEGF receptor 1 (Gerber et al, 1997), and ET-1 (Hu et al., 1998). Expression of VEGF and its receptor Fetal Liver Kinase 1 receptor (FLK-1) was enhanced in hypoxic pulmonary hypertensive rats (Christou et al., 1998). Rats maintained under hypoxic hypoxia (exposure to 10% oxygen) showed augmented volume and total length of pulmonary blood vessels (Howell et al. 2002, 2003; Ooi et al., 2000). In another study, rats exposed to hypobaric hypoxia, exhibited reduced Angiopoietin-1/Tie2 and VEGF expression, together with a diminished number of arterial blood vessels in the lung (Yamamoto et al., 2008).

Sands et al (2012) gave insight on the interaction of members of the VEGF family in hypoxic PH. They found that VEGFB and the placental growth factor (PGF) inhibit or potentiate the actions of VEGFA, according to their relative concentrations, which change in the lungs of rats subjected to hypoxic hypoxia. The same authors stated that the abovementioned effects *in vivo* depend on specific concentrations of VEGF and PGF within the alveolar wall during adaptation to hypoxia. In this context, human subjects developing acute mountain sickness had higher levels of plasma VEGF on ascend to altitude as compared to their own values obtained under normoxic conditions. This variation did not occur in healthy subjects (Tissot van Patot et al., 2005).

VEGF exerts its angiogenic effect through four different pathways, which include NO participation (proposed by Gramatikoff, 1999). In a previous study, adrenomedullin mRNA expression was greater in the lungs of chickens with pulmonary hypertension compared with those without pulmonary hypertension (Gómez et al., 2008) and Vadivel et al (2010) demonstrated that adrenomedullin promotes angiogenesis in the lung. Hence, two vasodilators of pulmonary blood vessels, NO and adrenomedullin, may also have angiogenic properties.

Fahra et al (2011) found that CD34(+) and CD133(+) progenitor cell numbers are higher in the bone marrow, blood, and pulmonary arteries of pulmonary hypertensive subjects as compared

to healthy controls. The blood levels of (hypoxia inducible myeloid-activating factors such as erythropoietin, the stem cell factor (SCF), and hepatocyte growth factor (HGF) were higher than normal levels in diseased individuals, and related to PH severity. Similarly, Davie et al (2004) found that progenitor cells from bone marrow origin contribute to neo-vascularization in pulmonary arteries of hypoxic calves, based on quantitative morphometric analyses of lung tissue from normoxic and hypoxic individuals. Their results showed adventitial growth in *vasa vasorum* of pulmonary arteries. This change was attributed to the transformation of cells from the bone marrow, which would be mobilized into the circulation, and differentiate into endothelial and smooth muscle cells. This model was supposed to entail an increase in the expression of c-kit(+), VEGF, fibronectin, and thrombin, as responses to hypoxia.

PH is linked to myeloid abnormalities, some of which may be related to increased production of HIF-inducible factors by a diseased pulmonary vasculature, but findings in non-affected subjects, suggest that myeloid abnormalities may be intrinsic to the disease process. Pro-angiogenic cell progenitors, and endothelial cells that have pathologic expression of hypoxia-inducible factor 1 alpha (HIF-1 alpha), were shown to be quantitatively higher in PH bone marrow, blood, and pulmonary arteries than in healthy controls. Also, the HIF-inducible myeloid-activating factors erythropoietin, stem cell factor (SCF), and hepatocyte growth factor (HGF) showed higher than normal levels in blood of pulmonary hypertensive subjects, and related to disease severity (Samar et al., 2011). In addition, endothelial progenitor cells with angiogenic capacity were found in the pulmonary circulation (Yoder, 2011). Epidermal growth factor promotes both angiogenesis and vascular remodeling (Janakidevi et al., 1995; Toby et al., 2010).

Bone morphogenetic protein (BMP) is compromised in endothelial repair within the pulmonary microvessels, and its expression is reduced in hypoxic PH, due to an increment in endothelial cell-derived Gremlin 1 and Gremlin 2, which antagonize BMP 2 type receptor expression (Cahill et al., 2012).

3. Inhibitors of angiogenesis

Angiogenesis is inhibited by thrombospondin-1 (TSP-1), a molecule produced by endothelial cells. Its secretion is induced by hypoxia and, experimentally, by NO inhibitors. In mesangial and smooth muscle cells, the cyclic guanosine monophosphate (GMPc) and kinase GMPc dependent protein, negatively regulate TSP-1 expression. CD36, a TSP-1 receptor, inhibits endothelial cell quimiotaxis (Isenberg el al., 2005). Low doses of NO stimulate endothelial cell proliferation, their motility and adhesion to collagen I matrixes, but the opposite is true for correspondent high doses. Exogenous TSP-1 treatment inhibits endothelial cell motility (Isenberg et al., 2005) and suppresses the angiogenic response provoked by low dose NO treatment (Ridnour et al., 2005).

Endogenous endostatin inhibits angiogenesis (Wickstrom et al., 2002; Paddenberg et al., 2006); it blocks the G1 phase of the cell cycle in endothelial cells, the attachment of $VEGF_{165}$ and $VEGF_{121}$ to the receptor VEGFR2 (Ribatti, 2009), and triggers endothelial cell apoptosis

http://dx.doi.org/10.5772/56054

(Dhanabal et al., 1999). Endostatin interferes with the assembly of the actin cytoskeleton of endothelial cells and inhibits their proliferation and participation in angiogenesis (Abdollahi et al., 2004; Skovseth et al., 2005). Endostatin expression on the surface of the smooth muscle cells of pulmonary arterioles in the mouse, is provoked by hypoxic exposure (Paddenberg et al., 2006). NO, in culture, induces endostatin production. Hence, endothelial apoptosis is also induced (Deininger et al., 2003). In human endothelial cell cultures, addition of endostatin reduces angiogenic activity by reducing HIF-1α, VEGF, VEGFR2, HGH and EGFR gene expression. Furthermore, expression of some anti-angiogenic genes, such HIF1An (a HIF antagonist), Kininogen, TSP-1 and a Vasostatin precursor is increased (Abdollahi et al., 2004). However, it appears that the mode of action of endostatin, in this context, needs to be fully elucidated (Deininger et al., 2003; Skovseth et al., 2005).

Adiponectin (ADPN) reinforces the vasodilatory action of NO in pulmonary blood vessels (Summer et al, 2009), and as stated, if NO contributes to the control of angiogenesis, ADPN will be a potential inhibitor of vascularization. Also, Le Cras et al (2003) found that alpha transforming growth factor (TGF-α) overexpression induced a diminution in pulmonary vascular development, which was accompanied by severe PH and vascular remodeling.

Oxidative stress is higher in the lungs of pulmonary hypertensive chickens (Iqbal y col., 2001a, b), which has been associated, in another species, with decreasing numbers of blood vessels (Murfee and Schmid-Schonbein, 2008). Oxidative stress, occurring in chronically hypoxic mice, can enhance endostatin production (Deininger et al., 2003) (Paddenberg et al., 2006).

4. Highlights on vascular development under hypoxia or normoxia in the chicken model

Angiogenesis in the mature pulmonary circulation could be a structural adaptation that may have important beneficial consequences for gas exchange (Howell et al., 2003). In contrast, Yamamoto et al (2008) reported a chronic hypoxia-induced pulmonary blood vessels loss, an event sometimes called “rarefaction” or “pruning”.

Although there is no definitive evidence of less vascularized areas in the avian lung, it is feasible that during hypoxia, vasculogenesis and angiogenesis occur to augment the gas exchange area. Hypoxia exposure for 3 to 4 weeks, does not affect pulmonary growth in current commercial chicken strains (Vásquez and Hernández, 2011; Areiza et al., 2011). These findings appear to be controversial, since alveolar and vascular neo-formation should both increase in the pulmonary response to hypoxia (reviewed by Bhattacharya, 2008). If this is not the case, less vascularized areas within the lung could gain new blood vessels, which might not significantly affect the whole organ´s weight. It should be noticed that arteriogenesis might also play a role in neo-vascularization (Deindi and Schaper, 2008)

The final goal of angiogenesis in the lung, should be to construct a complete functional arterio-venous tree. Therefore, vascular density would be represented by different types of blood vessels, such as arterioles or pre-capillaries. In this framework, using the chicken as a model,

quantitative and molecular studies were undertaken to test the potentiality of hypobaric hypoxia to induce angiogenesis, to detect possible differences in vascular density in pulmonary hypertensive chickens and to correlate these findings with mRNA expression of some key genes for angiogenesis. Under hypobaric hypoxic conditions (altitude 2638 m above sea level, (oxygen tension: approximately 111 mmHg), both, healthy and pulmonary hypertensive birds, had more blood vessels with diameter ranges between >100-200, >200-300 and >300-500 μm, as compared to healthy chickens maintained under relative, normobaric, normoxic conditions (460 meters above sea level (oxygen tension: approximately 152 mmHg). However, the opposite was encountered when comparing values obtained for blood vessels within the ≥50-100 μm range. Coincident with this result, decreased expression of hepatocyte growth factor (HGF), HIF-2α, VEGF, Flk-1, and HGFR genes was encountered in the lung of chickens exposed to hypoxia. The same mRNA expression pattern did not show coincidence with observations for blood vessels within the range of 100-500 μm (Areiza et al., 2011, 2012).

It is interesting that the mRNA expression of various genes compromised in both, angiogenesis and vascular remodeling, varies in pulmonary hypertensive (susceptible chickens) versus non-pulmonary hypertensive chickens, which indicates different degrees of resistance or susceptibility to hypobaric hypoxia (Gómez et al, 2007, 2008; Areiza et al., 2012). This coincidence reinforces the idea that among all the known compensatory mechanisms for low pO2 in the airways and hypoxemia, angiogenesis is one of them, but it might be a long term one.

5. Final remarks

Promoting angiogenesis, vasculogenesis, or arteriogenesis could represent a possible palliative treatment in individuals chronically exposed to hypobaric hypoxia. It is clear that it would be useful to establish some characteristics of vascular development in the lung of susceptible individuals subjected to chronic hypoxia, in order to design long term treatments, such as vascular neo-formation enhancement. In this context, it is desirable to further investigate the chronology of angiogenesis in the hypoxic lung.

Again, mesodermal derivatives particularly bone marrow cells, the endothelium, and fibroblasts, act in two different but possibly complementary ways, as a response to hypoxia: the remodeling process and pulmonary vascular neo-formation. However, as has been presently highlighted, some results are controversial, which could not be currently explained, since there is not enough information on the vascular development in the lung, as a result of hypoxic exposure of susceptible and non-susceptible individuals, which would allow for the understanding of the molecular framework of angiogenesis at different stages of development and possibly, the correspondent genes involved.

Angiogenesis is a distinct mechanism to compensate for the hypoxic conditions within the alveoli (or respiratory capillaries in birds). However, addition of new blood vessels to the lung as a compensatory mechanism, might be a time-dependent process, as it occurs in the placenta, where distinct factors intervene at different times during gestation (Hamilton et al., 1995; Athanassiades and Lala, 1998; Matsumoto et al., 2002; Wulff et al., 2002), and further studies

are needed in this matter. Evidence in this direction was given by Sands et al (2011), as related to the adapting process to hypoxia. They found that the *in vivo* actions of VEGFB and PGF can either inhibit or potentiate the actions of VEGFA. Those effects depend on their relative concentrations within the lung, which change in the hypoxic lung.

At this point, it is noted that ET-1, one the most studied molecules, has been chosen as a target molecule, in works aimed to design PH alleviation, by blocking its A receptor with bosentan (Weber et al., 1996; Lim et al., 2009). Also, vasoactive intestinal peptide was found to be a more potent ET-1 A receptor blocking agent than bosentan (Hamidi et al., 2011). Stromal derived factor 1 (SDF1), angiopoietin 2 (ANGPT2), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and stem cell factor (SCF) are also included as target molecules (reviewed by Rey and Semenza, 2010).

It is clear that both, angiogenesis and vascular remodeling as seen in PH, share common biological pathways and the endothelium appears to be the main participating structure in this regard. This coincidence might be an advantage in the design of therapeutic measures.

Some apparent differences in quantitative findings related to vascular neo-formation appear to depend on genetic differences and/or time of exposure to hypoxic conditions.

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