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Review

Cellular interplay in pulmonary arterial hypertension: Implications for new therapies

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a complex and multifactorial disease characterized by vascular remodeling, vasoconstriction, inflammation and thrombosis. Although the available therapies have resulted in improvements in morbidity and survival, PAH remains a severe and devastating disease with a poor prognosis and a high mortality, justifying the need of novel therapeutic targets. An increasing number of studies have demonstrated that endothelial cells (ECs), smooth muscle cells (SMCs) and fibroblasts of the pulmonary vessel wall, as well as platelets and inflammatory cells have a role in PAH pathogenesis. This review aims to integrate the interplay among different types of cells, during PAH development and progression, and the impact of current therapies in cellular modulation. The interplay among endothelial cells, smooth muscle cells and fibroblasts present in pulmonary vessels wall, platelets and inflammatory cells is regulated by several mediators produced by these cells, contributing to the pathophysiologic features of PAH. Current therapies are mainly focused in the pulmonary vascular tone and in the endothelial dysfunction. However, once they have not been effective, novel therapies targeting other PAH features, such as inflammation and platelet dysfunction are emerging. Further understanding of the interplay among different vascular cell types involved in PAH development and progression can contribute to find novel therapeutic targets, decreasing PAH mortality and morbidity in the future.

1. Introduction

Pulmonary arterial hypertension is a progressive and life-threatening disease, multifactorial in nature [1,2]. Although the "trigger" that leads to the disease is still unknown, a complex interplay among different types of cells occurs and multiple alterations are verified: (i) intimal hyperplasia; (ii) medial hypertrophy and hyperplasia; (iii) adventitia proliferation; (iv) neointima formation and (v) occurrence of plexiform lesions. In addition, these changes are accompanied by vasoconstriction, local inflammation and thrombosis in situ [3–6].

Endothelial cells, located in the inner layer of the pulmonary artery wall, have several O_2-sensing mechanisms, including O_2-sensitive NADPH oxidases, endothelial nitric oxide synthase, and heme oxygenases [7,8]. Vascular smooth muscle cells, located in the medial layer, have multiple stretch-sensing mechanisms, and are able to convert a mechanical stimulus into an intracellular signal that leads to modulation of gene expression and cellular function, such as contraction, proliferation, apoptosis and migration [9]. Fibroblasts, present in the adventitial compartment, may be considered the principal injury-sensing cells. They may experience functional changes due to stimuli such as vascular injury (Fig. 1) [10].

Abbreviations: 5-HT, serotonin; 5-HT_{1B}, serotonin type 1B receptor; 5-HT_{2A}, serotonin type 2A receptor; 5-HT_{2B}, serotonin type 2B receptor; 5-HTT, serotonin transporter; ADP, adenosine diphosphate; ATP, adenosine triphosphate; AVD, apoptotic volume decrease; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein receptor type II; cAMP, cyclic adenosine monophosphate; cav-1, caveolin-1; CCBs, calcium channel blockers; cGMP, cyclic guanosine monophosphate; DNA, deoxyribonucleic acid; ECM, extracellular matrix; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; ET-1, endothelin-1; ET_A, endothelin-1 type A receptor; ET_B, endothelin-1 type B receptor; FKN, fractalkine; HA, hyaluronic acid; MCT, monocrotaline; MMPs, matrix metalloproteinases; mRNA, messenger ribonucleic acid; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PAECs, pulmonary artery endothelial cells; PAH, pulmonary arterial hypertension; PAI-1, plasminogen activator inhibitor-1; PASMCs, pulmonary artery smooth muscle cells; PDE-5, phosphodiesterase type 5; PDGF, platelet-derived growth factor; PGI_2, prostacyclin; RANTES, Regulated upon Activation, Normal T-cell Expressed and Secreted; ROCK, Rho kinase; SMCs, smooth muscle cells; SM-MHC, smooth muscle-myosin heavy chain; SNAPs, soluble ATPase N-ethylmaleimide-sensitive factor association proteins; SNAREs, SNAP receptors; TASK-1, two pore-related acid-sensitive potassium channel-1; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; t-PA, tissue plasminogen activator; TXA_2, thromboxane A_2; VDCC, voltage-dependent calcium channels; α-SMA, α-smooth muscle actin

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Table 1
Current and novel therapies for pulmonary arterial hypertension.

Current therapies	Novel therapies
Endothelial cells	
Prostacyclin analogs	Rho kinase inhibitor
Epoprostenol	Fasudil
Treprostinil	
Iloprost	
Endothelin-1 receptor antagonists	Dual endothelin-1 receptor antagonist
Bosentan	Macitentan
Ambrisentan	Pyruvate dehydrogenase kinase inhibitor
	Dichloroacetate
	Endothelial progenitor cells
Smooth muscle cells	
Phosphodiesterase inhibitors	Soluble guanylate cyclase stimulator
Sildenafil	Riociguat
Tadalafil	
Calcium channel blockers	Prostacyclin receptor agonist
Nifedipine	Selexipag
Diltiazem	
Amlodipine	
Fibroblasts	
	Elastase inhibitors
Inflammatory cells	
	Rapamycin
	Tryptolide
	Thymulin
Platelets	
	Thromboxane synthesis inhibitors
	Ozagrel
	Furegrelate

Given the role of these cell types in the regulation of several vascular processes, the next points intend to detail various aspects of the contribution of these cell types to development and progression of PAH as well as of platelets and inflammatory cells. The mediators that regulate the interplay between these distinct cell types during PAH development and progression will also be analyzed envisioning novel therapeutic targets.

2. Endothelial cells

2.1. Role in pathophysiology of PAH

Endothelial damage is a key initial event in PAH. Although the mechanisms that mediate this damage are largely unknown, insults such as chronic hypoxia, inflammation, viral infection, mechanical stretch or shear stress, can activate the endothelial apparatus and induce cell apoptosis [11]. The death of ECs leads to the appearance of apoptotic-resistant and hyper-proliferative ECs, contributing to the vascular remodeling [12]. Endothelial cells not only contribute to the vascular remodeling linked to PAH development, but also regulate the related vasoconstriction and thrombosis processes, through the production and release of several mediators [2].

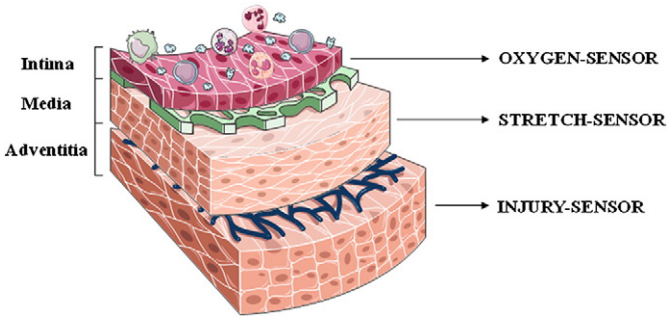

Fig. 1. "Sensing" mechanisms in the different layers of the pulmonary artery wall. Endothelial cells of the intima are equipped with mechanisms to sense differences in the oxygen supply. Medial smooth muscle cells have multiple stretch-sensing mechanisms that participate in the modulation of their functions. Fibroblasts, present in the adventitia, are considered the principal injury-sensing cells.

Endothelial cell migration and proliferation were thought to be responsible for the regeneration of injured endothelium. However, additional mechanisms were shown to replace the denuded or injured arteries. It has been reported that endothelial progenitor cells (EPCs) exert important functions in repairing and maintaining the integrity of the endothelial monolayer by replacing denuded parts of the artery [13]. After several pre-clinical studies demonstrate the beneficial effects of EPC administration, which was translated into a decrease of right ventricle systolic pressure, as well as a decrease in pulmonary and cardiac remodeling [14], clinical trials were performed. Autologous EPC administration in adult and pediatric idiopathic PAH patients showed an improvement on pulmonary hemodynamics and exercise capacity [15,16]. These results contrast with the finding that EPCs can contribute to PAH-related vascular remodeling [17]. However, given the promising results observed in clinical studies, the use of EPCs as a therapeutic option continues to be investigated. The results of the clinical trial PH (pulmonary hypertension) and Cell Therapy (PHACeT trial; ClinicalTrials.gov Identifier: NCT00469027), performed with the aim of evaluating the safety of administering autologous progenitor cells transduced with eNOS in idiopathic PAH patients are awaited. Furthermore, a combined therapy with autologous bone marrow-derived EPCs and sildenafil (a phosphodiesterase type 5 inhibitor) showed a superior efficacy than either bone marrow-derived EPCs or sildenafil alone in preventing monocrotaline (MCT)-induced PAH [18]. This combined therapy successfully abolished PAH-induced hemodynamic impacts on the right ventricle. Despite these encouraging results, it is yet unclear what is the EPC mechanism of action in PAH treatment. In this setting, a study reported that EPC infusion prevented MCT-induced PAH in athymic nude rats through a mechanism that requires the presence of natural killer cells [19].

2.2. Intracellular pathways modulated by PAH

The increase of endothelial cell permeability seems to have a huge contribution to PAH pathogenesis [20]. Stimuli that activate RhoA/ROCK, like thrombin, increase endothelial permeability. Stimuli that stimulate Rac1/p21-activated kinase, like prostacyclin (PGI_2), promote barrier integrity [20,21]. The net effect of EC permeability favors the release of mediators like endothelin-1 (ET-1), nitric oxide (NO), PGI_2 and thromboxane-A_2 (TXA_2) on SMCs and platelets. In addition, endothelial dysfunction in PAH can be reflected by a reduction in vasodilators/growth inhibitors like NO and PGI_2, and an increase in vasoconstrictors/co-mitogens like ET-1 and TXA_2 [20].

Another pathway that seems to contribute to EC permeability is the bone morphogenetic protein (BMP) signaling pathway. Recently, the loss of bone morphogenetic protein type II receptor (BMPRII) in ECs was reported to promote the extravasation of leucocytes into the pulmonary artery wall, increasing the susceptibility to inflammation [22]. Bone morphogenetic proteins are members of the transforming growth factor-β (TGF-β) family and signal via BMP type I and II receptors, which are serine/threonine kinase transmembrane receptors. This interaction with the receptors usually activates Smad1/5/8 that form complexes with Smad4 and translocate to the nucleus, regulating gene expression through the interaction with transcription factors. Besides the canonical Smad signaling, non-Smad pathways, such as the MAPK (mitogen-activated protein kinase), can also be involved [23,24]. As it is known, several gene mutations that lead to BMPRII loss of function have been associated to heritable PAH [25,26]. However, BMPRII expression is reduced in the pulmonary vasculature of patients with heritable and idiopathic PAH, independently of whether they present or not the BMPRII gene mutation [27]. In ECs, BMPRII activation seems to promote cell proliferation, migration and survival [28,29]. Loss of BMPRII function in these cells was reported to induce apoptosis, contributing to the

vascular remodeling associated to PAH [28], as described above. Recently, a study reported that BMPRII ligands, BMP2 and BMP4, stimulate eNOS (endothelial nitric oxide synthase) activity in PAECs (pulmonary artery ECs), inducing NO synthesis. Furthermore, eNOS activity stimulation was shown to be dependent on protein kinase A activation and BMP-stimulated PAEC migration needs eNOS activity. Importantly, BMP2 and BMP4 failed to stimulate eNOS phosphorylation/activation in PAECs collected from PAH patients with BMPRII gene mutations [30].

Endothelial cell mitochondrial metabolism is also affected in PAH. A shift from oxidative phosphorylation to glycolysis was reported [1]. Xu et al. [31] verified, through positron tomography scan, a significantly higher uptake of glucose in PAECs from patients with idiopathic PAH compared with ECs from controls. In addition, they demonstrated that oxygen consumption, the number of mitochondria *per* cell and the mitochondrial DNA content of idiopathic PAH endothelial cells were lower in comparison with ECs from control patients.

Alterations in the intracellular trafficking were also reported in PAH [32]. Histological and electron microscopy studies highlighted enlarged endoplasmic reticulum and Golgi stacks in pulmonary arterial lesions in human and in experimental (hypoxia and MCT) PAH [33,34]. In addition, the loss of the cell surface raft/caveolar protein caveolin-1 (cav-1) in ECs in MCT-induced PAH was shown [35]. Monocrotaline and hypoxia seem to disrupt the molecular machinery of vesicular trafficking at the level of Golgi tethers, SNAPS (soluble ATPase N-ethylmaleimide-sensitive factor association proteins) and SNARES (SNAP receptors) (Golgi blockade hypothesis) [36,37]. This disruption seems to result in the trapping of cav-1 in the Golgi of ECs, with consequent intracytoplasmic sequestration of eNOS and thus, reduction of NO production [36].

Currently, the endothelial dysfunction is a primary therapeutic target in PAH, being used drugs such as prostacyclin analogs, endothelin-1 receptor antagonists and phosphodiesterase type 5 (PDE-5) inhibitors to counteract this abnormality [38]. The first PGI_2 synthetic analog approved by the FDA was intravenous epoprostenol. Although being to date the only PAH therapy that was associated with a mortality benefit in a randomized clinical trial, epoprostenol administration might lead to catheter infections [38]. To overcome this, a more stable formulation of epoprostenol became recently available [39,40]. Treprostinil (subcutaneous, intravenous and inhaled) and iloprost (inhaled) were approved as PGI_2 analogs, also with some limitations. In the case of subcutaneous treprostinil, infusion site pain occurs in the majority of patients and inhaled iloprost needs to be administrated 6 to 9 times daily, up to 15 min each [38,41]. Currently, a phase III clinical trial is underway to evaluate the effect of an oral formulation of a PGI_2 receptor agonist named selexipag (GRIPHON trial; ClinicalTrials.gov Identifier: NCT01106014). Approved therapies targeting ET-1 pathway include the dual ET-1 receptor antagonist bosentan and the selective ET_A receptor antagonist ambrisentan, both available in oral formulations. In the case of bosentan, regular liver function tests are needed, once it is associated with an increased risk of liver dysfunction [38,41,42]. Macitentan is a new oral dual ET-1 receptor antagonist that presents an increased efficacy due to its high lipophilicity [38,43]. Sildenafil and tadalafil are oral PDE-5 inhibitors approved for the treatment of PAH. Side effects include headache, flushing, dyspepsia and myalgia [38,41]. Riociguat, a soluble guanylate cyclase stimulator, was evaluated in a phase III clinical trial and showed promising results [44]. Furthermore, fasudil [45,46] and dichloroacetate [47], a Rho kinase inhibitor and a pyruvate dehydrogenase kinase inhibitor, respectively, include drugs in development to the treatment of PAH. Dichloroacetate is in evaluation in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT01083524) (see Table 1).

3. Smooth muscle cells

3.1. Role in pathophysiology of PAH

Vascular SMCs can present different phenotypes, depending on their functions. These phenotypes are characterized by differences in cell morphology, proliferation and migration rates and in the expression of protein markers [48,49]. The different phenotypes are seen in SMCs of different vessels as well as among SMCs within the same vessel [48]. A contractile SMC phenotype is typified by elongated cells, with a slow growth and migratory rates. On the contrary, synthetic SMCs have a rhomboid morphology and higher growth and migratory activities [50]. Once the protein markers used to identify SMCs are also expressed in other cell types in normal and pathologic situations, the recognition of these cells is sometimes a difficult task. Currently, the two protein markers most used to characterize a mature contractile SMC phenotype are smooth muscle-myosin heavy chain (SM-MHC) and smoothelin. SM-MHC expression has never been detected in non-SMCs in vivo. Smoothelin complements SM-MHC as a contractile SMC marker and appears to be more sensitive [10,48]. Other markers expressed in a contractile SMC include α-smooth muscle actin (α-SMA), desmin, smooth muscle-calponin and h-caldesmon. Synthetic SMCs express proteins like cellular retinol binding protein-1 and SMemb/non-muscle MHC isoform-B [48,51]. During vascular injury, SMCs "switch" from a contractile to a synthetic, proliferative phenotype to support vascular repair. Importantly, this phenotypic switch is often reversible. However, deregulation of this process can contribute to the vascular remodeling associated to PAH [52,53]. Discovering the mechanisms involved in the SMC phenotype modulation can help to control this "switch" process. Examples include pathways/mediators such as TGF-β, PDGF (platelet-derived growth factor), angiotensin II and TNF (tumor necrosis factor)-α [48]. PDGF-A and PDGF-B induce, in adult SMCs, a more synthetic phenotype [48]. Increased proliferation and migration of SMCs were observed in pig coronary arteries treated with PDGF-B [54]. In addition, PDGF-A and PDGF-B inhibition resulted in reduced SMC proliferation and migration in injured human artery [55,56]. Furthermore, TGF-β isoforms induce a contractile SMC phenotype [48]. Importantly, a recent study investigated the crosstalk between ECs and SMCs during low shear stress induced vascular remodeling and identified the factors TGF-β1 and PDGF-BB as important players in this process. The authors used an EC/SMC cocultured parallel-plate flow chamber system and each cell type was grown on the opposite sides of a porous membrane. Then shear stress was applied to ECs and the effects were observed. They reported that low shear stress induces the production of PDGF-BB and TGF-β1 by ECs and the proliferation and migration of ECs and SMCs. PDGF-BB was involved in the paracrine control of SMCs by ECs, and TGF-β1 participates in the feedback control from SMCs to ECs [57]. More recently, microRNAs (miRNAs) emerged as important modulators of SMC phenotype, and consequently as possible therapeutic targets in PAH-related vascular remodeling. These tissue and cell specific non-coding single-strand RNA molecules with approximately 19–25 nucleotides negatively regulate gene expression by base pairing to the 3′-untranslated region of mRNAs, avoiding their translation [58–60]. Several miRNAs seem to be differentially expressed during PAH development in the hypoxia and MCT-induced PAH and also in patients [61,62]. MiR-143, -145 and -204 are examples of miRNAs reported to be involved in SMC phenotype regulation. MiR-204 levels were found down-regulated in MCT and hypoxic PAH animal models [62]. In addition, in PASMCs from idiopathic PAH patients, miR-204 expression was also found to be reduced and increased levels of proliferation and lower levels of apoptosis were verified in comparison with control PASMCs. A synthetic miR-204 already showed its beneficial effects in the pulmonary arteries of MCT rats [61]. The expression of miR-143/145 was reported to be elevated in hypoxic PAH model and also in patients with heritable or idiopathic PAH. The increased expression of miR-143/145 was found to be related with PAH associated with BMPRII mutation, and so with BMP receptor function loss [63]. Importantly, BMP signaling pathway is known to induce miR-143/145 expression, and so these observations can be explained by the compensatory increase in TGF-β signaling pathway in response to the decrease in BMP signaling pathway, once TGF-β mediators also induce miR-143/145 expression [64].

3.2. Intracellular pathways modulated by PAH

Potassium (K^+) and calcium (Ca^{2+}) ions have been reported to play pivotal roles in both vasoconstriction and vascular remodeling in PAH [65]. When K^+ channels are blocked (or K^+ channels are down-regulated), PASMC membrane depolarizes and opens voltage-dependent Ca^{2+} channels (VDCC), promoting a Ca^{2+} influx, increasing $[Ca^{2+}]_{cyt}$ and causing PASMC vasoconstriction. Conversely, when K^+ channels are activated (or K^+ channel gene expression is up-regulated), the membrane hyperpolarizes and closes VDCC, inhibiting Ca^{2+} influx, decreasing $[Ca^{2+}]_{cyt}$ and causing vasodilatation [65–67]. Calcium is also an important second messenger that leads to cell proliferation and migration through the activation of transcription factors. In apoptosis, K^+ channels mediate the K^+ efflux that is necessary for apoptotic volume decrease (AVD). K^+ efflux also leads to the decrease of $[K^+]_{cyt}$, releasing the inhibition of caspases [65,67]. A defect in gene expression with attenuated function of Kv channels was reported in PASMCs from patients with pulmonary hypertension [68]. Thus, a decrease in K^+ channels gene expression and/or function stimulates PASMC proliferation by increasing $[Ca^{2+}]_{cyt}$ and inhibits PASMC apoptosis by decelerating AVD and attenuating cytoplasmic caspase activity [68]. Although numerous studies have investigated ion channels in PAH, little is known about the association between the two-pore domain K^+ channel, TASK-1, and human PASMCs. Tang et al. [69] reported the involvement of TASK-1 in the ET-1-mediated depolarization in human PASMCs. The authors concluded that ET-1 depolarizes primary human PASMCs by phosphorylating (inhibiting) TASK-1 by a mechanism in which ET-1 binds to the ET_A receptor, leading to the protein kinase C-induced phosphorylation of TASK-1 channels through phospholipase C, phosphatidylinositol 4, 5-biphosphate and diacylglycerol.

Unfortunately, only 10–15% of idiopathic PAH patients could benefit from long-term therapy with calcium channel blockers (CCBs) (nifedipine, diltiazem and amlodipine) [70,71]. During diagnosis by cardiac catheterization, patients perform an acute vasodilator test with agents such as nitric oxide, prostacyclin or adenosine. If the response to this test is positive (decrease of at least 10 mm Hg in the mean pulmonary arterial pressure, to a value of less than 40 mm Hg with an increased or unchanged cardiac output), the patient can benefit from CCB therapy [72,73].

4. Fibroblasts

4.1. Role in pathophysiology of PAH

Adventitia, the principal "injury-sensing tissue" of the vessel wall, undergoes several changes during PAH. Adventitia fibroblasts are able to proliferate with greater propensity than SMCs in response to injury, increasing extracellular matrix (ECM) component deposition, and synthesizing and releasing molecules that act on SMCs and ECs, and facilitate the recruitment of circulating leucocytes and progenitor cells. Thus, fibroblasts seem to contribute to inflammation and vascular remodeling linked to PAH [74,75].

4.2. Intracellular pathways modulated by PAH

The identification of fibroblasts is a tricky task, because of the lack of specificity and sensitivity of the markers used (vimentin and α-SMA). Vimentin does not distinguish fibroblasts from other cells of mesenchymal origin and α-SMA is only generally observed in activated fibroblasts. In addition, this last marker is observed in SMCs and in circulating smooth muscle precursors [75]. Activation of fibroblasts results in their differentiation into myofibroblasts, leading to the production of ECM proteins, such as collagen, fibronectin, tenascin and elastin. The appearance of myofibroblasts expressing α-SMA was observed in the adventitia in hypoxia-induced PAH [75]. In addition, an excessive deposition of ECM proteins in the adventitia in PAH was shown [76].

Glycosaminoglycans, like hyaluronic acid (HA), are components of the ECM that control SMC proliferation and differentiation. A study performed with idiopathic PAH human lung tissue, showed an increase in the expression of HA associated with increased hyaluronan synthase-1 (responsible for the synthesis of HA) and decreased hyaluronoglucosaminidase-1 (important in the degradation of HA) gene expression [77]. Aytekin et al. [78] verified that patients with idiopathic PAH have higher circulating levels of HA. They also showed that the expressions of HA synthase-2 and hyaluronidase-2 decrease in idiopathic PAH compared with controls. Thus, the decreased HA degradation seems to have a higher impact than the increased HA production in the elevated HA levels in PAH. It is known that HA has a variety of biological functions, which differ according to the molecular weight. HA degradation in high molecular mass (HMM) fragments (20.000–500 kDa) manifests immunosuppressant and anti-angiogenic activities. Conversely, low molecular mass (LMM) HA fragments (lower than 500 kDa) have been shown to stimulate inflammation and to promote EC and SMC proliferation and migration [79]. Once the accumulation of pro-inflammatory and pro-proliferative HA fragments resulting from matrix degradation can be associated with PAH-related inflammation and vascular remodeling, Ormiston et al. [80] studied the potential role of HA degradation at different stages of PAH progression in MCT-induced PAH. The authors found a generation of LMM HA at the early stages and an enhanced synthesis of HMM HA in advanced disease. Thus, the early generation of LMM HA fragments can lead to the inflammatory and proliferative modulation associated with PAH.

Matrix metalloproteinases (MMPs), a family of zinc enzymes produced by fibroblasts and macrophages and responsible for degradation of the ECM components, are also important to SMC and EC migration and proliferation. Excessive expression of MMPs may contribute to the pathogenesis of PAH, because MMPs may lead to fibroblast migration into the media and intima [75]. MMP activity was found to be up-regulated in adventitial fibroblasts in hypoxia and MCT-induced PAH [75,81]. Furthermore, also serine elastases can induce the release of growth factors from the ECM, promoting PASMC proliferation. Elastase activity was reported to be increased in PAH and serine elastase inhibitors already showed its beneficial effects in hypoxia and MCT-induced PAH [82–85].

5. Inflammatory cells

5.1. Role in pathophysiology of PAH

Inflammatory processes have been recognized as major pathogenic components of pulmonary vascular remodeling [86]. There are several observations demonstrating that inflammation performs a role in PAH development: i) association of inflammatory conditions such as connective tissue disorders and certain infections like human immunodeficiency virus with PAH; ii) accumulation of T cells, B cells and macrophages in plexiform lesions; iii) detection of auto-antibodies to ECs and fibroblasts, and iv) elevation in the circulating levels of certain cytokines and chemokines [1,11,87]. In addition, a study described an experimental model of antigen-induced pulmonary arterial muscularization, demonstrating that $CD4^+$ T cells, the IL-4-induced Th2 response, and endogenous IL-13 can be related with the vascular remodeling process [88].

5.2. Intracellular pathways modulated by PAH

Mast cells are implicated in inflammation and tissue remodeling. When activated, they produce several mediators including serotonin (5-HT), cytokines IL-6 and IL-13 and serine proteases chymase and tryptase that are capable to activate MMPs [89]. It was reported that mast cells are a rich source of IL-4, as well as other factors that can stimulate B-cells to secrete auto-antibodies, including anti-endothelial cell antibodies and its degranulation had been linked to the development

of pulmonary vascular remodeling in chronic hypoxic rats [90]. In addition, a study demonstrated that the accumulation and activation (degranulation) of mast cells in the lungs contribute to the development of PAH in MCT-rats and mast cell population was increased in idiopathic PAH patients compared with controls [91]. Also, Farha et al. [92] reported that mast cells may promote vascular remodeling, contributing to PAH in patients. In addition to mast cells, several other inflammatory cells like T cells, B cells and dendritic cells showed to have a role in PAH development [11,93]. Increased levels of certain cytokines, including IL-1β, IL-6 and TNF-α were observed in plasma of PAH patients [11,86,94]. Importantly, Hagen et al. [95] reported a negative feedback loop between IL-6 and the BMP pathway, mediated through p38 MAPK activity. Given the low penetrance of BMPRII mutations, alterations in other pathways may be needed to initiate PAH development and thus, the IL-6 activation can be considered an inflammatory "second hit" associated with the loss of BMP signaling that predisposes to PAH.

With the involvement of inflammation in PAH development, several anti-inflammatory and immunosuppressant therapies have emerged. Examples include rapamycin [96], tryptolide [97] and thymulin [98] that showed beneficial effects in PAH experimental models.

6. Platelets

6.1. Role in pathophysiology of PAH

Platelet dysfunction is a crucial contributor to PAH-related thrombosis [74]. In addition, also coagulation cascade abnormalities and endothelial cells seem to have a role on it. Underlying these alterations are increased levels of von Willebrand factor, plasma fibrinopeptide A, plasminogen activator inhibitor (PAI)-1, 5-HT and TXA_2, and decreased levels of tissue plasminogen activator (t-PA), thrombomodulin, NO, and PGI_2 [99,100]. Furthermore, a study recently evaluated, in PAH patients, the expression of PAR-1 (protease activator receptor-1), an important mediator of platelet activation by thrombin [101]. The authors found that the subpopulation of PAH patients that presented lower platelet counts seems to have increased platelet membrane expression of PAR-1 and PAR-mediated surface exposure of P-selectin (an adhesion molecule involved in inflammation and thrombosis), which may represent increased propensity to thrombosis.

6.2. Intracellular pathways modulated by PAH

Platelets have secretory granules which contain stored proteins and small molecules that are released in a regulated manner upon stimulation [102]. Dense granules store molecules such as ATP, ADP, 5-HT, glutamate, calcium and pyrophosphate. Alpha granules contain an extensive list of proteins, such as platelet-factor 4 (PF4/CXCL4), RANTES (CCL5), IL-1α and IL-1β, TGF-β and TNF-α [102]. Thus, platelet activation in PAH not only promotes thrombosis but also leads to the release of granule content, including mitogenic agents and vasoconstrictive substances, such as serotonin [103].

The beneficial effects of several drugs targeting the mechanisms associated with thrombosis support the important role of this process in PAH development. Examples of these drugs include thromboxane synthesis inhibitors, such as ozagrel and furegrelate. The first showed positive effects in a patient with portopulmonary hypertension and the second was tested in a hypoxia PAH experimental model [104,105].

7. Interplay between cell types involved in PAH pathogenesis

Although the mechanisms that lead to pulmonary arterial hypertension are still poorly understood, it is known that the interaction between vascular cells, inflammatory cells and platelets is involved in the structural changes that culminate in an increased pulmonary arterial pressure. This interplay is regulated by several mediators, which are highlighted in Fig. 2.

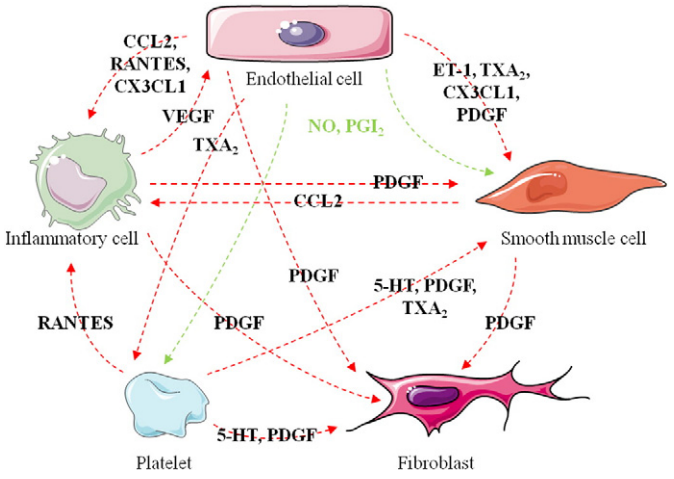

Fig. 2. Interplay between endothelial, smooth muscle and inflammatory cells, platelets and fibroblasts in pulmonary arterial hypertension. Green arrows denote the interaction between endothelial cells, platelets and smooth muscle cells, mediated by NO and PGI_2, which leads to vasodilator, anti-proliferative and anti-platelet aggregation effects. Red arrows show the interactions mediated by several mediators associated with vasoconstriction, proliferation, migration and platelet aggregation. Figure made with Servier Medical Art.

The vascular remodeling, mainly characterized by an excessive cell proliferation and a reduced apoptosis, is one of the most important features of PAH. For this structural change, ECs contribute with a pivotal role, producing and releasing mediators such as NO, PGI_2, ET-1 and TXA_2 that control platelet and SMC behavior. In addition to changes in the production levels of these mediators, also the increased endothelial barrier permeability counts to the PAH vascular alterations, by exposing the neighboring cells to these mediators [20,106].

Nitric oxide is synthesized in the endothelial cells from L-arginine by eNOS. Upon its release by endothelium, NO diffuses into vascular SMCs where it stimulates soluble guanylate cyclase (sGC) to produce the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), which has vasodilatory, anti-proliferative and anti-platelet aggregation properties [2]. The NO pathway is impaired in several ways in PAH. eNOS expression is depressed [107] and phosphodiesterase type 5 is increased in PASMCs, which leads to inactivation of cGMP [108]. The production of endogenous NOS inhibitors, asymmetrical and symmetrical dimethylarginines (ADMA and SDMA, respectively), is enhanced in PAH [109]. In addition, sGC expression is up-regulated in human PAH, as a compensatory mechanism [110]. In addition to eNOS, also iNOS (inducible NOS) seems to have a role in PAH, once a recent study reported that iNOS inhibition protected against PAH [111].

Prostaglandin, generated from arachidonic acid by fatty acid cyclooxygenase, is a substrate for both prostacyclin synthase and thromboxane synthase. Prostacyclin synthase generates PGI_2 in PAECs, which relaxes and inhibits the proliferation of vascular SMCs, and also inhibits platelet aggregation, via the stimulation of cAMP production [2]. Analysis of urinary metabolites of PGI_2 showed a decrease in the amount of excreted 6-ketoprostaglandin F1α, a prostacyclin inactive metabolite, in patients with idiopathic PAH [112]. Moreover, PAECs of PAH patients are characterized by reduced expression of prostacyclin synthase [113]. Prostacyclin receptor expression is also reduced in PASMCs [114]. Thromboxane synthase generates TXA_2, which stimulates SMC vasoconstriction and proliferation and platelet aggregation via thromboxane receptors [2]. An increase in the urinary excretion of 11-dehydrothromboxane B_2 (a stable metabolite of thromboxane A_2) [112] and in total body synthesis of TXA_2 in patients with idiopathic PAH was verified [115].

Endothelin-1 is mainly produced by ECs and acts via two receptors: ET_A and ET_B. Both receptors are found in PASMCs and mediate vasoconstriction and proliferation, whereas only the ET_B receptor is present in

ECs and mediates NO and PGI_2 release, leading to vasodilatation [116,117]. Lung and circulating ET-1 levels are increased in PAH patients [118]. Another mediator responsible for cell proliferation is PDGF, synthesized and released by several cell types (SMCs, ECs, macrophages and platelets). The expression of PDGF and its receptors was reported to be increased in the pulmonary arteries of PAH patients [119], contributing to a higher proliferation rate of SMCs and fibroblasts. Platelets also contribute to vascular remodeling through the release of factors such as PDGF, TXA_2 and 5-HT that act on fibroblasts and SMCs leading to vasoconstriction, proliferation and migration. Cellular migration is also reported in PAH and is associated with vascular wall thickness. Myofibroblasts are able to migrate from the adventitia to the media and to the intima, thus contributing to the thickening of these components. For this cell migration MMPs secreted by fibroblasts and macrophages might also contribute by degrading ECM components [75,120].

Thrombosis in small peripheral pulmonary arteries also contributes to PAH. In addition to platelets, ECs are directly involved in this process. Endothelial cells participate in the coagulation process by activating the factor X and the extrinsic pathway of coagulation via release of tissue factor. ECs also produce and release von Willebrand factor that attracts and activates platelets [121]. On the other hand, ECs can also inhibit thrombosis and promote fibrinolysis. They produce and release NO and PGI_2, two important inhibitors of platelet aggregation, preventing thrombosis. Endothelial cells participate in the fibrinolytic process through the synthesis and release of the profibrinolytic t-PA that activates plasminogen in the fibrinolytic cascade. In addition, ECs also produce the antifibrinolytic/prothrombotic PAI-1 [121]. Thus, ECs are important regulators of the balance between prothrombotic and antithrombotic processes.

PAH-related vasoconstriction also results from the interplay between several cells, including ECs, SMCs and platelets. Endothelial cells produce and release vasoconstriction mediators such as ET-1 and TXA_2 that act in SMCs and platelets [2]. On the other hand, platelets release 5-HT and TXA_2 that can act on SMCs and fibroblasts [121]. Serotonin is produced by the gastrointestinal tract enterochromaffin cells and pulmonary neuroepithelial bodies and stored in platelets [122]. It is taken up by the serotonin transporter (5-HTT or SERT) and the serotonin receptors ($5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$) in the pulmonary artery smooth muscle, endothelial cells and fibroblasts [123]. Serotonin is a vasoconstrictor that stimulates the proliferation of SMCs and fibroblasts [123,124]. A significant rise of 5-HT in plasma is observed in pulmonary hypertensive patients and the level in platelets is low [125].

In response to inflammatory/injury events, vascular cells (ECs and SMCs) can produce mediators such as cytokines and chemokines (soluble cytokines with chemoattractant function) that will be responsible for the recruitment of inflammatory cells (T cells, B cells, macrophages, dendritic cells, mast cells). These inflammatory cells can thereby continue the release of chemokines, cytokines and also growth factors, such as VEGF (vascular endothelial growth factor), which will promote EC proliferation and migration and resistance to apoptosis, contributing to the vascular remodeling [11]. Endothelial cells and SMCs are reported to release the chemokine CCL2/MCP-1 (monocyte chemotactic protein-1). The same study showed an increase in CCL2 levels in plasma and lung tissue of idiopathic PAH patients. In addition, it also showed that monocyte migration was higher in the presence of ECs from PAH patients in comparison with controls. Furthermore, ECs from PAH patients also seem to be responsible for the production and release of RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted)/CCL5 in PAH patients. An increased expression of RANTES mRNA was observed in lung tissue from PAH patients [126]. Pulmonary artery ECs from PAH patients also expressed the chemokine FKN (fractalkine/CX3CL1). Elevated FKN plasma levels and an increase in FKN mRNA expression in lung tissue were observed in PAH patients, in comparison with controls. In addition, the FKN receptor (CX3CR1) expression was found to be increased in vascular SMCs and in circulating T-cells from PAH patients. Fractalkine was found to induce proliferation but not migration of cultured rat PASMCs [127,128].

8. Conclusions

Despite the success of PAH stabilization with the current medical therapy targeting pulmonary vascular tone, this disease remains associated to a poor prognosis. Furthermore, most of the alternative/novel therapeutic approaches have been studied in experimental models, and once there is no ideal animal model, the translation of data to humans is challenging. Therefore, we are still tracking the best treatment for PAH. Considering that disease progression is driven by a cellular interplay at the pulmonary artery wall that leads to vasoconstriction, thrombosis, inflammation and vascular remodeling, we believe that an effective treatment must target PAH-related cellular features.

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