Formation of Plexiform Lesions in Experimental Severe Pulmonary Arterial Hypertension

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Background—The plexiform lesion is the hallmark of severe pulmonary arterial hypertension. However, its genesis and hemodynamic effects are largely unknown because of the limited availability of lung tissue samples from patients with pulmonary arterial hypertension and the lack of appropriate animal models. This study investigated whether rats with severe progressive pulmonary hypertension developed plexiform lesions.

Methods and Results—After a single subcutaneous injection of the vascular endothelial growth factor blocker Sugen 5416, rats were exposed to hypoxia for 3 weeks. They were then returned to normoxia for an additional 10 to 11 weeks. Hemodynamic and histological examinations were performed at 13 to 14 weeks after the Sugen 5416 injection. All rats developed pulmonary hypertension (right ventricular systolic pressure $>100$ mm Hg) and severe pulmonary arteriopathy, including concentric neointimal and complex plexiform-like lesions. There were 2 patterns of complex lesion formation: a lesion forming within the vessel lumen (stalk-like) and another that projected outside the vessel (aneurysm-like). Immunohistochemical analyses showed that these structures had cellular and molecular features closely resembling human plexiform lesions.

Conclusions—Severe, sustained pulmonary hypertension in a very late stage of the Sugen 5416/hypoxia/normoxia-exposed rat is accompanied by the formation of lesions that are indistinguishable from the pulmonary arteriopathy of human pulmonary arterial hypertension. This unique model provides a new and rigorous approach for investigating the genesis, hemodynamic effects, and reversibility of plexiform and other occlusive lesions in pulmonary arterial hypertension. (Circulation. 2010;121:2747-2754.)

Key Words: hypertension, pulmonary → pathology → remodeling → SU5416

Pulmonary arterial hypertension (PAH) comprises a multifactorial group of pulmonary vascular disorders that frequently lead to right heart failure and premature death. Major factors contributing to the complicated pathogenesis include sustained vasoconstriction and progressive fixed vascular remodeling. Histologically, patients with severe PAH have combinations of small pulmonary arterial medial and adventitial thickening, occlusive neointima, and complex plexiform lesions. Despite recent advances in our understanding of the pathogenesis of PAH, the genesis, hemodynamic effects, and reversibility of these lesions remain controversial. This is due mainly to the limited availability of serial lung tissue samples from patients with PAH and the lack of appropriate animal models.

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Although the plexiform lesion is regarded as the hallmark of severe PAH, it is not known whether the lesion is the cause or the effect of the hypertension. Furthermore, although there are several hypotheses, it is also not known exactly how and where in the pulmonary arterial tree the lesion is formed and how hemodynamically important it is. Human studies will probably never answer these questions because it is essentially impossible to obtain serial lung biopsy samples and matched hemodynamic data for detailed assessment of lung vascular morphology and its contribution to the increased vascular resistance. Thus, it is invaluable to establish an animal model that closely mimics the pathophysiology and pulmonary arteriopathy of human PAH. In addition, PAH patient recruitment for new clinical trials is becoming increasingly difficult. It is essential, therefore, to narrow down candidate therapeutic agents or their combinations by rigorous preclinical drug evaluations in such an animal model.

The major limitation of the frequently studied animal models of pulmonary hypertension (ie, chronically hypoxic...
and monocrotaline-injected rodents) is that they do not develop occlusive neointimal and plexiform lesions.9–11 Chronically hypoxic (3 to 4 weeks) rats do not show any such lesions. In addition, the existence of the complex lesion was not reported in 1 study that performed histological examination of lungs from rats exposed to longer periods of hypoxia (up to 11 weeks).12 Thus, it appears that chronic hypoxia alone is not sufficient to cause plexiform lesion formation, probably because the pulmonary hypertension is relatively mild compared with patients with severe PAH. In the case of the monocrotaline model, the rats possibly die of cardiac and renal dysfunction before these lesions form. A more recent rat model that better mimics the pulmonary arteriopathy of human PAH is a single subcutaneous implantation of the vascular endothelial growth factor (VEGF) receptor blocker Su5416, semaxinib, combined with exposure of the rats to 3 weeks of chronic hypoxia (10% O2) followed by 2 weeks of reexposure to normoxia.13,14 By 5 weeks (3 weeks of chronic hypoxia and 2 weeks of reexposure to normoxia) after the Su5416 injection, this “2-hit” model, but neither Su5416 nor hypoxia alone, develops severe progressive PAH associated with the formation of occlusive neointimal lesions in small pulmonary arteries and arterioles.13 However, the advanced neointimal (ie, concentric laminar) and plexiform lesions characteristic of human severe PAH have not been observed at this stage.

Limited human studies suggest that it takes some time to develop plexiform lesions after the establishment of significantly severe PAH.15 We therefore hypothesized that later stages of the Su5416/hypoxia/normoxia-exposed rat would develop plexiform lesions. In this study, we tested this hypothesis by examining hemodynamically and histologically a very late stage of the Su5416 plus hypoxia/normoxia-exposed rat (ie, 13 to 14 weeks after the Su5416 injection).

Methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. Adult male Sprague-Dawley rats weighing 180 to 220 g (n=12) were injected subcutaneously with Su5416 (20 mg/kg) and exposed to hypoxia (10% O2) for 3 weeks.2 They were returned to normoxia (21% O2) for an additional 10 to 11 weeks (total 13 to 14 weeks after Su5416 injection). An additional 15 rats were used for histological examination at various time points after Su5416 injection (plus 3 weeks of hypoxic exposure): 0 (normal control without Su5416 injection), 5, and 8 weeks (5 rats each; Harlan Laboratories, Indianapolis, Ind).

Hemodynamic Measurements in Catheterized Rats

Rats were placed on controlled heating pads after they were anesthetized with pentobarbital sodium (30 mg/kg IP). Hemodynamic measurements were performed under normoxic conditions. Polyvinyl catheters (PV-1, Saint-Gobain, Courbevoie, France; internal diameter, 0.28 mm) were inserted into the right ventricle (RV) via the right jugular vein for measurement of RV systolic pressure (RV systolic pressure instead of pulmonary arterial pressure was measured because we could not consistently catheterize the pulmonary artery as a result of extremely high pressures).14 A microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, Tex) was inserted into the right ventricle (RV) via the right jugular vein for measurement of RV systolic pressure (RV systolic pressure instead of pulmonary arterial pressure was measured because we could not consistently catheterize the pulmonary artery as a result of extremely high pressures).14 A microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, Tex) was inserted into the right carotid artery and advanced into the left ventricle (LV) as described previously.16 The signals were continuously recorded by the MPVS-300 system with PowerLab/4SP, A/D

Table 1. Hemodynamic Parameters and RV Hypertrophy Index

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<th>PAH Rats</th>
<th>Historical Normal Mean Values</th>
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<tr>
<td>RVSP, mm Hg</td>
<td>96±11</td>
<td>21</td>
</tr>
<tr>
<td>MSAP, mm Hg</td>
<td>128±11</td>
<td>117</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>349±18</td>
<td>372</td>
</tr>
<tr>
<td>CI, mL·min⁻¹·kg⁻¹</td>
<td>40±5</td>
<td>119</td>
</tr>
<tr>
<td>RV/LV+S</td>
<td>0.74±0.04</td>
<td>0.25</td>
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RVSP indicates RV systolic pressure; MSAP, mean systemic arterial pressure; HR, heart rate; and CI, cardiac index. RV/LV+S is an index of RV hypertrophy. Values are mean±SE of Su5416/hypoxia/normoxia-exposed PAH rats (n=11).

Figure 1. A, A representative low-magnification photomicrograph showing various types of pulmonary vascular lesions in a very-late-stage Su5416/hypoxia/normoxia-exposed rat lung. B through E, Higher-magnification photomicrographs of medial wall thickening (B), concentric cellular laminar neointimal lesion (C), plexiform lesion (arrow) adjacent to a small pulmonary artery with medial wall thickening and eccentric neointimal proliferation (D), and nearly complete occlusion of 2 small pulmonary arteries by concentric neointimal proliferation (E). Verhoeff–van Gieson stained.
converter (AD Instruments, Colorado Springs, Colo), and a personal computer. RV systolic pressure, heart rate, mean systemic arterial pressure, and cardiac output were measured. Cardiac index was calculated by dividing cardiac output by body weight. At the end of each hemodynamic study, the rat was euthanized by an overdose of pentobarbital sodium, and lungs and hearts were collected for histological and immunohistochemical evaluation and RV/LV+septum (RV/LV+S) weight ratio measurement.

Histopathology
The lungs were inflated with 1% formalin plus 0.5% agarose at 20 cm H2O pressure and fixed in 10% formalin overnight.17 The left lobe was blocked and embedded in paraffin. All sections were cut at 5 μm and were stained with hematoxylin and eosin or Verhoeff–van Gieson.

Immunohistochemistry
Immunohistochemical staining was performed with the Vectastain Universal Quick kit (Vector Laboratories, Burlingame, Calif).13 Antibodies used were von Willebrand factor (Dako, Glostrup, Denmark), α-smooth muscle actin (Abcam, Cambridge, Mass), Ki67 (Thermo Scientific, Asheville, NC), VEGF, and VEGF receptor-2 (Santa Cruz Biotechnology, Santa Cruz, Calif).

Human Lung Specimens
Lung specimens were obtained from autopsy material of patients with severe PAH. Informed consent to use the tissue for research purposes had previously been obtained. The tissue was fixed in 10% formalin and embedded in paraffin after routine processing. Slides were cut at 5 μm and stained with hematoxylin and eosin.18

Results
Hemodynamic Data and RV Hypertrophy
Compared with historical normal values of these parameters in control adult male rats at sea level (unpublished data), rats had very high RV systolic pressure (96±11 versus 21 mm Hg; n=7) with reduced cardiac index (40±5 versus 119 mL·min⁻¹·kg⁻¹; n=4) and normal mean systemic arterial pressure (128±11 versus 117 mm Hg; n=7). All rats showed severe RV hypertension as reflected by high RV/LV+S (0.74±0.04 versus 0.25; n=11; Table 1). SU5416/ hypoxia/normoxia-exposed rats at all other time points also developed severe PAH as evidenced by increased RV/LV+S ratio (0.76±0.04 and 0.74±0.02 for 5 and 8 weeks after SU5416 injection). All rats survived until the planned studies were performed except 1 rat in the 13- to 14-week group, which died at 5 weeks after SU5416 injection possibly as a result of severe PAH and right heart failure (RV/LV+S ratio, 0.82 at the time of necropsy).

Histological Findings
Histological examination revealed that pulmonary arteries in the 13- to 14-week SU5416/hypoxia/normoxia-exposed rat lungs had various forms of vascular remodeling (Figure 1A), including medial wall hypertrophy (Figure 1B and 1D), various degrees of neointimal thickening (eccentric [Figure 1D], concentric [Figure 1E], and concentric laminar [Figure 1C]), and complex hypercellular lesions (Figure 1D). There were no other obvious lung parenchymal abnormalities.

Two Types of Plexiform Lesions: Stalk-Like and Aneurysm-Like Patterns
We observed 2 different patterns of complex hypercellular lesions with respect to the sites where the lesions formed and their morphology. First, a complex stalk-like lesion formed within the blood vessel lumen (Figures 2 and 3). The longitudinal section of this pattern of lesion showed that it formed just distal to a dichotomous branching point (Figure 2A). The body of the lesion appeared to be a disorganized stalk-like mass comprising hyperchromatic and oval cells that were covered by von Willebrand factor–positive endothelial cells (Figure 2B and 2C). The stalk-like structure appeared to arise from the arterial wall and extend downstream into the lumen of the vessel. The cross section showed that it had many slit-like channels (Figures 2D and 3A) that were separated by the hyperchromatic and oval inner core cells. There were frequent observations of small pulmonary artery cross sections that showed a “bud-like” small mass of hyperchromatic and oval cells protruding from the arterial wall into the lumen. These buds
were covered by an endothelial layer (Figure 2E and 2F). As was recently suggested by Tuder et al19 in their review of human PAH arteriopathy, these lesions may represent an early stage of the intraluminal pattern of plexiform lesion.

Immunohistochemical analysis showed that the slit-like channels of the intraluminal lesions were lined by von Willebrand factor–positive endothelial cells (Figure 3A). These cells were also positive for VEGF receptor 2 (Figure 3D). The chromatin-rich inner core cells were partially positive for α-smooth muscle actin (Figure 3B), highly positive for VEGF (Figure 3C), and frequently highly positive for Ki67 (Figure 3E).

The second, aneurysm-like complex lesion extended outside the vessel lumen into the lung parenchyma (Figure 4). The longitudinal section of this pattern showed that a cluster of hyperchromatic and oval cells appeared to project out of the parent artery through a “window” to form an “aneurysm-like” or “sac-like” complex lesion (Figure 4A and 4D through 4F). It was not clear whether the window was an orifice of a supernumerary artery or something else, like a result of severe necrosis. The parent artery had no (Figure 4B and 4C) or mild (Figures 1E, 4A, and 4D through 4F) neointimal proliferation. The lesion comprised slit-like channels lined by von Willebrand factor–positive cells (Figure 4C; the endothelial lining was somewhat less complete than that seen in the stalk-like lesions) and hyperchromatic and oval core cells. The core cells were partially or weakly positive for α-smooth muscle actin (Figure 4D) and highly positive for VEGF (Figure 4E) and Ki67 (Figure 4F).

**Time-Dependent Histological Features of the Pulmonary Arteries and Arterioles**

Over time, the SU5416/hypoxia/normoxia-exposed severe PAH rats sequentially showed various types of histological changes in the pulmonary arteries and arterioles that follow the Heath-Edwards classification.20 As shown in Figure 5 and Table 2, the early-stage rats (5 weeks after SU5416 injection) showed medial wall thickening in all sizes of pulmonary arteries (grade 1) and cellular neointimal reaction/proliferation in small pulmonary arteries and arterioles (≈50 μm in diameter; grade 2). In addition to these changes, the later-stage rats (8 weeks after SU5416) developed a more complicated/advanced lesion, concentric laminar neointima (grade 3), that occurred mainly in slightly larger arteries. The typical complex lesions (plexiform lesions; grade 4) were not observed until a very late stage (13 to 14 weeks after SU5416).

**Morphological Similarity Between Lesions in Human PAH and the SU5416/Hypoxia/Normoxia-Exposed Rat**

Figure 6 shows the morphological similarity of aneurysm-like (Figure 6A and 6B) and stalk-like (Figure 6C and 6D) plexiform lesions and concentric laminar neointimal lesions (Figure 6E and 6F) between human and SU5416/hypoxia/normoxia-exposed rat model of PAH. There is also strong similarity in concentric laminar neointimal lesions between the human and rat PAH (Figure 6E and 6F).
Discussion

This study demonstrated that rats receiving a single injection of the VEGF receptor blocker SU5416 followed by exposure to 3 weeks of hypoxia (10% O₂) and an additional 10 to 11 weeks of reexposure to normoxia (13 to 14 weeks after the SU5416 injection) developed severe PAH accompanied by pulmonary arteriopathy strikingly similar to that observed in human severe PAH. The arteriopathy included concentric laminar neointimal and 2 different patterns of complex plexiform lesions.

The plexiform lesion is a characteristic structure of the pulmonary arteriopathy in severe PAH. It is easily identified in histological sections from patients with severe PAH because it has a distinct glomoid structure and the cells that form the inner core of the lesion are uniquely hyperchromatic and oval.5,6 Despite these unique morphological characteristics, its definition is relatively obscure, presumably because the lesion is dynamic (ie, early lesions are cellular and later become less cellular and more fibrotic), and more important, it is not known how it forms. The consensus seems to be that the plexiform lesion is a complex and disorganized pulmonary arterial proliferative lesion that consists of a network or plexus of channels lined by endothelial cells and separated by core cells.3–7 There are, however, many uncertainties. For example, it is not known whether the endothelium-lined channels are functional blood vessels or just slits. In addition,
it has not been determined whether the core cells are myofibroblasts, smooth muscle cells, endothelial cells, or undifferentiated cells.\(^3\)–\(^7\)

The characteristics of the complex lesions observed in very late stages of the SU5416/hypoxia/normoxia-exposed rat agree in every major aspect with those described as plexiform lesions in human PAH. The cross section of the stalk-like lesion shows a complicated channel or slit-like structure that is lined by von Willebrand factor–positive endothelial cells. The channels or slits are separated by chromatin-rich and oval core cells that are highly positive for the cell proliferation marker Ki67. Some but not all of these core cells are positive for the smooth muscle cell marker \(\alpha\)-actin, suggesting that these cells are a mixture of different types of cells or undifferentiated cells. Consistent with previous studies,\(^19\),\(^21\) VEGF and VEGF receptor 2 are highly expressed in the lesion, although VEGF receptor 2 expression appears to be restricted to the channel-lining endothelial cells. The longitudinal section of this lesion indicates it is a disorganized stalk-like structure arising from the arterial wall and growing downstream into the vessel lumen. Further detailed analyses, including a 3-dimensional reconstruction of the lesion, are required to confirm this observation. In agreement with previous findings in human PAH lungs,\(^18\) these lesions are often observed just distal to dichotomous branch points.

We have also observed similarly complex lesions at different vascular sites and with a different presentation; in other words, the complex lesion projecting out of the vessel lumen into lung parenchyma (aneurysm-like pattern; Figure 4) in contrast to that growing within the lumen (stalk-like pattern; Figure 2). This type of lesion also shows the basic characteristics of the human plexiform lesion (ie, a complex lesion consisting of slit-like channels lined by endothelial cells and chromatin-rich and oval core cells). The aneurysm- or sac-like lesion has been well documented in PAH associated with congenital heart disease.\(^20\) Although it has not been clearly described that there are different patterns of plexiform lesions, the aneurysm-like pattern and stalk-like intraluminal pattern have also been reported in idiopathic PAH (Figure

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Table 2. Sequential Appearance of Different Grades of Pulmonary Arterial Lesions

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<tr>
<th>Histological Grade</th>
<th>Weeks After SU5416</th>
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<tr>
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Figure 6. Morphological similarities in plexiform and concentric neointimal lesions between human PAH and a very-late-stage SU5416/hypoxia/normoxia-exposed rat model of PAH (B, D, and F; images in B and D are the same as those in Figures 1D and 3A, respectively). A and B, Cellular plexiform lesions (arrows) projecting into lung parenchyma of pulmonary arteries (aneurysm-like pattern). C and D, Cross-sectional views of plexiform lesions with luminal occlusion (arrows; stalk-like pattern). E and F, Concentric cellular neointimal lesions (arrows). A, C, E, and F, Hematoxylin and eosin stained; B, Verhoeff–van Gieson stained; D, von Willebrand factor stained.
It has been proposed that aneurysm-like lesions could arise from the orifice of supernumerary arteries, but there is also the possibility that it is a result of necrotizing arteritis. In fact, we have frequently observed fibrinoid necrosis-like lesions in small pulmonary arterial walls at an earlier time point (8 weeks after the SU5416 injection) in the SU5416/hypoxia/normoxia-exposed rats (unpublished data). Further investigations are needed to clarify how and where the aneurysm-like complex lesions are formed.

Other recent rodent models have been reported to develop pulmonary plexiform-like lesions. Greenway et al have observed development of pulmonary arterial changes resembling human plexogenic arteriopathy in a minority of mice overexpressing S100A4/Mts1. Although the occlusive neo-intimal lesions are striking, the plexiform-like characteristics of the lesions have not been described in detail. In addition, it is not clear how severe the pulmonary hypertension is in these mice. White et al have reported that monocrotaline given to left pneumonectomized young rats causes severe pulmonary hypertension accompanied by development of perivascular plexiform-like lesions. Although these lesions are intriguing, there is a major difference in the time course of lesion formation between this model and human PAH. In contrast to the development of plexiform lesions in advanced and severe stages of human PAH, the plexiform-like lesion in pneumonectomized rats develops very early (within 1 week) in the absence of pulmonary hypertension. This difference raises questions about the relevance of this model to severe human PAH and plexiform lesion formation.

The SU5416/hypoxia-exposed rats develop progressive pulmonary hypertension and vascular remodeling even after they were reexposed to normoxia. By 5 weeks (3 weeks of hypoxia and 2 weeks of reexposure to normoxia) after the SU5416 injection, the rats showed severe pulmonary hypertension and RV hypertrophy accompanied by proliferation of apoptosis-resistant endothelial cells and occlusive neo-intimal lesions in precapillary pulmonary arterioles (Heath-Edwards grade 2 lesion; Figure 5). However, at this time point and even at 8 weeks (3 weeks of hypoxia and 5 weeks of reexposure to normoxia) after the SU5416 injection, the rats have not developed obvious complex proliferative lesions (Heath-Edwards grade 4 lesion; Figure 5). We have found in this study that in a much later stage (13 to 14 weeks after the SU5416 injection) the rats have developed the complex plexiform lesions. This time course is consistent with that of PAH associated with congenital heart disease and probably with that of idiopathic PAH, although the duration required to develop plexiform lesions seems to depend on the progression and severity of the hypertension. One case report of an idiopathic PAH patient shows that it took 5 years to develop plexiform lesions after the establishment of severe pulmonary vascular disease. Assuming that the natural history of chronic disorders is proportional to the lifespan of the species, years of the “latent period of the plexiform lesion in severe PAH” in humans (life span ~80 years) translates into ~10 weeks in rats (lifespan ~3 years).

Our observation that it takes several weeks to develop plexiform lesions after the establishment of severe pulmonary hypertension in the SU5416/hypoxia/normoxia rat suggests that sustained exposure to very high blood pressure may be the major factor required for their development and that the lesion may be the consequence rather than the cause of the hypertension, considering that there are no longer direct effects of SU5416 and hypoxia at the time of their appearance. This speculation is supported by the fact that plexiform lesions are formed in lungs of patients with congenital left-to-right shunt heart disease, a pure hemodynamic disorder, only in late advanced stages or, in other words, after several months to years of exposure to high blood pressure and flow. Although we did not find convincing evidence that newly formed thrombi were present in von Willebrand factor–stained lung sections from various time points of the PAH rats, an important issue that needs to be investigated in the future is the potential role of thrombosis in the complex lesion formation.

Conclusions

This study demonstrated that pulmonary arteriopathy, including concentric laminar and plexiform lesions, in a very late stage of the SU5416/hypoxia/normoxia-exposed rat model of PAH has features that are strikingly similar to those of human severe PAH. This model also closely simulates the time course of the development of plexiform lesions in patients with PAH. We conclude that this model will be useful to investigate the pathogenesis of severe PAH, particularly that of the plexiform lesion. Considering that it will be virtually impossible to obtain lung tissue samples from medically untreated patients with PAH, the importance of an animal model that accurately mimics human severe PAH is substantial. We propose using this model for rigorous preclinical drug evaluations because it is obvious that the conventional animal models do not adequately mimic human severe PAH and because findings in preclinical studies with these models (ie, successful prevention and reversal of pulmonary hypertension by a large number of drugs and chemical compounds) are unfortunately not mimicked in human conditions.

Acknowledgments

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Disclosures

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References

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