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From Cell Biology to Tissue Engineering

Pulmonary haemodynamics are correlated with intimal lesions in a rat model of severe PAH: attenuation of pulmonary vascular remodelling with ambrisentan

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Summary. Rationale: Pulmonary arterial hypertension (PAH) is characterized by obstructive lesions and vasoconstriction of the pulmonary arteries. Early therapeutic interventions with vasodilator drugs are thought to be beneficial in PAH. However, it remains unknown whether the severity of intimal obstruction is associated with increased pulmonary arterial pressure and whether reduction of vasoconstriction in the earlier stage by these drugs has a beneficial effect. Therefore, the aims of this study were to investigate these issues in a rat model of severe PAH. Methods: A rat model of severe PAH was created by injection of a vascular endothelial growth factor receptor blocker in combination with hypoxia for the first 3 weeks followed by normoxia for the next 9 weeks. To assess intimal obstruction, "the pulmonary artery occlusion index (PAOI)" was developed to digitize all lesions. The small pulmonary arteries were assessed by this index, and the association between right ventricular systolic pressure (RVSP) and PAOI was investigated. An endothelin receptor antagonist, ambrisentan, was administered by gavage to rats during either hypoxia (Prevention study group, n=25) or normoxia (Early treatment group, n=15). Results: PAOI showed a positive correlation with RVSP, and both RVSP and PAOI increased gradually over time. There were no severe occlusive lesions in either group, but the density of partially occlusive lesions was significantly decreased in the Prevention study group. Conclusion: A novel PAOI index was developed, and this index was strongly correlated with RVSP. Furthermore, ambrisentan reduced luminal occlusive lesions more effectively when treatment was given during the first 2 weeks of hypoxia.

Key words: Pulmonary arterial hypertension, A rat model of PAH, Haemodynamics, Intimal lesions, Ambrisentan

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Introduction

It is generally believed that the progression of disease in patients with PAH is divided into three stages, including presymptomatic, clinical and advanced, and that the maximal increase in mean pulmonary arterial pressure (*P*pa) seems to occur during the early clinical stage (Rich, 1988; Howard, 2011; Sakao et al., 2015). This suggests that the maximum elevation of mean *P*pa is attributable to pulmonary arterial constriction and/or pathologically altered vascular lesions, which are completed during the early stage. However, the detailed features of pulmonary vascular lesions during this stage remain unclear, because the lack of signs and symptoms makes it difficult to diagnose early-stage PAH in patients, and open lung biopsies are contraindicated in patients with severe PAH (Rich, 1988; Brown et al., 2011; Howard, 2011). In fact, most of the features of pulmonary vascular lesions that have been described previously have originated from autopsy samples

obtained from end-stage PAH patients (Stacher et al., 2012).

After the introduction of three classes of vasodilator drugs, including prostacyclin analogues, endothelin receptor antagonists (ERAs) and phosphodiesterase type 5 (PDE5) inhibitors, the survival rate in patients with idiopathic and heritable PAH improved (Humbert et al., 2004; Sakao et al., 2012). These vasodilator drugs have a role to not only oppose abnormal vasoconstriction but also inhibit pulmonary vascular cell growth (Humbert et al., 2004). In human PAH, however, it remains unknown whether these drugs are able to reverse established stenoses and/or obstructive vascular lesions, which include both intimal and medial lesions. This is because no open lung biopsy can be obtained during the treatment period due to excessive bleeding. Recently, the histopathological investigation of explanted lung tissues from patients with severe PAH, who had been treated with the drugs mentioned above, demonstrated that the degree of medial thickening overlapped with the medial thickness observed in the control lung tissues, although intimal and plexiform lesions were still observed (Stacher et al., 2012). These findings suggested that these PAH-targeting drugs played a role in reversing medial lesions that consisted of pulmonary vascular smooth muscle cell proliferation, and that the residual elevated mean *P*pa in patients with advanced severe PAH was attributable to complex vascular lesions that consisted of intimal and plexiform lesions.

Recently, studies in the Sugen/Hypoxia rat model, which is based on an injection of a vascular endothelial growth factor (VEGF) receptor blocker (Sugen5416) in combination with chronic hypoxia demonstrated that the maximal increase in right ventricular systolic pressure (RVSP) was not achieved until 5 weeks after Sugen injection, and this maximum elevation was attributable to pulmonary arterial constriction and/or pathologically altered vascular remodelling (Abe et al., 2010; Toba et al., 2014). A dual endothelin receptor antagonist, macitentan, has also been shown to reverse pulmonary vascular remodelling only when this drug was administered from 3 to 5 weeks after Sugen injection, but not when it was given from 5 to 8 weeks (Shinohara et al., 2015). These findings suggest that this drug is more beneficial if given before the maximal increase in RVSP is achieved. In this model of severe PAH, continuous alveolar hypoxia plays a significant role in addition to VEGF receptor blockade, which induces hypoxic pulmonary vasoconstriction (HPV)-related high shear stress, resulting in medial wall thickening and muscularisation of non-muscularised arterioles (Heath et al., 1990). Therefore, it was our hypothesis that a reduction of HPV due to treatment with a standard PAH drug under hypoxic conditions would be of greater benefit in controlling the development of pulmonary vascular remodelling than if the drug were given under normoxic conditions.

The purpose of this study was to investigate whether the degree of intimal obstruction and/or stenosis was associated with RVSP in a rat model of severe PAH. An additional purpose was to determine whether the initial administration of ambrisentan, an endothelin receptor-A selective antagonist, under hypoxic conditions (0 to 2 weeks after Sugen injection) reduces pulmonary vascular remodelling more effectively than when the drug is given under normoxic conditions (3 to 5 weeks after Sugen injection).

Materials and methods

Animals

Five-week-old male Sprague-Dawley rats (100-120 g) were purchased from CLEA Japan Inc, Tokyo, Japan. The rats were housed in stainless steel cages in an animal room, where room temperature and relative humidity were set at 25°C and 50%, respectively. The rats had free access to food and water. All of the animal experiments were approved by the Review Board for Animal Experiments of Chiba University and were performed in accordance with the guidelines of the Animal Research Committee of Laboratory Animal Center, Graduate School of Medicine, Chiba University.

Experimental design

All rats (n=65) were given a subcutaneous injection of SU5416 (20 mg/kg) (Cayman Chemical Company, Michigan, USA), which was suspended in CMC (0.5% [w/v] carboxymethylcellulose sodium, 0.9% [w/v] sodium chloride, 0.4% [v/v] polysorbate 80, and 0.9% [v/v] benzyl alcohol in deionized water). After SU5416 injection, rats were divided into the following 3 groups: SU-5416 only (Su/Hx group); daily gavage with an endothelin receptor antagonist, ambrisentan, (20 mg/kg) on days 1-14 (Prevention study group); or daily gavage with ambrisentan (20 mg/kg) on days 22-35 (Early treatment group). All rats were exposed to chronic normobaric hypoxia (10% O_2) in a ventilated chamber for 3 weeks after SU5416 injection and were subsequently returned to normoxia (21% O_2) for up to 9 weeks after the hypoxic exposure. We examined rats at 5 different time points (4, 6, 8, 10, and 12 weeks) after SU5416 injection as shown in Fig. 1.

Hemodynamic measurements in catheterized rats

All rats were placed on controlled heating pads after they were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). Hemodynamic measurements were performed under normoxic conditions, as previously described with minor modifications (Abe et al., 2010). Briefly, a polyethylene catheter (size-3; internal diameter: 0.5 mm, Hibiki, Tokyo, Japan) was inserted into the right ventricle (RV) via the right jugular vein for measurement of RV systolic pressure (RVSP) under anaesthesia. RVSP instead of pulmonary arterial pressure was measured, because the pulmonary artery (PA) could not be routinely catheterized. In addition to RVSP, heart rate was also

monitored. A physiological transducer connected to an amplifier system (NEC Sanei Co., Ltd. Tokyo, Japan) was used to monitor the right heart catheterization (RHC) signals, and those signals were also displayed on a recorder (Nihon Kohden, Tokyo, Japan). 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 evaluation (Fig. 1).

Histopathology

The lungs were inflated with 4% paraformaldehyde phosphate solution at 20 cm H_2O pressure and fixed with the same solution. The left lobes were blocked and embedded in paraffin wax. All sections were cut at 2.5 μm intervals and then stained with haematoxylin and eosin (HE stain), or Elastica van Gieson (EVG stain). To compare the pathological features among the 3 groups, the degree of intimal obstruction and/or stenosis was assessed by scoring EVG-stained slides using a previously described method (Toba et al., 2014) as follows: no evidence of neointimal formation (grade 0); partial (<50%) luminal occlusion (grade 1); and severe (>50%) luminal occlusion (grade 2). All small pulmonary arteries (20 μm<OD<100 μm) from two slices of each left lung lobe were graded using this method. To assess whether the degree of intimal obstruction and/or stenosis has an impact on increases in pulmonary arterial pressure, "the pulmonary artery occlusion index (PAOI)" was developed to digitize all histopathologic vascular alterations. The formula used to calculate this index is shown below:

$$PAOI=(n0 \times 0 + n1 \times 1 + n2 \times 2)/(n0 + n1 + n2)$$

where n0, n1 and n2 represent the number of vessels with grades of 0, 1 and 2, respectively. All small pulmonary arteries in the Su/Hx group at each of the 5 time points after SU5416 injection (n=5 at each time point) (Fig. 1) were assessed using this index, and the correlation between RVSP and the PAOI was calculated.

Statistical analysis

Quantitative data are presented as the mean ± standard error of mean. Comparison of groups was performed using an unpaired Student's t-test. The Spearman's rank correlation coefficient was used to determine the relationship between RVSP and PAOI. A p value of <0.05 was considered significant. All statistical analyses were performed using JMP® 9.0.0 (Japanese version, SAS Institute Inc., Tokyo, Japan).

Results

The relationship between RVSP and PAOI

PAOI values were calculated in the Su/Hx group and the Prevention group at each of 5 time points after SU5416 injection (n=5 at each time point) (Fig. 1) and in the Early treatment group at each of 3 time points after ambrisentan treatment (n=5 at each time point), and the relationship between PAOI and RVSP in these groups was assessed. When the data obtained at all time points were combined (n=65), PAOI was positively correlated with RVSP in these groups (Fig. 2).

Hemodynamic measurements

As shown in Fig. 3, the maximal elevation of RVSP

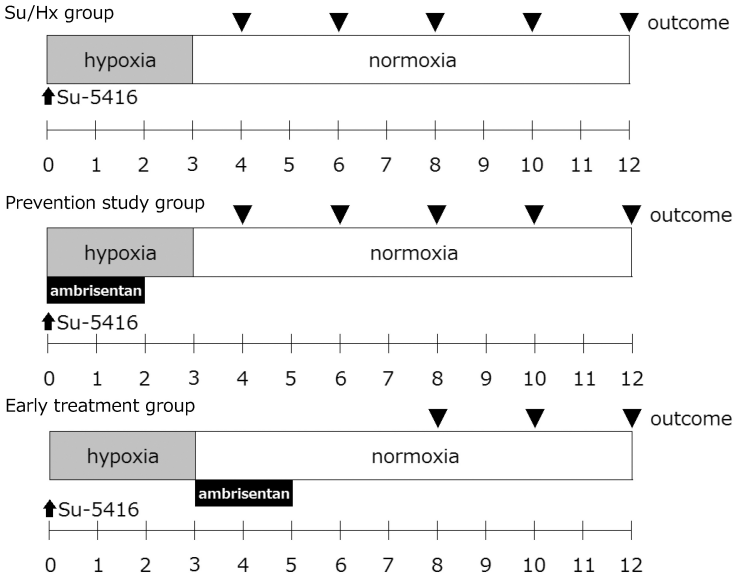

Fig. 1. Panel A shows the time line in the Su/Hx group (n=25); panel B, the Prevention study group (n=25); panel C, the Early treatment group (n=15). The black arrowhead indicates the date of the harvest in each study (each n=5). Su/Hx indicates combined exposure to Su-5416 and hypoxia for 3 weeks.

was not achieved until 8 weeks after SU5416 injection, supporting the results of previous reports (Abe et al., 2010; Toba et al., 2014). Furthermore, ambrisentan reduced RVSP at each time point, not only in the Prevention study group, but also in the Early treatment group. Additionally, there were significant differences in RVSP at 8 to 12 week after SU5416 injection between the Su/Hx and both the Prevention study and Early treatment groups ($P<0.05$). Furthermore, there were significant differences in RVSP between the Prevention study and Early treatment groups at both 10 and 12 weeks (Fig. 3).

Pulmonary artery intimal stenosis and occlusion

The lung tissues from each rat were stained with HE and EGV, and the degree of pulmonary arterial intimal obstruction and/or stenosis was assessed. Pulmonary arterial intimal lesions did not appear until 4 weeks in the Su/Hx rats, and these lesions developed gradually over time (Fig. 4A-C). However, in the two ambrisentan-treated groups, these lesions were reduced at each time point compared with the Su/Hx group. In addition, there was a tendency for ambrisentan to reduce intimal remodelling more effectively in the Prevention study group than in the Early treatment group (Fig. 4A-C).

The density of partially (grade 1) and severely (grade 2) occlusive and/or stenotic pulmonary arterial lesions developed gradually over time, and the density of these lesions peaked at 10 weeks after SU5416 injection in the Su/Hx group (Fig. 5). There were no severely occlusive and/or stenotic lesions in either of the

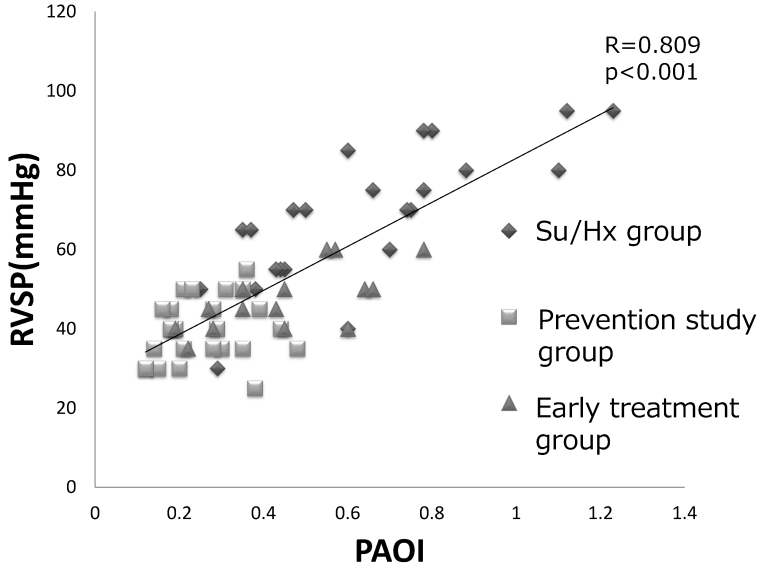

Fig. 2. Correlation between RVSP and PAOI in the Su/Hx model. Each dot indicates the RVSP and PAOI of each Su/Hx model and ambrisentan-treated rats at each time point. The color and form of dots show the different 3 groups. PAOI was positively correlated with RVSP in these groups (n=65, $p<0.001$, R=0.809). Both RVSP and PAOI increased in a time-dependent manner.

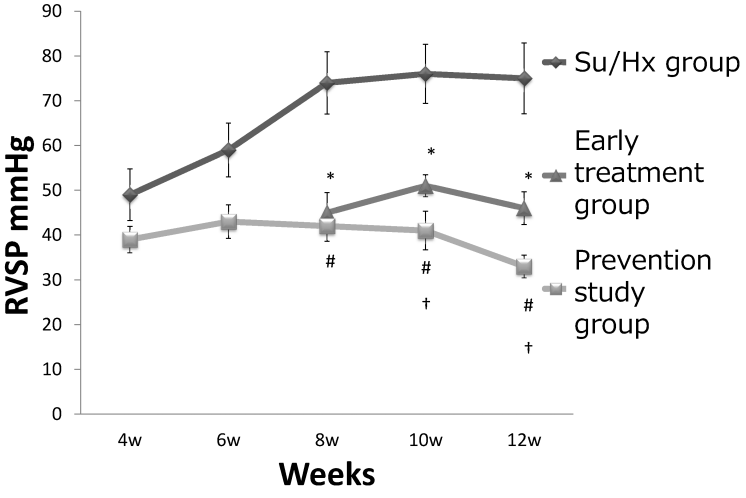

Fig. 3. RVSP changes during the entire observation period in the 3 groups (n=65). In the Su/Hx model, RVSP gradually increased until week 8, and then stayed elevated until week 12. From week 8 to 12, both the Prevention study and the Early treatment groups had a lower RVSP than the Su/Hx group. From week 10 to 12, the Prevention study group had a lower RVSP than the Early treatment group. # $p<0.05$ Su/Hx group vs. Prevention study group. * $p<0.05$ Su/Hx group vs. Early treatment group. † $p<0.05$ Prevention study group vs. Early treatment group.

ambrisentan-treated groups, and the density of partially occlusive and/or stenotic lesions was significantly reduced in the Prevention study group compared with the Early treatment group (Fig. 5).

Discussion

In this study, a novel formula, designated as the pulmonary artery occlusion index (PAOI), was developed to quantify the degree of intimal obstruction and/or stenosis, and this index had a positive correlation with RVSP in a model of severe PAH (Fig. 2). In addition, the endothelin receptor-A selective antagonist, ambrisentan, improved pulmonary hemodynamic and reduced luminal occlusive lesions in the pulmonary arteries in this rat model (Figs. 3, 4A-C, 5). This pulmonary vasodilator drug seemed to have a greater effect when it was given under hypoxic conditions (0 to 2 weeks after Sugen injection) rather than under normoxic conditions (3 to 5 weeks after Sugen injection) (Figs. 3, 4A-C, 5).

HPV-related high fluid shear stress alone has been shown to induce pulmonary vascular remodelling, including medial wall thickening and muscularisation of non-muscularised arterioles, but not to cause intimal lesions (Sakao et al., 2010). It is generally known that a combination of HPV due to chronic hypoxia and a VEGF receptor blockade by SU5416 causes both medial

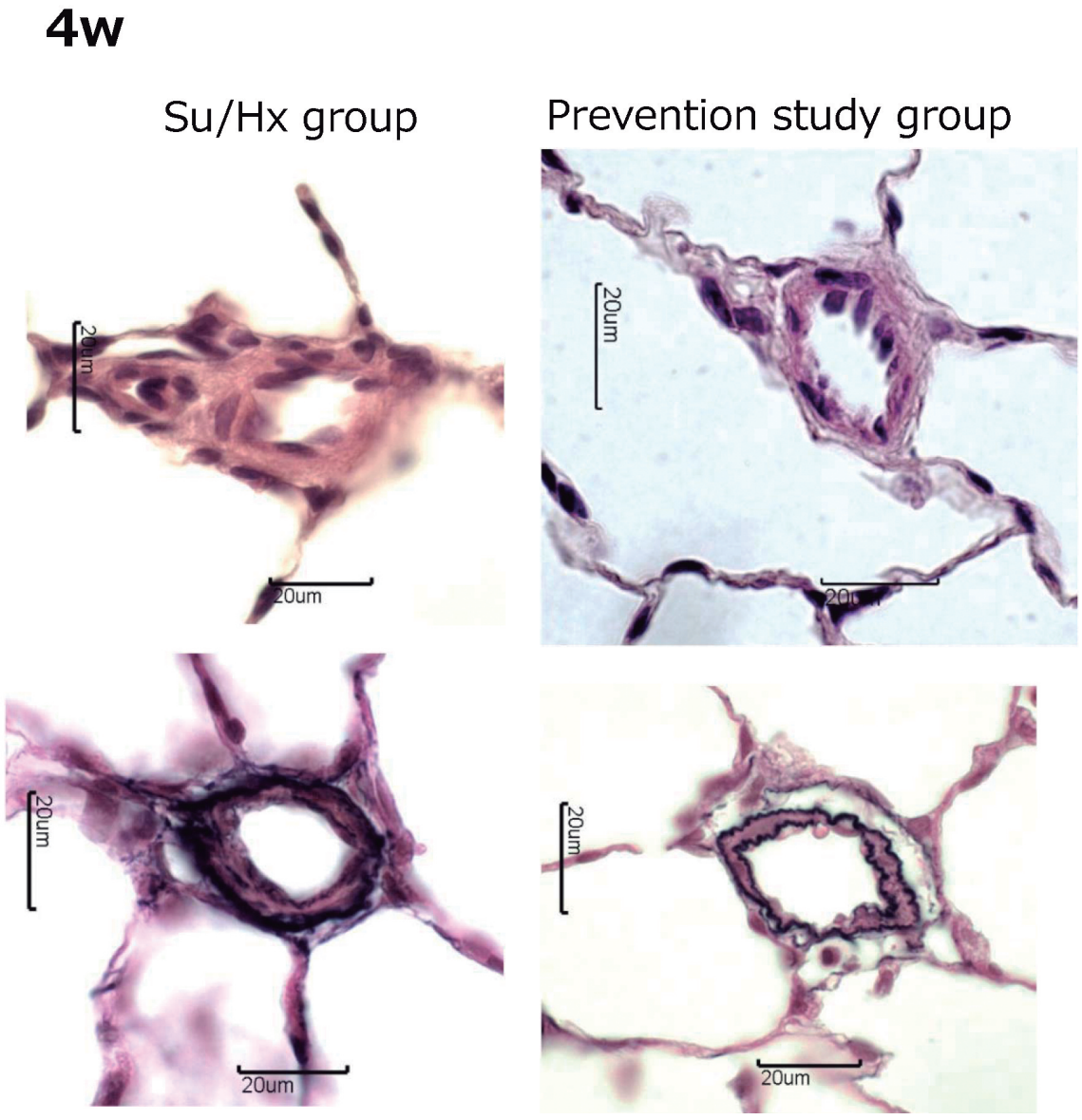

Fig. 4A. Representative photomicrographs of pulmonary arteries at different time points. All sections are stained with Hematoxylin-Eosin (HE, top) or elastic van Gieson (EVG, bottom). 4 weeks. There were no significant differences between the Su/Hx and Prevention study groups.

and intimal lesions (Abe et al., 2010), although SU5416 alone under normoxic conditions is not able to induce intimal lesions at least in this Sprague-Dawley rat strain (Jiang et al., 2016). In the current study, a reduction in the pulmonary arterial vasoconstrictive reaction due to ambrisentan reduced the density of obstructed and/or stenotic pulmonary arteries, indicating that preventative effect against HPV by ERA may be essential etiology for better outcome of late stage vascular lesions in the Prevention study group. However, HPV is also associated with the generation and secretion of inflammatory cytokines and growth factors in the microenvironment around the pulmonary vasculature (Stenmark et al., 2006). It remains to be elucidated whether vasodilator usage under hypoxic condition actually has a role in controlling the generation and secretion of growth factors and cytokines.

Although a positive correlation between the hemodynamic values and pulmonary artery wall thickness had been shown in a previous study (de Raaf et al., 2014), the present study is the first to show a correlation between hemodynamic values and the degree of intimal obstruction and/or stenosis in the Su/Hx rat model of severe PAH. As it is generally thought that both vasoconstriction and obliterative lesions are essential determinants of an increased *P*pa (Sakao et al., 2015), the current study suggests that the luminal obstruction and/or stenosis of pulmonary arteries may be an independent determinant of an elevated *P*pa.

Although the maximal elevation of RVSP in a previous study (Toba et al., 2014) was not achieved until 5 weeks after SU5416 injection, the maximal increase in RVSP in the present study required 8 weeks to develop (Fig. 3). Moreover, the maximal RVSP in this study (around 80 mmHg) (Fig. 3) tended to be lower than that in the previous study (around 100 mmHg) (Toba et al.,

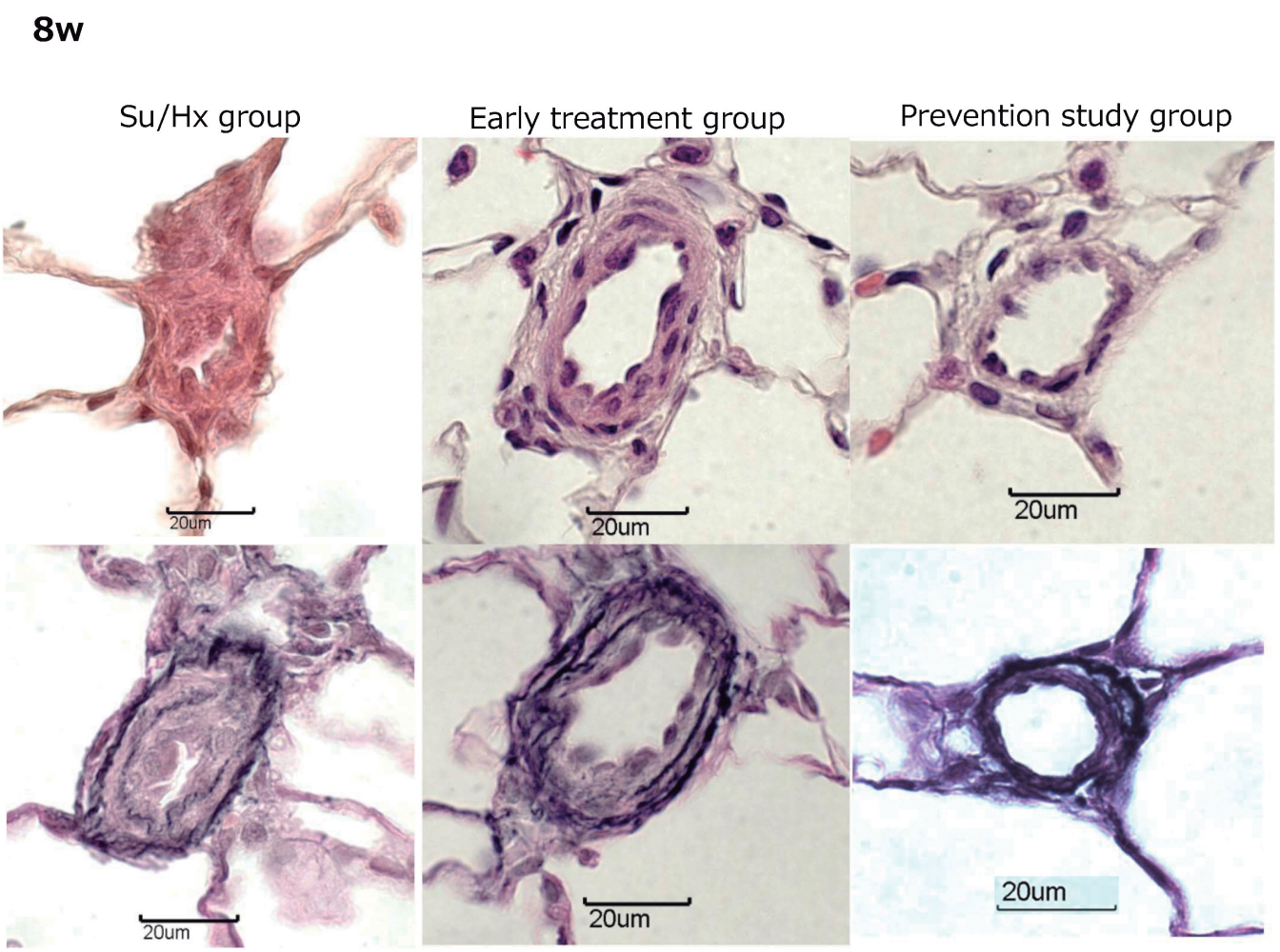

Fig. 4B. Representative photomicrographs of pulmonary arteries at different time points. All sections are stained with Hematoxylin-Eosin (HE, top) or elastic van Gieson (EVG, bottom). 8 weeks. Severe intimal obstruction appears in the Su/Hx group. On the other hand, only mild lesions were found in both the Prevention study and Early treatment groups.

2014). Recently, strain and colony differences in rats have been shown to significantly impact the severity of the response to SU5416 (Jiang et al., 2016). The difference in the severity of haemodynamics between the current and previous studies may be attributable to differences in the strain and colony of rats used to develop the PAH model.

In Su/Hx rat model, inflammatory cell infiltration with upregulation of inflammatory genes including IL6, MCP1, cathepsin S, and MMP9, is closely related to progression of pulmonary vasculopathy (Otsuki et al., 2015). In the current study, however, it remains unknown how vascular inflammation is altered by ERA. Recently, it has been demonstrated that ERA can play an anti-inflammatory role in alveolar macrophages through reducing Endothelin (ET)-1 release, which results in CCL-2, IL-6 and MMP-9 expression (Gerlach et al., 2015). Although it remains unknown whether this applies to perivascular macrophages in the PAH model as well as to alveolar macrophages, this result may be a possible explanation for anti-inflammatory effects of ERA.

ET is generally recognized to be a vasoconstrictor, and its expression increases in PAH patients and in animal models of PAH (Giaid et al., 1993; Miyauchi et al., 1993). There are two types of ET receptors, ET_A and ET_B. The former is mainly expressed in smooth muscle cells, and the latter is expressed in both smooth muscle and endothelial cells (Galié et al., 2004). The activation of both receptors results in the proliferation of smooth muscle cells and pulmonary arterial vasoconstriction, whereas ET_B activation in smooth muscle cells inhibits cell growth and its activation in endothelial cells induces vasodilation (Seo et al., 1994). This indicates that ET_B has a role to neutralize the deleterious effects of ET-1. This is the reason why selective ET_A receptor inhibition

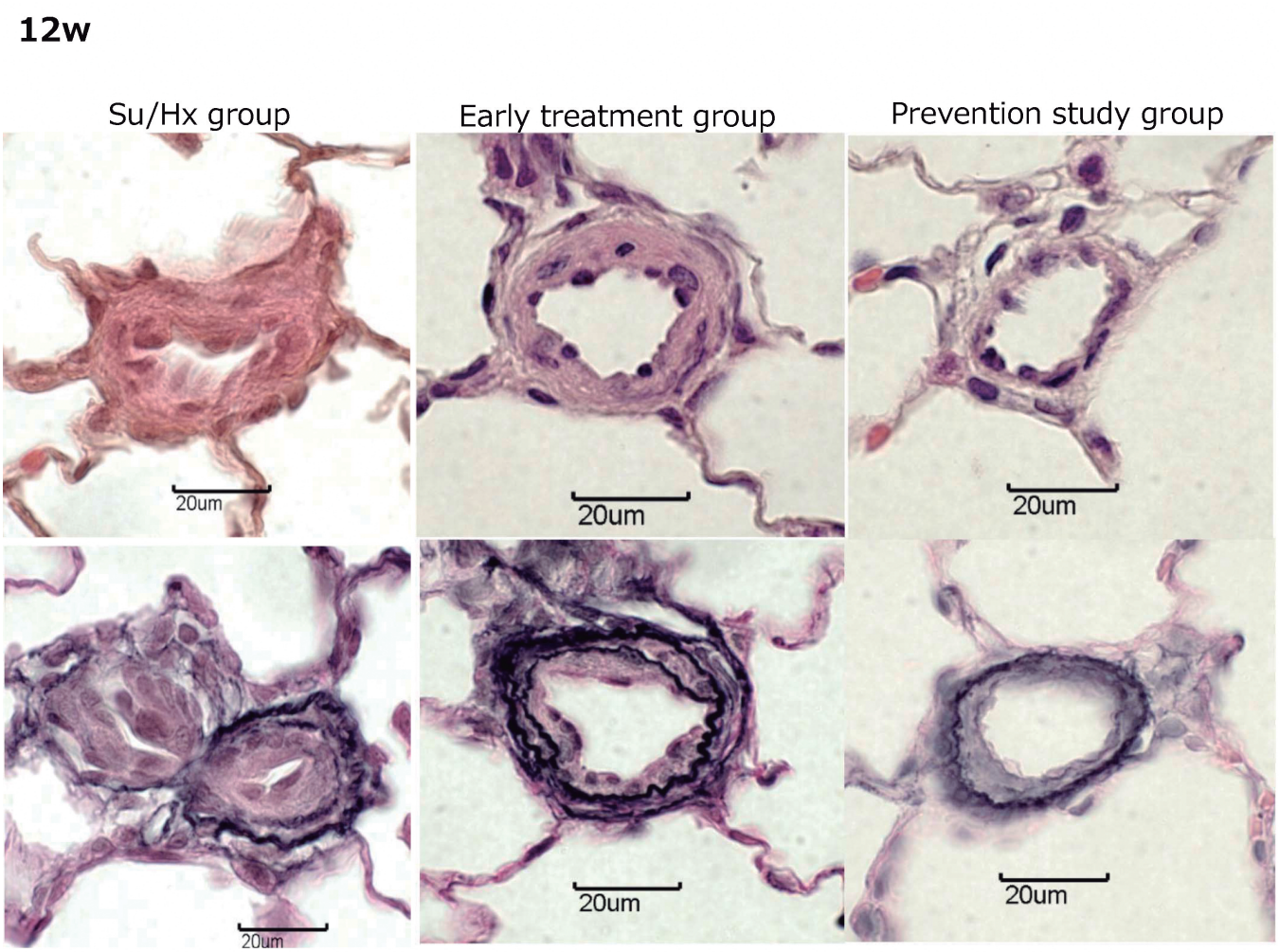

Fig. 4C. Representative photomicrographs of pulmonary arteries at different time points. All sections are stained with Hematoxylin-Eosin (HE, top) or elastic van Gieson (EVG, bottom). 12 weeks after the injection of Su-5416. Severe intimal obstruction appears in the Su/Hx group. On the other hand, only mild lesions were found in both the Prevention study and Early treatment groups.

is supposed to be more beneficial than inhibition of both receptors. However, there have been few reports that investigated the role of a selective ET_A receptor antagonism in the Su/Hx rat model of severe PAH. In this rat model, there is medial muscular thickening and muscularisation of proliferating smooth muscle cells, as well as intimal obliteration with phenotypically-altered, proliferated endothelial cells (Oka et al., 2007; Abe et al., 2010; Toba et al., 2014). Therefore, a selective ET_A receptor antagonist, Ambrisentan, was selected for use in the present study. In addition, it has proven efficacy in the treatment of human PAH (Galié et al., 2005) and has high bioavailability and a half-life of about 9-15 hours, which makes once-daily dosing possible (Billman, 2002). However, there is no difference in the efficacy between the dual and the selective compounds treatments in patients with PAH (Galié et al., 2013).

Conclusion

The current study demonstrated that the degree of intimal obstruction and/or stenosis had a positive correlation with RVSP in a rat model of sever PAH. Furthermore, the initial administration of ambrisentan under hypoxic conditions had a role in reducing pulmonary vascular remodelling in this severe PAH model. However, it remains unknown how initial vasodilator treatment under hypoxic conditions prevents the progression of vascular lesions to obstruction and/or stenosis more effectively than the administration of this drug later during normoxic conditions. Although early diagnosis and therapeutic intervention does not seem to be easy in patients with PAH (Sakao et al., 2015), the control of the vasoconstrictive reaction in the earlier stage may be essential to reduce the development of the

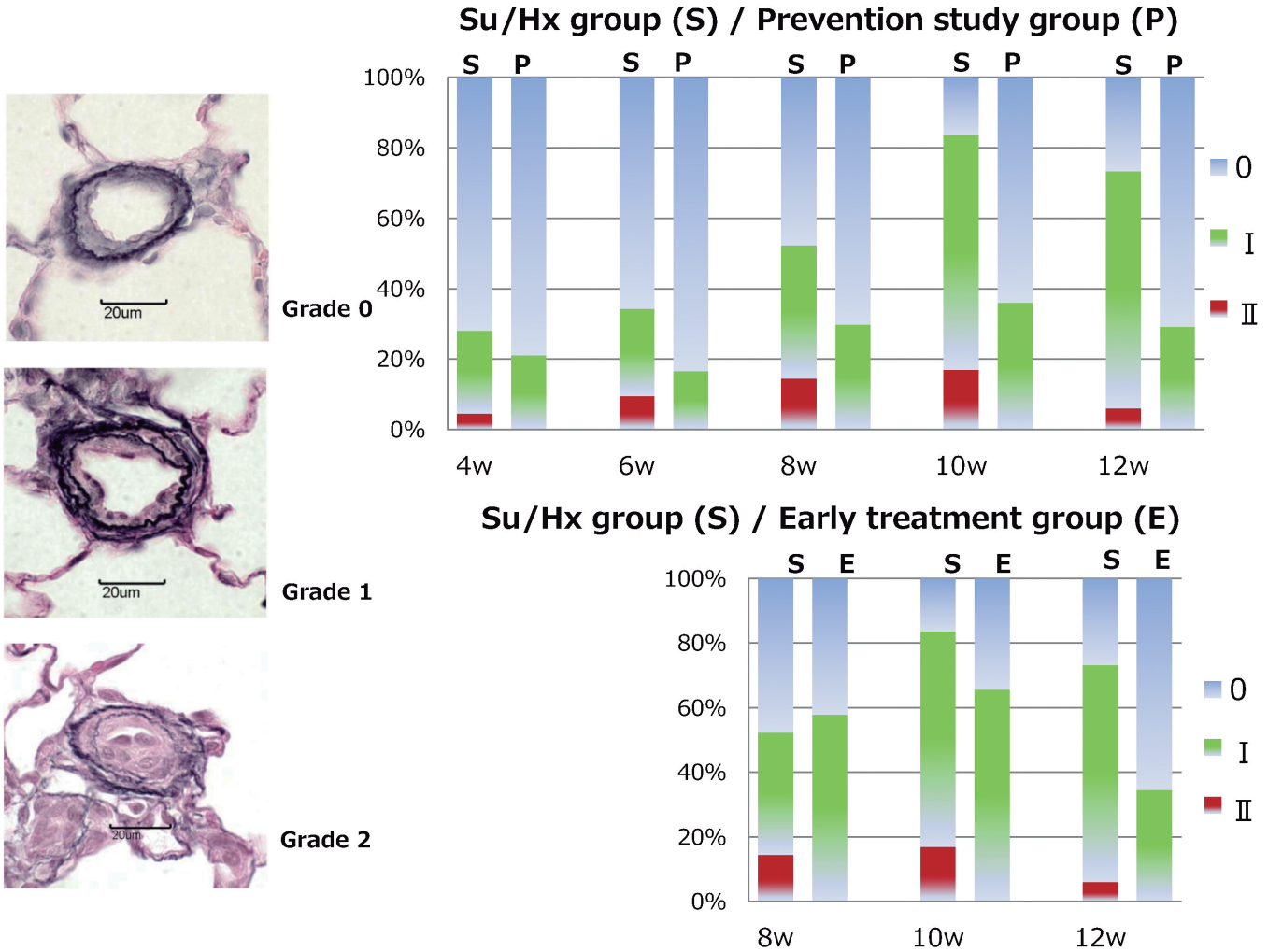

Fig. 5. Percentage of grade 0, 1, and 2 pulmonary arteries at different time points. Representative photomicrographs of pulmonary arteries of grade 0,1 and 2 are shown on the left. All bar scales indicate 20 μm. In the Su/Hx group, lesions of Grade 1 and 2 increased gradually until week 10. Grade 2 lesion could not be found in either of the ambrisentan-treated groups, and Grade 1 lesions significantly decreased in the Prevention study group compared with the Early treatment group.

subsequent vascular lesions.

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