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Targeted activation of endothelin-1 exacerbates hypoxia-induced pulmonary hypertension

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（課程博士関係）

学 位 論 文 の 内 容 要 旨

Targeted activation of endothelin-1 exacerbates hypoxia-induced pulmonary hypertension

エンドセリン－１過剰発現マウスにおける低酸素誘発性肺高血圧症に関する研究

神戸大学大学院医学研究科医科学専攻

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a severe and fatal disease which eventually leads to right heart failure and death. Endothelin-1 (ET-1), a 21-amino acis peptide, has strong vasoconstrictive effects and is known to have important role in the development of PAH. Several drugs have been developed based on ET-1 system such as endothelin receptor antagonists and these drugs could improve the long term outcomes in PAH patients. However, until recent, there are still no drugs which could completely cure PAH. Because the importance of ET-1, gene modified mice based on ET-1 system have been generated. ET-1 transgenic (ETTG) mice were established by transferring human ET-1 gene into germline of the mice under transcriptional control of its native promoter. These mice have increased ET-1 level in plasma and tissues, especially brain, lungs, and kidney. One main obstacle in the PAH studies is the difficulty to obtain the human lung sample as seen in severe PAH. Therefore, condition mimicking human PAH is continued to be generated. In rats, vascular endothelial growth factor receptor (VEGF-R) inhibitor SU5416 injection has been reported to induce severe PAH. But experiments using mice model still could not develop severe PAH mainly because the results were unstable, reversible, and also not well established. Therefore, we try to make severe PAH in mice. We hypothesize that using ETTG mice combined with SU5416 and chronic hypoxia could induce severe PAH.

METHODS

In this study, we induced PAH in ETTG mice and wild type (WT) littermates by 3 or 6 chronic hypoxia using hypoxic chambers containing 10% of O_2. SU5416 injection was given to ETTG mice and combined with chronic hypoxia. The dose of SU5416 was based on previous study and was administered at 20 mg/kg three times a week. After exposure of chronic hypoxia, we tried to evaluate hemodynamic perimeter using right ventricular systemic pressure (RVSP) polygraph machine and Fulton's index to measure right ventricular hyperthrophy. Fulton's index was calculated using the formula of (right ventricle)/(left ventricle+septum). Histological analysis was done by hematoxylin eosin (HE) staining and immunohistochemical staining of von Willebrand Factor (vWF) and interleukin (IL)-6. HE staining was done to analyze wall thickness in the small pulmonary arteries. Quantitative RT-PCR used these primers: ET-1, ETAR, ETBR, IL-6, IL-6 receptor and GAPDH was used as reference. Immunoblotting was done for IL-6 and GAPDH antibodies. In cell culture study, we used human pulmonary arterial endothelial cells (HPAEC) and stimulated with 100 nM of ET-1 for 24 hours.

RESULTS

Our results showed that after induction to chronic hypoxia for 6 weeks, both WT and ETTG mice had increased RVSP. ETTG mice had higher RVSP compared to WT mice, in hypoxia and normoxia condition. However, ETTG mice did not develop increased systemic blood pressure, as compared to WT mice. Fulton's index did not differ between ETTG and WT mice. Nevertheless, our result demonstrated the increase of pulmonary arterial wall thickness in ETTG mice compared to control group. Increase of ET-1 expression was found after chronic hypoxia and significantly increased more in ETTG mice. We observed that chronic hypoxia induction in ETTG mice resulted in significant but only modest increase in RVSP. Thus, we decided to treat ETTG mice with SU5416 in conjunction with 3-week hypoxia exposure. The result showed that treatment with SU5416 significantly increased RVSP, Fulton's index, and pulmonary arterial wall thickness. Treatment with SU5416 further enhanced ET-1 expression after chronic hypoxia. SU5416 also further enhanced the increase of ETA receptor expression and further enhanced the decrease of ETB receptor. However, treatment with SU5416 with chronic hypoxia failed to produce severe PAH characterized by no detected plexiform lesions. Furthermore, we analyzed the expression of IL-6 as one important marker in the progression of PAH. Our result demonstrated that IL-6 expression was upregulated in lung after chronic hypoxia and SU5416 treatment further enhanced it in ETTG mice. Expression of IL-6 receptor also increased during chronic hypoxia. We found similar increasing pattern in the protein expression of IL-6 in the lung after chronic hypoxia. Our immunohistochemical staining of IL-6 in the lung showed the increase expression of IL-6 in pulmonary arterial endothelial cells after chronic hypoxia and SU5416 further enhanced it. We also tried to elucidate the correlation between IL-6 and ET-1 by performing in vitro experiment using HPAEC stimulated with ET-1 in 24 hours. The result showed that stimulation with ET-1 enhanced IL-6 expression in HPAEC.

DISCUSSION

In the current study, we described for the first time the direct role of endothelin system in the development of PAH by using ETTG mice. We demonstrated that our ETTG mice have higher RVSP compared to WT littermates, even in normoxia condition. We also confirmed previous finding showing that ETTG did not develop systemic hypertension. Our results highlighted that pulmonary artery is more prone toward ET-1 vasoconstrictive activity

as compared to systemic resistance arteries. Thus, emphasizing the importance of ET-1 in PAH development.

Our results showed that chronic hypoxia increase RVSP and pulmonary arterial vessel wall thickening but no effect observed on the proliferation of endothelial cells. Recent publications showed that SU5416 administration could induce severe PAH in rats but could not induce the same condition in mice. Our previous study using SV129 mice combined with SU5416 could induce relatively severe PAH and formation of plexogenic lesion. However severe PAH model and its plexiform lesion has not been generated and established. We presumed that combining SU5416 and chronic hypoxia in ETTG mice could induce severe PAH and its plexiform lesions. Unfortunately, the result showed no sign of severe PAH and plexiform lesions. Therefore, further studies are needed to elucidate the role of ET-1 in the formation of plexiform lesions.

We tried to analayze IL-6 as one of the most important interleukins in the development of PAH as well as the induction of ET-1 because interaction between ET-1 and IL-6 is still elusive, particularly in endothelial cells. Our previous study showed that IL-6 expression was enhanced in endothelial cells. Other study showed that ET-1 could also induce adipocytes. Our study demonstrated that, in pulmonary arterial endothelial cells, ET-1 enhanced the expression of IL-6. Moreover, we assume that ET-1 and IL-6 might synergistically aggravate PAH by stimulating each other.

Finally, our study also showed and provided experimental evidence and rationale for the inhibition of endothelin system in PAH, which has been accepted widely.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

受付番号	甲 第 2538 号	氏 名	MUHAMMAD GAHAN SATWIKO
論文題目 Title of Dissertation	Targeted activation of endothelin-1 exacerbates hypoxia-induced pulmonary hypertension エンドセリン－１過剰発現マウスにおける低酸素誘発性肺高血圧症に関する研究		
審査委員 Examiner	主 査 Chief Examiner 西村 善博 副 査 Vice-examiner 古屋敷智之 副 査 Vice-examiner 小川 渉		

（要旨は１，０００字～２，０００字程度）

Pulmonary arterial hypertension (PAH) is a severe and fatal disease which eventually leads to right heart failure and death. Endothelin-1 (ET-1), a 21-amino acids peptide, has strong vasoconstrictive activity and important role in PAH. Endothelin receptor antagonists are known to improve long term outcomes in PAH patients. In rats, vascular endothelial growth factor receptor (VEGF-R) inhibitor SU5416 injection has been reported to induce severe PAH. However, in mice, there is no model available which demonstrates severe PAH and plexiform lesions. Therefore, Dr. Gahan hypothesized that ET-1 transgenic (ETTG) mice mice combined with SU5416 and chronic hypoxia could induce severe PAH.

In this study, Dr. Gahan induced PAH in ETTG mice and wild type (WT) littermates by 3 or 6 weeks chronic hypoxia using hypoxic (10% of O2). SU5416 injection (20 mg/kg) thrice a week was administrated to ETTG mice and combined with chronic hypoxia. Dr. Gahan analyzed hemodynamic parameter using right ventricular systemic pressure (RVSP) and Fulton's index (right ventricular hyperthrophy index). Immnohistological, cell biological and molecular biological analyses were performed.

After 6 weeks chronic hypoxia, both WT and ETTG mice had increased RVSP. ETTG mice had higher RVSP compared to WT mice, in hypoxia and normoxia. Pulmonary arterial wall thickness was increased in ETTG mice compared to control group. Dr. Gahan observed that chronic hypoxia induction in ETTG mice resulted in moderate increase in RVSP. Thus, Dr. Gahan decided to treat ETTG mice with SU5416 in conjunction with 3-week hypoxia exposure. The result showed that treatment with SU5416 significantly increased RVSP, Fulton's index, and pulmonary arterial wall thickness. However, treatment with SU5416 with chronic hypoxia did not generate severe PAH and plexiform lesions. In addition, Dr. Gahan analyzed the expression of IL-6 and demonstrated that IL-6 expression was upregulated in lung after chronic hypoxia and SU5416 treatment enhanced it furthermore. Immunohistochemical staining of IL-6 in the lung showed the increase expression of IL-6 in pulmonary arterial endothelial cells after chronic hypoxia and SU5416 further enhanced it. In vitro results showed that stimulation with ET-1 enhanced IL-6 expression in HPAEC after 24 hours.

In the present study, Dr. Gahan demonstrated the direct role of endothelin system in the development of PAH by using ETTG mice. Dr. Gahan showed that ETTG mice have higher RVSP compared to WT littermates, even in normoxia condition. Dr. Gahan's results highlighted that pulmonary artery is more prone toward ET-1 vasoconstrictive activity as compared to systemic resistance arteries, emphasizing the importance of ET-1 in PAH development.

Dr. Gahan's results showed that chronic hypoxia increase RVSP and pulmonary arterial vessel wall thickening but no effect observed on the proliferation of endothelial cells. Therefore, further studies are needed to elucidate the role of ET-1 in the formation of plexiform lesions.

The candidate, having completed studies on animal model of severe pulmonary arterial hypertension, with a specialty in the effects of Endothelin-1, and having advanced the field of knowledge in the area of pulmonary arterial hypertension, is hereby recognized as having qualified for the degree of Ph.D. (medicine).