The Expressions of Some Growth Factors as the Progressive Indicators of Pulmonary Arterial Hypertension

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Abstract

Vasoconstriction is commonly regarded as the cause of pulmonary arterial hypertension (PAH), but another cause that cannot be ignored is artery wall remodeling marked by the thickening of tunica intima, media, and adventitia. Several parameters' expressions indicating PAH include basic fibroblast growth factor-2 (bFGF-2), transforming growth factor-ß1 (TGF-ß1), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), and type 1 collagen. This study aims to verify the progressive increase in the expression of bFGF-2, TGF-β1, TIMP-1, and type 1 collagen, the decrease of MMP-2 expression, as well as the MMP-2:TIMP-1 ratio in the tunica adventitia of pulmonary arteries.

The samples of paraffin blocks in rat models' lung tissue of pulmonary arterial hypertension were made in a particular time span. The immunohistochemical method was employed to examine the expressions of bFGF-2, TGF-β1, MMP-2, TIMP-1, and type 1 collagen. The data analysis was carried out using the Pearson and Spearman statistical test.

After 28-day observation, the number of cells expressing bFGF-2, TGF-β1, TIMP-1, progressively increased and had a strong, positive and significant correlation with the time reaching p-value of 0.005, 0.000, and 0.000 respectively. Meanwhile, MMP-2 and MMP-2:TIMP-1 ratio had a weak negative and insignificant correlation with the time.

In tunica adventitia of pulmonary arteries on rat models of pulmonary arterial hypertension induced with monocrotaline, it is proven that there is a progressive increase in the expression of bFGF-2, TGF-β1, TIMP-1, and type 1 collagen. In addition, MMP-2 expression suggests a progressive decrease, while the MMP-2:TIMP-1 ratio also decreases.

Introduction

Pulmonary hypertension (PH) is associated with a variety of heart, lung, and systemic diseases in neonates, infants, and older children, and contributes to significant morbidity and mortality[1]. Vasoconstriction is commonly regarded as the cause of pulmonary arterial hypertension (PAH), but another cause that cannot be ignored includes artery wall remodeling marked by the thickening of *tunica intima, media*, and *adventitia*[2,3]. These

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maladaptive responses result in an increase in pulmonary vascular resistance, which causes sustained pulmonary hypertension[4].

The current incidence of PAH is not yet clear, eighty percent of congenital heart disease (CHD) population live in developing countries, and it is estimated that only 2-15% of patients receive curative treatment[5]. Several reports mentioned that PAH in children reached 2.2-3.7:1,000,000 with a male:female sex ratio of 1.8:1. As many as 25-50% of cases suffer from Eisenmenger syndrome[6].

In developing countries like Indonesia, the incidence is greater due to the lack of facilities and infrastructure, as a result, the curative treatment against anatomic defects is often delayed, even it can cause right-sided heart failure and premature death[7,8].

Excessive vasoconstriction is associated with endothelial dysfunction causing impaired

production of nitric oxide (NO) vasodilator[9], prostacyclin, and excessive expression of vasoconstrictorsi.e. Endothelin-1 (ET-1). Prostacyclin and NO are antiproliferative through cyclic Adenosine Monophosphate (cAMP) and cyclic Guanosine Monophosphate (cGMP), respectively [3]. The loss of cGMP/cAMP mechanism, the deregulation of transforming growth factor (TGF)-β, and growth factor activation, such as basic Fibroblast Growth Factor-2 (bFGF-2) cause an increase in the inflammatory responses and fibrosis in PAH. Patients with PAH will undergo an increase in serum cytokines, including IL-1, IL-6, and IL-8, as well as chemokines, such as chemokine (C-C motif) ligand 2 (CCL2)/Monocyte Chemotactic Protein (MCP)-1 [10]. TGF-β is cytokines that can induce the transformation of fibroblasts to myofibroblasts through the expression of alpha-smooth muscle actin (α-SMA) stimulation and collagen production [11,12]. TGF-β reduces Matrix Metalloproteinase-2 (MMP-2) production, stimulates the Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) expression, causes complete inhibition on ECM degradation and excessive matrix accumulation [13].

As a result of these complex changes, the pathological structural remodeling of pulmonary arteries causes severe PAH, which often does not respond to therapy and eventually leads to *cor pulmonale* and death [8]. This study focuses on proving a progressive increase in the expressions of bFGF-2, TGF-β1, TIMP-1, and type 1 collagen, as well as the decrease of MMP-2 expression and MMP-2:TIMP-1 ratio in the *tunica adventitia* of pulmonary arteries. This study used rat models of pulmonary arterial hypertension.

Materials and methods

The population was the paraffin blocks in PAH rat models, particularly in male white rats (*Rattus norvegicus*) Sprague Dawley strain aged 12 weeks with a bodyweight of 300-500 grams. A 12-week-old rat is equivalent to 7.5 years of human age [14].

The samples were the total paraffin blocks in lung tissues, which were estimated to have pulmonary arterial hypertension after receiving an intraperitoneal monocrotaline injection at a dose of 60 mg/kg body weight. The sample size in this study was the total samples of paraffin blocks in rat models' lung tissues of the pulmonary arterial hypertension made on days 0, 3, 7, 10, 14, 17, 21, 24, and 28. Each sample of the paraffin blocks was made each day.

Paraffin block in rat's lung tissue was made on day 0 for the rats that did not receive monocrotaline. Meanwhile, for the rats receiving monocrotaline, the paraffin blocks were made on day 3, 7, 10, 14, 17, 21, 24, and 28 after monocrotaline administration. The materials for making preparations in this study examined with the immunohistochemical techniques included lung tissue, monoclonal antibodies against bFGF-2, monoclonal antibodies against TGF-β1, monoclonal antibodies against MMP-2, monoclonal antibodies against TIMP-1, and monoclonal antibodies against type I collagen. In the process of immunohistochemical preparations making for the examination of bFGF-2 expression, TGF-β1, MMP-2, TIMP-1, and type I collagen, lung tissues and pulmonary arteries were fixed with 10% formalin buffer for 24 hours. Subsequently, the tissue was cut and embedded with paraffin, then cut with a thickness of 5 μm and fixed on the glass object as slides. The immunohistochemical staining technique used was a streptavidin-biotin technique.

Data collection was carried out in a controlled and monitored environment with an assumption that all conditions were attempted to be the same and can be controlled. Data analysis was performed using computer program software. The statistical test of Pearson correlation was used for normally distributed data, while the Spearman correlation test was used for data that were not normally distributed.

Results

Figure 1 shows the bFGF-2 expression. In Figure A, the black arrows indicate the bFGF-2 expression in the *tunica adventitia* fibroblasts of pulmonary arteries on PAH rat models without monocrotaline injection. The figure also shows that the number of cells expressing bFGF-2 was smaller than in other figures. In Figure C, black arrows indicate the number of cells expressing bFGF-2 on day 7 that increased progressively.

Figure 2 presents the TGF-β1 expression. In Figure A, the black arrows mark TGF-β1 expression in the *tunica adventitia* fibroblasts of pulmonary arteries on PAH rat models without monocrotaline injection. The figure also

describes that the number of cells expressing TGF-β1 was smaller than in other figures. In Figure E, the black arrows mark the number of cells expressing TGF-β1 on day 14 that started to increase progressively.
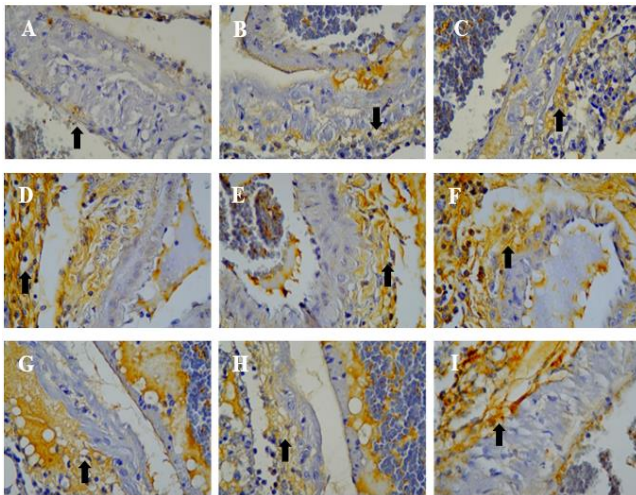

Figure 1. The black arrows indicate bFGF-2 expression in the *tunica adventitia* of pulmonary arteries on the rat models of Pulmonary Arterial Hypertension. (A) Observation on day 0, (B) Day 3, (C) Day 7, (D) Day 10, (E) Day 14, (F) Day 17, (G) Day 21, (H) Day 24, and (I) Day 28. Enlargement 400x. The black arrows indicate cells that express bFGF-2.
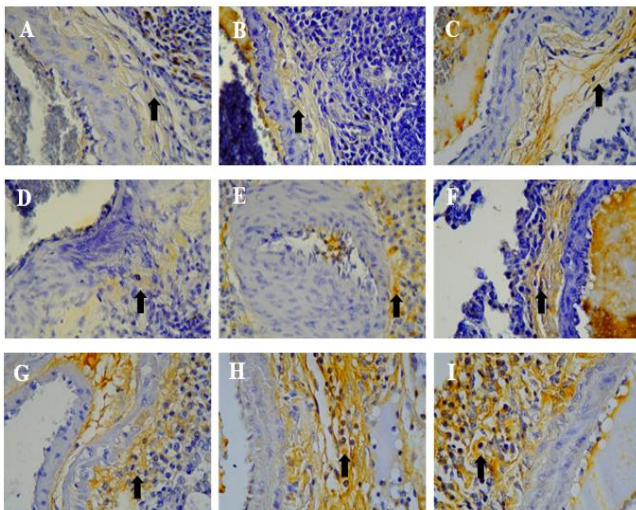

Figure 2. The black arrows indicate TGF-β1 expression in the *tunica adventitia* of pulmonary arteries on rat models of Pulmonary Arterial Hypertension. (A) Observation on day 0, (B) Day 3, (C) Day 7, (D) Day 10, (E) Day 14, (F) Day 17, (G) Day 21, (H) Day 24, and (I) Day 28. Enlargement 400x. The black arrows indicate cells that express TGF-β1.
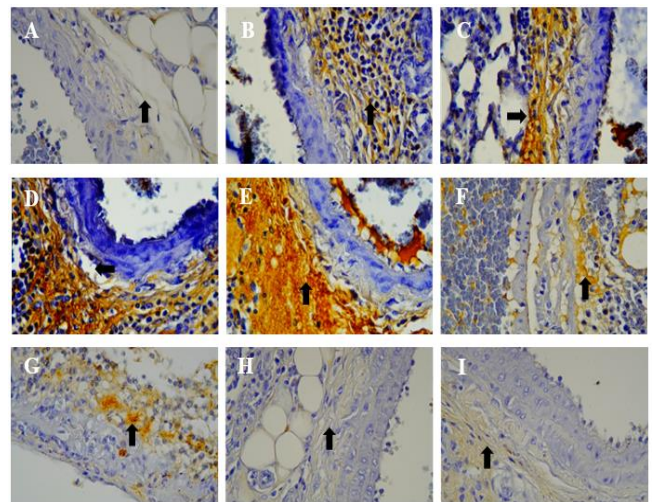

Figure 3. The black arrows indicate MMP-2 expression in the *tunica adventitia* of pulmonary arteries on the rat models of Pulmonary Arterial Hypertension. (A) Observation on day 0, (B) Day 3, (C) Day 7, (D) Day 10, (E) Day 14, (F) Day 17, (G) Day 21, (H) Day 24, and (I) Day 28. Enlargement 400x. The black arrows indicate cells that express MMP-2.
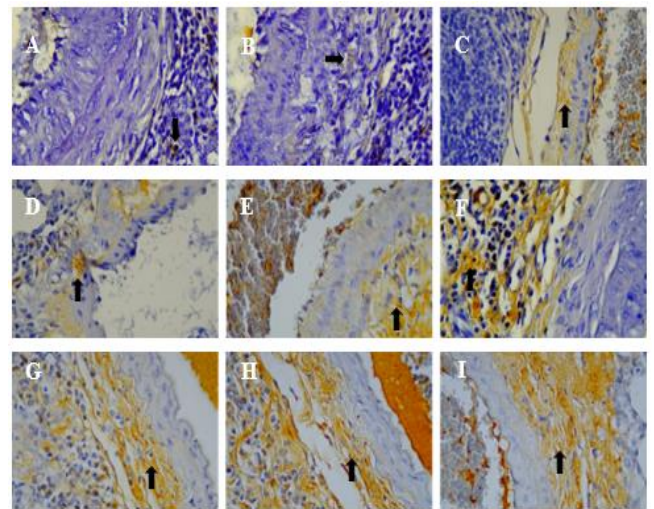

Figure 4. The black arrows indicate TIMP-1 expression in the *tunica adventitia* of pulmonary arteries on the rat models of Pulmonary Arterial Hypertension. (A) Observation on day 0, (B) Day 3, (C) Day 7, (D) Day 10, (E) Day 14, (F) Day 17, (G) Day 21, (H) Day 24, and (I) Day 28. Enlargement 400x. The black arrows indicate cells that express TIMP-1.

MMP-2 expression is shown in Figure 3. In Figure A, the black arrows indicate MMP-2 expression in the *tunica adventitia* fibroblasts of pulmonary arteries on PAH rat models without monocrotaline injection. The figure also suggests

that the number of cells expressing MMP-2 was smaller than in other figures. In Figure B, C, and D, the black arrows mark the number of cells expressing MMP-2 that increased with the highest number obtained on day 10. It appears that the number of cells expressing MMP-2 was progressively decreased with the lowest number in Figure I.

Figure 4 describes the TIMP-1 expression. In Figure A, the black arrows indicate TIMP-1 expression in the *tunica adventitia* fibroblasts of pulmonary arteries on PAH rat models without monocrotaline injection. The figure also presents the number of cells expressing TIMP-1 that was smaller than in other figures. In Figure B, the black arrow shows the number of cells expressing TIMP-1 on day 3 which started to increase progressively.
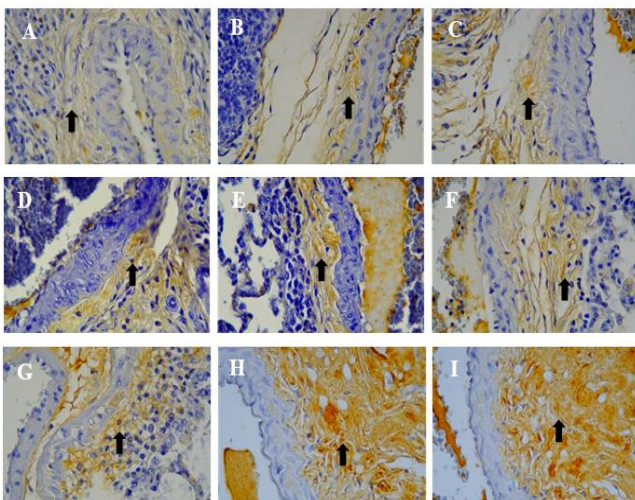

Figure 5. The black arrows indicate type 1 collagen expression in the *tunica adventitia* of pulmonary arteries on rat models of Pulmonary Arterial Hypertension. (A) Observation on day 0, (B) Day 3, (C) Day 7, (D) Day 10, (E) Day 14, (F) Day 17, (G) Day 21, (H) Day 24, and (I) Day 28. Enlargement 400x. The black arrows indicate cells that express Collagen type 1.

Figure 5 suggests type 1 collagen expression. In Figure A, the black arrows mark the type 1 collagen expression in *tunica adventitia* fibroblast of the pulmonary artery of PAH rat models that did not get monocrotaline injections. It presents that the number of cells expressing type 1 collagen was more visible than in figures B and C. In Figure E, the black arrows indicate the number of cells expressing type 1 collagen on day 14 which started to increase

progressively.

Data normality test for the number of cells expressing TGF-β1, MMP-2, TIMP-1, MMP-2:TIMP-1 ratio, and type I collagen using Shapiro-Wilk suggested that the data obtained were normally distributed, while in bFGF-2, the data obtained was not normally distributed. The correlation results among variables are described in Table 1.

After 28-day observation, the number of cells expressing bFGF-2 on PAH rat models increased progressively and had a strong, positive and significant correlation with time (p of 0.005). The number of cells expressing TGF-β1 on PAH rat models increased progressively and had a strong, positive and significant correlation with time (p of 0.000). The number of cells expressing MMP-2 on PAH rat models decreased progressively and had a weak, negative and insignificant correlation with time (p of 0.381). The number of cells expressing TIMP-1 on PAH rat models increased progressively and had a strong, positive and significant correlation with time (p of 0.000). The cell ratio expressing MMP-2 and TIMP-1 had a moderate, negative and insignificant correlation with time (p of 0.212). The number of cells expressing type 1 collagen on PAH rat models increased progressively and had a strong, positive and significant correlation with time (p of 0.001).

Discussion

The study results were in accordance with several studies, which suggested that the patients with PH had increased the levels of FGF-2 or basic FGF-2 (bFGF-2). bFGF-2 also induced the proliferation of Pulmonary Arterial Smooth Muscle Cells (PASMC), and its inhibition could prevent PH development in rats induced with monocrotaline [15]. Pulmonary artery examination in humans with PAH suggested the increase of bFGF-2 immunostaining on endothelial cells and smooth muscle[16].

The increase of TGF-β activities correlated with the number of macrophage recruitment in PAH rat models with monocrotaline. The inhibition of TGF-β signaling through activin-like kinase-5 (ALK-5) receptors inhibited the development and progression of monocrotaline-model PAH and the inhibition of pulmonary artery smooth muscle cell migration. TGF-β isoforms are multi-functional cytokines

that play a role in the cellular and molecular processes of blood vessel remodeling on lungs. TGF-β isoform expression in IPAH patients and in the lungs of PAH rats with monocrotaline increased [17].

The study results were in line with several studies that exposed that TIMP-1 serum level in PAH patients becauseleft-to-right shunt congenital heart disease were higher than controlled patients without PAH. TIMP-1 serum level in PAH patients was three times higher than controlled patients without PAH [18]. MMPs' activities were regulated through enzyme activity inhibition by the tissue inhibitors of metalloproteinase (TIMP), which strictly regulated the MMPs' expression and activity, and produced a balancing mechanism to prevent excessive ECM degradation [19]. The increasing of MMP activity in rats' pulmonary arteries after being moved from hypoxic space followed by active collagen resorption and remodeling repair suggested that MMP played a role in the breakdown of excess collagen during recovery.

In the MMP-2:TIMP-1 ratio, the influence of MMP-2 was greatly strong and significant. MMP is a degradation enzyme involved in the regulation of MES in migration and proliferation of smooth muscle cells and endothelial cells. MMP expression and activity increased on experimental PAH [20]. Slight changes in collagen synthesis and metabolism caused large changes in collagen content, the buildup of elastin and collagen during the PAH process. Smooth muscle cells and fibroblasts in the larger arteries produced collagen and elastin in *tunica media* and *adventitia*. Protein degradation in ECM was caused by MMPs, collagenase and elastase, activities, and these activities were regulated in turn by tissue inhibitors of matrix metalloproteinases orTIMPs [4].

Observations at day 0-10 of this study revealed an increase in the expression ratio of MMP-2:TIMP-1. It was related to the increasing number of cells expressing MMP-2. After day 10, there was an increase in the number of cells expressing TIMP-1 and a decrease in MMP-2 expression, thus the MMP-2:TIMP-1 ratio decreased until the end of observation. This result was in accordance with studies on MMP-3, which found TIMP imbalance in the smooth muscle cells of pulmonary arteries, in which TIMP-1 tended to increase, while MMP-3 decreased. The total of MMP-2 and the proportion of active MMP-2 increased, especially in smooth muscle cells and elastic fibers [20].

At the beginning of the observation, there was a decrease in the number of cells expressing type 1 collagen. It correlated with an increase in the number of cells expressing MMP-2 and MMP-2:TIMP-1 ratio. After day 10, the number of cells expressing type 1 collagen increased. This condition was caused by an increase in cell activities expressing bFGF-2, TGF-β1, TIMP-1, and a decrease in MMP-2:TIMP-1 ratio.

This study used rat models of pulmonary arterial hypertension induced with monocrotaline. The developing process of PAH in rat models was not the same as the PAH process in children with congenital heart disease induced by increased flow in the pulmonary arteries or shear stress. The use of rats as animal models could not represent the disease process in humans. The study results cannot be directly applied to humans, especially in children with congenital heart disease who experience pulmonary arterial hypertension.

Conclusions

In *tunica adventitia* of pulmonary arteries on rat models of pulmonary arterial hypertension induced with monocrotaline, it is revealed that there is a progressive increase in the expression of bFGF-2, TGF-β1, TIMP-1, and type 1 collagen. In addition, MMP-2 expression suggests a progressive decrease, thus MMP-2:TIMP-1 ratio also decreases.

Declaration of Interest

The authors report no conflict of interest.

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