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VEGFmRNA and eNOSmRNA expression in immature rabbits with bleomycin-induced pulmonary hypertension*

GONG Fang-qi (龚方戚)†, LIN You-min (林幼敏), TANG Hong-feng (汤宏峰), GU Wei-zhong (顾伟忠), WANG Wei (汪 伟), KANG Man-li (康曼丽)

(*Affiliated Children's Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China*)

†E-mail: gongfangqi@zju.edu.cn

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Abstract: Objective: To investigate the evolution of pulmonary hypertension, the pathological changes of pulmonary arteries, and the expression of VEGFmRNA and eNOSmRNA of pulmonary arterial endothelial cells in immature rabbits treated with intratracheal bleomycin (BLM). Methods: Immature rabbits were divided into control and BLM group. Two and four weeks after intratracheal normal saline or BLM injection, the systolic, diastolic and mean pulmonary artery pressure (PASP, PADP, MPAP) were measured by micro-catheter; the pathological changes and the expression of VEGFmRNA and eNOSmRNA of endothelial cells in pulmonary arteries were evaluated by HE and in situ hybridization. Results: Two and four weeks after intratracheal injection of BLM, the PASP, PADP and MPAP increased 53%, 49%, 52% in 2 weeks, and 43%, 89%, 56% in 4 weeks; the wall thickness increased and the cavity in middle and small pulmonary arteries became narrow; the Thickness Index (TI) and Area Index (AI) increased 25%, 14% in 2 weeks, and 22%, 24% in 4 weeks; the level of VEGFmRNA and eNOSmRNA expression decreased 46%, 43% in 2 weeks, and 43%, 51% in 4 weeks. There was no significant difference between 2 weeks and 4 weeks BLM groups. Conclusion: The pulmonary artery pressure was elevated, the thickness of wall increased and the cavity became narrow in middle and small pulmonary arteries, and the level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells decreased in immature rabbits after 2 weeks and 4 weeks of intratracheal 4 U/kg BLM injection.

Key words: Bleomycin, Pulmonary hypertension, VEGF, eNOS, mRNA
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INTRODUCTION

Pulmonary hypertension is not rare in human with some heart and lung diseases. Chronic pulmonary hypertension leads to structural alterations of the lung vessels. The pathophysiology of this remodeling process is still poorly understood. Furthermore, the structural damage of the lung vessels limits the clinical success of vasodilator treatment. Assuming genetic susceptibility, shear stress and inflammation are the principal pathogenetic factors involved in lung vessel remodeling (Voelkel and Tuder, 1995).

Vascular endothelial growth factor (VEGF) was first discovered as a potent and specific mitogen for endothelial cells (Ferrara and Henzel, 1989) and a vascular permeability factor (Leung *et al*., 1989) and was found to play an important role in normal as well as pathological angiogenesis (Plate *et al*., 1992). Nitric oxide (NO) has many important

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roles in vascular biology, including regulation of vascular tone and inhibition of platelet aggregation and leukocyte adherence (Faraci and Brian, 1994; Moncada and Higgs, 1993; Moncada *et al*., 1991). NO is considered to be an important modulator in pulmonary hypertension by virtue of its antiplatelet effect and its vasodilatory and antiproliferative actions on pulmonary vascular smooth muscle cells (Garg and Hassid, 1989; Roberts *et al*., 1995; Heath, 1993; Radomski *et al*., 1987; Assender *et al*., 1991; deGraaf *et al*., 1992; Williams *et al*., 1992). The main source of NO in normal vessels is endothelial nitric oxide synthase (eNOS). VEGF and eNOS have been demonstrated to be related closely with pulmonary hypertension.

Many studies showed that pulmonary hypertension could be induced by intratracheal injection of BLM in many animals (Phan *et al*., 1981; Snider, 1983; Michel *et al*., 1988). In order to reveal the pathophysiology of pulmonary hypertension in immature animals, we investigated the evolution of pulmonary hypertension, the pathological changes of pulmonary arteries, and the expression of VEGF-mRNA and eNOSmRNA of pulmonary arterial endothelial cells in immature rabbits treated with intratracheal bleomycin (BLM) injection.

METHODES

Animals

One month old rabbits were used in this study. Thirty three immature rabbits were randomly chosen to receive intratracheal injection of normal saline or BLM by tracheal puncture. This protocol resulted in the creation of 2 groups:

Control group: 14 immature rabbits treated with intratracheal injection of 0.2 ml/kg normal saline.

BLM group: 19 immature rabbits treated with intratracheal injection of 4 U/kg BLM (diluted by normal saline, 2 U/0.1 ml).

Experimental protocol

Hemodynamic studies were conducted 14 days (Control group 8, BLM group 10) or 28 days (Control group 6, BLM group 9) after rabbits were randomly assigned to receive intratracheal injection of normal saline or BLM. After the rabbits were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg), a catheter (Hydrocath 20 G/1.1, Flow rate 13 ml/min, OHMEDA, U.K.) was inserted through the right jugular vein into the pulmonary artery for measurement of systolic pulmonary artery pressure (PASP), diastolic pulmonary artery pressure (PADP) and mean pulmonary artery pressure (MPAP). These hemodynamic variables were measured with a pressure transducer connected to a monitor (Hewlett-Packard). After completion of the above measurement, cardiac arrest was induced by draining blood through the catheter. The lungs were excised, dissected free, and washed in 4 °C normal saline and 4 °C 0.1 mol/L PBS+DEPC.

Morphometric Analysis of Pulmonary Arteries

Paraffin sections 4 μm thick were obtained from the low region of the left lung and stained with hematoxylin and eosin for examination by light microscopy. The external and internal diameter, and the vessel area and vessel cavity area were measured in middle and small pulmonary arteries per lung section. For each artery, the arterial well thickness was expressed as follows: Thickness Index (TI)=1−internal diameter/external diameter. The arterial wall area was expressed as follows: Area Index (AI)=1−vessel cavity area/vessel area.

In situ hybridization

The VEGFmRNA and eNOSmRNA expression of endothelial cells in pulmonary arteries were evaluated by in situ hybridization which was performed as kit described in the VEGF kit.

The VEGF probe sequences of oligonucleotides (VEGF kit, Boster Co., Cat. NO. MK1142) were

5'-TGGGACCACTTGGCATGGTGGAGGTAGAGC-3' for oligonucleotide 1,

5'-GGGTACTCCTGGAAGATGTCCACCAGGGTC-3' for oligonucleotide 2,

5'-CTGCAAGTACGTTCGTTTAACTCAAGCTGC-3' for oligonucleotide 3.

The eNOS probe sequences of oligonucleo-

tides (eNOS kit, Boster Co., Cat. NO. MK1058) were

5'-AGGGCCCATCCTGCTGCGCCTGGGC-GCTGA-3' for oligonucleotide 1,

5'-GGACAGGAAATAGTTGACCATCTCC-TGATG-3' for oligonucleotide 2,

5'-CTCTGGGTGCGTATGCGGCTTGTCA-CCTCC-3' for oligonucleotide 3.

The level of VEGFmRNA and eNOSmRNA expression were evaluated by the colour in the endothelial cells of middle and small pulmonary arteries.

Statistical analysis

All data were expressed as mean±SEM. Comparisons of parameters among 2 groups were made by student test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Body weight

The body weight of control group and BLM group in pre-experiment and post-experiment of two weeks are summarized in the Fig.1. There was no significant difference in body weight between the two groups in pre-experiment and post-experiment at two weeks.

The body weight of control group and BLM group in pre-experiment and post-experiment of four weeks are summarized in the Fig.2. There was no significant difference in body weight between the two groups in pre-experiment and post-experiment at four weeks.

Pulmonary artery pressure

The PAP of control group and BLM group in post-experiment at two and four weeks are summarized in Fig.3 and Fig.4. Two and four weeks after intratracheal injection of BLM into immature rabbits, the PAP increased significantly. The PASP, PADP and MPAP increased 53%, 49% and 52% in post-experiment at two weeks respectively (all $P<0.05$ vs Control group), and increased 43%, 89% and 56% in post-experiment at four weeks respectively (all $P<0.05$ vs Control group). There was no

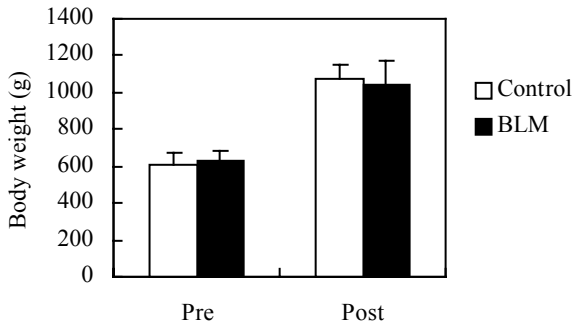

Fig.1 Body weight of control group and BLM group in pre-experiment and post-experiment at two weeks

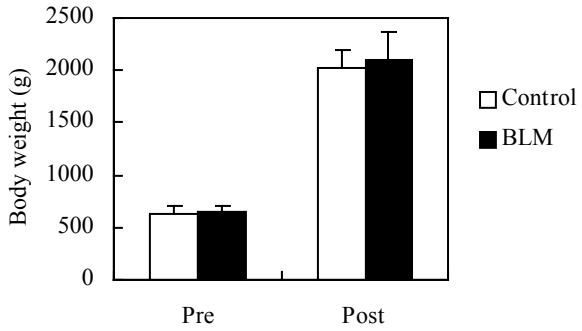

Fig.2 Body weight of control group and BLM group in pre-experiment and post-experiment at four weeks

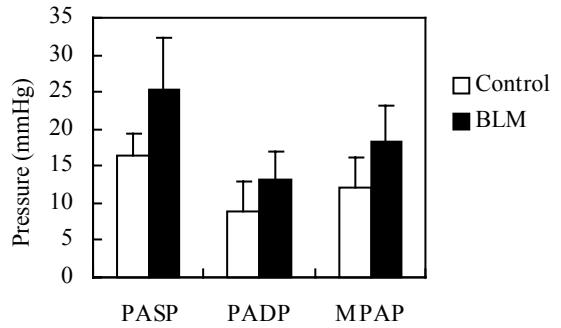

Fig.3 Pulmonary artery pressure of control group and BLM group in post-experiment at two weeks

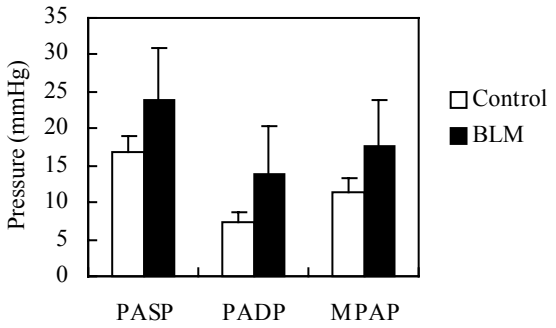

Fig.4 Pulmonary artery pressure of control group and BLM group in post-experiment at four weeks

significant difference in PASP, PADP and MPAP between two weeks and four weeks BLM groups.

Morphometric analysis of pulmonary arteries

The Thickness Index (TI) and Area Index (AI) of control group and BLM group in post-experiment at two and four weeks are summarized in Fig.5 and Fig.6. Two and four weeks after intratracheal injection of BLM into immature rabbits, the thickness of wall increased and the cavity became narrow in middle and small pulmonary arteries, and the TI and AI increased significantly. The TI and AI increased 25% and 14% in post-experiment at two weeks respectively (all $P<0.05$ vs Control group), and increased 22% and 24% in post-experiment at four weeks respectively (all $P<0.05$ vs Control group). There was no significant difference in TI and AI between two weeks and four weeks BLM groups.

VEGFmRNA and eNOSmRNA expression of pulmonary arteries

The level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells of control group and BLM group in post-experiment at two and four weeks are summarized in Fig.7 and Fig.8. Two and four weeks after intratracheal injection of BLM into immature rabbits, the level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells decreased significantly. The level of VEGFmRNA and eNOSmRNA expression decreased 46% and 43% in post-experiment at two weeks respectively (all $P<0.05$ vs Control group), 43% and 51% in post-experiment at four weeks respectively (all $P<0.05$ vs Control group). There was no significant difference in the level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells between two weeks and four weeks BLM groups.

DISCUSSION

In the present study, we demonstrated that two and four weeks after intratracheal injection of BLM

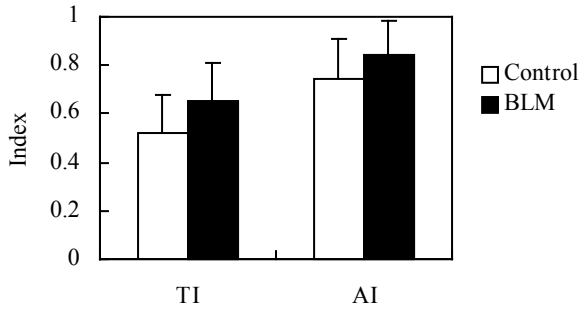

Fig.5 Thickness Index (TI) and Area Index (AI) of control group and BLM group in post-experiment at two weeks

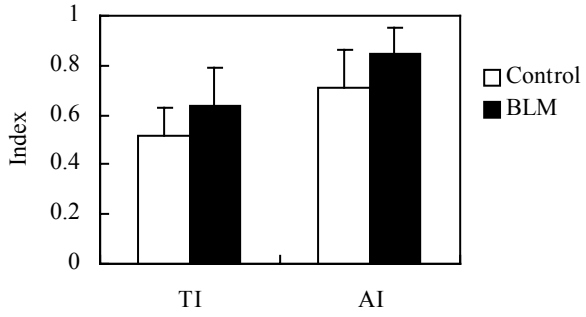

Fig.6 Thickness Index (TI) and Area Index (AI) of control group and BLM group in post-experiment at four weeks

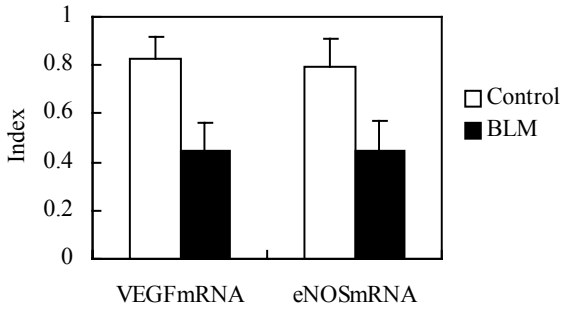

Fig.7 The level of VEGFmRNA and eNOSmRNA expression of control group and BLM group in post-experiment at two weeks

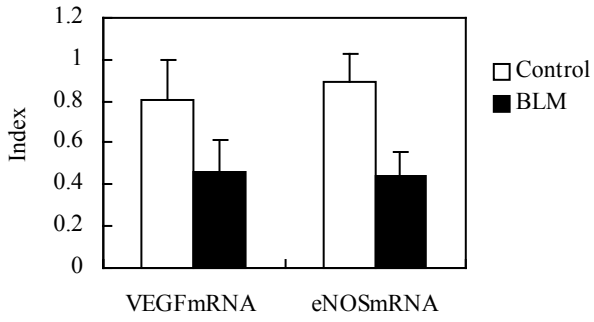

Fig.8 The level of VEGFmRNA and eNOSmRNA expression of control group and BLM group in post-experiment at four weeks

into immature rabbits, the thickness of wall increased and the cavity became narrow in middle and small pulmonary arteries, Thickness Index, Area Index, PAP increased significantly, the level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells decreased significantly. There was no significant difference in TI, AI, PAP, and the level of VEGFmRNA and eNOSmRNA expression between two weeks and four weeks BLM groups.

BLM exposure had been shown to result in significant decrease in the overall number of pulmonary microvessels, which, together with the hypertrophic remodeling of the remaining arteries and arteriols, led to the characteristic decrease in pulmonary vascular conductance observed in this condition. Michel *et al*.(1988) found that vascular compliance decreased, resistance of arterial, middle segment increased, capillary bed was severely obliterated, arteries showed increased percentage of medial and intimal thickening and peripheral muscularization in experimental fibrosis induced by bleomycin and radiation. In this study, two weeks after intratracheal injection of BLM into immature rabbits, the thickening of wall and the narrowing of cavity in middle and small pulmonary arteries, and increasing of PAP probably resulted from enhanced collagen deposition in the pulmonary artery. Bishop *et al*.(1990) found that collagen synthesis rates of about 3%/day were found in the control pulmonary artery and aorta, and about one-half of the newly synthesized collagen was degraded rapidly. Fourteen days after bleomycin treatment, there was a five-fold increase in collagen synthesis rate and a marked decrease in the percentage of newly synthesized collagen degraded rapidly. There was no change in collagen metabolism in the aorta of these animals. Pulmonary artery collagen from control rabbits consisted of 26.5%±1.0% type III collagen. There was no change in composition in bleomycin-treated animals. This study demonstrated quite rapid turnover rates for collagen in normal blood vessels, and that remodeling of arterial connective tissue matrix during pulmonary hypertension involved marked but commensurate increases in type I and III collagens brought about by changes in both synthesis and degradative processes.

The relationship of pulmonary hypertension with eNOS and VEGF gene had been demonstrated by many studies. Chung *et al*.(2003) reported that histological changes and collagen increases consistent with lung injury/fibrosis induced by BLM. The eNOS knockout animals had prolonged fibrosis. The gene transfer of eNOS to the lung could prevent the developing of pulmonary hypertension induced by BLM in mouse (Champion *et al*., 1999). Fehrenbach *et al*.(1999) found VEGF and VEGF receptor Flk1 were related with lung fibrosis and pulmonary hypertension induced by BLM. In this study, two weeks after intratracheal injection of BLM into immature rabbits, the level of VEGFmRNA and eNOSmRNA expression in pulmonary arterial endothelial cells decreased significantly; probably because of the thickening of wall and the narrowing of cavity in middle and small pulmonary arteries, and increasing of PAP. But the cause of the decrease of VEGFmRNA and eNOSmRNA expression level in pulmonary arterial endothelial cells is not clear so far.

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