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Original Article

Angiotensin II induces apoptosis of human pulmonary microvascular endothelial cells in acute aortic dissection complicated with lung injury patients through modulating the expression of monocyte chemoattractant protein-1

Zhiyong Wu, Feifeng Dai, Wei Ren, Huagang Liu, Bowen Li, Jinxing Chang

Department of Cardiovascular Surgery, Wuhan University Renmin Hospital, Wuhan, China

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Abstract: Patients with acute aortic dissection (AAD) usually showed acute lung injury (ALI). However, its pathogenesis is still not well defined. Apoptosis of pulmonary microvascular endothelial cells (PMVECs) is closely related to the alveolus-capillary barrier injury and the increased vascular permeability. In this study, we aim to investigate the human PMVECs (hPMVECs) apoptosis induced by angiotensin II (AngII) and monocyte chemoattractant protein-1 (MCP-1) and their potential interaction in the pathogenesis of AAD complicated with ALI. Fifty-eight newly diagnosed AAD, 12 matched healthy individuals were included. Pulmonary tissues of AAD complicated with lung injury were obtained from 2 cadavers to determine the levels of AngII type 1 receptor (AT1-R) and MCP-1. Serum AngII was measured using commercial ELISA kit. H&E staining and immunohistostaining were performed to determine the expression of AT1-R and MCP-1. For the in vitro experiment, hPMVECs were divided into control, AngII group, AngII+Bindarit group and Bindarit group, respectively. Flow cytometry was performed to analyze the apoptosis in each group. Reverse transcription-polymerase chain reaction was performed to determine the mRNA expression of MCP-1. Western blot analysis was performed to evaluate the expression of MCP-1 and apoptosis related protein. Apoptosis of hPMVECs was observed in the lung tissues in the cadavers with AAD complicated with ALI. Besides, the expression of AT1-R and MCP-1 was remarkably elevated. Compared with normal individuals and the non-lung injury AAD patients, the expression of serum AngII was remarkably elevated in AAD patients complicated with ALI. In vitro experiments showed AngII contributed to the apoptosis and elevation of MCP1 in hPMVECs. Besides, it involved in the down-regulation of Bcl-2 protein, and up-regulation of Bax and Caspase-3. Such phenomenon was completely reversed after administration of MCP-1 inhibitor (Bindarit). The production of MCP-1 and cellular apoptosis induced by AngII in hPMVECs are closely related to the pathogenesis of AAD complicated with ALI. The association between MCP-1 and AngII is crucial in the apoptosis of hPMVECs.

Keywords: Aortic dissection, lung injury, MCP-1, angiotensin II, apoptosis

Introduction

Acute aortic dissection (AAD), the most frequent and catastrophic manifestation of acute aortic syndrome, is frequently reported to occur accompanied by acute lung injury (ALI) featured by severe lung oxygenation impairment [1, 2]. Until now, the pathogenesis of AAD complicated with ALI is still not well defined, and the treatment outcome is still far from satisfactory in most patients [3, 4]. Systemic inflammatory reactions were proposed to play crucial roles in this condition [5, 6]. These reactions may induce alveolus-capillary barrier injury, and finally lead to increased vascular permeability in ALI patients [7, 8]. As previously described, apoptosis of pulmonary microvascular endothelial cells (PMVECs) induced by inflammatory factors is closely related to the alveolus-capillary barrier injury [9, 10]. This leads us to investigate the potential roles of apoptosis of PMVECs in the pathogenesis of AAD complicated with ALI.

Recently, elevation of angiotensin II (AngII), a key factor in the inflammatory diseases, has

been frequently reported in AAD patients [11, 12]. AngII could induce apoptosis through regulating the expression of nucleolin and Bcl-xL by SHP-2 in primary lung endothelial cells [13, 14]. Besides, it contributes to the crosstalk with MAPK protein through modulating the production of MCP-1 in vascular endothelial cells [15, 16]. As a chemotactic for monocytes, MCP-1 involves in the recruitment of macrophages to the lesion sites [17]. At the same time, it could induce apoptosis of vascular endothelial cells. On this basis, we hypothesize that there might potential interaction between AngII and MCP-1 in the pathogenesis of AAD complicated with ALI.

In this study, we investigate the roles of apoptosis of PMVECs in the AAD complicated with ALI. In addition, the efficiency of AngII and MCP-1 in the prediction of AAD complicated with ALI was determined. In vitro studies were performed to illustrate the potential interaction between AngII and MCP-1 in the apoptosis of hPMVECs.

Materials and methods

In vivo study

Patients and sample: Fifty-eight newly diagnosed AAD patients admitted in the intensive care unit (ICU) of our hospital from September 2014 to July 2015 were included in this study. Besides, 12 matched individuals were registered. The diagnosis of AAD was based on the computed tomography (CT) scan and ultrasonic examination. ALI was defined as $PaO_2/FiO_2 \leq 300$ mmHg in the first 24 hour after definited diagnosis according to the diagnostic criteria by American-European consensus conference [11]. Patients admitted to our hospital 7 days or more after the onset of disease were excluded from the study. Those with cancer, chest trauma and pulmonary infection within one month before including in this study were excluded. PaO_2/FiO_2 was determined within 1 h after sample collection. Among the 58 AAD patients, 21 showed concurrent hypoxemia before the surgery. Written informed consent was obtained from each subject. This study was approved by the Ethical Committee of Wuhan University Renmin Hospital.

Pulmonary tissues of AAD complicated with lung injury were obtained from 2 cadavers to observe the ultrastructural changes and determine the levels of AT1R and MCP-1. Pulmonary tissues obtained from the organ donors (n=2) served as the control. The study protocols were approved by the Ethical Committee of Wuhan University Renmin Hospital.

Electron microscopic examination of lung tissue: The lung tissues obtained from the cadaver were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Three hours later, specimens were placed in 2% OsO_4 for 2 h. Subsequently, the mixture were hydrated in a decreasing series of ethanol solutions and embedded in Epon-Araldite. The samples were stained with uranylacetate and lead citrate, and were examined by an H-7700 transmission electron microscope (Hitachi, Tokyo, Japan).

ELISA: Serum AngII was measured using commercial ELISA kit (category No. EK0459, Biofavor Biotech Service Co., Ltd. Wuhan, China) according to the manufacture's instructions. All tests were carried out at least in triplicate.

Histopathological examination: The lung tissues were fixed and embedded, followed by cutting into 4 μm sections. Afterwards, H&E staining and immunohistostaining were performed to determine the expression of AT1-R and - MCP-1 (category No. PB0492 and BA1254, Boster Co., Ltd. Wuhan, China) according to the previous description [12, 13]. The images were observed using a CKX41SF light microscope (Olypus Corporation, Tokyo, Japan).

In vitro study

Cell culture: The hPMVECs of passages 2~8 were purchased from Biofavor Biotech Service Co., Ltd. (category No. CP-H001, Wuhan, China). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.5% fungizone (Invitrogen, Carlsbad, CA, USA), followed by culturing at 37°C in a humidified atmosphere of 5% CO_2 in air. The growth of the cells was arrested by replacing 10% FBS RPMI 1640 with FBS-free RPMI 1640 for 24 h.

Experimental design: The cells were divided into four groups, including: control, the cells were incubated with low-serum RPMI 1640 supplemented with 2% FBS; AngII group, incu-

Table 1. Clinical features of the patients

Variable	Control (n=12)	AAD	
		Complicated with lung injury AAD (n=21)	Non lung injury AAD (n=37)
Age, yr	40±9	47±6	51±6
Male sex, n%	8 (66.7)	17 (81.0)	30 (81.1)
Average duration from onset, h	N/A	9.2	10.7
Smoking, n (%)	4 (33.3)	11 (52.4)	19 (51.4)

bated with low-serum RPMI 1640 supplemented with 2% FBS and AngII (1 μM Sigma-Aldrich, St. Louis, USA); AngII+Bindarit group, incubated with low-serum RPMI 1640 supplemented with 2% FBS, AngII (1 μM Sigma-Aldrich, St. Louis, USA) and Bindarit (300 μM, Selleck Chemicals LLC. USA); and Bindarit group, incubated with low-serum RPMI 1640 supplemented with 2% FBS and Bindarit (300 μM, Selleck Chemicals LLC. USA). The cells were incubated for 24 h before the subsequent analysis as previously described [14, 15].

Flow cytometry: All cells were immunostained according to the protocol of the Annexin V/PI apoptosis kit. The apoptosis ratio was analyzed using flow cytometry.

Reverse transcription-polymerase chain reaction: Total RNA was isolated from hPMVECs using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Reverse transcription was performed using the Tetro Reverse Transcriptase (Bioline, London, UK) according to the manufacturer's instructions. The specific primers for human MCP1 were as follows: sense, 5'-AACTGAAGCTCGCACTCTCG-3'; antisense, 5'-TCAGCACAGATCTCCTTGGC-3'. GAPDH served as the control, and the primer sequences were: sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense, 5'-TCCHCCACCCTGTTGCTGTA-3'. The sequencing of PCR products was performed by Sangon Biotech (Shanghai, China). The amplified production of human MCP1 and GAPDH were in a length of 258 and 452 bp, respectively.

Western blotting

After 72 hours, cells were harvested in cell lysis solution (BioDev-Tech. Company, Beijing, China), and then protein was extracted. The resultant protein concentrations were determined by BCA Protein Assay reagents (Beyotime Biotechnology, Jiangsu, China) and the MCP1, Bcl-2, Bax and Caspase-3 were detected using a standard Western blot protocol. The transferred membrane was blocked with 10% skimmed milk for 1 h at room temperature, and then the blocked membrane was incubated with the primary antibody against MCP1 (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 (dilution 1:700; Santa Cruz Biotechnology), Bax (dilution 1:700; Santa Cruz Biotechnology), Caspase-3 (dilution 1:700; Santa Cruz Biotechnology), and β-actin (dilution 1:700; Santa Cruz Biotechnology) overnight at 4°C, respectively. After incubation with the horseradish peroxidase-conjugated secondary antibody (dilutions of 1:5000; Beijing Zhong Shan-Golden Bridge Biological Technology Company, Beijing, China) for 1 h at room temperature, the immunoblotting signals were visualized using a Western Luminescent Detection kit (Vigorous Biotechnology, Beijing, China).

Statistical analysis

All data are expressed as the mean ± standard error of mean (SEM). All the tests were performed at least in triplicate. Data analysis was performed using one-way ANOVA. $P<0.05$ was considered as statistical significance.

Results

In vivo study

Patient characteristics: The patient characteristics were listed in **Table 1**. No statistical difference was noticed in the age and gender in the AAD complicated with ALI patients, AAD without ALI and normal individuals. AAD patients showed remarkable decrease of the PaO_2/FiO_2 ratio.

Compared with the normal individuals, the PaO_2 and SpO_2 was remarkably decreased in AAD patients (**Figure 1**). Among the 58 AAD patients, 21 (36.2%) showed hypoxemia with a PaO_2/FiO_2 of ≤300 mmHg. This revealed patients with AAD showed lung injury mainly featured by hypoxemia.

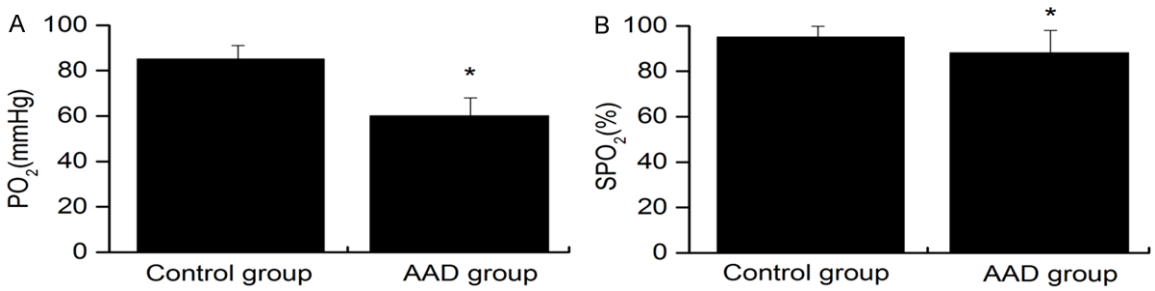

Figure 1. The levels of PO_2 and SpO_2 were remarkably decreased in the patients with AAD. *P<0.05 versus control.

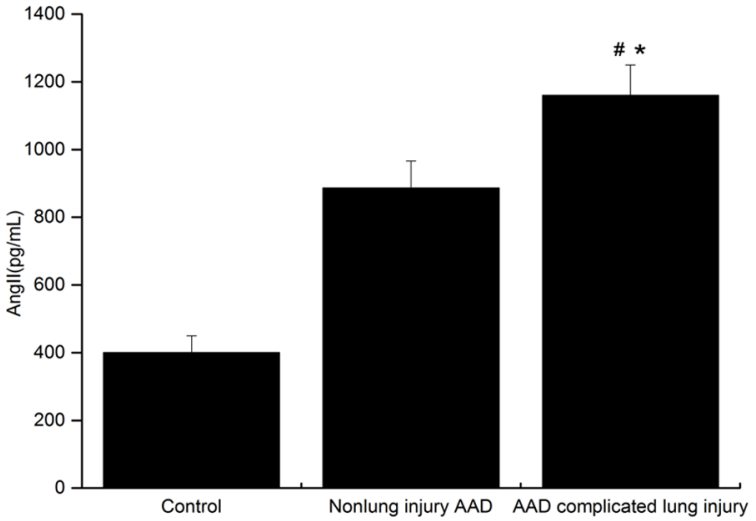

Figure 2. Circulating AngII is elevated in the blood samples from AAD complicated with lung injury patients. AngII was assayed by the systems for each marker in the human peripheral blood samples from healthy control volunteers (n=12); AAD patients without ALI (n=37) or AAD complicated with ALI patients (n=21). *P<0.01 versus control, #P<0.05 versus AAD patients without ALI. AngII, angiotensin II; AAD, acute aortic dissection; ALI, acute lung injury.

Elevation of serum AngII in patients with AAD complicated with ALI: The concentration of AngII in the patients with AAD complicated with ALI was remarkably elevated compared with those of the normal individuals and the AAD patients without ALI (**Figure 2**). On this basis, it is reasonable to imply that AngII may be associated with, or at least partly, play important roles in the onset of AAD complicated with ALI.

Electron microscopic examination of lung tissue in patients with AAD complicated with ALI: In this study, electron microscopic scanning was performed to the lung tissue in two cadavers with AAD complicated with ALI and organ donors. The results indicated accumulation of macrophages, together with oedema in lung tissues, and nuclear alteration related to apoptosis in hPMVECs (**Figure 3**). Taken together, we concluded apoptosis of hPMVECs may be involved in the AAD complicated with ALI.

Pathological and immunohistochemical analysis in lung tissues from AAD complicated with ALI patients: Immunohistochemistry for the lung tissue showed that the expression of AT1-R in hPMVECs in AAD patients complicated with ALI was significantly higher than that of normal individuals. This indicated the over-expression of serum AngII involved in the pathogenesis of AAD complicated with ALI through acting with AT1-R. In addition, immunolocalization showed over-expression of MCP-1 in AAD complicated with ALI patients. However, the expression of MCP-1 was extremely low in lung tissues of normal individuals. Based on this, we implied the MCP-1 were also involved in the pathogenesis of AAD complicated with ALI (**Figure 4**).

In vitro study

AngII contributed to the apoptosis of hPMVECs in vitro: The early stages of apoptosis in the hPMVECs treated above were detected by Flow cytometry. Compared with the Control group, apoptosis was enhanced in AngII group. The apoptosis ratio was significantly decreased in AngII+Bindarit group compared with that of AngII group. No obvious differences between the control group and Bindarit group (**Figure 5**).

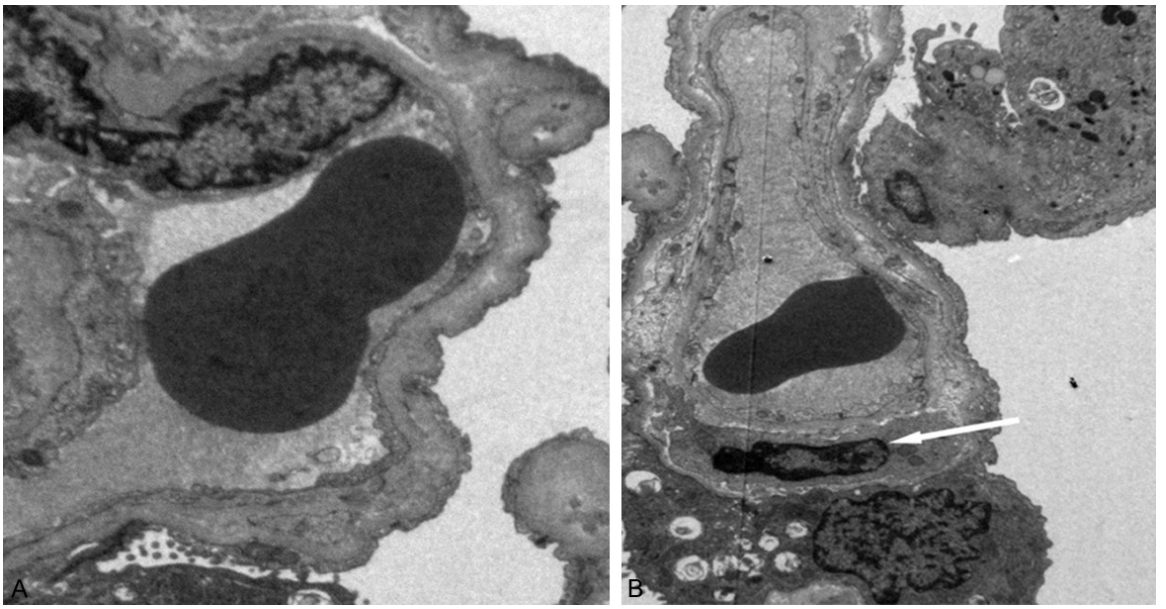

Figure 3. The lung tissues of normal donor (A) and cadavers from AAD complicated with ALI patients (B) observed by transmission electron microscopy under a magnification of 1500×. The nucleus of cadavers from AAD complicated with ALI showed pleomorphism, pyknosis, and chromatin margination. Also, macrophage infiltration and adhesion with hPMVECs were observed. AAD, acute aortic dissection; ALI, acute lung injury.

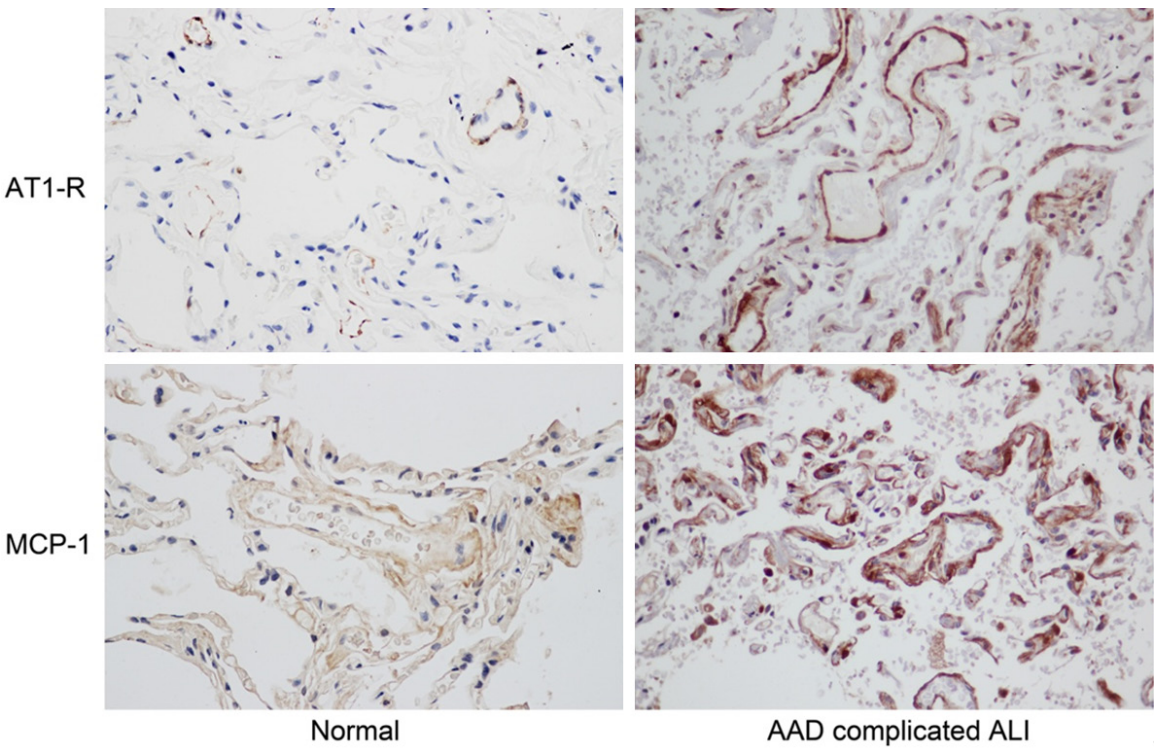

Figure 4. Immunohistochemical images of ATIR and MCP-1 in lung tissues of patients with AAD complicated with ALI and normal individuals.

It is reasonable to conclude that inhibiting the expression of MCP-1 could inhibit the apoptosis of hPMVECs induced by AngII.

AngII inducated elevation of MCP1 in hPMVECs: MCP1 was reported to play crucial roles in the chemotaxis of monocytes in the presence of

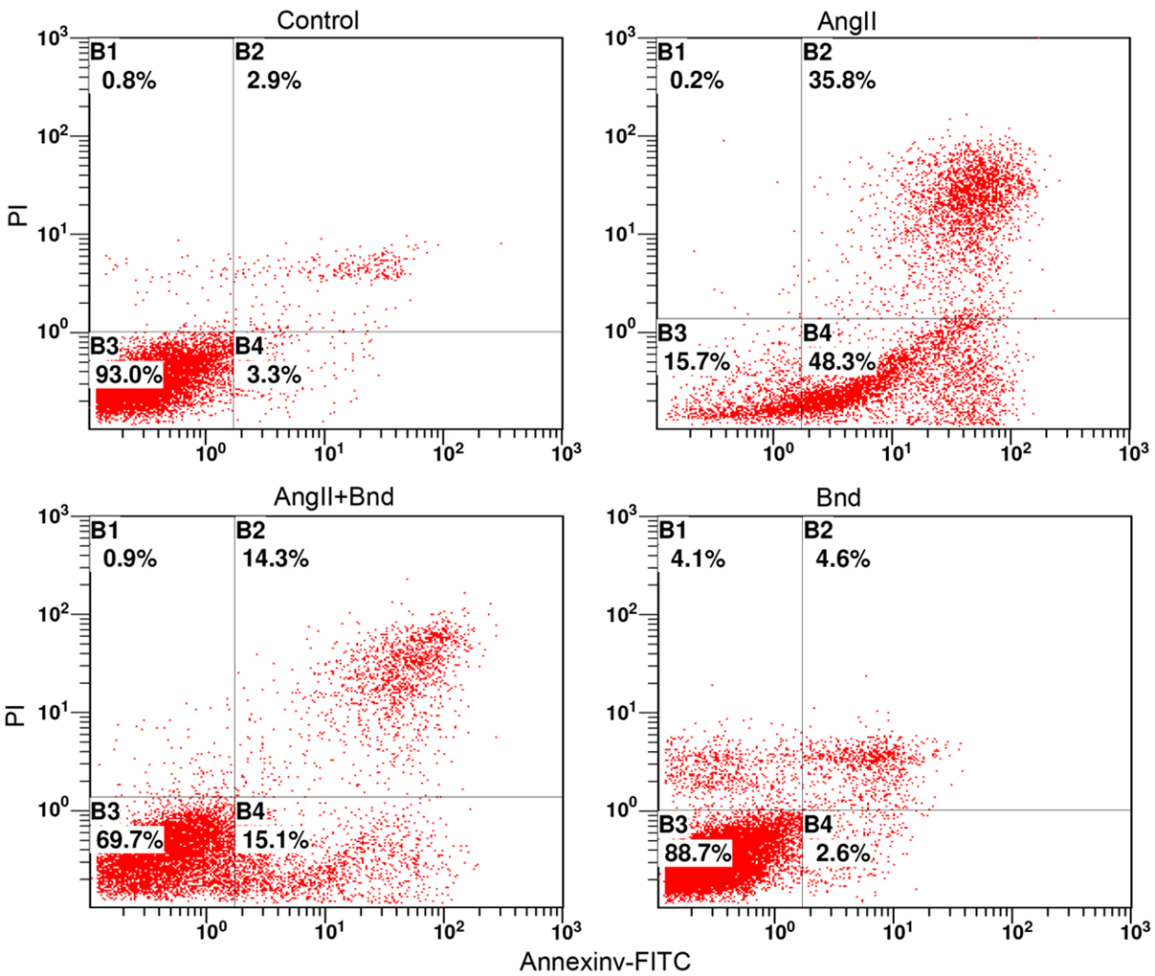

Figure 5. Bindarit reduced the apoptosis in AngII induced hPMVECs. hPMVECs, human pulmonary microvascular endothelial cells; AngII, angiotensin II; PI, Propidium Iodide. FITC, fluoresceine isothiocyanate. Bnd, bindarit.

inflammation. In this study, we determined the expression of MCP1 mRNA and protein in hPMVECs after AngII interference. The expression of mRNA and protein of MCP1 was significantly increased after AngII interference (**Figure 6**). Taken together, we implied that the overexpression of MCP1 in hPMVECs stimulated by AngII played an important role in the recruitment of macrophages. Such phenomenon was completely reversed after inhibiting the MCP-1 activity by Bindarit.

Determination of the apoptosis related proteins in hPMVECs: MCP1 was supposed to play a key role in the processes of AngII induced hPMVECs apoptosis. As shown in **Figure 7**, Western blots analysis showed that the protein expression of Bcl-2 in AngII induced hPMVECs showed remarkable decrease compared with the control group. However, the expression of Bax and caspase-3 was increased significantly in AngII group compared with that of the control group. After interference of MCP1 inhibitor (Bindarit), the expression of Bcl-2 was remarkably increased, while the expression of Bax and caspase-3 was significantly decreased in AngII+Bindarit group. These results revealed MCP1 involved in the up-regulation of Bax and caspase-3, as well as the down-regulation of Bcl-2.

Discussion

Systemic inflammatory reaction involves in the AAD complicated with ALI. Besides, impairment of the alveolar-capillary barrier is the pathological basis for such condition, especially the injury of PMVECs. Therefore, it is necessary to investigate the association between inflammatory factors and apoptosis of hPMVECs in the AAD complicated with ALI.

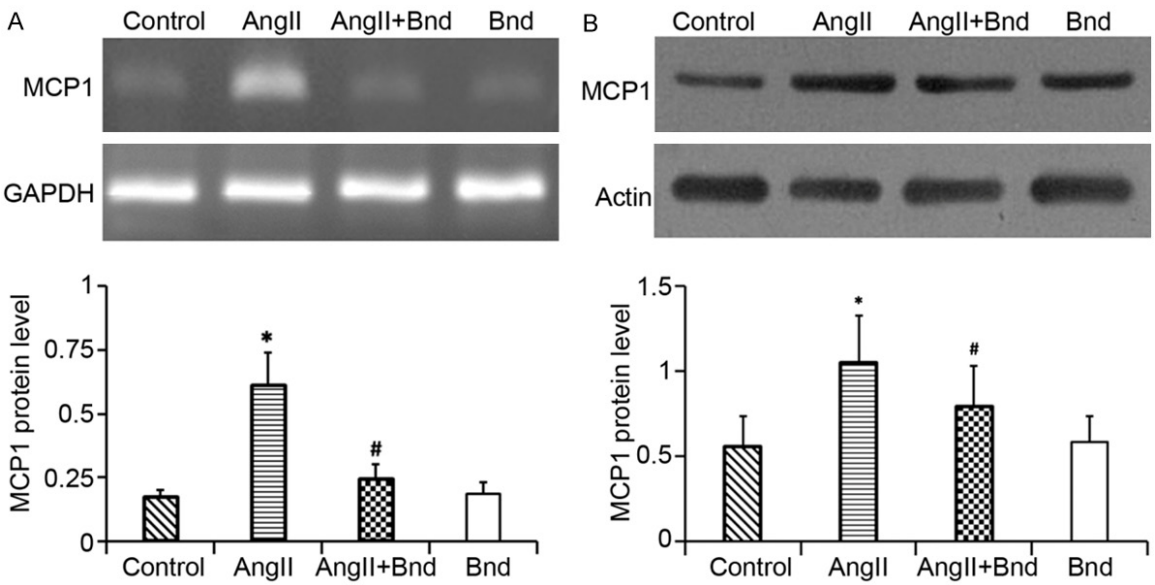

Figure 6. AngII up-regulated the expression of MCP1 mRNA (A) and of MCP1 protein (B) in hPMVECs. GAPDH served as the internal standard for mRNA analysis. Beta-actin served as the internal standard for the Western blot analysis. *p<0.01 vs. control, #p<0.01 vs. AngII. PMVECs, pulmonary microvascular endothelial cells; MCP1, Monocyte chemotactic protein 1; AngII, angiotensin II; Bnd, bindarit.

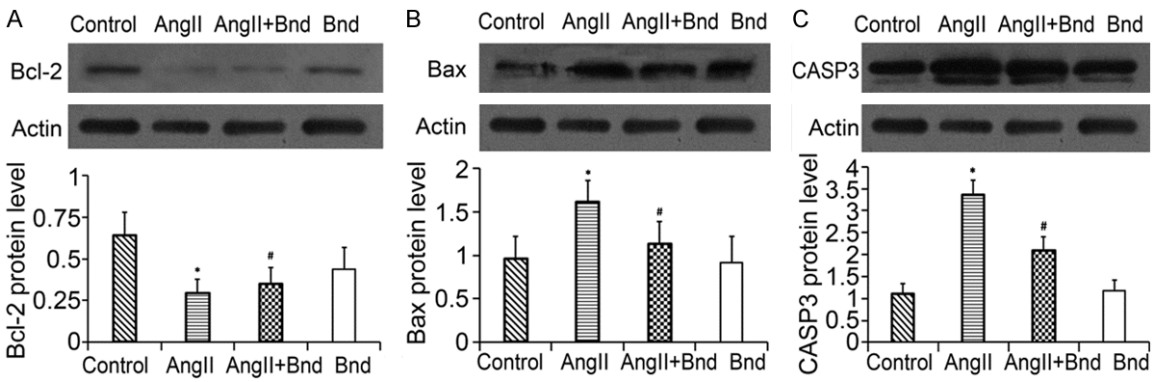

Figure 7. AngII involved in the down-regulation of Bcl-2 (A), and up-regulation of Bax protein (B) and CASP3 protein (C) in hPMVECs. Data, normalized to the amount of actin, are expressed as mean ± SEM (n=7). *p<0.01 vs. control, #p<0.05 vs. AngII. PMVECs, pulmonary microvascular endothelial cells; AngII, angiotensin II; SEM, standard error of mean; Bnd, bindarit.

AngII, the main effector of the renin-angiotensin system, plays an essential role in the inflammatory response, cell proliferation, migration and apoptosis [16-18]. In our study, we found that the AngII levels in patients with AAD complicated with ALI were remarkably elevated compared with the normal individuals, and the AAD patients without ALI. This indicated AngII may be associated with the onset of AAD complicated with ALI. Electron microscopic scanning and immunohistochemisty of lung tissue in cadavers with AAD complicated with ALI showed that pyknosis and chromatin margination in hPMVECs and abundant macrophage aggregated in the lung tissue, together with up-regulation of MCP1 expression. Based on these results, we concluded that hPMVEC apoptosis and MCP1 may play an important role in the pathogenesis of AAD complicated with ALI.

Apoptosis of PMVECs is a key pathologic feature in ALI, which contributes to the impairment of the alveolar-capillary barrier and the final ALI [19, 20]. At present, PMVECs apoptosis related genes are considered to include Bcl-2, Bax and caspase-3 [21, 22]. Bcl2 could suppress cell

apoptosis, while Bax functions as an apoptotic activator [21, 22]. Liu *et al*. showed that lipopolysac-charide (LPS) could induce the apoptosis of PMVECs by up-regulating the protein expression of Bax and down-regulating the protein expression of Bcl-2 [21]. In addition, activation of caspase played an important role in the execution-phase of cell apoptosis [16]. Our results revealed AngII could induce the apoptosis of PMVECs through activating the caspase-3 and increasing the Bax/Bcl-2 ratio. In a previous study, Hernandez *et al*. showed that AngII could induce apoptosis through regulating AMPK/eNOS/p53 pathway and mediating activation of caspase 3, as well as down-regulation of Bcl-2 and up-regulation of p53 [16]. However, blocking this pathway could not inhibit the apoptosis completely, which indicated that there existed another mechanism mediating cell apoptosis.

In our study, the expression of MCP1 in hPMVECs was significantly increased after AngII interference, which indicated that the overexpression of MCP1 may closely relate to the AngII induced hPMVECs apoptosis. MCP-1, a main chemotactic factor for monocyte aggregation and migration [23], is closely related to the development of ALI [12, 24]. Besides, AngII could induce the expression of *MCP1* gene through modulating the expression of NFκB and activating AP-1 protein, as well as up-regulating the expression of c-jun and c-fos [8, 25]. In addition, MCP1 may induce hUVECs apoptosis via evoking the imbalance between anti-apoptotic Bcl-2 protein and proapoptotic Fas/Bax [26]. Bindarit is demonstrated to have a dose-dependent, inhibitory effect on MCP1 production [15]. In our study, the expression of Bcl-2 was increased, while the expression of Bax and caspase-3 was significantly decreased when MCP1 was inhibited by bindarit. The apoptosis ratio was significantly decreased in AngII+Bindarit group compared with that of AngII group. On this basis, we implied MCP1 was involved in the AngII induced PMVECs apoptosis.

In conclusion, AngII plays an important role in the development of AAD complicated with ALI through inducing the expression of MCP1 in PMVECs. MCP-1 involves in the inflammatory reaction through mediating the recruitment of macrophages to the lesion. Also, it induces the apoptosis of hPMVECs through up-regulating the expression of caspase-3 and Bax, and down-regulating the expression of Bcl-2, which lead to impairment of air-blood barrier.

Address correspondence to: Dr. Zhiyong Wu, Department of Cardiovascular Surgery, Wuhan University Renmin Hospital, No. 238, Zhangzhidong Road, Wuhan, China. Tel: +86-27-88041911; E-mail: zhiyongwu889@sina.com

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