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The mechanisms of Ang II-induced hypertensive vascular remodeling under suppression of CD68 in macrophages

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Abstract. – OBJECTIVE: High blood pressure (hypertension) is one of the most common cardiovascular diseases. In recent years, there were more and more studies on the function of inflammation in hypertension. CD68 mainly mediates the activation of cytokine interleukin-17 (IL-17) signaling pathway and participates in inflammatory responses. It has been studied the function of CD68 and IL-17 in hypertension, but it has not been reported whether it affected hypertension and vascular remodeling when macrophage CD68 expression inhibited. In this study, antisense-CD68 mice were used to study the effect and mechanism of angiotensin II-induced hypertensive vascular remodeling under specific suppression of macrophage CD68.

MATERIALS AND METHODS: Fifty 8-week-old male antisense-CD681 and C57 mice were divided into control and experimental group (angiotensin II group, 1000 ng·kg^{-1}·min^{-1}). After infusion of angiotensin II for 28 days, hematoxylin-eosin (HE) staining and immunohistochemical staining were used to observe the remodel of vascular. The changes of aortic inflammatory factors were detected by Real-time PCR (RT-PCR) and Western blotting.

RESULTS: By specifically inhibiting the expression of macrophage CD68, macrophage infiltration was mitigated in Ang II-induced hypertensive vascular remodeling model mouse, which also down-regulated the expression of vascular tissue inflammatory factor and activation of vascular smooth muscle cell p65.

CONCLUSIONS: CD68 regulates the Ang II-induced hypertensive vascular remodeling through mediating macrophage inflammatory factor release.

Key Words:
Hypertensive, Vascular Remodeling, CD68.

Introduction

Vascular remodeling (VR) is a change in vascular structure and function caused by chronic hypertension. Initially, it was thought that vascular remodeling was a compensatory response to increased stress on blood vessels and a result of elevated blood pressure in hypertensive patients. However, recently studies have found that vascular remodeling and blood pressure rise existed in a cause and effect relationship, leading to a vicious circle. In addition, others studies indicated that elevation of blood pressure and vascular remodeling did not show a consistent change. Clinical and experimental studies showed that there were non-blood pressure-dependent changes in vascular remodeling[1-3]. It has been observed that Ang II would not only cause elevation of blood pressure, but also cause vascular hypertrophy, thickening of the vessel wall, increasing wall/cavity ratio, finally occurring the vascular remodeling[4,5]. The mechanism of vascular remodeling is complex and includes changes in hemodynamics, vasoactive substances, vascular inflammatory responses, oxidative stress, and abnormal activation of renin-angiotensin system, vascular endothelial dysfunction, vascular smooth muscle cells (VSMC) and extracellular matrix deposition. Inflammation has played a key role in proliferation, apoptosis and remodeling of VSMC in vascular remodeling[6]. Macrophages are important regulators in immune inflammation, including intrinsic macrophages and monocytes differentiated macrophages in blood vessels. Current studies suggest that monocytes can immediately differentiate into macrophages once they enter the tissue from the circulatory system[7]. It has been reported that macrophages played an important role in hypertension and hypertensive vascular remodeling. Macrophage dysfunction inhibited the Ang II or DOCA-induced elevation of blood pressure and vascular remodeling in mcsf (Csf1) mutant mice[8]. Deletion of mononuclear macrophage inhibited the Ang II induced blood pressure and reduced the vascular dys-

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function[9]. It is clear that hypertension is able to activate the renin-angiotensin-aldosterone system (RAAS), thereby stimulating superoxide anion and activating redox sensitivity factor-mediated inflammatory response, thus involving in vascular remodeling[10]. We established hypertension model on the gene engineering antisense-CD68 mice with inhibited macrophage CD68 to observe the effect on the change of blood pressure, vascular structure and function after suppression macrophage CD68, and to explore the molecular mechanism of CD68 in hypertension.

Materials and Methods

Establishment of Hypertension Model

Configure the Ang II micropumps: the experiments were performed on 8 week-old antisense-CD68 and C57BL/6J mice for control that were treated with Ang II for 28 days (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 1000 $ng \cdot kg^{-1} \cdot min^{-1}$ (200 L) by using ALZET mini-osmotic pumps (Cupertino, CA, USA) by subcutaneously implanted. Mice were anesthetized by 0.1 mL ketamine (Sigma-Aldrich, St. Louis, MO, USA) with an intraperitoneal injection and fixed on a special fixation frame, following with complex iodine or alcohol disinfection of the skin. A length of about 1 cm was crosscut in the neck, and free back skin with subcutaneous tissue was blunted. The prepared Ang II micro-pump was implanted into the layer-by-layer subcutaneous tissue. After 28 days of treatment, subsequent histological studies were performed following the histochemical procedures described in the sections that follow. After anesthetized, the blood vessels and other tissues were extracted for the next experiment.

The experimental animals received humane care, and the study protocols were in accordance with the guidelines the Ethics Committee of The Affiliated Hospital of Qingdao University.

HE staining

Before beginning this procedure, the tissues should be embedded in paraffin, sectioned to 3-5 μm, floated on a water bath, picked up onto glass slides, and placed in slide racks. After the samples were hydrated, they were stained in hematoxylin solution for 1 min and placed at room temperature for at least 5 min. The samples were stained in working eosin Y solution for 10 s. After that, they were dehydrated and cleaned for three times with xylene for 2 min each. The tissue on each slide was added to a coverslip and viewed using a microscope (Olympus Life Science, Tokyo, Japan).

Immunohistochemistry Staining

At the end of the treatment, the tissues were fixed by transcranial perfusion with 100 mL 4% (w/v) paraformaldehyde (pH 7.4). The slices were prepared according to the above described. After the paraffin was clear, the samples were washed with phosphate-buffered saline (PBS) for 5 min three times. The samples were placed in citric acid buffer solution for high-pressure boil for 5 min, then washed with phosphate-buffered saline (PBS) for 5 min three times. The samples were soaked with 0.3% hydrogen peroxide at room temperature for 30-60 min and washed with phosphate-buffered solution (PBS) for 5 min three times. The sample was placed in fetal bovine serum (FBS) 10% for 30 min, and incubated with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. After the samples were washed with PBS for 5 min three times, they were incubated in the secondary antibody (1:100) at 37°C for 1 hour. After termination of the reaction, a drop of paramount was placed over the tissue on each slide and a coverslip was added. The slides were viewed using a microscope (Olympus Life Science, Tokyo, Japan).

Western Blotting Analysis

Tissue proteins were extracted using general protein kits (Beyotime, Haimen, Jiangsu, China). All protein samples were adjusted to equal concentrations, followed by addition of bromophenol blue. Equal amounts of proteins were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE). Then, the protein was transferred to the nitro-cellulose membranes and blotted with primary antibodies at a dilution of 1:1000, followed by secondary antibodies and analyzed by the LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Lincoln, NE, USA).

RT-PCR

Fresh tissue was placed in a mortar and the liquid nitrogen and rapidly ground to a powder. Total RNA extraction was performed using the TRIzol method (Qiagen, Valencia, CA, USA). The RNA was converted into a complementary DNA (cDNA) using a reverse transcriptase or stored at -80°C (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

All experimental data were expressed as Mean ± SD and analyzed by Image-Pro-Plus 6.0 and Graph Pad Prism 5. The one-dimensional variance analysis was used to compare the multi-group comparisons. The paired *t*-test was used to compare the two groups. $p < 0.05$ represents a significant difference.

Results

Effect on Vascular Remodeling in Hypertensive Mice After Specific Suppression of Macrophage CD68

After 28 days of Ang II treatment, wild type (WT) and CD68 mice were sacrificed, and the diaphragmatic myocardium (1 cm) and mesenteric artery (1 cm) were extracted for HE staining. In the Sham group, blood vessels of WT and CD68 mice had no significance change. In Ang II treated group, the vessel wall of the thoracic aorta (Figure 1A) and mesenteric artery (Figure 1B) was significantly thickened. Compared with WT mice, CD68 mice had significantly reduced vascular remodeling.

Effect on Macrophage Infiltration and Expression of Vascular a-SMA After Specific Suppression of Macrophage CD68

Flow cytometry was used to detect macrophage infiltration in aorta. Macrophage infiltration of vascular tissue has been promoted post Ang II perfusion, while after inhibition of macrophage CD68, the macrophage infiltration

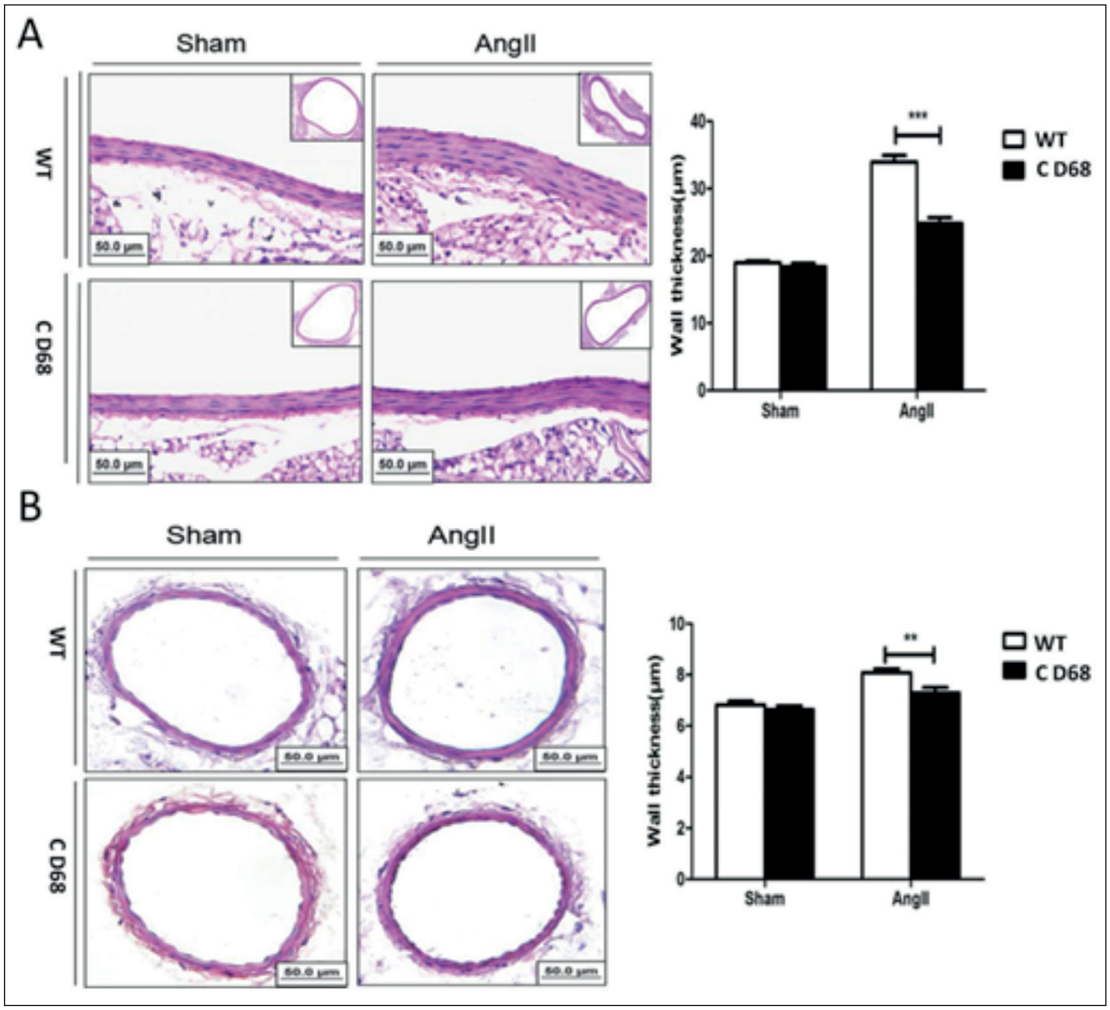

Figure 1. Effect on hypertensive vascular remodeling after specific suppression of CD68 in macrophages. ***A,*** HE staining image of the thoracic aorta; ***B,*** HE staining image of superior mesenteric artery. $^{**}p < 0.01$, $^{***}p < 0.001$.

was significantly reduced (Figure 2A). It was observed that suppression of macrophages CD68 could relieve hypertensive vascular remodeling, so the expression of a-SMA in vascular tissue was detected by immunohistochemical staining. It was indicated that the expression of a-SMA in the vessel was significantly higher than that in the control group (Figure 2C). Compared with the WT mice, the expression of a-SMA in the vessel was significantly lower than that in the control group.

The Effect on Expression of Vascular Inflammatory Factors After Specific Suppression of Macrophage CD68

The above studies have shown that the suppression of macrophage CD68 could inhibit the Ang II-induced macrophage migration and reduce infiltration of vascular macrophage, as well as reduce the vascular remodeling. The mechanism of these changes is unclear. In order to further clarify the molecular mechanism, the total RNA of aortic vascular tissue was extracted from WT and CD68 mice, and the levels of inflammatory factors in vascular tissue were detected using Mouse cytokines and chemokines PCR Array (Qiagen, Hilden, Germany). The mRNA levels of Ccl1, C12, IL15, IL18, IL1b, IL6 etc. are significantly down-regulated in angiotensin-induced hypertensive vascular tissue, while the mRNA levels of Ccl1, Ccl2, Cxcl13, Cxcl16, Gpi1, Mif etc. are significantly up-regulated (*p <0.05, **p < 0.01, CD68 *vs.* C57) (Table I).

Mechanism of Suppressing CD68 in Macrophages

Inflammatory cells are closely related to nuclear factor-kappa B (NF-κB) signaling. In order to further clarify the molecular mechanism of inhibition of VSMC proliferation after suppression macrophage CD68, the mesenteric artery smooth muscle cells of WT mice are isolat-

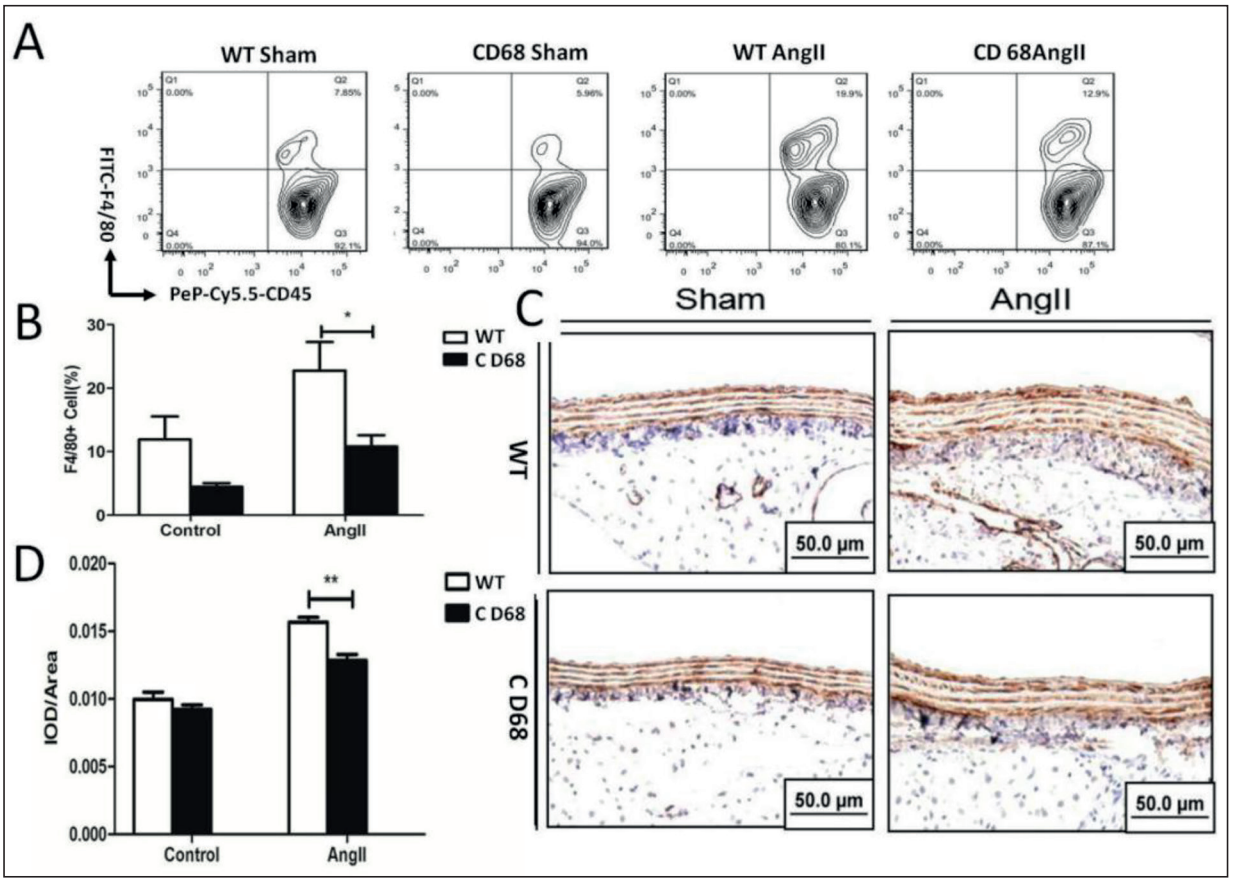

Figure 2. The effect of specific inhibition of macrophage CD68 expression on hypertensive vascular tissues of mice macrophage infiltration and expression of vascular a-SMA. ***A,*** Flow cytometry detection of expression of the macrophage; ***B,*** the statistics of A; ***C,*** a-SMA expression in vascular remodeling; ***D,*** the statistics of C. *p < 0.05, **p < 0.01.

Table I. Expression of cytokines and chemokines in hypertensive vascular tissue.

IL1b	11.50**	Mif	4.02*	Ccl5	1.09	Ifnγ	2.57	Pf4	1.27
IL1m	7.00**	Gpi1	6.42**	Ccl7	1.35	IL11	2.78	Ppbp	2.23
IL4	3.26*	Cxc19	3.2	Cd401g	1.11	IL12b	2.64	Spp1	2.08
IL6	7.71**	Ccl19	3.94*	Cd70	1.19	IL13	1.12	Tgfb2	1.99
IL7	6.66**	Ccl2	3.43*	Csf1	1.11	IL16	1.26	Thpo	1.96
IL12a	3.86*	Adipoq	1.53	Csf2	1.13	IL17f	2.38	Tnf	2.58
IL15	4.77*	Bmp2	1.03	Ctf1	1.25	IL1a	2.03	Tnfsd11b	2.16
IL15	11.48**	Bmp4	1.16	Cxcl1	2.13	IL23a	1.27	Tnfsd10	2.26
Lif	4.16*	Bmp6	1.28	Cxcl10	1.49	IL27	1.20	Tnfsd13b	1.79
Ltb	3.04*	Bmp7	1.08	Cxcl11	1.45	IL10	2.23	Vegfa	2.07
Cxcl1	3.5*	Ccl17	1.47	Cxcl12	2.72	IL9	3.04	Xcl1	2.73
Ccl11	4.67*	Ccl22	1.03	Cxcl5	1.95	IL3	1.05		
Ccl12	3.98*	Ccl24	1.22	Fasl	2.78	IL5	2.47		
Cxcl13	6.32**	Ccl3	1.27	Hc	1.17	Mstn	1.21		
Cxcl16	7.52**	Ccl4	1.43	Ifna2	1.97	Osm	2.63		

ed and cultured. The culture medium of bone marrow-derived macrophages of WT mice was treated with Ang II. CD68 mice were used as conditioned medium; primary mesenteric vascular smooth muscle cells of WT mice were treated for 24 hours. WT-C: WT, BMDM + Ang II conditioned medium + 50% Dulbecco's Modified Eagle Medium (DMEM)/F12 + 10% fetal bovine serum (FBS), CD68-C: CD68, BMDM + Ang II conditioned medium + 50% Dulbecco's Modified Eagle Medium (DMEM)/F12 + 10% fetal bovine serum (FBS). The results show that expression of CD68 in CD68-C was decreased (Figure 3A). Compared with C57 group, the expressions of

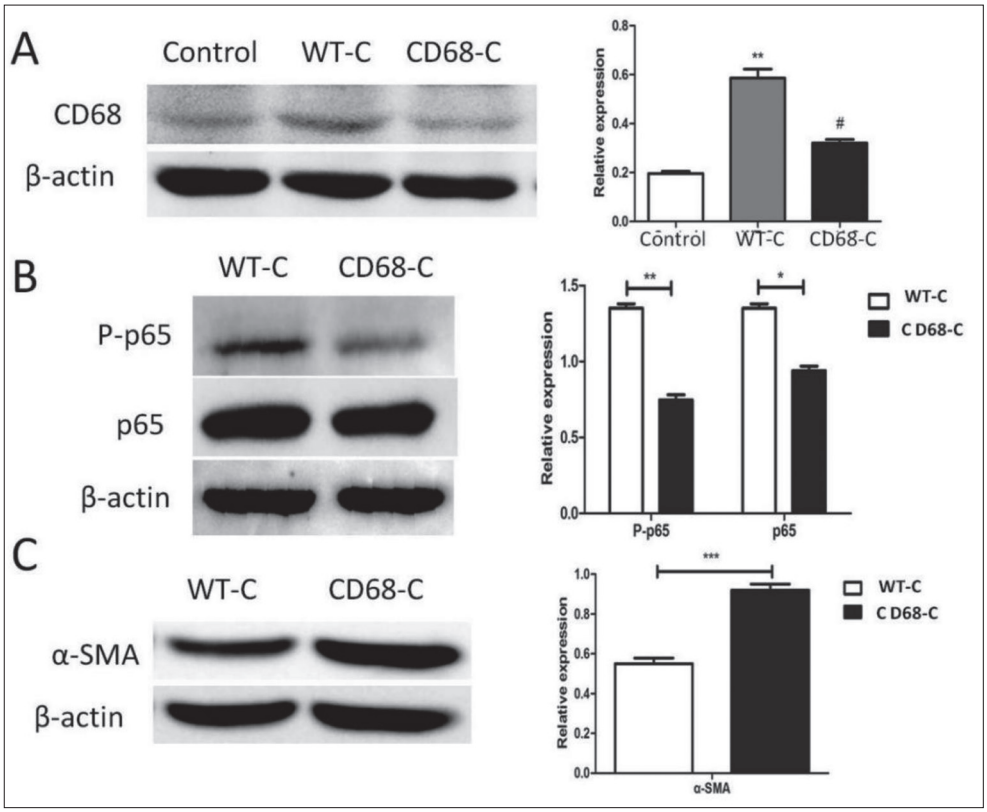

Figure 3. Study on mechanism of suppression of CD68 in macrophages. ***A.*** The expression of CD68 in cells was detected by Western-blot; ***B.*** NF-κB signaling related molecules were detected by Western-blot; C. The expression of a-SMA in two kinds of cells was detected by Western-blot. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

P-p65 and p65 were significantly down-regulated in mesenteric vascular smooth muscle cells treated with CD68 conditional medium (Figure 3B). The level of a-SMA in CD68-C group mesenteric VSMC was significantly higher than that WT-C group. It inhibited the activation of NF-κB in vascular smooth muscle cells, as well as inhibited the phenotypic transformation of vascular smooth muscle cells after suppression of macrophage CD68.

Discussion

Hypertension is a chronic low-grade inflammatory response. Inflammation and hypertension affect each other, which mutual cause and effect. By changing the biological activity of nitric oxide, the inflammation reduces the endothelium-dependent vasodilator factor, promoting the occurrence of hypertension. Macrophages in blood vessels mainly include inherent macrophages and those differentiated by monocytes. Hypertension causes endothelial injury, surface adhesion molecules and inflammatory mediators expressed by impaired ECs involved in regulating monocytes to accumulate in the endothelium and migrate to the vascular membrane. The step of infiltration of macrophages includes: (1) due to activation of ECs, the expression of chemokines is increased causing monocytes from the blood into the vascular tissue; (2 vascular inflammatory microenvironment promotes the differentiation and activation of macrophages; (3) the inflammatory response expands by the activated macrophages[11-13].

Vascular inflammation involves the interaction between inflammatory cells (neutrophils, lymphocytes, monocytes, and macrophages), endothelial cells, vascular smooth muscle cells and extracellular matrix. Studies have shown that Ang II could induce the secretion of proinflammatory cytokines (such as TNF-α, MCP-1 and IL-1β) to promote vascular inflammation and vascular injury; vascular inflammation and cytokines (such as IL-1β, IL-6) could stimulate VSMCs, promoting the proliferation and migration of VSMCs, leading to vascular remodeling[14-16]. In order to explore the molecular mechanism of reduction of Ang II-induced hypertensive vascular remodeling after suppression of macrophage CD68, two mice hypertensive vascular tissues of cytokines and chemokines expression were analyzed by PCR array. After suppression of CD68, the level of IL-1β, IL-6 and IL-18 in Ang II-induced hypertensive vascular tissue was significantly down-regulated, indicating that suppression of CD68 expression in macrophages could inhibit the expression of VSMC related vascular inflammatory factors, and the proliferation of vascular smooth muscle cells. As an important adapter protein in downstream of IL-17, CD68 could mediate the activation of NF-κB and mitogen-activated protein kinases (MAPKs) signaling pathway[17,18]. Ang II could act on the vascular wall, stimulate the inflammatory response through the cytoplasmic NF-κB transcription factor, inducing the production of reactive oxygen species and inflammatory factors and gene expression of adhesion molecules, thereby enhancing vascular inflammation, inducing EC dysfunction, increasing hypertension and vascular remodeling[19,20]. We found that the expression of P-p65 and p65 was significantly decreased in mesenteric VSMCs treated with CD68-C, which indicated that suppression of CD68 expression in macrophages could inhibit the activation of NF-κB to reduce vascular inflammation, thereby reducing vascular remodeling.

Conclusions

The typical characteristics of vascular remodeling included vascular endothelial cell damage, middle smooth muscle cell proliferation and phenotypic transformation, and lumen diameter decrease resulted from extracellular matrix deposition. The proliferation and migration of vascular smooth muscle cells play a key role in vascular remodeling. In this study, we found that the level of a-SMA in CD68-C treated mesenteric vascular smooth muscle cells was significantly higher than the WT-C group, indicating that inflammatory factors secreted by macrophages inhibit the phenotypic transformation of VSMC after suppression of macrophage CD68.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Anderson PG, Bishop SP, Digerness SB. Vascular remodeling and improvement of coronary reserve after hydralazine treatment in spontaneously hypertensive rats. Circ Res 1989; 64: 1127-1136.

2) Chan SL, Umesalma S, Baumbach GL. Epidermal growth factor receptor is critical for angiotensin II-mediated hypertrophy in cerebral arterioles. Hypertension 2015; 65: 806-812.
3) Mieth A, Revermann M, Babelova A, Weigert A, Schermuly RT, Brandes RP. L-type calcium channel inhibitor diltiazem prevents aneurysm formation by blood pressure-independent anti-inflammatory effects. Hypertension 2013; 62: 1098-1104.
4) Javeshghani D, Sairam MR, Neves MF, Schiffrin EL, Touyz RM. Angiotensin II induces vascular dysfunction without exacerbating blood pressure elevation in a mouse model of menopause-associated hypertension. J Hypertens 2006; 24: 1365-1373.
5) Patel VB, Zhong JC, Fan D, Basu R, Morton JS, Parajuli N, Mcmurtry MS, Davidge ST, Kassiri Z, Oudit GY. Angiotensin-converting enzyme 2 is a critical determinant of angiotensin II-induced loss of vascular smooth muscle cells and adverse vascular remodeling. Hypertension 2014; 64: 157-164.
6) Intengan HD, Schiffrin EL. Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. Hypertension 2001; 38: 581-587.
7) Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Key K. Development of monocytes, macrophages, and dendritic cells. Science 2010; 327: 656-661.
8) De Ciuceis C, Amiri F, Brassard P, Endemann DH, Touyz RM, Schiffrin EL. Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin II-infused macrophage colony-stimulating factor-deficient mice: evidence for a role in inflammation in angiotensin-induced vascular injury. Arterioscler Thromb Vasc Biol 2005; 25: 2106-2113.
9) Wenzel P, Knorr M, Kossmann S, Stratmann J, Hausding M, Schuhmacher S, Karbach SH, Schwenk M, Yogev N, Schulz E, Oelze M, Grabbe S, Jonuleit H, Becker C, Daiber A, Waisman A, Munzel T. Lysozyme M-positive monocytes mediate angiotensin II-induced arterial hypertension and vascular dysfunction. Circulation 2011; 124: 1370-1381.
10) Renna NF, De Las Heras N, Miatello RM. Pathophysiology of vascular remodeling in hypertension. Int J Hypertens 2013; 2013: 808353.
11) Fenyo IM, Gafencu AV. The involvement of the monocytes/macrophages in chronic inflammation associated with atherosclerosis. Immunobiology 2013; 218: 1376-1384.
12) Touyz R, He G, Ei Mabrouk M, Schiffrin EL. p38 Map kinase regulates vascular smooth muscle cell collagen synthesis by angiotensin II in SHR but not in WKY. Hypertension 2001; 37: 574-580.
13) Touyz RM. Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. Exp Physiol 2005; 90: 449-455.
14) Chae CU, Lee RT, Rifai N, Ridker PM. Blood pressure and inflammation in apparently healthy men. Hypertension 2001; 38: 399-403.
15) Crowley S, Song YS, Lin EE, Ruiz P. Lymphocyte responses exacerbate angiotensin II-dependent hypertension. Am J Physiol Regul Integr Comp Physiol 2010; 298: 1089-1097.
16) De Miguel C, Das S, Lund H, Mattson DL. T lymphocytes mediate hypertension and kidney damage in Dahl salt-sensitive rats. Am J Physiol Regul Integr Comp Physiol 2010; 298: 1136-1142.
17) Brasier AR, Recinos A, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. Arterioscler Thromb Vasc Biol 2002; 22: 1257-1266.
18) Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004; 84: 767-801.
19) Rensen SS, Doevendans PA, Van Eys GJ. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth Heart J 2007; 15: 100-108.
20) Nyberg M, Gliemann L, Hellsten Y. Vascular function in health, hypertension, and diabetes: effect of physical activity on skeletal muscle microcirculation. Scand J Med Sci Sports 2015; 25 Suppl 4: 60-73.