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Original Article

Endothelial dysfunction induced by antibodies against angiotensin AT_1 receptor in immunized rats

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Aim: To investigate the association between autoantibodies against angiotensin AT1 receptor (AT1-AAs) and endothelial dysfunction *in vivo*.
Methods: Rat models with AT1 receptor antibodies (AT1-Abs) were established by active immunization for nine months. Lactate dehydrogenase (LDH) activity was regarded as an indicator of cell necrotic death. Endothelin-1 (ET-1) in the sera of rats was determined and endothelium-dependent vasodilatation was detected in isolated thoracic aorta. Endothelial intercellular adhesion molecule-1 (ICAM-1) expression in aorta endothelium was assessed using confocal microscopy. Coronary artery endothelial ultrastructure was observed.
Results: IgGs in the immunized group significantly increased the LDH activity (0.84±0.17 *vs* 0.39±0.12, $P<0.01$ *vs* vehicle group IgGs) in incubated human umbilical vein endothelial cells through AT1 receptor. Higher content of ET-1 occurred in the immunized rats than that of the vehicle group, and reached two peaks at month 3 (27±4 ng/L, $P<0.01$) and month 7 (35±5 ng/L, $P<0.01$), respectively. In addition, aortic endothelium-dependent vasodilatation was attenuated; endothelial ICAM-1 level was markedly increased and cardiac capillary endothelium was damaged following immunization.
Conclusion: Our study demonstrated that AT1-Abs contributed to endothelial dysfunction *in vivo*, which was a potential mechanism through which the antibodies play vital roles in related diseases.

Keywords: angiotensin II type 1 receptor; antibodies; endothelium; endothelin-1; endothelial intercellular adhesion molecule-1; endothelium-dependent vasodilatation

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Introduction

The renin-angiotensin system (RAS) is a very important hormone system that regulates blood pressure and water balance. Angiotensin II is the major bioactive product of this system, which can cause the release of aldosterone, constrict blood vessels, and promote the proliferation of vascular smooth muscle cells (VSMCs) through AT1 receptor[1]. The vital roles of RAS in hypertension have attracted research attention for many years. Lots of experiments[2, 3] show that in various types of hypertension, there is excessive AT1R activation, performing strong vessel contractions, water natrium retention, and sympathetic system excitement. But what is interesting is that over-activated AT1R cannot be fully explained by angiotensin II.

At the end of the twentieth century, different research groups had found that there was a high level of autoantibodies against AT1R (AT1-AAs) in preeclampsia[4], malignant hypertension[5], refractory hypertension[6] and renal transplantation hypertension[7]. Researchers have paid a lot of attention to this phenomenon. It has been proven that the antibodies can specifically identify the second extracellular loop of AT1R ($AT1R\text{-}EC_{II}$, the sequence homology between human and rat is 92.6%[8]) and show receptor agonist-like effects, such as promoting the proliferation of VSMCs[9], activating NF-κB and ROS[10], increasing the serum level of soluble fms-like tyrosine kinase-1[11], and causing human mesangial cells to secrete plasminogen activator inhibitor-1 and interleukin-6[12]. These suggest that AT1-AAs may be a reason for excessive AT1R activation in hypertension. Nonetheless, values of the antibodies in these diseases should be further studied.

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More research has proven that, endothelial injury is the common trigger of the series of vascular lesions in both pregnancy-related and pregnancy-unrelated hypertension[13, 14]. Now it has been accepted that endothelial injury should be the result of multiple harmful factors, including known and unknown ones. Therefore, exploring more potential adverse agents associated with endothelial disorder has become one of the "hot" points in this field. In the vascular system, AT1R expresses on endothelial cells except on VSMCs[15]. It has been found that[16] AT1R activation can result in increased endothelial permeability. Recently, LaMarca[17] *et al* have found that the renal cortices and placental preproendothelin mRNA levels were significantly higher in pregnant rats when injected with AT1-AAs, suggesting endothelial cells were activated. All these phenomena show that AT1-AAs may be involved in the aggravation of autoantibody-positive diseases by influencing endothelial function, but at present there is not enough sufficient direct support regarding the relationship between AT1-AA and endothelial injuries.

Therefore, this study intended to establish long-term positive AT1-Ab rat models by active immunization with synthetic human AT1R-EC_{II} peptide. On this basis, whether AT1-Abs could directly destroy endothelial cells was determined firstly *in vitro*, and LDH was treated as a marker of cell death. Then, how the antibodies affected the structure and function of endothelium were observed *in vivo*.

Materials and methods

Animals

The experiments were performed in adherence to the National Institutes of Health "Guidelines on the Use of Laboratory Animals" (publication No 85-23, revised 1996) and were approved by Shanxi Medical University Committee on Animal Care. Healthy AT1-AA-negative male Wistar rats weighing 0.18-0.20 kg [6-8 weeks old, Grade II, certificate number of the breeder: SCXK (Jin) 2009-0001] were selected for establishing an immunization model.

Active immunization model

Sixteen healthy Wistar rats were randomly divided into two groups: immunized group and vehicle group. The peptide corresponding to the sequence of the second extracellular loop of the human AT1 receptor (165-191, I-H-R-N-V-F-F-I-E-N-T-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T, 95% purity) was synthesized by GL Biochem (Shanghai) Ltd. At first, the immunized group was actively immunized using 400 g per kg body weight of the corresponding peptide which was emulsified in the same volume of complete Freund's adjuvant and injected subcutaneously at multiple points. Two weeks later, a booster injection of a mixture of 400 g per kg body weight of the corresponding peptide in the same volume of incomplete Freund's adjuvant was injected subcutaneously at one point, once every two weeks. After three months, rats received a booster immunization every four weeks until the ninth month. The vehicle group rats were injected using adjuvant and saline without peptides following the same procedure as the immunized group. The blood samples of all rats were collected for detection of serum AT1-Abs.

Enzyme linked immunosorbent assay (ELISA)

Modified ELISA was used to detect the titers of AT1-Abs in sera[18]. The procedure was as follows, the synthesized peptide was dissolved in a Na_2CO_3 solution (pH 11.0). The peptide solution (1 mg/L, 50 μL) was coated on 96-well plates and incubated overnight at 4 °C. The wells were then blocked with PMT [0.1% (*w/v*) albumin bovine V, 0.1% (*v/v*) Tween 20 in phosphate-buffered saline (PBS-T), pH 7.4] for 1 h at 37 °C. After the wells were washed three times using PBS-T, 50 μL of serum was added [dilutions in PMT, 1:10 (*v/v*)] to each well and incubated for 1 h at 37 °C. After another three washings, biotinylated goat anti-rat IgG was added at 1:4500 dilution. The plates were incubated for 1 h at 37 °C, then washed and incubated with PMT containing streptavidin-peroxidase (Sigma, USA) at 1:2000 for 1 h. Finally, 2,2'-azino-di (3-ethylbenzothiazoline) sulphonic acid (ABTS)-H_2O_2 (Roche, Basel, Switzerland) was added as the substrate. Half an hour later, absorbance values were detected at 405 nm using a microplate reader (Spectra Max Plus, Molecular Devices Corp, CA, USA).

Preparation of the immunoglobulin G

As the immunization process finished, rats in both the immunized group and vehicle group were anesthetized intraperitoneally with 10% chloral hydrate (3 mL/kg) and the sera were collected. Then total IgGs in the sera were purified by IgG affinity column (Mab Trap Kit, Amersham). Briefly, the column was renaturated with 3 mL binding buffer. Then 1 mL serum sample diluted in equal volume of binding buffer was added, and the column was washed with 7 mL binding buffer followed by 5 mL elution buffer. The eluate (IgG solution) was stored in neutral buffer.

Culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were cultured on 6-well plates with cell culture medium (RPMI-1640, Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, at 37 °C in a humidified incubator containing 5% CO_2. The endothelial cells assumed a spindle-shaped or polygon appearance under the inverted microscope. After the growth fusion, cells became monolayer and displayed a cobblestone or paving stone arrangement. The original culture medium was discarded when the cells confluence reached 80%. The cells were then washed twice by D-Hanks' liquid and digested with 0.125% trypsin. The digestion liquid was discarded when cells contracted to a round shape and the intercellular space widened. Finally, the fourth generation HUVECs were treated with 0.1 μmol/L of IgGs purified from two groups (Mab Trap Kit, Amersham) and angiotensin II (0.1 μmol/L, Sigma, USA) , respectively. The inhibitory effect of specific AT1R antagonist losartan (1 μmol/L, Sigma, USA) was observed. Medium samples were collected at 6, 24, and 48 h, and LDH activity was determined according to the protocol reported by Korzeniewski[19] *et al*. The procedure was as follows: the reaction monitored is that

the lactate is oxidized to pyruvate (coupled to the reduction of cytosolic NAD^+ to NADH) in the presence of a tetrazolium salt (INT) as an oxidizing agent and phenazine methosulphate (PMS) as an electron transfer agent. Reaction buffer was prepared by mixing 4.17 mg INT, 1.07 mg PMS, 10.76 mg NAD with 60 mg lactic acid [dissolved in 12 mL 0.2 mol/L Tris (pH 8.2)]. Conditioned medium sample (50 μL) was added to each well, then 100 μL of the reaction buffer was added to start the reaction. The change in absorbance was determined by a microplate reader under kinetic mode at 490 nm for 5 min. LDH activity was defined as optical density (*OD*) value at the end point.

Detection of serum ET-1 content

ET-1 concentration was determined by the corresponding Quantitative EIA Kit (R&D, USA) following the manufacturer's instructions. Briefly, standard substances and the samples (50 μL) were added into the wells which had been coated with anti-endothelin monoclonal antibodies. After being incubated at 37 °C for 1 h, the plates were washed with cleaning buffer five times. Then anti-rat endothelin-1 IgG/HRP was added and reacted at 37 °C for 1.5 h. Following five washings, *o*-phenylenediamine (OPD) at 100 μL was added to the wells and incubated in the dark at 37 °C for 0.5 h. Finally, stop buffer was added. The optical densities were measured at 492 nm in an ELISA reader. The standard curve was made on coordinate paper.

Thoracic aorta preparations and *in vitro* vasodilatation

When the rats were anesthetized, thoracic aortas were quickly removed and placed in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution(in mmol/L: NaCl 144.0, KCl 5.8, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2$ 2.5, glucose 11.0, Hepes 5.0, pH 7.4). The surrounding tissue was cleaned and the aortas were cut into rings of 3–4 mm in length. The rings were suspended on steel hooks in tissue baths containing 10 mL of HEPES solution bubbled with 100% O_2 and maintained at 37°C. The changes in isometric force were recorded by a PowerLab system (AD Instruments, Australia). Passive tension was adjusted to 2.0 g. Then a 1.5 h equilibration period was allowed before any experimental intervention, and the HEPES was replaced every 20 min. After equilibration, the rings were constricted with HEPES-buffer containing 60 mmol/L KCl (in mmol/L: NaCl 89.8, KCl 60.0, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2$ 2.5, glucose 11.0, Hepes 5.0, pH 7.4) followed by extensive washing. Then the thoracic aorta was contracted by 10^{-6} mol/L NE, the integrity of endothelium was ensured by the relaxation induced by 10^{-9} to 10^{-6} mol/L acetylcholine (ACh). 10^{-9} to 10^{-6} mol/L sodium nitrate (SNP) was added to observe the non-dependent relaxation of endothelium.

ICAM-1 detection by laser scanning confocal microscopy (LSCM)

Thoracic aorta slides were washed by PBS and followed by microwave antigen retrieval (0.01 mol/L sodium citrate buffer, pH 6.0, 100 °C, 15 s). After cooling for 30 min, ICAM-1 antibody (goat anti-rat polyclonal IgG, Santa Cruz, USA) was added and incubated overnight at 4 °C. Then, FITC-labeled rabbit anti-goat IgG (H+L) was used and was reacted with the samples for 30 min at 37 °C. The slides were mounted with glycerol and observed with LSCM. The excitation wavelength of FITC is 495 nm, and the emission wavelength is 519 nm.

Transmission electron microscope (TEM)

Heart tissue (1 mm×1 mm) was removed quickly and stored in 2.5% glutaric dialdehyde for 2 h at 4 °C. After washing with phosphate buffer (pH 7.4), the sample was post-fixed in 1% osmium tetroxide for 2 h at 4 °C. Gradient dehydration by 50% to 100% ethanol was performed, and the sample was then embedded in epoxies at 37 °C overnight. Ultrathin sections were cut at 50 nm, and stained with lead citrate solution. Then a TEM was used for observing the structural changes of coronary endothelium.

Statistical analysis

All of the data were expressed as mean±SD. The statistical analysis was calculated using independent-samples *t*-test for two groups and one-way ANOVA for multiple groups using SPSS 13.0. $P<0.05$ was considered statistically significant.

Results

The rat models actively immunized with AT1R-EC_{II} were successfully established

The titers of AT1-Abs in the sera of two rat groups were detected by using the ELISA method. As shown in Figure 1, 7 of 8 active immunized Wistar rats generated increased serum levels of AT1-Abs at the 1st month after initial immunization. And the concentrations of AT1-Abs maintained a constant high level from the 2nd month to the end of immunization (*OD* value at the last month, 1.79±0.29 *vs* 0.45±0.16, $P<0.01$ *vs* vehicle group at the same time point), however, AT1-Ab was

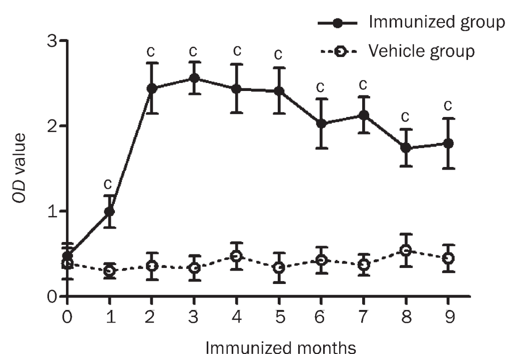

Figure 1. High titers of AT1-Abs were generated in the rats immunized with human AT1R-EC_{II}. Healthy Wistar rats were actively immunized with synthetic human AT1R-EC_{II}. ELISA method was used to assess the generation rule of serum AT1-Abs. The titer of antibody was defined by *OD* value. Data were expressed as means±SD. *n*=7−8 rats per group. $^cP<0.01$ *vs* vehicle group at the same period. *OD*, optical density; AT1R-EC_{II}, epitope peptide of extracellular second loop of AT1 receptor; AT1-Ab, AT1 receptor antibody.

not detected in the concurrent control, suggesting that the active immunization models were successfully established. The remaining one immunized rat failing to produce AT1-Ab was excluded.

LDH activation in cultured HUVECs treated with AT1-Ab-positive IgGs

Total IgGs from the sera of the two rat groups were purified and added into cultured HUVECs, and LDH activity was detected after 6 h, 24 h, and 48 h. As illustrated in Figure 2, compared with vehicle group IgGs, 0.1 μmol/L total IgGs from AT1-Ab-positive sera of the immunized group caused a noticeable increase of LDH activity in cocultured HUVECs after 48 h (*OD* value, 0.84±0.17 *vs* 0.39±0.12, $P<0.01$, *vs* 0.1 μmol/L vehicle group IgGs), which was completely blocked by losartan (*OD* value, 0.28±0.13, $P<0.01$), an AT1-receptor antagonist. The effect of immunized group IgGs was almost identical with that observed in angiotensin II-treated HUVECs (*OD* value, 1.04±0.16), which indicated that there was necrotic cell death.

Immunization with synthetic AT1R-EC$_{II}$ peptide caused a significant increase of serum ET-1 *in vivo*

As detected by ELISA kit, a persistent increase of ET-1 in the immunized group started from the 3rd month after initial immunization till the end of the experiment. Two peaks occurred at month 3 (27±4 ng/L) and month 7 (35±5 ng/L), respectively. Both of them were significantly different with the vehicle group at the same time point (12.4±2.4 ng/L, 12.1±2.9 ng/L, $P<0.01$, $P<0.01$). The data were presented in Figure 3.

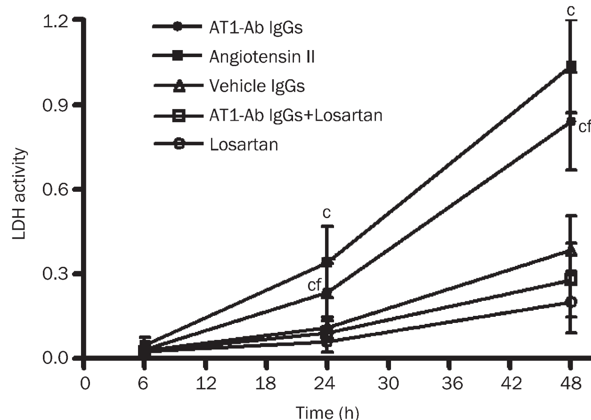

Figure 2. The effect of IgGs from immunized group on LDH activity in cultured HUVECs. Total IgGs purified from immunized group (0.1 μmol/L) or vehicle group (0.1 μmol/L) was added into cultured HUVECs, respectively. LDH activity was detected after 6, 24, and 48 h. The effects of AT1 receptor agonist angiotensin II (0.1 μmol/L) and the blocker losartan (1 μmol/L) were also observed. Data were expressed by means±SD. n=6 cells per group. $^cP<0.01$ *vs* vehicle IgGs. $^fP<0.01$ *vs* AT1-Ab IgGs+losartan. *OD*, optical density.

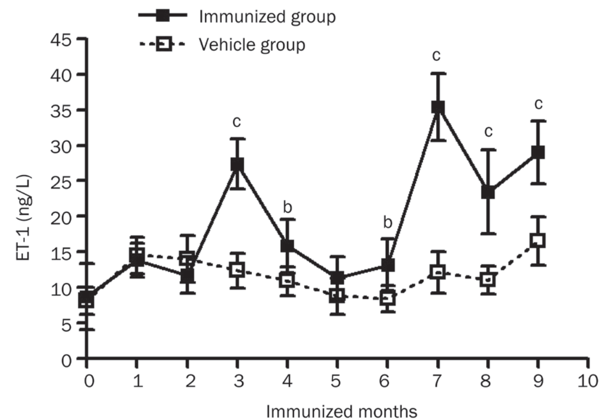

Figure 3. Persistent high content of ET-1 occurred in the sera of immunized rats. In the immunization process, the concentrations of sera ET-1 in two groups were monitored dynamically. Data were expressed by means±SD. n=6−8 rats per group. $^bP<0.05$, $^cP<0.01$ *vs* vehicle group at the same period. ET-1, endothelin-1.

The AT1R-EC$_{II}$ immunization resulted in attenuated endothelium-dependent vasodilatation

As summarized in Figure 4, vascular dysfunction as evidenced by reduced acetylcholine-dependent aortic vasodilatation was observed in the immunized group which was introduced with human AT1R-EC$_{II}$ for nine months. Thoracic aortas were pre-contracted by 10^{-6} mol/L norepinephrine, then endothelium-dependent vascular relaxation was performed using 10^{-9}–10^{-6} mol/L acetylcholine. Compared with the vehicle group, the diastolic range significantly decreased in the immunized rats (relaxation percentage of the pre-contraction, 51%±6% *vs* 62%±7%, $P<0.05$, *vs* vehicle group, Figure 4A). However, there was no difference in 10^{-9} mol/L–10^{-6} mol/L sodium nitroprusside-induced endothelium-independent vasodilatation between the two groups (Figure 4B).

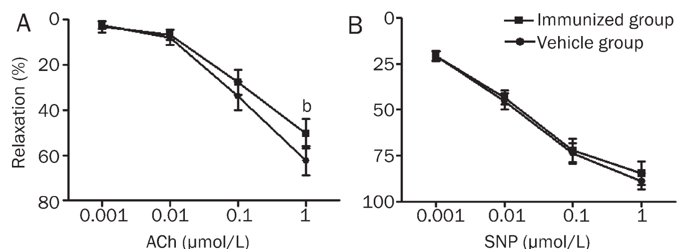

Figure 4. Endothelium-dependent diastolic function of thoracic aorta declined in the immunized rats. When the rats have been immunized for nine months, endothelium-dependent (A) and in-dependent (B) diastolic function of thoracic aorta in the rats were detected *in vitro*. Diastolic function was defined by the percentage of the pre-contraction. Data were expressed by means±SD. n=6−8 rats per group. $^bP<0.05$ *vs* vehicle group at the same period. ACh, acetylcholine; SNP, sodium nitroprusside.

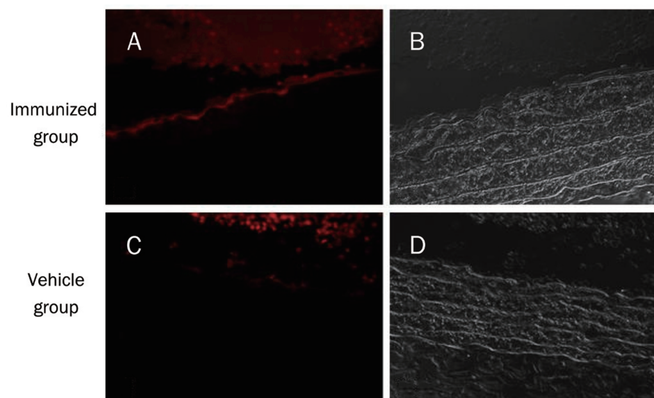

Figure 5. Upregulation of the expression ICAM-1 in thoracic aortic endothelium in the immunized rats. At the end of the immunization, the expressions of thoracic aortic endothelial ICAM-1 in the two groups of rats were measured under a laser scanning confocal microscope. A FITC-labeled anti-rat ICAM-1 antibody produced the red stain (5A, 5C). 5B and 5D were the corresponding structure charts of vascular lying on left, respectively.

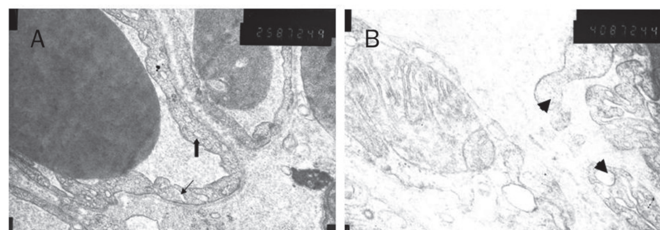

Figure 6. Ultrastructural changes of coronary artery endothelium in the immunized rats were observed by electron microscopy. Coronary artery ultrastructure in rats of immunized group was determined by transmission electron microscopy. The magnification was 250 000. Thin arrow and thick arrow represented penetrating vesicle-channels and the pinocytotic vesicles, respectively (A). Arrowhead meant the effervesced vascular endothelium (B).

Immunization with synthetic AT1R-EC_{II} peptide contributed to increased ICAM-1 in endothelial cells

The expression of endothelial ICAM-1 was determined by laser scanning confocal microscopy. Compared with the vehicle group (Figure 5C, 5D), enhanced fluorescence intensity were observed in immunized rats at month 9 after initial immunization (Figure 5A, 5B), which indicated that expressions of ICAM-1 increased in rats that contained high levels of AT1-Abs for a long term.

Endothelial structural damage of coronary artery in rats from AT1-Ab-positive group

Coronary artery structure in rats of the immunized group was determined by transmission electron microscopy. It was clear that penetrating vesicle-channels were shaped between the double-membrane of the capillary endothelial cells (thin arrow), and the number of pinocytotic vesicles increased (thick arrow) (Figure 6A, original magnification×250 000). In addition, the cytoplasmic membranes of the vascular endothelium effervesced, then the small vesicles were formed and shed. The shedding vesicle membrane was a lipid bilayer and the intramembrane component was cytoplasm (arrowhead) (Figure 6B, original magnification×250 000), suggesting that the permeability of cardiac capillary endothelial cell membrane increased.

Discussion

There were several considerable findings by active immunization rat model in the current study: (1) AT1-Abs in the immunize rats could cause necrotic cell death of HUVECs *in vitro*; (2) in the AT1-Ab positive immunized rats, the content of serum ET-1 raised significantly; the endothelium-dependent relaxation of the thoracic aorta declined; the expression of ICAM-1 in endothelial cells was up-regulated and the permeability of coronary endothelial cells increased.

Vascular endothelium is not only largest endocrine/paracrine organ in the human body, but also the target organ of many active substances. It performs diverse important physiological functions in some aspects such as regulating the systolic and diastolic states of blood vessels, maintaining the balance of coagulation and fibrinolytic systems, inhibiting the adhesion of the inflammatory cells and regulating vascular smooth muscle cell growth. A large number of studies have shown that endothelial injury may play a key role in initiating hypertensive vascular lesions[20]. And recent studies suggest that endothelial injury may be caused by oxidative stress[21], malnutrition[22], and the hidden white blood cells or platelet membrane molecule[23], *etc*. However, there are aslo many unknown injury factors being explored now.

In 1999, Wallukat[4] *et al* first reported that AT1-AAs were present in preeclamptic patients. The antibody plays an agonist-like role by recognizing AT1R-EC_{II} specifically. It has been demonstrated that AT1-AAs increased the expression of tissue factor[24], induced Ca^{2+} release[25] and activated NADPH oxidase[10] in VSMCs[9]. Our research group also found[18] that AT1-AAs could contract capacity blood vessels (thoracic aorta) and resistance blood vessels (coronary, middle cerebral artery) of rats. These studies suggested that AT1-AAs in patients may be closely related to vascular activities. In view of the important role of endothelium in the vascular homeostasis, this paper focused on the relationship between AT1-Ab and endothelial activity using a traditional active immunization model, which may provide useful lab evidences for elucidating the potential significances and pathways of AT1-AAs that participated directly in vascular damage in complicated high blood pressure related diseases.

Active immunization is a classic method for studying a variety of antibodies. Fu *et al*[26] have reported that high level and specific AT1-Abs would be generated in rats actively immunized with synthesized human AT1R-EC_{II} peptides. This antibody showed an agonist-like effect, which was similar to

the effect of AT1-AA in patients. Our previous research also found that both AT1-Abs got from the actively immunized rats and AT1-AAs obtained from preeclamptic patients could increase the beating frequency of spontaneously contracting cardiomyocytes and induce thoracic aorta contraction *in vitro* via the AT1 receptor. Therefore, AT1-Ab in the current study could be considered as AT1-AA.

In this study, we firstly observed whether AT1-Abs played the direct role in the endothelium injury. LDH is one kind of cytoplasm containing enzymes of the living cell. In normal circumstances, it cannot permeate the cell membrane. When the target cells are attacked and damaged, cell membrane permeability changes, then LDH may be released to the medium. So the increase of LDH is often viewed as one of the hallmarks of cell disintegration and necrosis. In order to reduce the interference caused by serum, total IgGs from AT1-Ab positive anti-sera of the immunized rats were purified according to previous studies[18]. Then the immunoglubulins were added to isolated cultured HUVECs, and LDH activity significantly increased after a certain time. This phenomenon was similar with the role of angiotensin II and could be blocked apparently by losartan, while IgGs in the vehicle group had a smaller influence on the changes of LDH activity, which revealed that from the cellular level AT1-Abs may directly lead to the necrotic death of endothelium through activating AT1R. However, whether other cell death pathways were involved in the process had not been explored.

Nevertheless, autoantibodies in clinical patients will be produced slowly and maintained for a long time, so whether the injurious effect caused by AT1-Abs *in vitro* still works *in vivo* warrants further study. ET-1 is the strongest and most lasting effective vasoconstriction agent, and there are three forms: ET-1, ET-2, and ET-3. Among them, ET-1 is the only one generated by vascular endothelium, and it plays important roles in contracting vessels, positive inotropic action and promoting the proliferation of VSMCs[27]. The increase level of ET-1 is mostly due to vascular endothelial injury or accidental activation, and to a certain extent reflects the degree of endothelial integrity[28]. It has been reported in clinic-based studies that high levels of ET-1 could be accompanied with AT1-AAs in patients[29]. This study demonstrated that using EIA test kit, from the third month after immunization the content of ET-1 in the sera of immunized rats was higher than that of the vehicle group and reached two peaks on the third month and the seventh month, respectively, suggesting that the vascular endothelium may exhibit discontinuous injury because of the long-term effect of AT1-Abs, and the damage was particularly serious on the third month and the seventh month. It may be the result of dynamic equilibrium regulation between the possible injury factor, AT1-Abs, continuous stimulation and cell regeneration.

Besides barrier function, the endothelial cell is also largest endocrine organ in the body. Through autocrine, it releases vasoactive substances and NO, endothelin, prostacyclin and so on to regulate vasomotion. Mediated by the non-neuronal M receptor, acetylcholine induces endothelium-dependent vascular relaxation. So, endothelium-dependent vasodilatation reaction is regarded as a classic indicator which can reflect the damage of endothelium. The present study found that at the ninth months immunization, endothelium-dependent vasodilatation reaction of capacity vessels (thoracic aorta) in the antibody positive group showed an obvious decline, which was consistent with the phenomenon of attenuated endothelium-dependent vasodilatation observed in patients with preeclampsia[30], malignant hypertension[31] *etc*. Accordingly, from the functional point of view, it increased endothelial cell injury.

In addition to the changes of serum indicator and function, endothelial cell activation is exhibited on many other aspects, and among them the increase of adhesion molecules expression is important. ICAM-1 belongs to the immunoglobulins superfamily, widely distributed on the surface of endothelial cells, monocytes and neutrophils. Under normal circumstances, ICAM-1 in endothelial cells shows weak expression. But it will be up-regulated when the endothelium was stimulated by the tumor necrosis factor, interferon-γ, endotoxin or other injury factors, which will then plays a key role in accumulation and infiltration of circular leukocytes, thus causing inflammatory lesions. Therefore, people pay more attention to ICAM-1 as an important immunological index[32] of endothelial activation in many diseases. At the present, whether AT1-AA is influencing the expression of ICAM-1 in endothelial cells has not been reported. This study demonstrated that through confocal laser technology, the ICAM-1 expression in the endothelial cells of thoracic aorta was higher than in the vehicle group when the rats were immunized for nine month, suggesting that endothelial cells may have inflammatory lesions in long-term AT1-Ab positive rats.

Compared with the function of the aorta, the small arteries seem more important in regulating tissue perfusion and blood pressure. To further confirm communication between AT1-Abs and endothelium, we checked the ultrastructural changes of coronary endothelium using electron microscopy. The pictures in Figure 6 displayed the increased permeability and damaged organelles of cardiac capillary endothelium in the immunized group. Considering the pathological changes of small vessels caused the dysregulation of contraction/relaxation, increase in vascular tone, and decrease in tissue perfusion, we suppose that these results may partly interpret the close relationship between AT1-AA and heart diseases in clinic[33].

In addition, this article also detected blood pressure changes by tail-cuff plethysmography in the two groups. Unexpectedly, no significant difference was observed between the two groups during the entire immunization process (data not shown). And the reasons may be: (1) the formation and regulation of blood pressure was the result of multiple factors. AT1-Ab, which could damage blood vessels, was not enough to cause change in blood pressure by itself; (2) the formation of high blood pressure was a relatively long pathological process. We carried out a nine-month rat active immunization model that may be too short for observing pathological changes of

the blood pressure in rats.

Collectively, this study gave a comprehensive analysis for the first time of the function, structure and other aspects of endothelium in the immunized rats induced by AT1-Ab from serum, which would lay the foundation for clarifying the importance of autoimmune mechanisms in the related complex types of hypertensions. Nevertheless, there are several shortcomings in the article: (1) considering the fluctuation of ET-1, we supposed that the endothelial injury caused by AT1-Abs may be a discontinuous process, in which the injury and repair-phase alternate. However, the changes of other key indicators were only investigated at the end of the immunization. So we cannot analyze the time course of this antibody; (2) the method of active immunization we used in this study is the classical research tool for studying antibodies, and also the first step in testing autoimmune diseases in basic research[34]. It provides us with an important reference for defining the function of antibodies. However, it has some unavoidable disadvantages. For example, the titer of antibody cannot be controlled easily, and it is usually much higher than the actual amount in clinic. Therefore, further studies using other animal models (such as passive immunized rat models) are necessary to draw firm conclusions.

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Author contribution

Hui-rong LIU designed the research; Su-li ZHANG, Li-hong YANG, Yun-hui DU, Rong-hua ZHENG performed the research; Xiao-li YANG contributed new reagents; Ming-sheng ZHANG contributed experiment tools; Su-li ZHANG, Jin WANG, Ye WU analyzed data; Su-li ZHANG, Yun-hui DU wrote the paper; Ke WANG corrected this paper.

References

1 Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. Pharmacol Rev 2000; 52: 11–34.
2 Roberts JM. Angiotensin-1 receptor autoantibodies: a role in the pathogenesis of preeclampsia? Circulation 2000; 101: 2335–7.
3 Fleming S. Malignant hypertension - the role of the paracrine renin-angiotensin system. J Pathol 2000; 192: 135–9.
4 Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jupner A, *et al*. Patients with preeclampsia develop agonistic antibodies against the angiotensin AT1 receptor. J Clin Inves 1999; 103: 945–52.
5 Nakashima H, Suzuki H, Ohtsu H, Chao JY, Utsunomiya H, Frank GD, *et al*. Angiotensin II regulates vascular and endothelial dysfunction: recent topics of angiotensin II type-1 receptor signaling in the vasculature. Curr Vasc Pharmacol 2006; 4: 67–78.
6 Liao YH, Wei YM, Wang M, Wang ZH, Yuan HT, Cheng LX. Autoantibodies against AT1-receptor and alpha1-adrenergic receptor in patients with hypertension. Hypertens Res 2002; 25: 641–6.
7 Dragun D, Müller DN, Bräsen JH, Fritsche L, Nieminen-Kelhä M, Kintscher DR, *et al*. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. N Engl J Med 2005; 352: 558–69.
8 Fu ML, Leung PS, Wallukat G, Bergström G, Fu H, Schulze W, *et al*. Agonist-like activity of antibodies to angiotensin II receptor subtype 1 (AT1) from rats immunized with AT1 receptor peptide. Blood Press 1999; 8: 317–24.
9 Sun YX, Zhang HY, Wei YM, Zhu F, Wang M, Liao YH. The mechanism of signal transduction during vascular smooth muscle cell proliferation induced by autoantibodies against angiotensin AT1 receptor from hypertension. Chin Med J (Engl) 2008; 121: 43–8.
10 Dechend R, Viedt C, Muller DN, Ugele B, Brandes RP, Wallukat G, *et al*. AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase. Circulation 2003; 107: 1632–9.
11 Irani RA, Zhang Y, Zhou CC, Blackwell SC, Hicks MJ, Ramin SM, *et al*. Autoantibody-mediated angiotensin receptor activation contributes to preeclampsia through tumor necrosis factor-alpha signaling. Hypertension 2010; 55: 1246–53.
12 Bobst SM, Day MC, Gilstrap LC 3rd, Xia Y, Kellems RE. Maternal autoantibodies from preeclamptic patients activate angiotensin receptors on human mesangial cells and induce interleukin-6 and plasminogen activator inhibitor-1 secretion. Am J Hypertens 2005; 18: 330–6.
13 Chen Q, Chen L, Liu B, Vialli C, Stone P, Ching LM, *et al*. The role of autocrine TGFbeta1 in endothelial cell activation induced by phagocytosis of necrotic trophoblasts: a possible role in the pathogenesis of pre-eclampsia. J Pathol 2010 Jan 21. doi: 10.1002/path.2690.
14 Versari D, Daghini E, Virdis A, Ghiadoni L, Taddei S. Endothelium-dependent contractions and endothelial dysfunction in human hypertension. Br J Pharmacol 2009; 157: 527–36.
15 Suzuki H, Eguchi K, Ohtsu H, Higuchi S, Dhobale S, Frank GD, *et al*. Activation of endothelial nitric oxide synthase by the angiotensin II type 1 receptor. Endocrinology 2006; 147: 5914–20.
16 Newton CR, Curran B, Victorino GP. Angiotensin II type 1 receptor activation increases microvascular permeability via a calcium dependent process. J Surg Res 2005; 123: 33–9.
17 LaMarca B, Parrish M, Ray LF, Murphy SR, Roberts L, Glover P, *et al*. Hypertension in response to autoantibodies to the angiotensin II type I receptor (AT1-AA) in pregnant rats: role of endothelin-1. Hypertension 2009; 54: 905–9.
18 Yang X, Wang F, Chang H, Zhang S, Yang L, Wang X, *et al*. Autoantibody against AT1 receptor from preeclamptic patients induces vasoconstriction through angiotensin receptor activation. J Hypertens 2008; 26: 1629–35.
19 Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983; 64: 313–20.
20 Vasudevan H, Nagareddy PR, John H. Gonadectomy prevents endothelial dysfunction in fructose-fedmale rats, a factor contributing to the development of hypertension. Am J Physiol Heart Circ Physiol 2006; 291: H3058–H3064.
21 Schulz E, Jansen T, Wenzel P, Daiber A, Münzel T. Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension. Antioxid Redox Signal 2008; 10: 1115–26.
22 Houston MC. Nutraceuticals, vitamins, antioxidants, and minerals in the prevention and treatment of hypertension. Prog Cardiovasc Dis 2005; 47: 396–49.
23 Walsh SW. What causes endothelial cell activation in preeclamptic women? Am J Pathol 2006; 169: 1104–6.
24 Dechend R, Homuth V, Wallukat G, Kreuzer J, Park JK, Theuer J, *et*

al. AT1 receptor agonistic antibodies from preeclamptic patients cause vascular cells to express tissue factor. Circulation 2000; 101: 2382–7.

25 Thway TM, Shlykov SG, Day MC, Sanborn BM, Gilstrap LC 3rd, Xia Y, *et al*. Antibodies from preeclamptic patients stimulate increased intracellular Ca^{2+} mobilization through angiotensin receptor activation. Circulation 2004; 110: 1612–9.

26 Fu ML, Leung PS, Wallukat G, Bergström G, Fu H, Schulze W, *et al*. Agonist-like activity of antibodies to angiotensin II receptor subtype 1 (AT1) from rats immunized with AT1 receptor peptide. Blood Press 1999; 8: 317–24.

27 Levin ER. Endothelins. N Engl J Med 1995; 333: 356–63.

28 Moldes O, Sobrino T, Millán M, Castellanos M, Pérez de la Ossa N, Leira R, *et al*. High serum levels of endothelin-1 predict severe cerebral edema in patients with acute ischemic stroke treated with t-PA. Stroke 2008; 39: 2006–10.

29 Ariza AC, Bobadilla NA, Halhali A. Endothelin 1 and angiotensin II in preeclampsia. Rev Invest Clin 2007; 59: 48–56.

30 Knock GA, Poston L. Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. Am J Obstet Gynecol 1996; 175: 1668–74.

31 Mizia-Steck K. Cytokines and adhesive molecules in detection of endothelial dysfunction. Pharmacol Rep 2006; 58 Suppl: 21–32.

32 Intengan HD, Schiffrin EL. Vasopeptidase inhibition has potent effects on blood pressure and resistance arteries in stroke-prone spontaneously hypertensive rats. Hypertension 2000; 35: 1221–5.

33 Qiu Y, Zhu F, Liao YH, Li ZZ, Wang M. Association between positive autoantibodies against AT1-receptor and cardiac remodeling in patients with hypertension. Zhonghua Xin Xue Guan Bing Za Zhi 2007; 35: 1141–4.

34 Jahns R, Boivin V, Hein L, Triebel S, Angermann CE, Ertl G, *et al*. Direct evidence for a β1-adrenergic receptor–directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. J Clin Invest 2004; 113: 1419–29.