Inhibition of experimental abdominal aortic aneurysm in a rat model by the angiotensin receptor blocker valsartan

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Abstract. Angiotensin (Ang) II exerts direct effects on the arterial wall to influence atherosclerosis and aneurysm development with the induction of vascular inflammation. Therefore, we examined the hypothesis that the inhibition of Ang II would decrease the expansion of abdominal aortic aneurysm (AAA) in a rat model. We used the Ang II receptor blocker (ARB) valsartan to inhibit the effect of Ang II. Additionally, we employed a dosage of valsartan (1 mg/kg/day) that does not affect blood pressure, to avoid the effect of blood pressure lowering. Notably, progression of elastase-induced AAA was inhibited in rats treated with valsartan ($P \leq 0.05$). To clarify the mechanism, we focused on matrix metalloproteases (MMPs) and inflammatory related factors. Western blot analysis demonstrated that the expression of MMPs was significantly decreased in an AAA model treated with continuous ARB infusion compared to an AAA model treated with vehicle ($P \leq 0.05$), through suppression of nuclear factor κB activation ($P \leq 0.05$). Consistently, valsartan significantly inhibited infiltration of macrophages into the aortic wall, accompanied by a reduction of protein expression of intercellular adhesion molecule-1. Importantly, the inhibitory effect of valsartan on MMP-2 and MMP-9 expression was also confirmed using isolated peritoneal macrophages from a rat AAA model. Moreover, treatment with valsartan protected against the destruction of elastic fibers. Overall, the present study demonstrated that treatment with valsartan, significantly prevented the progression of experimental AAA in a rat model. These data suggest that blockade of Ang II has an inhibitory effect on the development of AAA, independent of its antihypertensive effect.

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Introduction

Abdominal aortic aneurysm (AAA) is a common degenerative condition associated with aging and atherosclerosis (1). Basic phenomena in the pathogenesis of AAA are degradation of extracellular matrix components and loss of structural integrity of the aortic wall (2). AAA typically involves tissue inflammation as evidenced by the presence of inflammatory cells, which are considered to participate in the immunopathogenesis of AAA, leading to destruction of the aortic matrix (3,4). The mechanisms of AAA related to several inflammatory cytokines, including IL-ß, IL-6, and TNF-α (3-6). In addition, inflammatory cells are considered to be those predominant in secreting matrix metallopreteases (MMPs) including MMP-2 and MMP-9. MMPs are prominent proteinases of extracellular matrix protein and thought to contribute to aneurysm development (7). As these MMPs and pro-inflammatory genes are known to be regulated by the transcription factor, nuclear factor κB (NFκB), it would be reasonable to establish a new strategy for the treatment of AAA focusing on NFκB activation. We previously reported the activation of NFκB in human aneurysm wall, and demonstrated that the inhibition of NFκB activation by a decoy strategy resulted in the inhibition of the progression of aneurysms in a rat model (8).

In contrast, angiotensin (Ang) II is well known to promote arterial inflammation, which has emerged as central to the initiation and progression of atherosclerosis (9). Evidence from animal models as well as patients suggests that Ang II exerts pro-inflammatory actions through an increase in the expression of several mediators including leukocyte adhesion molecules, chemokines, specific growth factors, heat shock proteins and endothelin-1 (10,11). Since Ang II induces NFκB activation (12-14), we hypothesized that one of the mechanisms of vascular injury by Ang II would be mediated by the activation of NFκB. Moreover, since hypertension accelerated AAA progression via NFκB activation (15), it is be important for hypertensive patients

with AAA to receive anti-hypertensive drugs that suppress not only blood pressure, but also NFκB activation followed by the induction of Ang II. Thus, in the present study, we employed a rat normotensive AAA model to test our hypothesis that the Ang II receptor blocker (ARB) valsartan has an inhibitory effect on aneurysm progression, independent of its antihypertensive effect.

Materials and methods

Procedure of AAA model. Male Wistar rats (350-400g, Charles River Breeding Laboratories) were anesthetized and underwent laparotomy, as previously reported, with modification (16,17). Briefly, the abdominal aorta was isolated from the level of the left renal vein to the bifurcation. The right femoral artery was exposed, and a PE-10 polyethylene tube (Baxter Healthcare Corp., IL) was introduced through the femoral artery into the distal aorta. The aorta was clamped above the level of the tip of the PE tube and ligated with a silk suture near the aortic bifurcation (~15 mm in length), followed by perfusion with 0.2 ml saline containing 50 units type I porcine pancreatic elastase (Sigma Chemicals, Australia). Aortic perfusion with 2 ml saline containing 25 units elastase was performed for 30 min at 100 mmHg. After perfusion, the clamp and ligatures were removed and the PE tube was withdrawn. Valsartan (Novartis Pharma) was given at doses of 1 mg/kg/day, via osmotic mini-pumps implanted, from operation date, to 4 weeks after operation. Systolic blood pressure was measured by tail cuff technique. Untreated rats (without elastase perfusion and administration of drug or vehicle) were analyzed as sham.

This study was performed under the supervision of the Animal Research Committee in accordance with The Guidelines on Animal Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law (No. 105).

Electrophoretic mobility shift assay (EMSA). Rats were sacrificed at 1 week after the operation, and nuclear extracts were prepared from the aneurysm walls. Oligonucleotides (ODNs) containing the NFκB binding site (5'-CCTTGA AGGGATTTCCCTCC-3'; only sense strands are shown) were labeled with [γ-^{32}P]-ATP at the 3' end using a 3' end-labeling kit as a primer. After end-labeling, ^{32}P-labeled ODN were purified by application to a Nick column (GE Healthcare). Binding mixtures (10 μl) including ^{32}P-labeled primers (0.5-1 ng, 10,000-15,000 cpm) and 1 μg polydeoxyinosinic-deoxycytidic acid (Sigma Chemicals) were incubated with 10 μg nuclear extract for 30 min at room temperature and then loaded onto 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and pre-incubated with parallel samples for 10 min before the addition of the labeled probe. As a control, samples were incubated with an excess (x100) of non-labeled ODN, which completely abolished binding. Gels were analyzed by autoradiography.

Measurement of an aortic diameter by ultrasonography. Ultrasonography was used to demonstrate dilatation of the abdominal aorta. A cardiovascular ultrasound system (Power Vision 6000, Toshiba, Japan) and a linear transducer (15 MHz) were used to image the abdominal aorta non-invasively in anesthetized rats. Rats were scanned transversely to obtain images for the measurement of the luminal diameter of the aneurysm at the segment with maximum diameter. The aortic size was measured before and after incubation with elastase once a week up to 4 weeks after the operation.

Histological studies. Rats were sacrificed 4 weeks after the operation. The excised aorta was fixed in 10% neutral buffered formalin, and processed for routine paraffin embedding. Aortic tissue cross-sections (5 μm) were stained with Miller's elastin and Van Gieson's (EVG) stain in a standard manner. The surface area occupied by elastic fibers stained with EVG was quantified using a computerized morphometry system, MacScope Ver. 2.2 (Mitani Corporation, Japan), and expressed as a percentage of the surface area occupied by elastic fibers.

Immunohistochemical studies. Mouse anti-rat CD68 (MCA-341R, Serotec Ltd., UK) was used for analysis of macrophage infiltration in the aneurysm wall at 1 week after the operation. Immunohistochemical staining was performed using an immunoperoxidase avidin-biotin complex system with nickel chloride color modification. After blocking endogenous peroxidase activity, diluted primary antibodies (1:100) were applied to the sections, and these sections were incubated overnight at 4˚C. Sections were serially incubated with biotinylated anti-mouse IgG (Vector Laboratories, CA) in PBS for 30 min and avidin-biotinylated horseradish peroxidase complex in PBS for 30 min, according to the manufacturer's specifications (Vectastain Elite ABC kit, Vector Laboratories). For negative control experiments, the primary antibody was omitted. Immune complexes were visualized using 0.05% 3,3'-diaminobenzidine (Vector Laboratories), and slides were counterstained with hematoxylin.

Western blotting. Rats were sacrificed 1 week after the operation. Aortic tissues were homogenized and total proteins were extracted. Samples (20 μg) were electrophoresed in SDS-PAGE acrylamide gels, transferred onto nitrocellulose membranes, and incubated for 24 h in PBS, 5% non-fat milk, and 0.2% Tween-20 at 4˚C. Membranes were then incubated for 24 h at 4˚C with rabbit anti-rat MMP-9 (Torrey Pines Biolabs Inc., 1:2000 dilution), anti-rat intercellular adhesion molecule-1 (ICAM-1, Seikagaku, Japan), anti-MMP-2, anti-MMP-3, or anti-MMP-12 goat polyclonal antibody (Santa Cruz Biotechnology, 1:200 dilution), washed in PBS and 0.1% Tween-20, incubated for 2 h at room temperature with donkey anti-rabbit IgG secondary antibody for MMP-9 (Amersham Biosciences; 1:20,000), sheep anti-mouse IgG for ICAM-1 (Amersham Biosciences; 1:10,000), or donkey anti-goat IgG for MMP-2, MMP-3, and MMP-12 (Promega, 1:10,000), then visualized using an ECLplus chemiluminescent kit (Amersham Biosciences) following the manufacturer's instructions and exposed to XAR-5 X-ray film (Eastman Kodak Co.) To quantify and compare the levels of proteins, the density of each band was measured by densitometry (Shimazu, Japan).

RT-PCR analysis in peritoneal macrophages. Cells in rat peritoneal exudates were harvested at 3 days after elastase infusion by peritoneal lavage using cold Hanks' solution

Table I. Effect of valsartan on systolic blood pressure (mmHg).

	Pre-administration	4 weeks after	P-value
Vehicle	116±6	118±7	N.S.
Valsartan (1 mg/kg/day)	117±11	123±10	N.S.
P-value	N.S.	N.S.	

N, 15 per group; N.S., not significant.

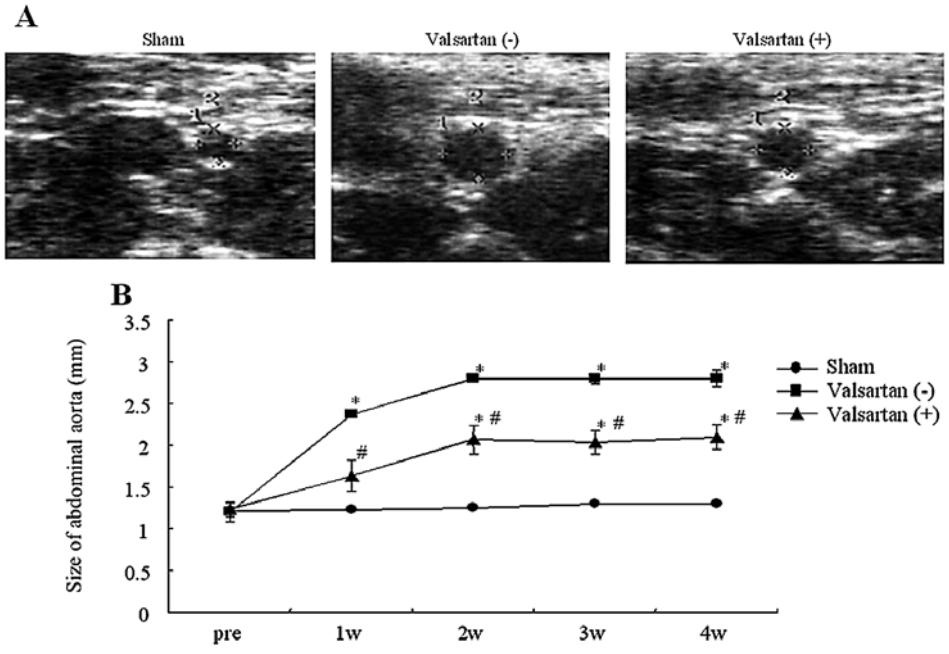

Figure 1. Prevention of AAA development by valsartan. (A) Representative ultrasound of aortic dilatation. (B) Time course of aortic size after elastase perfusion as assessed by ultrasound. Sham, untreated aorta without perfusion of elastase, valsaltan(-), treated with vehicle, valsartan(+), treated with valsartan at 1 mg/kg/day, pre, before elastase infusion, n=15 per group. *P≤0.05 vs. sham, #P≤0.05 vs. valsaltan(-).

containing 5% fetal calf serum, as previously reported (18). Cells were washed twice and re-suspended in RPMI-1640 medium containing 10% fetal calf serum. Monolayers of peritoneal macrophages were prepared by plating cell suspensions ($2x10^9$/l) in plates and incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 3 h to allow macrophage adherence. Non-adherent cells were removed by washing with warm RPMI-1640 medium three times. mRNA was purified from cultured cells using an RNeasy Mini Kit (Qiagen). Equal amounts (0.2 μg) of mRNA from each sample were reverse transcribed into cDNA for 30 min at 55˚C. Polymerase chain reaction amplification used the following conditions: denaturation for 18 sec at 94˚C, annealing for 45 sec at 68˚C, elongation for 1 min at 68˚C for 50 cycles. PCR products were run on 1.5% agarose gels and stained with ethidium bromide. The expression of MMP-2 and -9 was determined relative to the expression of tubulin. Primer sequences for rat MMP-2 (F-5' CTGATAACCTGGAT GCAGTCGT 3', R-5' CCAGCCAGTCCGATTTGA 3') and MMP-9 (F-5' CAGACCAAGGGTACAGCCTGTT 3', R-5 'AGCGCATGGCCGAACTC 3') were used.

Statistical analysis. Samples were coded, allowing analysis to be performed without knowledge of which treatment each group had received. The observer was blinded to other data concerning the samples. All values are expressed as mean ±SEM. Analysis of variance with ANOVA was used to determine the significance of differences in multiple comparisons. P≤0.05 was considered significant.

Results

To avoid the effect of blood pressure lowering, we employed a dosage of valsartan that does not affect blood pressure. Blood pressure was not markedly changed after 4 weeks of administration in the group treated with 1 mg/kg/day valsartan (Table I).

Ultrasound analysis demonstrated that AAA was already established at 1 week after incubation with elastase. No significant regression was observed in the natural course. Notably, the progression of the size of AAA was significantly decreased from 1 week to 4 weeks after elastase incubation in rats treated with valsartan compared to vehicle treatment (Fig. 1, P≤0.05).

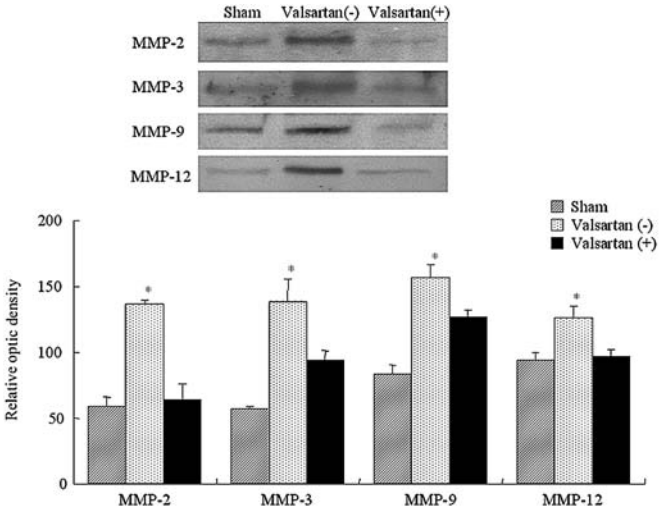

Figure 2. Effects of valsartan on MMP expression. Typical examples of Western blots of MMPs and quantitative analysis by densitometry at 1 week after perfusion. N=3 per group, *P≤0.05 vs. sham & valsartan(+).

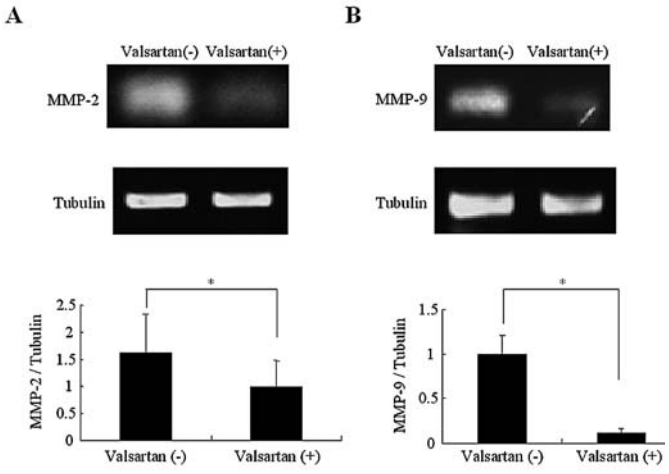

Figure 3. Suppression of MMP-2 and MMP-9 gene expression in peritoneal macrophages by valsartan. (A) Semiquantitative RT-PCR analysis of MMP-2. (B) Semiquantitative RT-PCR analysis of MMP-9. N=3 per group, *P≤0.05.

Therefore, we further studied how an ARB, valsartan, inhibited the progression of AAA. We measured MMPs, which destroyed elastic fibers and are considered as key molecules in the pathogenesis of AAA. Western blot analysis demonstrated that expression of MMP-2, -3, -9 and -12 within the aneurysm wall was up-regulated in a rat AAA model treated with vehicle, while the expression of these MMPs was significantly decreased in valsartan-treated rats compared to control rats (Fig. 2). Furthermore, we focused on MMP expression in peritoneal macrophages, since peritoneal macrophages may play a pivotal role in the expansion of AAA. Notably, the inhibitory effect of valsartan on MMP-2 and -9 gene expression was also confirmed using isolated peritoneal macrophages from a rat AAA model (Fig. 3).

Infiltration of monocytic leukocytes into the aortic wall is also considered to be a key mechanism in the progression of AAA in animal models as well as in humans. Western blot analysis demonstrated that the expression of ICAM-1, a major adhesion molecule, was significantly decreased in valsartan-treated rats compared to control rats (Fig. 4A). In addition, the immunohistochemical study revealed that macrophage infiltration was significantly inhibited in rats treated with valsartan, while many macrophages migrated into the aorta of rats treated with vehicle (Fig. 4B). Importantly, the binding activity of NFκB, as assessed by EMSA, was significantly decreased in the aorta of valsartan-treated rats as compared to vehicle treatment (Fig. 5, P≤0.05).

Finally, we focused on the vascular structure, as marked destruction was observed in the human aneurysm wall. Destruction of the matrix is dependent on the expression of MMPs, and elastic fibers maintain the structure of the vascular wall against hemodynamic stress. EVG staining demonstrated that treatment with valsartan significantly inhibited proteolysis of elastic fibers, while vehicle treated rats demonstrated marked loss of elastin in the aortic wall (Fig. 6).

Discussion

In vascular cells, NFκB has been shown to regulate the expression of adhesion molecules as a part of the inflammatory response (19). The contribution of the inflammatory process is important in the pathogenesis of AAA, since macrophages from the intravascular or retroperitoneal space, induced by inflammation, are the predominant MMP-9 secreting cells (20,21). Besides inflammation, as NFκB directly regulates transcription of MMPs, its up-regulation is considered to promote aortic dilatation in AAA. Our previous study demonstrated that inhibition of NFκB by a decoy strategy resulted in the prevention and regression of experimental AAA (8,22), suggesting that NFκB must be a key transcription factor in aneurysm formation. Activation of NFκB is observed by a wide range of extracellular stimuli and Ang II is well known to up-regulate NFκB (10-12). The renin-angiotensin system (RAS) plays important roles, not only in the regulation of blood pressure, but also in several cardiovascular pathological conditions, including cardiac and vascular hypertrophy and remodeling (23,24). These findings strongly suggested that RAS involved the mechanisms of AAA development based on atherosclerosis. Sustained infusion of Ang II leads to aneurysmal lesions in the atherosclerosis-prone apoE-/- mouse without systemic hypertension (25). This report demonstrated that Ang II is one of the important factors which accelerate aortic dilation, independent of its raising effect on blood pressure. Moreover, increasing evidence suggests the importance of tissue RAS in local peripheral tissues, including the kidney, adrenal, brain, vasculature, and cardiac tissues (26,27). ACE activity was increased in human AAA tissues (28). These data support the report showing that an ACE inhibitor suppressed aortic dilation in an experimental AAA model (29). From these viewpoints, suppression of RAS would be an attractive strategy as medical treatment for AAA.

Therefore, we hypothesized that an ARB would decrease the expansion of AAA via not only the reduction of blood pressure, but also inhibition of NFκB followed by inhibition

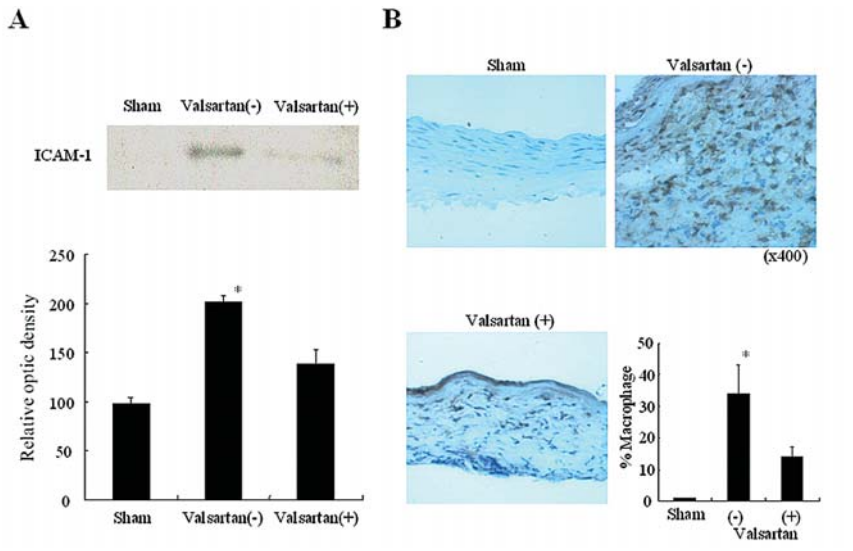

Figure 4. Anti-inflammatory effects of valsartan at 1 week after operation. (A) Typical examples of Western blots of ICAM-1 and quantitative analysis by densitometry. (B) Representative pictures of immunohistochemical staining for CD68 and quantitative analysis of the number of the recruitment macrophages. N=3 per group, *P≤0.05 vs. sham and valsartan(+).

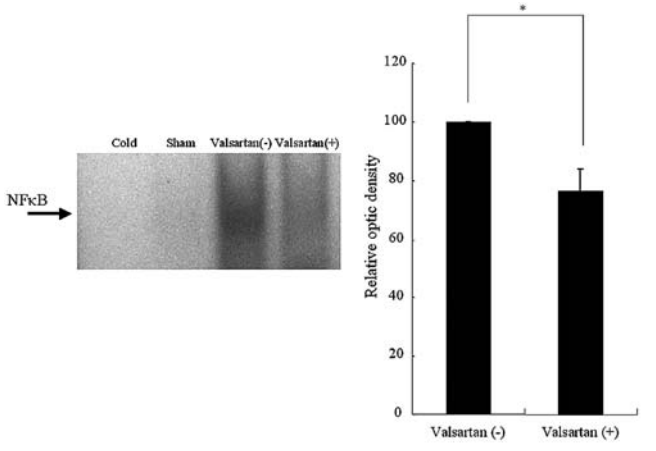

Figure 5. Effects of valsartan on NFκB activity. Gel-mobility shift assay for NFκB and quantitative analysis by densitometry at 1 week after operation. N=3 per group, Cold, non-labeled NFκB (x100 excess), *P≤0.05.

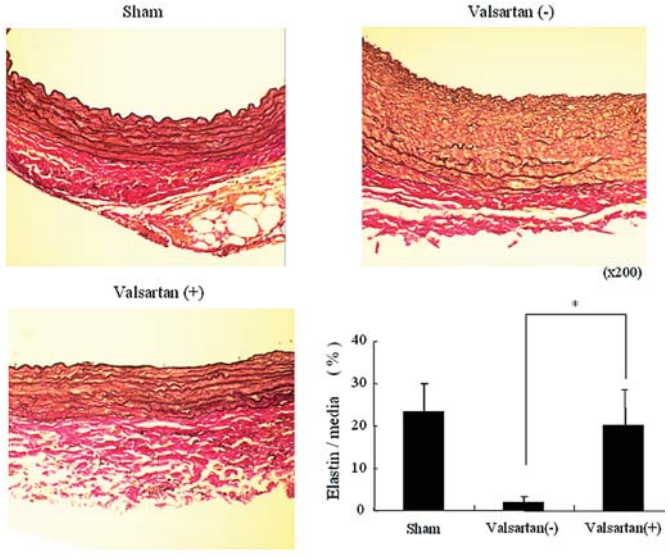

Figure 6. Preservation of elastic fiber by valsartan. Histological sections of rat aorta stained with EVG (x200) and percentage of area positive for elastic fibers at 4 weeks after operation. Elastin is stained dark brown. N=3 per group, *P≤0.05.

of the Ang II action. To test our hypothesis, in this study, we employed an elastase-infused rat AAA model. Since the phenomena such as inflammation, macrophage recruitment, induction of MMPs expression and degradation of elastic fiber occurred in this model, the model is considered to reflect the pathogenesis of human AAA. Many previous reports demonstrated that elastase activity plays an important role in progression of human AAA. There was significantly greater activity of elastase in the aneurysm tissue obtained from patients with AAA, and furthermore, elastase activity was significantly increased in tissue from ruptured AAA as compared to that of AAA in patients who underwent an elective procedure for AAA (30,31). These data suggest that elastase activity is a key mechanism of human AAA development.

Notably, the present study demonstrated that valsartan significantly decreased the expansion of experimental AAA at a low dose that did not lower blood pressure. Furthermore, administration of valsartan inhibited the expression of MMPs, accompanied by the inhibition of NFκB activation, and protected against the degeneration of elastic fibers. Valsartan also inhibited the recruitment of peritoneal and circulating macrophages, which are considered to be the major cell secreting MMPs, accompanied by a reduction of ICAM-1 expression. ICAM-1, which plays an important role in mediating the localization of monocytes at the intimal surface, is also well known to be induced by Ang II (32). The effect of valsartan on macrophages was also confirmed in isolated macrophages from a rat AAA model. Expression of mRNA, MMP-2 and MMP-9 in peritoneal macrophages was inhibited by valsartan treatment.

Thus, the present study suggests that the decrease in the expansion of AAA by valsartan is mediated by two pathways;

1) direct inhibition of MMPs gene expression driven by NFκB, 2) indirect inhibition of MMPs secretion, accompanied by the inhibition of the migration of macrophages. It is noteworthy that these effects were independent of its anti-hypertensive effect. In contrast, a previous study demonstrated that another ARB, losartan, did not suppress aortic dilation in a rat experimental AAA model (29). These findings and our provided data suggest that different ARBs may have different actions in the mechanisms of AAA progression.

Overall, the present study demonstrated that valsartan significantly decreases the expansion of experimental AAA independent of its blood pressure-lowering effect. The beneficial effect of valsartan on the progression of AAA is suggested to be mediated by a decrease in MMPs and ICAM expression and infiltrating macrophages through the inhibition of NFκB activation. ARBs are potentially useful drugs for hypertensive patients with aortic aneurysms.

Acknowlegements

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