Angiotensin II Type 1 Receptor Blocker Reduces Monocyte Adhesion to Endothelial Cells in Spontaneously Hypertensive Rats

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Abstract. Monocyte adhesion to arterial endothelial cells is the initial step in atherosclerosis. Whereas angiotensin II is known to elicit leukocyte adhesion, it is not clear whether blockade of the angiotensin II receptor signaling reduces monocyte adhesion to endothelial cells beyond its antihypertensive action. This study compared the effect of two different antihypertensive drugs on monocyte adhesion to thoracic aorta endothelium in spontaneously hypertensive rats (SHR): the angiotensin II receptor blocker, valsartan (20 mg · kg⁻¹ · day⁻¹) and the vasodilator, hydralazine (0.75 mg · kg⁻¹ · day⁻¹). The effects were quantitated in vivo using an enface method that optimizes the observation of endothelial surfaces after immunohistochemical staining for CD68. Both agents significantly and comparably reduced blood pressure over 4-week treatment course. Both valsartan and hydralazine profoundly reduced monocyte adhesion compared with nontreated controls, with valsartan having a modestly more reductive effect. Both agents also reduced the intima and medial thickening with valsartan reducing the mean thickness modestly more than hydralazine. Our data confirms that the reduction of blood pressure is effective method to reduce monocyte adhesion. Also, our date demonstrates that valsartan has a modest beneficial effect on monocyte adhesion to endothelial cells and arterial intima-medial vessel thickening beyond its action as an antihypertensive agent.

Key words: Monocyte-endothelial interaction, Hypertension, Cardiovascular disease, NEMOes

(Hypertension is an established risk factor for the progression of atherosclerosis. An enhanced renin-angiotensin system in the arterial wall plays at least partly an role in the mechanism underlying this link [1–4]. Epidemiological data suggest that hypertensive patients with enhanced renin-angiotensin systems are at higher risk of myocardial infarction than patients with other types of hypertension [5]. Furthermore, treatment of patients with angiotensin II receptor blocker (ARB) or angiotensin-converting enzyme inhibitors reduces the onset of cardiovascular disease [6].

Thus, angiotensin II inhibition is one of the preferred therapeutic strategies for preventing cardiovascular diseases in hypertensive patients. However, according to a recent data, an ARB does not have more beneficial effects on preventing cardiovascular diseases than a Ca channel blocker [7]. The importance of the effect of ARB beyond its action as an antihypertensive agent has not been fully elucidated [8, 9].

The adherence of circulating monocytes to the endothelial lining of large arteries is one of the earliest detectable events in human and experimental atherosclerosis. It is proposed that the subsequent transendothelial migration of these adherent monocytes, as well as their accumulation in the intima and transformation into lipid-engorged “foam cells” leads to the formation of atherosclerotic plaques [10]. Although monocyte-endothelial cell interactions in postcapillary venules...
are induced by a wide range of stimuli, monocyte adhesion in arteries is only induced by certain risk factors for atherosclerosis including angiotensin II [11]. Angiotensin II induces endothelial dysfunction and vascular remodeling in several experimental models [12], and is known to promote both mitogenic signals and oxidative stress in the vasculature [13]. These signals result in arterial inflammation, which in turn upregulates monocyte recruitment [11, 14–16]. However, it remains unclear, whether inhibition of angiotensin II reduces monocyte adhesion to endothelial cells beyond the effect of simply reducing blood pressure.

To quantitate particularly monocyte adhesion to arterial endothelial cells, we recently established a new *enface* method for optimal observation of endothelial surface (NEMOes). This method provides a clear focused image of the entire endothelial surface, enabling quantitation of monocytes that adhere to a specific region of rat thoracic aorta after immunostaining for the monocyte/macrophage-specific protein, CD68 [17–21]. Here, we used this method to investigate the effect of ARB on monocyte adhesion to endothelial cells in spontaneously hypertensive rats (SHR). To investigate its effect under the clinical relevant condition, we chose to use enough dose of ARB to reduce blood pressure, rather than the dose that does not affect the level of blood pressure. Our results confirmed the importance of reducing blood pressure levels on the reduction of monocyte adhesion. Also, our data suggests the presence of the beneficial effect of ARB on monocyte adhesion to endothelial cells beyond its antihypertensive action.

**Methods**

**Experimental Protocol**

All procedures were conducted in accordance with institutional guidelines for animal research. Specific-pathogen-free 10-week-old male SHR/Izm rats (n = 27) were purchased from Japan SLC (Hamamatsu, Japan). All rats were housed in stainless steel wire cages (two to three animals per cage) in a temperature-controlled clean room with a 12-hour light-dark cycle. The animals were provided with standard rat chow (CRF-1; Oriental Yeast Co, Tokyo, Japan) and autoclaved tap water *ad libitum*. Blood pressure and heart rate were measured once a week by the tail-cuff method (BP-98A; Softron, Tokyo, Japan). The ARB, valsartan or the vasodilator, hydralazine dissolved in polyethylene glycol (PEG) 400 was administered through an implanted osmotic pump (Alzet; Muromachi kikai Co., Ohmuta, Japan), at the following dosages: valsartan, 20 mg/kg/day (n = 9), and hydralazine, 0.75 mg/kg/day (n = 9). Valsartan and hydralazine were kindly provided by Novartis Pharm AG (Basel, Switzerland) PEG 400 alone was administered to some SHR rats through the implanted osmotic pump as a control (n = 9). All treatments were continued for 4 weeks.

**Laboratory data**

Urine 8-hydroxydeoxyguanosine (8-OHdG) was measured by enzyme-linked immunosorbent assay (ELISA) using an anti-8-OHdG monoclonal antibody (8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan). The value of urine 8-OHdG divided by urine creatinine was used as a marker of oxidative stress.

**NEMOes**

At the end of the treatment, monocyte adhesion to the thoracic aorta was quantified by NEMOes, as described previously [17–21]. Briefly, rats were sacrificed under anesthesia induced by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Nembutal, Abbott Laboratories, Abbott Park, IL). Then, rats were perfused with normal saline followed by 10% buffered formalin. After fixation, the aortic arch to the lower thoracic region was dissected and immersed in 10% buffered formalin. Next, the aorta was divided into segments 8–12-mm long and the proximal end of each segment was marked to confirm flow direction. Each segment was then placed in 0.05% hydrogen peroxidase in methanol for 20 min at room temperature. These specimens were placed into 0.2-ml microcentrifuge tubes (Treff AG, Degersheim, Switzerland) for incubation with the mouse anti-rat CD68 antibody (1 : 100 dilution: Serotec, Raleigh, NC) in phosphate-buffered saline (PBS) for 60 min at 37°C. The specimens were incubated with biotinylated anti-mouse IgG for 30 min at room temperature in a new tube, followed by reaction with horseradish peroxidase-conjugated streptavidin with the aid of LSAB2 kit (Dako Corporation, Carpinteria, CA). Staining was completed after incubation with a substrate-chromogen.
solution and counterstaining with hematoxylin. The segments were then cut open longitudinally along the ventral side with scissors. Each specimen was simply placed on a slide glass with the intimal side up, and covered with a coverslip by surface tension. Specimens were viewed under a microscope (E800; Nikon, Tokyo) connected to an XYZ controller and a digital camera (Media Cybernetics Inc., Silver Spring, MD). Pictures were captured at various focal lengths with an automatically regulated Z-stepper and the clearest images were selected automatically to produce a composite image of the whole thoracic aorta by Image-Pro4.5J (Planetron Co., Tokyo).

Cell counting

To quantitate the number of monocytes adhering to the endothelium, we set the area as a rectangle with sides that were twice the length of the long and short diameters of the vessel opening, respectively, and which was centered on the opening. We defined the area bounded by this rectangle as the area around the branching site (15). The total number of CD68-immunopositive cells around the branching site was counted in each aorta. The cell density in each area was then calculated as the cell count divided by the total area by examiners blinded to the treatment regimen.

Morphometry

After immunostaining and recording the en face view, the specimens were paraffin-embedded. Four cross-sections of each aorta spaced at approximately 4-mm intervals were stained with penta-chrome. The cross sectional intima-medial areas of a lesion in a given photomicrograph were measured using image-analysis software (Image-Pro4.5J, Planetron Co., Tokyo) by examiners blinded to the treatment regimen. The average intima-medial area was then calculated for each artery.

Real-time quantitative RT-PCR

Total RNA was extracted from the abdominal aorta of each rat with an ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan), according to the instructions provided by the manufacturer. Then, cDNAs were synthesized with Superscript II RNase H reverse transcriptase and oligo-dT primers, and amplified using a SYBER Green PCR kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed on an ABI PRISM 7700 sequence detection system (Perkin Elmer Life Sciences Inc.). The relative abundance of mRNAs was calculated by the comparative cycle of threshold ($C_T$) method using β-actin mRNA as the invariant control. The primer sequences used in this study were described previously [18].

Statistical analysis

All data are expressed as mean ± SEM (n = 9 per group). Statistical significance was determined with one-way analysis of variance. A $P$ value less than 0.05 denoted the presence of a statistically significant difference.

Results

Effects of hydralazine and valsartan on blood pressure and urine 8-OHdG level in SHR

As shown in Table 1, treatment with either hydralazine or valsartan markedly reduced blood pressure compared to control SHR. The blood pressure-lowering effect of hydralazine was stronger than valsartan one week after the treatment, although mean blood pressure over the entire treatment course was comparable for the two drugs (Table 1). We also measured urine 8-OHdG level 4 weeks after the treatment as an index of oxidative stress. Although mean 8-OHdG concentration was the lowest in the valsartan-treated group, the differences among groups were not significant (Fig. 1).

Effects of hydralazine and valsartan on monocyte adhesion to endothelium

Four weeks after each treatment, we quantitated monocyte adhesion to the aortic endothelium of each group by NEMOes. As shown in Fig. 2, the number of adhering monocytes was markedly suppressed by both hydralazine and valsartan treatment. Furthermore, valsartan had a modestly but significantly stronger effect on reducing monocyte adhesion than hydralazine.
Next, we quantitated the mean intima-medial thickness of each group at 4 weeks after each treatment. As observed with the monocytes adhering to aorta, intima-medial thickness was reduced by both hydralazine and valsartan treatment, with valsartan again inducing a significantly larger decrease than hydralazine.

Expression of adhesion molecules in aorta was suppressed by each treatment

To identify molecules involved in the drug-induced reduction of monocyte adhesion, we examined the gene expression levels of three major cell adhesion molecules and the macrophage chemoattractant protein-1 (MCP-1), a major chemokine involved in the formation of atherosclerotic lesions, using RNA isolated from the abdominal aorta of each rat at 4 weeks after each treatment. Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin all play major roles in monocyte-endothelial cell interactions. VCAM-1 and MCP-1 expressions were significantly and comparably decreased by both valsartan and hydralazine, while the expression of ICAM-1 was only significantly reduced by hydralazine, although both drugs had an effect, and the expression level of E-selectin was not affected by either drug treatment (Fig. 4). Thus, the expression levels

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Data are expressed as mean ± SEM.

*P<0.05 vs. Control groups.  ^P<0.05 vs. Hydralazine groups.

Urinary 8-OHdG concentrations in each group. Oxidative stress marker, urine-8OHdG was measured at the end of the treatments. Data are expressed as mean ± SEM.

Effect of hydralazine and valsartan on intima-medial thickness

Next, we quantitated the mean intima-medial thickness of each group at 4 weeks after each treatment. As
HYPERTENSION AND MONOCYTE ADHESION

of MCP-1 and VCAM-1 can be correlated with the reduced monocyte adhesion by the reduction of blood pressure.

Discussion

To the best of our knowledge, this is the first study to investigate how different antihypertensive agents affect monocyte adhesion to arterial endothelial cells in SHR. To elucidate the effect of ARB beyond its antihypertensive properties, we compared the ARB agent, valsartan, with a vasodilator, hydralazine, and used each drug at a dosage to comparably reduce blood pressure. Applying this method rather than using the dose that does not affect the blood pressure enabled us to compare the effect of reducing blood pressure with the effect beyond its blood pressure lowering effect. The present study demonstrated that reduction of blood pressure...
pressure by both treatments profoundly reduced monocyte adhesion to the endothelium with an associated decrease in intima-medial thickness. On the other hand, despite the comparable blood pressure reduction, valsartan affected monocyte adhesion and intima-medial thickness to a greater extent than hydralazine. Thus, our data indicates that ARB might have an additional protective effect acting via additional mechanisms, though the effect is modest compared to the effect of reducing blood pressure.

This is the first study to investigate the monocyte adhesion to endothelial cells by en face method in SHR, which is a well established model rat showing systematic hypertension with several arterial changes [22, 23]. The number of monocyte adhesion to endothelial cell by NEMOes in non treated SHR at the age of 14 weeks was 452 ± 7 cells/mm² (Fig. 2). On the other hand, that of Whistar rat at the similar age was 62 ± 3 cells/mm² and that of SD rat was 22 ± 3 cells/mm² (Azuma K. and Watada H. unpublished observation). The number of monocyte adhesion in SHR was remarkably higher than non-hypertensive rats. Thus, our data demonstrated that SHR has increased monocyte adhesion to endothelial cells in aorta.

Previous studies demonstrated an association between high arterial pressure and inflammation in the vascular wall [24, 25]. This is at least in part due to the enhanced renin-angiotensin system in arterial walls under hypertensive conditions. Angiotensin II stimulates the inflammatory process via the angiotensin II receptor type I, and monocyte recruitment requires the expression of cell adhesion molecules such as ICAM-1 and VCAM-1. The increased expression of these molecules is induced by the local inflammation in the arterial wall [12]. In addition to cell adhesion molecules, chemokines such as MCP-1 help to regulate leukocyte trafficking. Previous data demonstrated that angiotensin II induces the expression of ICAM-1, VCAM-1, and MCP1 [11, 16], although this does not necessarily mean that treatment of hypertension by ARB will decrease the expression levels of ICAM-1, VCAM-1, and MCP-1 more than other therapies. In fact, the reduction in blood pressure in this study by either valsartan or hydralazine induced a similar decrease in the expression of VCAM-1 and MCP-1. With regard to ICAM-1, hydralazine, not valsartan significantly reduced its expression. Our results demonstrated that reduction of blood pressure per se results in reduced expression of these genes, clearly suggesting that the most efficient prevention of monocyte adhesion was derived from the antihypertensive action and not indirectly via another mechanism.

One week after the treatment, we found the significant decrease of blood pressure in hydralazine group compared with valsartan group, although the mean blood pressure during whole study period in each group was comparable. This difference might be preferable for the prevention of arterial changes in hydralazine group, and might be related to the reduction of ICAM-1 expression in artery. Notwithstanding, valsartan group showed significantly more reduction of monocyte adhesion and intimal thickening than hydralazine group, suggesting the presence of its beneficial effect beyond blood pressure lowering.

We also found that valsartan reduced monocyte adhesion modestly but significantly more than hydralazine, and that this difference was not associated with the expression levels of ICAM-1, VCAM-1, E-selectin, or MCP-1. In support of this finding, Alvarez et al. [11] demonstrated that monocyte adhesion to arterial endothelial cells in vivo induced by the infusion of angiotensin II was not affected by functional blockade of these same molecules. It remains likely therefore that other players might be involved in the reduced monocyte adhesion following ARB treatment.

In the present study, we also showed that valsartan and hydralazine similarly reduced intima-medial thickness, although again, valsartan reduced the mean thickness more efficiently than hydralazine. Numerous studies have shown that angiotensin II mediates mitotic signaling pathways through activation of the angiotensin II receptor type I, and suggest that this mechanism may be involved in the vascular remodeling caused by hypertension [1]. The present findings confirmed that ARB has an additional and further effect on reducing intima-medial thickness beyond its effect on blood pressure.

In summary, our data clearly confirmed that reducing blood pressure efficiently reduced monocyte adhesion to endothelium and intima-medial thickness in vivo. In addition, our data showed the presence of the beneficial effect of ARB beyond its effect as an antihypertensive drug. Extrapolation of the results of these animal studies to hypertensive patients suggests that the use of ARB is a potentially effective approach to preventing cardiovascular disease, if blood pressure is to be well controlled.
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References


