Plaque-Stabilizing Effect of Angiotensin-Converting Enzyme Inhibitor and/or Angiotensin Receptor Blocker in a Rabbit Plaque Model

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Aim: Previous studies have revealed that blockade of the renin angiotensin system attenuates plaque vulnerability and reduces cardiovascular events; however, few studies have compared the effects of an angiotensin-converting enzyme inhibitor (ACEI) with an angiotensin receptor blocker (ARB) and evaluated combination therapy. The objective of this study was to compare the efficacy and mechanisms of plaque stabilization by ACEI or ARB and to determine the effects of combination therapy.

Methods: Twenty-eight male Japanese white rabbits were fed a high-cholesterol diet after balloon injury of the carotid arteries, then separated into ACEI (n = 7; imidapril 0.5 mg/kg/day), ARB (n = 7; TA606 4.5 mg/kg/day), combination (n = 7; imidapril 0.5 mg/kg/day + TA606 4.5 mg/kg/day), and vehicle (n = 7) groups.

Results: No difference in plaque volume was identified among the 4 groups. ACEI or ARB increased the thickness of the fibrous cap, collagen content and the number of smooth muscle cells in the intima (% smooth muscle cell in intima: ACEI, 36.3%; ARB, 36.4%; vehicle, 14.9%), and reduced the accumulation of macrophages (% macrophages in intima: ACEI, 20.1%; ARB, 24.0%; vehicle, 37.9%), suggesting the plaque-stabilizing effects of each drug. ACEI reduced matrix metalloproteinase (MMP)-9 expression and gelatinolytic activity in the intima. While ARB did not change gelatinolytic activity, accumulation of T cell in the intima was suppressed. Combination therapy did not show additive effects.

Conclusion: These results suggest that ACEIs and ARBs have similar, but not additive, plaque-stabilizing effects. Each agent showed specific effects, with ACEIs decreasing gelatinolytic activity and ARBs suppressing T cell accumulation.


Key words: Plaque stabilization, Angiotensin-converting enzyme inhibitor, Angiotensin receptor blocker

Introduction

Involvement of the renin angiotensin system (RAS) has been identified in every stage of the development of cardiovascular disease1–2. Several clinical studies have demonstrated that RAS blockade, such as by an angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB), reduces acute coronary events3–5. Whether ACEIs and ARBs have similar anti-atherogenic effects remains controversial6,7. More recently, the Blood Pressure Lowering Treatment Trialists’ Collaboration (BPLTTC) meta-analysis revealed that ACEIs achieved a blood pressure (BP)-independent reduction in the relative risk of coronary heart disease (CHD), whereas ARBs did not8.

Previous studies have documented that ACE, angiotensin II (AngII) and AngII type 1 receptor (AT1R) are upregulated in atherosclerotic plaque in
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Patients with acute coronary syndrome\textsuperscript{a}, \textsuperscript{b}. These observations suggest that the activated RAS is profoundly involved in plaque rupture. Several animal studies have demonstrated that ACEIs or ARBs attenuate plaque vulnerability. The molecular mechanisms of such effects have been proposed to involve the suppression of inflammatory cytokines or reactive oxygen species, and upregulation of nitric oxide production\textsuperscript{11-14}; however, few studies have compared the plaque-stabilizing effects of ACEI to those of ARB\textsuperscript{15,18}.

We have recently developed a useful rabbit model showing the change from stable to unstable plaque\textsuperscript{19}. To address the above question, we examined the use of ACEI, ARB and combination therapy in this animal model.

Methods

Drugs

TA606\textsuperscript{20, 21}, a selective AT1R blocker, and imidapril, an ACE inhibitor, were kindly donated by Mitsubishi Tanabe Pharma, Osaka, Japan.

Animal Experimental Protocols

Twenty-eight male Japanese white rabbits (Kita-yama Laves, Nagoya, Japan) weighing 3.0 to 3.5 kg were housed at room temperature with a 12-h light-dark cycle and provided with ad libitum access to tap water. All animal studies were performed according to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The Osaka Medical College Animal Care and Use Committee approved all animal experiments.

Rabbits were anesthetized with pentobarbital (25 mg/kg), and the right carotid arteries were injured using a 2.75-mm percutaneous transluminal coronary angioplasty balloon-catheter (Boston Scientific, Natick, MA, USA), as described previously\textsuperscript{19}. Rabbits were fed normal standard diet for 4 weeks after injury and sacrifice, as previously reported\textsuperscript{22}. Blood samples were collected thereafter, and total plasma cholesterol and triglycerides were measured enzymatically (SRL, Tokyo, Japan). At the end of the protocol, all rabbits were sacrificed by venous injection with an overdose of sodium pentobarbital.

Tissue Preparation

After blood collection, heparinized saline was introduced at a standardized pressure (100 mmHg) into the left ventricle via an 18-gauge needle and allowed to flow out of a cut point at the right atrium. When the perfusate became clear, the right carotid artery was dissected and rinsed in phosphate-buffered saline. Thereafter, samples for histological and immunohistochemical analyses were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan), and snap-frozen in liquid nitrogen.

Histological and Immunohistochemical Staining

Frozen sections were cut into 3-μm slices and stained with hematoxylin and eosin (H&E). The interstitial collagen content of carotid arteries was evaluated by Sirius red staining with a polarized microscope. Briefly, frozen sections were incubated with 0.1% Sirius red in saturated picric acid for 90 minutes. After incubation in 1% acetic acid for 30 minutes and rinsing, slides were counterstained in hematoxylin. Slides were visualized under both a bright-field and polarized light microscope, and photographed with identical exposure settings for all sections.

Immunohistochemical studies were performed using the following primary antibodies: mouse monoclonal antibody against rabbit macrophages (RAM-11, 1:1,000 dilution; DAKO, Carpinteria, CA, USA); human α-smooth muscle actin (α-SMA) (1A4, 1:1,000 dilution; Sigma-Aldrich, St. Louis, MO, USA); human matrix metalloproteinase (MMP)-9 (56-2A4, 1:10 dilution; Daichi Fine Chemical, Takaoka, Japan); mouse monoclonal antibody against rabbit T cells (KEN-5, 1:2 dilution; AbD Serotec, Oxford, UK); and goat polyclonal antibody against human monocyte chemoattractant protein (MCP)-1 (sc-1304, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Frozen sections were air-dried, fixed in acetone, and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Tissue sections were incubated overnight with primary antibodies at 4°C, and then with biotinylated anti-mouse immunoglobulin (E0354, 1:500 dilution; DAKO) at
room temperature for 30 min. Sections were visualized using 3,3-diaminobenzidine (LY. 186; Dojin Chemical, Kumamoto, Japan) with hematoxylin or methyl green as the nuclear counterstain.

H&E staining, Sirius red staining and immunohistochemistry were quantified by computer-assisted image analysis using Image J version 1.4.3.67 software (National Institutes of Health). Positive areas are expressed as percentages (%) of the intimal area.

**Gelatin Zymography**

To detect gelatinolytic activity in the protein extract of the carotid artery, zymographic analysis with a 7.5% acrylamide gel containing 0.2% gelatin was performed according to the method of Aikawa et al.23). Briefly, after electrophoresis under non-reducing conditions, the substrate gels were soaked twice with Triton-X-100 solution (2.5%) to remove sodium dodecyl sulfate. The gels were then incubated in 50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% NaN₃, and 0.05% Brij 35 for 24 h at 37°C. Lysis of substrates in the gels was visualized by staining with 2.5% Coomassie brilliant blue (Sigma-Aldrich).

**Statistical Analysis**

Quantitative data are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed using the JMP7.0.1 software package (SAS Institute, Cary, NC, USA). Unpaired t-tests were used for comparisons between 2 groups. To compare more than two groups on a continuous variable, we used one-way ANOVA followed by Dunnett's post-hoc analysis. *P* < 0.05 was considered to indicate statistical significance.

**Results**

**Serum Lipid Levels and Blood Pressure**

After 8 weeks on the high-cholesterol diet, serum levels of total cholesterol and triglycerides were increased. No significant differences in cholesterol levels were seen among the 4 groups (vehicle, 2327 ± 177 mg/dL; ACEI, 2277 ± 248 mg/dL; ARB, 1992 ± 320 mg/dL; combination, 1930 ± 199 mg/dL).

Blood pressure was not significantly different among the 4 groups at baseline. At 12 weeks, systolic blood pressure (SBP) in the ACEI and ARB groups did not differ from that in the vehicle group (88.0 ± 4.0 mmHg in ACEI, 92.7 ± 3.6 mmHg in ARB, 96.7 ± 3.8 mmHg in vehicle). SBP was significantly lower in the combination group (84.3 ± 3.6 mmHg, *p* = 0.01) than in the vehicle group.

**Histological Evaluation**

**Fig. 1.** Plaque volume in each group. Intimal area (a) and intima/media ratio (b) in carotid artery sections from rabbits receiving ACEI (imidapril), ARB (TA606) or combination (ACEI + ARB). Bars represent SEM.

**Plaque Volume**

Intimal area was not found to differ among the 4 groups (vehicle, 2.0 ± 0.4 mm²; ACEI, 2.1 ± 0.4 mm²; ARB, 1.2 ± 0.3 mm²; combination, 1.5 ± 0.3 mm²). In addition, the intima/media ratio did not differ among the 4 groups (Fig. 1).

**Fig. 2** shows immunohistochemical analysis of plaque cell composition in rabbit carotid arteries from the 4 groups. In the vehicle group, most of the infiltrating cells in the intima were identified as macrophages, but a-SMA-positive cells were only occasionally observed (Fig. 2a, 2e). In the ACEI, ARB, and combination groups, the extent of macrophage accumulation in the intima seemed to be decreased, but a-SMA-positive cells were, in contrast, increased and located on the luminal side (Fig. 2b-d, 2f-h). Quantitatively, the macrophage-positive area in the intima was significantly decreased in the ACEI (20.1%, *p* = 0.013), ARB (24.0%, *p* = 0.047), and combination groups (23.7%, *p* = 0.049) as compared with the vehicle group (36.3%), as shown in Fig. 2i. In contrast,
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Red staining was increased, suggesting that interstitial collagen content increased in these groups. Quantitative analysis revealed that the Sirius red-positive area in the intima was significantly increased in the ACEI, ARB and combination groups (Fig. 3i). Compared with treatment, in the vehicle group, T cells were accumulated in the plaque (Fig. 4). The number of T cells in the plaque was similar in the ACEI group (Fig. 4b), but was reduced in the ARB and combination groups (Fig. 4c, d). Quantitative analysis revealed that the number of T cells in the plaque was significantly lower in the ARB group (p = 0.029) and tended to be lower in the combination group (p = 0.052) as compared with the vehicle group (number of T cells in plaque: 10 ± 2/ high power field in vehicle; 7 ± 3/ high power field in ACEI; 3 ± 2/ high power field in ARB; 4 ± 3/ high power field in combination).

The SMA-positive area in the intima was significantly increased in the ACEI (36.3%, p = 0.0049), ARB (36.4%, p = 0.0001) and combination groups (37.1%, p = 0.004) as compared with the vehicle group (14.9%) (Fig. 2j).

We defined a “fibrous cap” as a luminal α-SMA-positive cell layer, overlying a lipid- and macrophage-rich region in this model. Thickness of the fibrous cap was increased in ACEI (197 ± 13.5 μm, p = 0.009), ARB (206 ± 29.8 μm, p = 0.008) and combination groups (197 ± 17.2 μm, p = 0.009) as compared with the vehicle group (88.8 ± 22.5 μm).

Fig. 3 shows interstitial collagen content examined by Sirius red staining. Carotid artery in the vehicle group showed only sparse positive staining in the intima (Fig. 3a), demonstrating a low content of interstitial collagen in the intima. In the ACEI, ARB, and combination groups (Fig. 3b-d), the intensity of Sirius red staining was increased, suggesting that interstitial collagen content increased in these groups. Quantitative analysis revealed that the Sirius red-positive area in the intima was significantly increased in the ACEI, ARB and combination groups (Fig. 3i).
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groups than in the ACEI and combination groups (Fig. 5e).

MCP-1 Immunohistochemistry

MCP-1 protein was abundant in the intima of the vehicle group (Fig. 5a). Decreased MCP-1 expression was observed in the ACEI, ARB and combination groups (Fig. 5b-d). Quantitative analysis revealed that the MCP-1-positive area in the intima was decreased more markedly in the ACEI and combination groups (vehicle, 22.8%; ACEI, 12.4%, \( p = 0.004 \) vs. vehicle; ARB, 13.2%, \( p = 0.013 \), vs. vehicle; combination, 11.4%, \( p = 0.003 \) vs. vehicle).

MMP-9 Immunohistochemistry and Gelatin Zymography

MMP-9 protein was abundant in the intima of the vehicle group (Fig. 5a). Decreased MMP-9 expression was observed in the ACEI, ARB and combination groups (Fig. 5b-d). Quantitative analysis revealed that the MMP-9-positive area in the intima was decreased more markedly in the ACEI and combination groups (vehicle, 38.0%; ACEI, 21.1%, \( p = 0.0009 \) vs. vehicle; combination, 25.5%, \( p = 0.013 \), vs. vehicle) than in the ARB group (ARB, 31.5%, \( p = 0.045 \) vs. vehicle). Gelatin zymography revealed that gelatinolytic activities at 92 kDa (pro-MMP-9), 80 kDa (an activated form of MMP-9), 72 kDa (pro-MMP-2), and 68 kDa (an activated form of MMP-2) were higher in the carotid arteries in the vehicle and ARB groups than in the ACEI and combination groups (Fig. 5e).

MCP-1 Immunohistochemistry

MCP-1 was abundant in the intima of the vehicle group (Fig. 6a). MCP-1 expression was decreased in the ACEI, ARB and combination groups (Fig. 6b-d). Quantitative analysis revealed that the MCP-1-positive area in the intima was significantly decreased in the ACEI, ARB and combination groups (vehicle, 22.8%; ACEI, 12.4%, \( p = 0.004 \) vs. vehicle; ARB, 13.2%, \( p = 0.013 \), vs. vehicle; combination, 11.4%, \( p = 0.003 \) vs. vehicle).
Previous studies comparing the plaque-stabilizing effects of ACEIs and ARBs mostly used apolipoprotein E (apoE)-deficient mice, or high cholesterol rabbits such as Watanabe heritable hyperlipidemic (WHHL) rabbits, as animal models. There is a marked difference in plaque progression between these animal models and humans. In the human coronary artery, one of the most atherosclerosis-prone arteries, diffuse intimal thickening composed of SMCs develops.

**Discussion**

The present study showed that the potency of plaque-stabilizing effects, such as increasing the thickness of the fibrous cap and collagen content in the plaque, and decreasing the number of plaque macrophages, was equivalent for ACEI and ARB, but each drug showed specific effects. Whereas ACEI suppressed gelatinolytic activity more effectively than ARB, ARB, but not ACEI, reduced T cell accumulation in the plaque.

![Fig. 4](image1.png)

Fig. 4. T cell accumulation in plaque intima of each group.

a-d) Representative immunohistochemical staining with anti-T cell antibodies in carotid artery sections from each group. a, vehicle group; b, ACEI (imidapril) group; c, ARB (TA606) group; d, combination (ACEI + ARB) group. Closed arrowhead indicates positive staining of T cells in brown. Scale bar, 200 μm.

![Fig. 5](image2.png)

Fig. 5. Matrix metalloproteinase (MMP)-9 in plaque lesions of each group.

a-d) Representative immunohistochemical staining with MMP-9 antibody in carotid artery sections from each group. a, vehicle group; b, ACEI (imidapril) group; c, ARB (TA606) group; d, combination (ACEI + ARB) group. Positive cells are stained brown. Closed arrowhead indicates internal elastic lamina. Scale bar, 200 μm. e) Gelatin zymography of gelatinase activities in protein extracts of carotid arteries from each group. Gelatinolytic activity was detected at 92 kDa (pro-MMP-9), 80 kDa (an activated form of MMP-9), 72 kDa (pro-MMP-2), and 68 kDa (an activated form of MMP-2).
Plaque Stabilizing with RAS Blockade

Accumulating lines of evidence suggest that RAS blockade has a beneficial effect on plaque stability by suppressing inflammatory cytokines and reactive oxygen species through inhibition of the common Ang II-AT1R signaling pathway. Among these inflammatory cytokines, MCP-1 expression was markedly decreased by both ACEI and ARB to a similar extent in the present study. Because MCP-1 is a critical molecule of monocyte/macrophage accumulation in the vascular wall, we speculate that both ACEI and ARB decrease macrophage accumulation in the plaque through inhibition of MCP-1 expression.

Increased plaque vulnerability is histologically characterized by an enhanced content of plaque macrophages and reductions in fibrous cap thickness and intraplaque collagen content. We report here that both ACEI and ARB reduced the number of macrophages in the plaque and increased the thickness of the fibrous cap and collagen content in the plaque to a similar extent. Accumulating lines of evidence suggest that RAS blockade has a beneficial effect on plaque stability by suppressing inflammatory cytokines and reactive oxygen species through inhibition of the common Ang II-AT1R signaling pathway. Among these inflammatory cytokines, MCP-1 expression was markedly decreased by both ACEI and ARB to a similar extent in the present study. Because MCP-1 is a critical molecule of monocyte/macrophage accumulation in the vascular wall, we speculate that both ACEI and ARB decrease macrophage accumulation in the plaque through inhibition of MCP-1 expression. Moreover, it has been reported that AT1R expression is upregulated in the artery wall in hypercholesterolemic rabbits. We suggest that inhibition of the common Ang II-AT1R signal was one of the main mechanisms of plaque stabilization by both ACEI and ARB in the present study.

The present study demonstrated that ACEIs or ARBs could increase plaque stability without BP reduction. In the present study, BP was directly measured by catheter cannulation into the central artery of the ear, which has been reported to reflect the cen-
tral blood pressure closely in rabbits. Several animal studies have already reported that ACEIs or ARBs inhibit atherosclerosis progression independent of BP reduction. As molecular mechanisms of these BP-independent effects, the suppression of inflammatory cytokines and reactive oxygen species in the atherosclerotic vasculature through inhibition of local RAS has been proposed. From the clinical aspect, RAS blockade of BP-independent effects is controversial: ACEIs, but not ARBs, have shown evidence of BP-independent effects on the risk of major coronary disease events in BPLTTC meta analysis. Further elucidation of this point is required in the clinical setting.

Increased activity of MMPs, especially MMP-2 and -9, has been associated with enhanced plaque vulnerability. In addition to the fact that both MMPs and ACE belong to the same zinc-dependent endopeptidase, it has been reported that ACEIs, but not ARBs, are able to inhibit MMP-2 and -9 activity directly by stabilizing hydrophobic interactions in the active domain. More recently, imidapril has been reported to reduce oxidized LDL-induced MMP-9 activity in macrophages. Thus, we speculate direct inhibition of MMP-2 and -9 activities with imidapril as the main mechanism whereby ACEI, not ARB, suppressed gelationolytic activity in atheromatous plaque in the present study. This might explain, at least partly, why distinct clinical efficacy in ACS inhibition is supposed to be present between ACEIs and ARBs, which has been speculated in the BPLTTC.

T cells have been suggested to be involved in plaque vulnerability by activating macrophages or producing proinflammatory cytokines. Recently, T cells have been reported to possess functional RAS, such as ACE and AT1R. AngII increased T cell proliferation and ARB reduced its proliferation in cultured human blood cells. In addition, endogenous AngII is involved in T cell activation and AT1R expression is upregulated about 10 times in activated T cells. These results strongly suggest that AngII can influence the activation of T cells and its proliferation in a paracrine or autocrine manner. In the present study, accumulation of T cells in plaque was significantly reduced in the ARB group. Although we did not detect the T cell activation status in the present study, it has been reported that most T cells in atheromatous plaque are activated. Thus, we speculate that ARB affects AT1R of T cells directly and reduces both the number of T cells and lymphokines released by activated T cells in atherosclerotic plaque, leading to stabilization of plaque conformation; however, there is a possibility that some cytokines involved in T cell accumulation are reduced by ARB more greatly than by ACEI. As far as we know, this is the first report in which ARB inhibited T cell accumulation in atheromatous plaque.

Only combination therapy with ACEI and ARB brought a significant reduction in SBP as compared with monotherapy. Nevertheless, combination therapy does not provide an additive effect on plaque stability compared with monotherapy. There is a discrepancy between the present study and other reports in which combination therapy with ACEI and ARB has additive effects of plaque stability over each monotherapy. This may be explained by the difference in the duration or timing of drug administration or by differences in the specific RAS inhibitor beyond a class effect. In clinical study, the majority of reports, including the most recent and largest study, The Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ON-TARGET), have revealed that combination therapy with ACEI and ARB does not provide additive effects for the prevention of ACS compared with each monotherapy.

Some limitations must be considered in this study. First, plaque rupture did not develop in our rabbit models. Other factors seem to be required for plaque rupture, such as BP elevation. Second, we used TA606 as the ARB but it is commercially unavailable. The active metabolite of TA606, 606A, appears to interact with AT1R with high affinity (Ki = 13 nM), whereas binding affinity to AT2R was negligible (Ki > 100 μM), indicating that the selectivity for AT1R is similar to or higher than that of valsartan or telmisartan. We observed similar results such as plaque-stabilizing effects or inhibitory effects on T cell accumulation in the plaque by olmesartan, a commercially available ARB in the same rabbit model (unpublished result by Hoshiga).

In conclusion, the potency of the plaque-stabilizing effect of ACEI and ARB was similar, but not additive. Specific effects were found, such as decreasing gelatinolytic activity by ACEI or suppressing T cell accumulation by ARB.

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Conflicts of Interest

None.
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