1.3. Elderly patients are at high risk of receiving fragmented and redundant medical care from multiple care providers because of multimorbidity.[10,26–28] Conversely, elderly patients are also at high risk of undertreatment because care provision, including admission to facilities and surgical treatment, tends to be limited for reasons of age, and decreased physical, mental and social function.[29–33] Care providers should keep in mind that there exist medical treatments for which beneficial effects in the elderly have been established in clinical trials, and make efforts to provide medical care balancing the benefit against the risk.[34,35]

2. Care towards maintenance and improvement of quality of life

- Care providers should try to maintain and improve quality of life (QOL) by preserving the remaining daily living functions and alleviating symptoms.

2.1. The decline in physiological reserve with aging makes elderly patients vulnerable. Conditions that are usually temporary and do not result in any long-term sequelae in younger patients, such as back pain or pneumonia, can cause long-term adverse outcomes, such as a decline in activities of daily living (ADL) in elderly patients, leading to poor QOL.[36–38] Because complete recovery is hard to achieve once ADL decline,[39,40] it is imperative to prevent diseases that can trigger ADL decline by measures such as fall prevention intervention,[41–44] vaccination programs[45–48] and oral hygiene.[49–51] It is also important to preserve ADL by early mobilization and rehabilitation to restore physical function when the patient has an illness.[52,53]

2.2. Geriatric syndromes;[54] that is, common medical conditions in the elderly, such as dementia, delirium, depression, frailty, sarcopenia, malnutrition, dysphagia, falls, urinary incontinence, constipation, decubitus ulcer and dehydration, frequently cause decreased ADL and poor QOL.[55–57] Comprehensive screening and assessment are required for prevention of these geriatric syndromes, and early detection and treatment are required once they occur. Dementia requires special attention and broad screening for early detection, diagnostic workup at specialized facilities if indicated, and early intervention is crucial.

2.3. Many conditions commonly observed in the elderly are chronic and unlikely to be cured completely.[3] In managing such chronic conditions, it is vital to focus on the alleviation of symptoms rather than futile, intensive treatment aiming towards a complete cure. To preserve and improve QOL, through integrated medical, public health and social welfare services, care providers should provide healthcare including environmental modification, mental health services, nutrition management and oral care in addition to palliative care, in order to alleviate symptoms that could worsen QOL.

3. Healthcare provision in daily life setting

- Care providers should understand the importance of where elderly patients spend their daily life in maintaining their QOL, and should provide support to enable elderly patients and their families to choose an appropriate place to live and receive healthcare.
- Care providers should understand issues that may occur during the transition between different healthcare settings and take appropriate preventive measures.

3.1. Care providers should provide comprehensive care in cooperation with Community General Support (*chiiki hokatsu shien*), integrating medical, nursing, long-term care and welfare services, so that an elderly patient can live in a place where they feel comfortable and are able to maintain their QOL.[58] In cases where elderly patients require admission to acute care medical facilities, care providers should initiate discharge support early, to help facilitate their return to their place of residence. Care providers should closely communicate with patients and their family members, and provide support to enable them to choose an appropriate setting where they receive healthcare, when the current healthcare setting is no longer appropriate.[59,60]

3.2. The quality of care may be compromised during patients' transition between different healthcare settings as a result of poor communication between care providers.[61,62] In addition, transition across sites of care is associated with an increased risk of psychological symptoms, such as delirium[63] and disuse syndrome, and subsequent functional decline.[36–38] Care providers should understand these risks associated with healthcare transition, promote communication between healthcare settings and take appropriate preventive measures.[64]

3.3. Care providers should consider medical care provision at long-term care facilities or at home, utilizing medical resources in the community, such as Home-visit Nursing Services (*houmonkango*) and Dementia Support Doctors (*nintishosapo-to i*), as a valid alternative to inpatient or outpatient care.

4. Basic concepts of pharmacotherapy for elderly patients

- Care providers should understand the principles of pharmacotherapy, which require consideration of

risk of adverse drug reactions, medication adherence and patients' priorities of healthcare outcomes, and put the principles into practice.

4.1. Elderly patients are at increased risk of adverse drug reactions.[65,66] Care providers should understand age-related changes in pharmacokinetics and pharmacodynamics,[67,68] and, as a general rule, start medication at the lowest feasible dose and titrate the dose upward slowly and gradually, monitoring the treatment response and adverse reactions to medication.[69,70] Polypharmacy, or use of multiple medications, should be avoided as much as possible, because polypharmacy, particularly when the number of medications is six or more, is associated with an increased risk of unexpected drug–drug reactions and adverse drug reactions.[6,71–76] In addition, several medications are known for their tendency to cause adverse drug reactions in elderly patients,[77,78] and particular attention should be paid to the indication and management of these medications.[79]

4.2. Various factors contribute to poor medication adherence, including cognitive impairment, fine motor impairment, dysphagia, limited access to pharmacy services, financial problems and polypharmacy.[80] Care providers should collect detailed information on medication adherence from patients as well as their family members and caregivers on a regular basis, and screen for factors that could lead to poor adherence in order to intervene and modify such factors, and prevent poor adherence.[81,82] Care providers should simplify medication regimens by use of combination drugs, single-dose packaging or changes in dosage forms.[83]

4.3. Although elderly patients often have multiple chronic conditions and geriatric syndromes, clinical guidelines for such elderly patients are still scarce.[9] However, application of clinical guidelines intended for younger patients may not necessarily result in good outcomes in the elderly.[10–12] It may also be inappropriate to consider pharmacological treatment separately for each medical condition and symptom. Care providers should evaluate the indications for medications and decide the priority of each medication depending on the therapeutic goals for patients and their family, comprehensively taking into consideration individual patients' medical conditions, their severity, organ function, physical, cognitive and daily function, and the family situation. Care providers should choose high-priority medications,[84] and consider discontinuing medications with low priority.[66,85,86]

4.4. Care providers should try non-pharmacological treatment first and avoid pharmacological treatment as long as alternative measures are available.[69,70] Care providers should regularly review medication using patients' medication records to identify all the medications patients take including vitamins, Chinese herbal medicines and over-the-counter drugs.[87,88] Care providers should avoid prescribing new medications if possible when a complete list of medications and dosages is not available. Care providers should understand that the absolute need for medications could alter over time as a result of age-related changes in pharmacokinetics and pharmacodynamics[67,68] or changes in healthcare settings, and should be re-evaluated regularly.[66,89–92]

5. Support for decision making

- Care providers should understand the importance of supporting the decision-making process and achieve a consensus on the treatment plan.

5.1. In geriatric medicine, the therapeutic goals may differ depending on the person's position and values. For example, a study on health outcome prioritization in geriatric medicine showed that the elderly considered effective treatment of diseases and improvement of physical function as the most important goals of care, whereas physicians prioritized improvement in QOL.[93] Therefore, it is essential to support the decision-making process by providing evidence regarding the treatment options and information on prognosis, and help build a consensus on the goals of care in line with values of both patients and their families.[94]

5.2. Care providers should respect and put the highest priority on the patient's personal wishes and values in the process of achieving a consensus on the treatment plan. Even if the patient is not able to express their wishes and values because of cognitive impairment or terminal illness, their family and the medical team should make an attempt to presume the patient's values and reach a decision on the treatment goals that best serve the patient's interests.

6. Providing support for caregivers, such as family members, as well

- Care providers should acknowledge the burden and distress experienced by caregivers, such as family members, and provide appropriate support for them from early on.

6.1. Caregivers experience mental and physical distress in care provision, and are at increased risk of developing depression and experiencing low QOL.[95–98] Therefore, care providers should actively provide information to help caregivers access social resources, such as long-term care services, and

propose interventions, such as respite care, to reduce their burden.[25,99-102] Caregivers should consider making a recommendation for caregivers to receive medical attention if they experience significant mental or physical distress.

6.2. Because of the low birth rate, the rapid aging population and the trend towards a nuclear family, the phenomena called "elderly living alone" in which an elderly person lives alone, "elderly-to-elderly care (*rou-rou kaigo*)" in which an elderly person provides care for another elderly person (usually a spouse) at home and "dementia-to-dementia care (*nin-nin kaigo*)" in which an elderly patient with dementia provides care to another patient with more severe dementia at home, are increasing in number and have become a public concern.[103] Such family situations warrant particular attention, and should prompt care providers to assess the caregiver's ability to provide care and initiate interventions, such as implementing long-term care insurance services.

7. Patient-centered team medicine

- Care providers should recognize that a patient is a part of the care team and provide patient-centered multidisciplinary care.

7.1. Team medicine is defined as "a care delivery system in which medical staff from diverse professional backgrounds work closely together and provide health services appropriate to the needs of patients, utilizing the expertise of each team member, while sharing common goals and information".[104] Team medicine in the care of elderly patients is effective in improving quality of care and safety, and reducing the burden on medical staff.[105-112] Care providers should understand and acknowledge the expertise of other team members from medical, nursing, long-term care and welfare fields, and engage in multidisciplinary team medicine.[113]

7.2. Team medicine should be patient-centered.[113] Care providers should encourage patients and their family members to participate in team meetings in addition to providing counsel and information. The active participation of patients and their family members in the care planning process may improve the quality of care,[114] and subsequently prevent functional decline and admission to acute care facilities.[59,60,115]

Conclusion

As society is facing the challenges of a "super-aged society", healthcare for the elderly is assuming greater importance, but is nonetheless fraught with problems. Inflating healthcare costs threaten to collapse the healthcare system. Opinions from care providers working in clinical settings will be increasingly considered important in order to establish a sustainable healthcare system for the elderly. Close collaboration between care providers, the local community and local government is crucial to foster a life environment conducive to the elderly population. In addition, evidence regarding the efficacy and safety of therapeutic interventions for elderly patients remains scarce, and only basic principles underlying geriatric medical care are presented in this guidance statement. It is paramount to promote clinical research, leading to establishment of evidence-based clinical guidelines.

In the practice of geriatric medicine, advanced medical skills are required to care for elderly patients with multimorbidity and heterogeneous nature in various care settings through multidisciplinary care teams while taking into account patients' values. Ideally, geriatricians with adequate experience and extensive knowledge in the field of geriatrics should provide care for elderly patients. However, the current number of geriatricians will not meet the continuous surge in demand for geriatric care. It is critical to improve the education system to produce more geriatricians, and create a framework to enlighten primary care physicians on the knowledge and skills of geriatric medicine.

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Original Article

Pravastatin and Olmesartan Synergistically Ameliorate Renal Failure-Induced Vascular Calcification

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Aim: Vascular calcification is a critical problem in patients with chronic kidney disease (CKD). In this study, we examined the effects of a HMG Co-A reductase inhibitor (statin) and an angiotensin II type 1 receptor blocker (ARB) on renal failure-induced vascular calcification.
Method and Result: Severe renal failure was induced in rats by feeding a 0.75% adenine diet for six weeks. These rats had hyperphosphatemia, hypertension and hypercholesterolemia. A histological assessment showed extensive linear calcification in the aortic media and a significant increase in the aortic content of calcium and phosphorus. Oral administration of pravastatin (a statin; 1-10 mg/kg/day) or olmesartan (an ARB; 1-10 mg/kg/day) dose-dependently inhibited the aortic calcification in parallel with their renoprotective, lipid-lowering and blood pressure-lowering effects. Of note, the lowest dose of pravastatin inhibited aortic calcification with no influence on the renal function, BP and cholesterol, suggesting that it has direct vasoprotective properties. Intriguingly, the combined administration of pravastatin and olmesartan at the lowest doses synergistically ameliorated the aortic calcification, and the protective effect was at least partly attributable to the inhibition of RF-induced apoptosis in the aortic wall. An *in vitro* model of inorganic phosphate (Pi)-induced vascular smooth muscle cell calcification mimicked these effects of pravastatin and olmesartan, and the beneficial effect of the combination was attributable to the inhibitory effects on Pi-induced apoptosis via the restoration of the Gas6/Axl-mediated anti-apoptotic pathway.
Conclusion: A statin and an ARB exerted potent protective effects against vascular calcification due to CKD, probably through their pleiotropic effects. In addition, combination therapy with pravastatin and olmesartan may provide a new therapeutic strategy for the prevention of vascular calcification.

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Key words: Vascular calcification, Chronic kidney disease, Statin, ARB, Combination therapy

Introduction

Vascular calcification is frequently associated with cardiovascular (CV) events and mortality[1, 2]. It has been shown that the high rates of CV events in patients with chronic kidney disease (CKD) cannot be completely explained by the excess of traditional risk factors, and these CKD patients have also been shown to possess a disproportionate burden of vascular calcification with several unique clinical findings, such as isolated systolic hypertension and orthostatic hypotension[3]. Therefore, the inhibition of vascular calcification is essential for preventing CV events in these patients, and would lead to more stable hemodynamics. In general, vascular calcification occurs at two anatomical sites in the vessel wall, the arterial media and the intima. The medial calcification frequently seen in CKD patients on dialysis as inappropriate biomineralization is observed as continuous linear deposits along the internal elastic lamina, in contrast to the intimal

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calcification seen as patchy scattered deposits occurring only within atherosclerotic plaques[4, 5].

Recent evidence has shown that vascular calcification is attributable to an active cell-mediated process resembling osteogenesis in bone, rather than passive mineral precipitation[6, 7], because the expression of bone-associated proteins, such as osteopontin, matrix Gla protein and the bone-specific transcription factor, Runx2/Cbfa-1, was found in the calcified medial area[8]. The mechanism responsible for the vascular calcification has been thought to be due to the induction of osteoblastic phenotypic changes, which result from an imbalance in the serum calcium and phosphorus levels in vascular smooth muscle cells (VSMC)[9]. Although hyperphosphatemia, which is frequently seen in patients with severe CKD, has been shown to be associated with the development of vascular calcification, the precise mechanisms underlying the development of vascular calcification in CKD have not been fully elucidated.

It is well known that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors (statins) and angiotensin II type 1 receptor blockers (ARBs) have potent anti-atherogenic effects via not only lipid-lowering and/or blood pressure (BP)-lowering effects, but also their unique properties that are independent of these effects[10, 11]. In addition, the synergistic beneficial effects of combination therapy with both types of drugs on BP control have been reported[12]. We also previously reported that several statins protected against the calcification of cultured VSMCs by inhibiting apoptosis as an initial step of nucleation[13]. However, thus far, the effects of statins and ARBs on vascular calcification have not been fully evaluated in *in vivo* and clinical studies.

Aim

Further pathophysiological understanding is necessary to design effective therapeutic strategies against the vascular calcification in CKD; therefore, it is of interest to identify new preventive therapies that can abrogate vascular calcification. In this study, we examined whether a statin or ARB could prevent aortic calcification in a rat model of severe renal failure. In addition, we hypothesized that the additive inhibitory effects of combination therapy might be more effective than the effects of either single therapy on the renal failure-induced aortic calcification.

Methods

Experimental Renal Failure Rats

Renal failure was induced by feeding rats a 0.75% adenine-containing diet as described previously[14]. In brief, 12-week-old male Wistar rats (Nippon Clea Inc., Tokyo, Japan) were pair-fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus; Nippon Clea Inc.) in the control group or CE-2 chow containing 0.75% adenine (Sigma) in the renal failure group for six weeks. The diet was then returned to normal chow, and the rats were fed the normal diet for an additional two weeks. The adenine-fed rats were given oral pravastatin (1-10 mg/kg/day; Daiichi Sankyo Co., Ltd., Tokyo, Japan), olmesartan (1-10 mg/kg/day; Daiichi Sankyo Co., Ltd.) or both pravastatin (1 mg/kg/day) and olmesartan (1 mg/kg/day), in their drinking water throughout the eight weeks of the experiments ($n=10$ in each group). In preliminary experiments, one rat each with and without renal failure was sacrificed every two weeks, and the BP and biochemical parameters were measured. The food and water consumption were regularly checked every three days, and the total volume of drug administration was adjusted over the whole period. All procedures and animal care were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo.

Measurement of the Blood Pressure and Biochemical Parameters

The blood pressure (BP) and heart rate were measured in conscious rats by tail-cuff plethysmography (BP-98A, Softron Co, Tokyo, Japan) every two weeks. To measure the biochemical parameters, such as the renal function, lipid profile and mineral metabolism, blood samples were collected by cardiac puncture under diethyl ether anesthesia. The serum levels of creatinine (Cre), calcium (Ca), inorganic phosphorus (P), total cholesterol (T-chol) and triglycerides (TG) were measured with an autoanalyzer (Hitachi-7180, Japan). In addition, urine was collected from each rat simultaneously to measure the excretion of urinary albumin as another parameter of renal damage.

Assessment of Calcification in the Aortic Wall

The aortic calcification induced by renal failure was evaluated using several procedures. In addition, each whole aorta was stained with Alizarin red, which could detect mineralization in a preliminary experiment. In addition, the aortic calcification was also evaluated by ultrasound examination (Agilent Co.

Ltd). As a next step, two different methods were carried out to compare the degree of aortic calcification between the groups.

First, as a histological assessment, the aorta was perfusion-fixed *in situ* with 10% buffered formalin at a constant, non-pulsatile pressure of 100 mmHg. The aortas were embedded in paraffin and sequentially cut into cross-sections with 5-μm thickness from the ascending aorta to the aortic arch. To detect calcification in the aortic wall, each cross-section was evaluated by von Kossa staining. To compare the extent of calcification in each group, the areas of calcified lesions and the aortic media between the internal and external elastic laminae were measured using a computerized histological analysis system (Scion Image Software), and then the ratio of the calcified area to the area of the aortic media was calculated. The data were collected from each of six sub-serial cross-sections at 100-μm intervals, and the average was taken as the value of each animal ($n=5$ in each group). This histological analysis was performed in a manner where the investigators were blinded to the treatment groups of the animals.

Second, to quantitate the mineral deposition in the aorta, the content of calcium (Ca) and phosphorus (P) in the carbonized aorta was measured using the o-cresolphthalein complexone method (C-Test, WAKO, Tokyo, Japan) after the determination of the weight of the dried aorta ($n=5$ in each group).

Determination of Apoptosis

The level of apoptosis in the aortic wall was detected by a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay with ApopTag Plus obtained from Chemicon International, Ltd. (Hampshire, UK), according to the manufacturer's instructions. To evaluate the Pi-induced apoptosis in the VSMC, cytoplasmic histone-associated DNA fragments were evaluated with a cell-death detection ELISA kit (Roche, Manheim, Germany) as a quantitative index of apoptosis.

VSMC Calcification *in Vitro*

To examine the mechanism(s) underlying the vascular calcification in an *in vitro* model, VSMCs were cultured with a statin and/or ARB. Calcification was induced in human aortic smooth muscle cells by treatment with a high dose (2.6 μmol/L) of inorganic phosphate (Pi; a mixed solution of Na_2HPO_4 and NaH_2PO_4, pH was adjusted to 7.4) for six days, as described previously[10]. The cells were simultaneously treated with pravastatin (50 μM) or olmesartan (100 μM) in the presence of 2.6 μmol/L Pi in the culture medium. The effect of each drug on the Ca deposition in the cells was determined by the *o*-cresolphthalein complexone method and by von Kossa staining to visualize the mineralization. In addition, to examine the synergistic effects of combined treatment with both drugs on the Ca deposition and the apoptosis-related cascade, the expression of Gas6, Axl and phosphorylated Akt (p-Akt), as a downstream signal of this pathway, was examined on day 6 by a Western blotting/SDS-PAGE analysis using each specific primary antibodies. The experiments were performed with at least three different cell populations.

Statistical Analysis

All results are presented as the means ± standard error (SE). The differences between the groups were analyzed using an ANOVA, followed by Bonferroni's test. A stepwise (forward) multiple regression analysis was performed to clarify the relationships among the aortic calcification, treatments and other measurements. A value of $p<0.05$ was considered to be significant.

Results

Aortic Calcification in Renal Failure Rats

After the induction of severe renal failure, calcification in the aorta was markedly observable by the naked eye at eight weeks (**Fig. 1A**). Ultrasound examinations showed high echoic signals in the aortic wall of the renal failure rats, suggesting the presence of massive calcification. In addition, Alizarin red staining showed significant calcification in the whole aorta (**Fig. 1B**). Histological assessment using von-Kossa staining demonstrated that the renal failure rats had extensive linear calcification, which was localized in the aortic media (**Fig. 1C**). Neointimal plaque formation was not found even in the renal failure group (data not shown), thus suggesting that the renal failure rats showed a typical Mönckeberg's sclerosis pattern.

Beneficial Effects of Statin and/or ARB Treatment on the Laboratory Parameters

Continuation of the adenine diet for six weeks induced severe renal failure with hyperphosphatemia and massive albuminuria (**Fig. 2**). In addition, the adenine-fed rats also showed a significant increase in the BP and serum T-chol. Olmesartan significantly ameliorated the increase in serum Cre in a dose-dependent manner, suggesting that it had renoprotective effects. The beneficial effect of pravastatin was very slight. Similar to the improvement of the serum Cre, the albuminuria was also inhibited by drug inter-

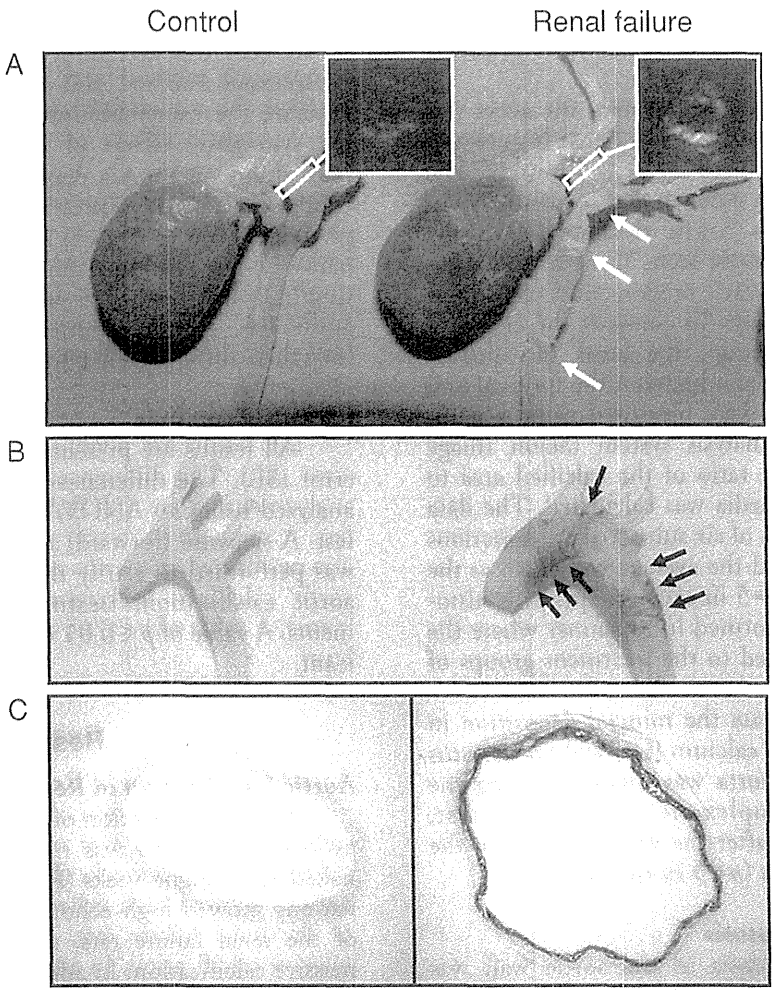

Fig. 1. Extensive aortic calcification in rats with renal failure induced by adenine feeding.

(A) Representative photographs of aortas from control (left) and renal failure (right) rats just after sacrifice. Massive calcification (arrow) was found in the aorta of the renal failure rats. Ultrasound examinations demonstrated high echoic signals in the aortic wall of adenine (AD)-fed rats, suggesting the presence of massive calcification. (B) The mineral deposition in whole aorta of control rat or renal failure rat was visualized by Alizarin red staining. (C) The histopathological assessment of calcification using von Kossa staining showed extensive linear calcification in the aortic media, so-called Mönckeberg's sclerosis.

vention. Especially compared to the pravastatin group, a significant improvement of albuminuria was found in the olmesartan group, which was similar to its preventive effects on the elevation in the serum Cre and the BP. Pravastatin and olmesartan ameliorated hyperphosphatemia by 17% and 35%, respectively. The highest dose of pravastatin (50 mg/kg/day) significantly improved the hypercholesterolemia, although no lipid-lowering effect was found at the lower doses of pravastatin. Intriguingly, a partial decline in the elevated T-chol level was unexpectedly observed even in rats administered the high dose (10 mg/kg/day) of olmesartan. The elevated BP was dose-dependently attenuated not only by olmesartan, but also by pravastatin. Notably, combined administration of the lowest doses (1 mg/kg/day) of pravastatin and olmesartan led to significant improvement of the renal function (especially albuminuria) and hypertension, and the

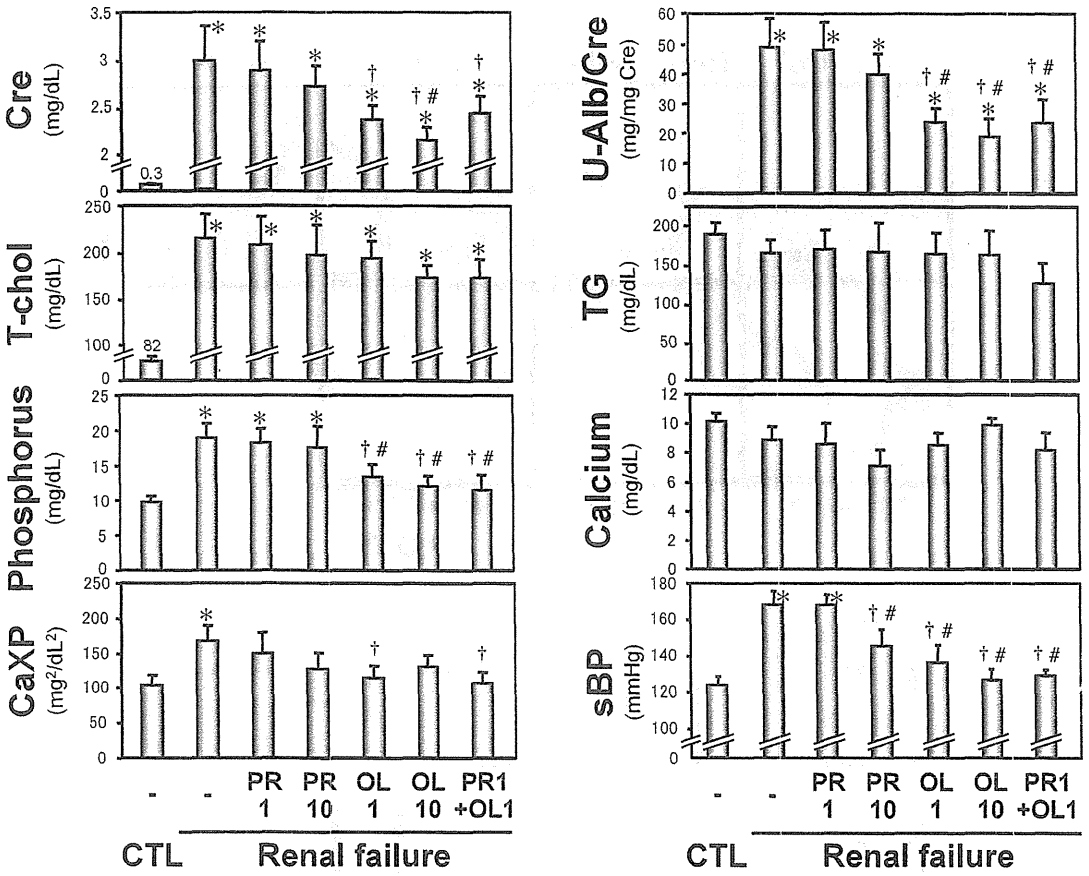

Fig. 2. The renoprotective, lipid-lowering and BP-lowering effects of the statin and/or ARB in renal failure rats.

Serum parameters, such as the creatinine (Cre; mg/dL), calcium (Ca; mg/dL), inorganic phosphorus (P; mg/dL), calcium-phosphorus product (CaXP; mg^2/dL^2) and total-cholesterol (T-chol; mg/dL) level, as well as the systolic blood pressure (sBP; mmHg), were measured at the end of the experiments (eight weeks). The urinary albumin excretion relative to the urinary creatinine excretion (U-Alb/Cre; mg/mg) was also measured at the same time. Pravastatin (PR; 1, 10, 50 mg/kg/day) and olmesartan (OL; 1, 10 mg/kg/day) each dose-dependently showed potent renoprotective effects, including inhibition of albuminuria, and combined administration (PR1 + OL1) demonstrated additive beneficial effects even when both were administered at the lowest doses. Each value indicates the mean ± SE.
*: $p<0.05$ vs control rats without renal failure and drug administration. †: $p<0.05$ vs adenine-induced renal failure rats without drug administration. #: $p<0.05$ vs adenine-induced renal failure rats treated with pravastatin (1 mg/kg/day).

beneficial effects of combination therapy were greater than those of either single therapy.

Synergistic Protection by Combination Therapy

The quantitative measurement of the aortic calcification was performed using two distinct methods, von Kossa staining and determination of the aortic Ca/P content. Pravastatin and olmesartan significantly inhibited aortic calcification in a dose-dependent manner, and the combination of the lowest doses of both drugs inhibited the calcification more effectively than either drug alone (**Fig. 3A**). A statistical analysis of the ratio of the calcified area showed synergistic inhibition by the combined administration (**Fig. 3B**). The quantitative assessment of aortic mineralization showed that the Ca content in the dried aorta was markedly increased (18.7 ± 2.2 μg/mg, $p<0.001$) by the adenine diet compared with the control rats (0.1 ± 0.1 μg/mg) (**Fig. 4A**), and the aortic P content was also similarly increased (**Fig. 4B**). Each drug dose-dependently reduced the aortic Ca content, and the maximal reduction was 94% in the highest-dose pravastatin group and 77% in the high-dose olmesartan group, compared with untreated renal failure rats.

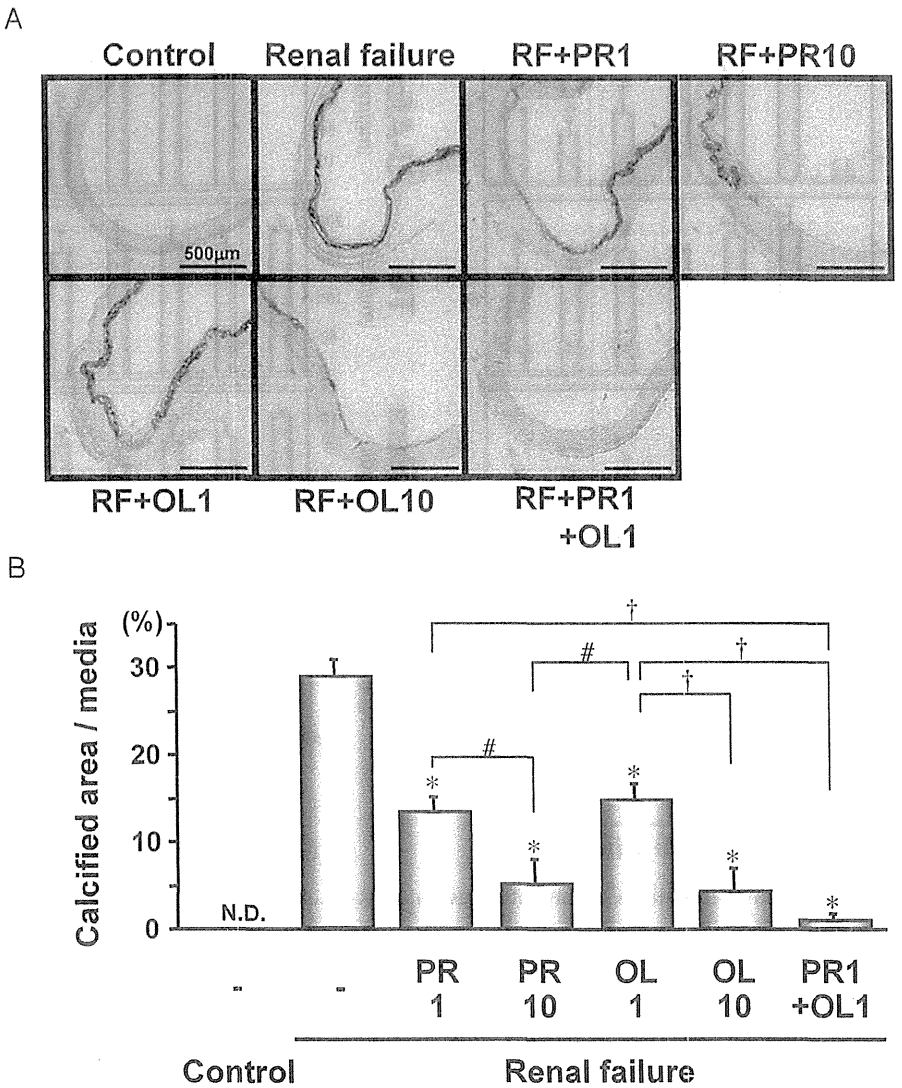

Fig. 3. The inhibitory effects of the statin or ARB and their synergistic benefit on renal failure-induced calcification in the aorta.

Two quantitatively distinct assessments of the aortic calcification were carried out. A. The ratio of the calcified area to the aortic media in each section from the ascending aorta to the aortic arch was calculated ($n=5$ each group). Pravastatin (PR; 1, 10 mg/kg/day) and olmesartan (OL; 1, 10 mg/kg/day) each dose-dependently inhibited the aortic calcification. Significant synergistic inhibition by combined administration (PR1 + OL1) was found even at the lowest doses.

The reduction of the aortic P content by drug intervention was comparable to the reduction of the Ca content. These changes in the aortic Ca and P content were consistent with the histological changes detected by von Kossa staining. Notably, partial inhibition of the aortic calcification by pravastatin was found even at the lowest dose, with no change in the serum Cre, T-chol, P or BP. In addition, combined administration of low-dose olmesartan with low-dose pravastatin additively reduced the elevated Ca/P content in the aorta, and this decrease was markedly higher than that induced by treatment with pravastatin alone. To dissect the renoprotective, BP-lowering and lipid-lowering effects of the statin and ARB, a stepwise multiple regression analysis was performed. The statistical results showed that the use of pravastatin and olmesartan was independently related to the aortic Ca content ($\beta=-0.605$ and -0.398, respectively, $p<0.01$), but

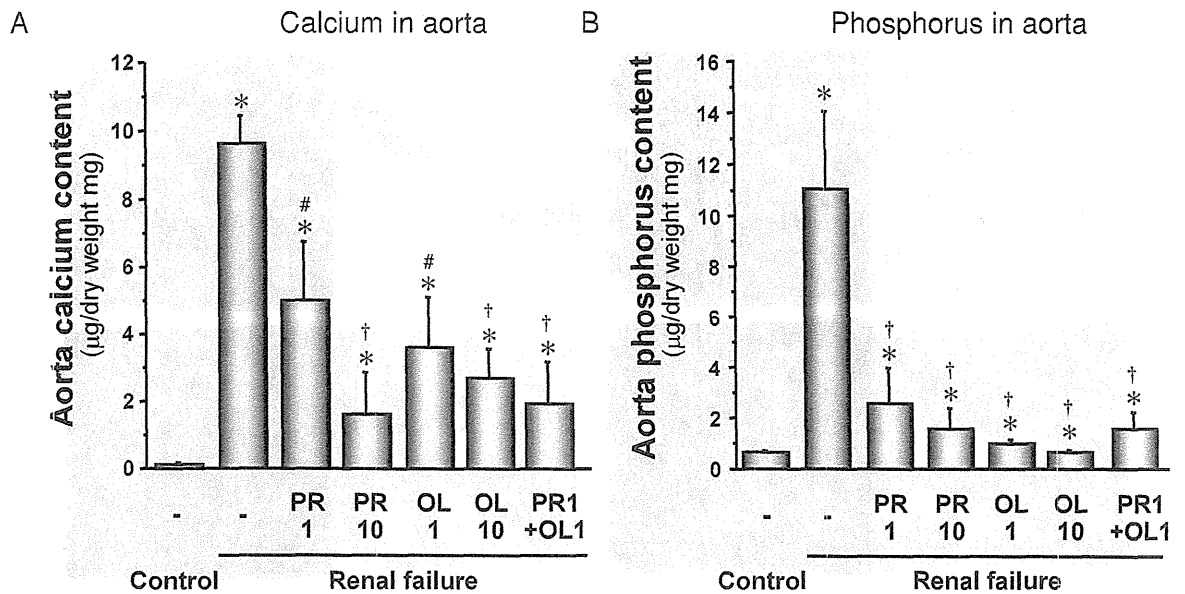

Fig. 4. The quantitative comparison of the inhibitory effects of the statin and/or ARB on renal failure-induced calcification in the aorta.

To quantitatively measure the inhibitory effects of pravastatin (PR) and/or olmesartan (OL) on renal failure-induced aortic calcification at eight weeks, the content of calcium (A) and phosphorus (B) in the dried aorta was measured. Pravastatin and olmesartan each dose-dependently and significantly inhibited the aortic Ca and P contents. The combination of pravastatin and olmesartan demonstrated synergistic inhibitory effects on the deposition of both Ca and P in the aorta.
Data are shown as the means ± SE.
*: $p<0.01$ vs adenine-induced renal failure rats without drug administration.
#: $p<0.05$, †: $p<0.01$. N.D.: not detected.
*: $p<0.05$ vs adenine (AD)-induced renal failure rats without drug administration. †: $p<0.05$ vs adenine-induced renal failure rats treated with pravastatin (1 mg/kg/day).

the serum Cre, T-chol, systolic BP and urinary albumin/Cre were not.

Inhibition of Apoptosis in the Aortic Wall

The extent of apoptosis in the aortic wall was evaluated by TUNEL staining (**Fig. 5**). Massive apoptosis was found in the renal failure rats, and the localization of the fluorescent signal was consistent with the calcified area. Low-dose pravastatin significantly inhibited the cell apoptosis similar to its effects on calcification. Notably, the combined treatment with pravastatin and olmesartan at the lowest dose completely inhibited the apoptosis.

Additive Inhibitory Effects of Combination Treatment on VSMC Calcification *in Vitro*

We have previously shown that the Gas6/Axl-mediated survival pathway plays an essential role in VSMC calcification via Pi-induced apoptosis[10]. To further understand the precise mechanism by which both drugs inhibited the aortic calcification induced by renal failure-based hyperphosphatemia, we decided to perform mechanistic experiments *in vitro* using human aortic smooth muscle cells to assess the inhibitory effects of pravastatin and olmestatin.

As shown in **Fig. 6**, each drug significantly inhibited the Pi-induced apoptosis and the subsequent Ca deposition in human VSMCs, and this inhibition was additively augmented by the combined treatment. In the assessment of the anti-apoptotic signals, single treatment with each drug significantly restored the downregulated expression of Gas6, Axl and p-Akt by Pi stimulation. The partial restoration of Axl and p-Akt, but not Gas6, was similar to the degree of inhibition of apoptosis and the subsequent calcification. The statistical analysis showed an additive benefit of combined treatment, which was more effective than either single treatment.

Discussion

The present study showed that treatment with a statin and an ARB prevented renal failure-induced aortic calcification, and the combined administration

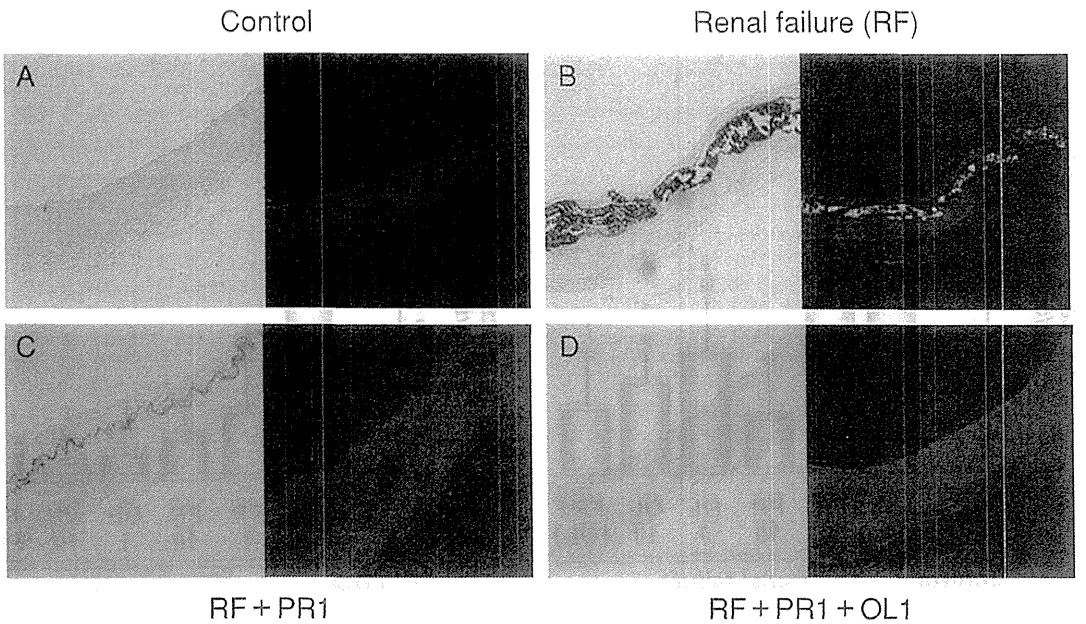

Fig. 5. The induction of apoptosis in the aortic wall of renal failure rats and the inhibitory effects of combination therapy.

The induction of apoptosis in the aortic wall was assessed by TUNEL staining. (A and B) Apoptosis was strongly induced by renal failure, and the apoptotic findings (right panel) were localized to the calcified area, as detected by von Kossa staining (left panel). (C) The administration of pravastatin at the lowest dose (PR1) showed significant inhibition of not only calcification, but also apoptosis. (D) Treatment with the combination of pravastatin (PR1) and olmesartan (OL1) even at the lowest doses showed complete inhibition of the apoptosis and subsequent calcification.

of both drugs synergistically exerted beneficial effects. The possible mechanisms by which combination therapy can abolish aortic calcification more effectively than each single therapy may include not only renoprotective and antihypertensive effects, but also direct effects of the drugs on the vasculature. Thus far, the clinical effects of statins on calcification of CV system are still controversial, and there are no clinical or basic reports showing the effects of ARBs on vascular calcification. This study explored the efficacy regarding the preventive potential of using a combination of a statin and an ARB against vascular calcification.

The CV-associated mortality in CKD patients on dialysis has been shown to be 10 to 30 times higher than that in the general population[15]. In particular, extensive calcification in the arterial media is strongly associated with the increased CV event rates in CKD patients[16, 17]. In the present study, adenine-fed rats exhibited severe renal failure with massive albuminuria and subsequent hypertension and hypercholesterolemia. A histopathological assessment of the kidneys showed progressive proximal tubular dilation and atrophic glomeruli (data not shown), similar to a previous report[14]. The renal failure rats showed extensive linear calcification in the media of the aortic wall, resembling the typical Mönckeberg's pattern frequently seen in CKD patients.

Inhibitory Effects of the Statin

With respect to the clinical efficacy of statins for CV calcification, several retrospective studies have demonstrated inhibitory effects on calcific changes in the aortic valve[18, 19] and coronary arteries[20, 21]. However, a recent prospective study did not show significant inhibitory effects[22]. Therefore, it is currently unclear whether statins directly affect the genesis of vascular calcification in the clinical setting, including in patients with CKD. In this study, renal failure-induced aortic calcification was significantly inhibited by pravastatin in a dose-dependent manner. Pravastatin administration showed strong renal protection and a dose-dependent reduction in the blood pressure and serum cholesterol level, suggesting that the blood pressure reduction might be a consequence of the improvement of renal function by pravastatin.

Especially in CKD patients, recent reports have shown cardioprotective effects[23] (an improvement in flow-mediated vasodilation) and renoprotective

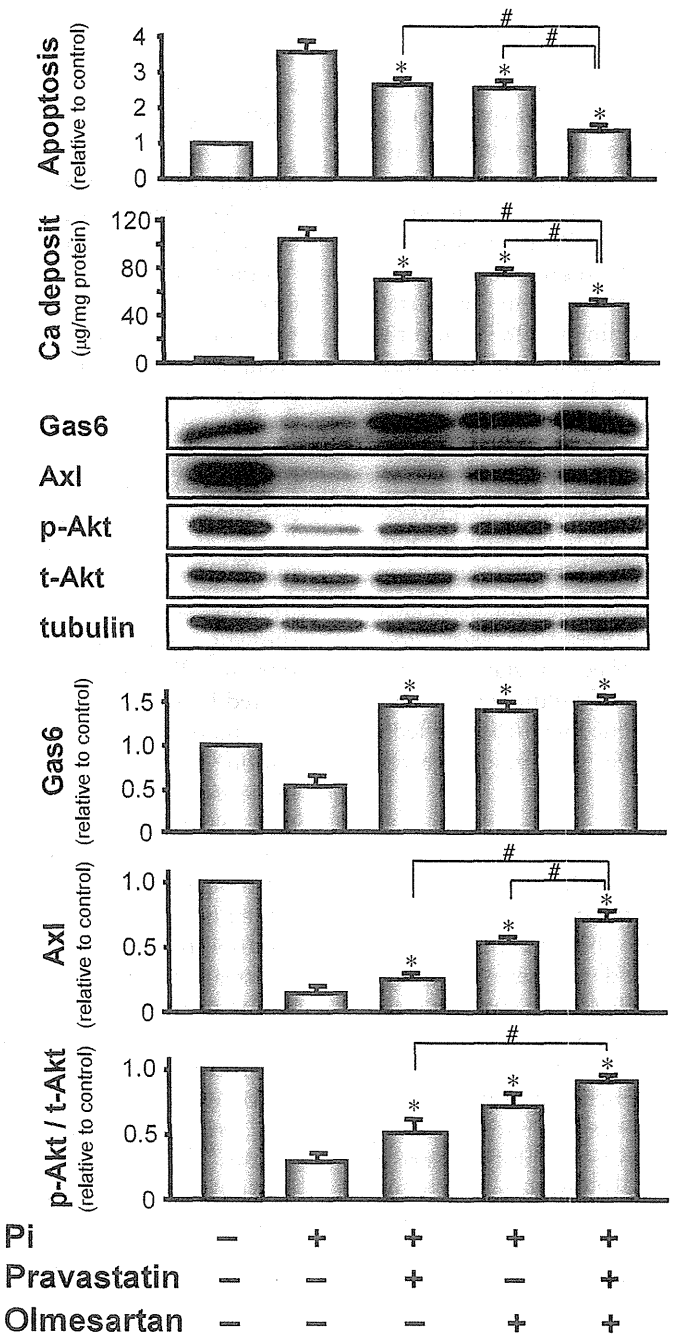

Fig. 6. The synergistic inhibitory effects of the statin and ARB on the phosphate-induced VSMC apoptosis and subsequent calcification.

Human VSMCs were treated with or without high-dose (2.6 μM) inorganic phosphate (Pi) for six days. Pi stimulation significantly increased the apoptosis and the subsequent Ca deposition, as shown. To assess the Gas6/Axl/Akt-mediated cell survival pathway, cell lysates extracted after six days were evaluated by a SDS-PAGE/Western blot analysis and representative results are shown. To compare each group, the expression level was presented as the relative value to the control without Pi treatment. The statistical analysis was performed on the results of three independent experiments. Pravastatin (50 μM) and olmesartan (100 μM) each partially inhibited the Pi-induced apoptosis and Ca deposition (upper panel), and restored the expression of Gas6, Axl and phosphorylated Akt (p-Akt) (lower panel). Combined treatment demonstrated not only additive inhibitory effects on apoptosis and the subsequent Ca deposition, but also additive restoration of this survival pathway.
*: $p<0.05$ vs. Pi stimulation without drug treatment. #: $p<0.05$ vs. Pi stimulation plus combined treatment.

effects[24-26], and also the efficacy of statin administration[27]. These findings strongly support our current findings. Notably, significant inhibition of aortic calcification was found in the pravastatin-treated rats, even at the lowest dose (1 mg/kg/day), which did not affect the renal function, lipid parameters or BP, suggesting that statins can attenuate aortic calcification through their direct vasoprotective effects. In addition, pravastatin at the middle dose (10 mg/kg/day) reduced the aortic Ca content more effectively than olmesartan (1 mg/kg/day), although the decline in the BP and the renal protection induced by pravastatin at that dose were smaller than those induced by olmesartan. This result implies that the inhibitory effects of pravastatin on aortic calcification are associated with its pleiotropic effects.

It has been shown that hypertension and hypercholesterolemia, accompanied by renal failure, augment the levels of several inflammatory cytokines, and statins exert an anti-inflammatory response associated with direct vasoprotection[28-30]. Based on these findings, the beneficial inhibitory effects of statins on the aortic calcification in cases of renal failure may, at least in part, result from their unique pleiotropic effects, including direct renoprotective and vasoprotective actions.

Inhibitory Effects of the ARB

We demonstrated dose-dependent effects of olmesartan on the inhibition of aortic calcification. In this study, the effects of the ARB beyond BP-lowering were not clear from the present data, because a significant reduction in the BP by olmesartan was observed even at the lowest dose (1 mg/kg/day). The severe renal dysfunction and subsequent hypertension were significantly improved by olmesartan; however, hypercholesterolemia was not. It is possible that the inhibitory effects of olmesartan on aortic calcification are in part attributable to its direct BP-lowering and renoprotective effects, which are independent of its effects on cholesterol. Strong renoprotection by ARBs, but not other antihypertensive drugs, via a reduction in proteinuria is well known, in addition to direct BP-lowering effects[31, 32]. In addition, several reports have shown that ARBs improve the arterial stiffness, as assessed by the pulse wave velocity[33, 34]. Especially in CKD patients, superior preventive effects for olmesartan on nocturnal hypertension and proteinuria have been suggested compared to other ARBs[35, 36]. Furthermore, the detailed medical prescription[37] or combined used of olmesartan with other medications[38] was also recently suggested. The underlying benefits of ARB treatment may also be attributable to direct vasoprotection independent of the effects on the BP. Therefore, our data suggest that ARBs, or at least olmesartan, may be beneficial for the prevention of aortic calcification in CKD.

Benefits of Combination Therapy

We explored the effects of combined administration of a statin and an ARB in our renal failure model. A significant decline in the BP and potent renal protection, with improvement of hyperphosphatemia, were observed in both the lowest-dose olmesartan group and the combination group. Although the lowest dose of pravastatin showed no significant effect on the laboratory parameters and BP, the combined use of olmesartan with pravastatin even at the lowest doses showed more effective inhibitory effects on aortic calcification not only in the histological assessment, but also in the quantitative assessment of the aortic Ca and P content, suggesting the increased benefit from the combined use from an early phase. Our results showing an additive improvement of the renal function and BP by the combination treatment are supported by some clinical and experimental reports[39, 40]. One report revealed an additive benefit of the combination via an improvement of the endothelial function (as assessed by flow-mediated dilation) and a reduction of inflammatory markers (as assessed by the plasma monocyte chemoattractant protein-1 and malondialdehyde levels) in hypertensive, hypercholesterolemic patients[39]. In addition, it has also been reported that combined treatment was superior to single treatment for inhibiting neointimal formation in rats with insulin resistance[41]. These findings strongly support our results showing the usefulness of combination therapy. It is well known that both statins and ARBs exert multiple effects, including organ protection, via their anti-oxidative and other pleiotropic actions[42-44]. Therefore, additional benefits of the combination therapy might contribute to the marked prevention of aortic calcification, together with stable hemodynamics, and provide a new therapeutic strategy for the management of aortic calcification in CKD.

Synergistic Protection from Apoptosis-based SMC Calcification by Combination Therapy

Hyperphosphatemia has been shown to be associated with vascular calcification in severe CKD[45]. In fact, pravastatin and olmesartan partially improved the hyperphosphatemia in the renal failure rats, presumably via renal protection. We have previously reported that Pi-induced VSMC apoptosis played an essential role in vascular calcification[10]. In our animal experi-

ments, there was remarkable induction of apoptosis in the aortic wall found in the renal failure rats. The apoptosis was completely ameliorated by the combination treatment in the present study. In addition, the direct effects of these drugs on VSMC calcification in an *in vitro* model were evaluated. The Pi-induced apoptosis and subsequent calcification were partially inhibited by each drug alone, and significant synergistic inhibition was found with combined treatment. The protective effects of each drug were associated with the restoration of the Gas6/Axl survival pathway and its signaling, and the anti-apoptotic function was augmented by the combined treatment.

However, this manuscript is associated with one limitation, namely we were unable to detect Gas6 expression in the rat aorta. However, we confirmed that this pathway is crucial using a VSMC *in vitro* model for the inhibitory effects of both drugs. Our previous data showed that the expression of thrombomodulin, one of upstream molecules regulating Gas6-mediated apoptosis, was upregulated in the calcified aortas from the same rat model[46], therefore, we believe that Gas6 was downregulated in the calcified aortas from rats. Although these additive benefits of combination therapy observed in our culture model strongly support the results from our animal experiments, the causal role of Gas6-related apoptosis in our rat model of vascular calcification needs to be confirmed in future studies.

Conclusion

We herein demonstrated that a statin and an ARB could each exert beneficial inhibitory effects on renal failure-induced aortic calcification not only due to their renoprotective effects, but also by their pleiotropic effects. Combined administration of pravastatin with olmesartan was superior to single-drug treatment for attenuating the vascular calcification. Our present work suggests that combination therapy with a statin and an ARB may thus be recommended as a new therapeutic strategy against vascular calcification in CKD patients.

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Conflicts of Interest

M.A. and Y.O. received lecture fees and research funding from Daiichi Sankyo Co., Ltd. The other authors declare that they have no financial interests regarding this study.

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