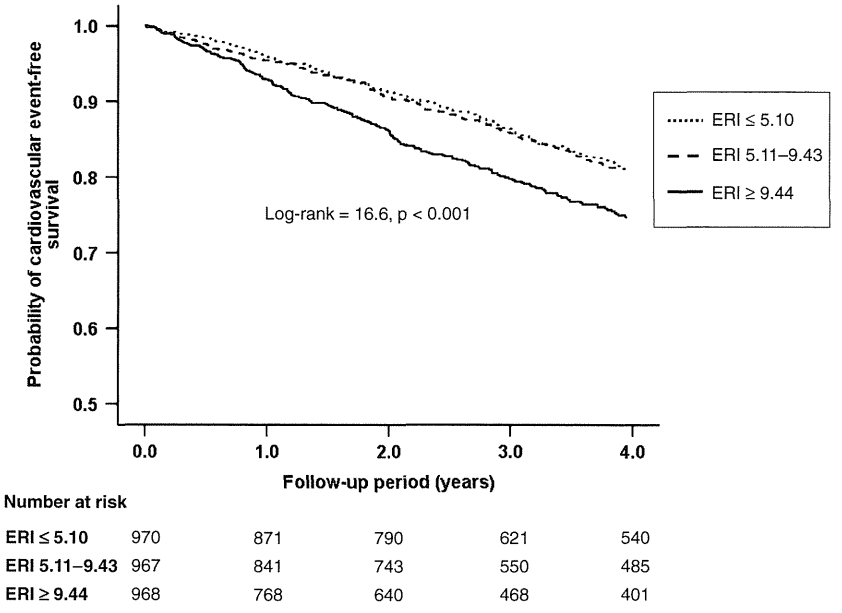

Fig. 2 Event-free survival rates for major cardiovascular disease according to the erythropoietin resistance index levels during the 4-year follow-up period. *ERI* erythropoietin resistance index

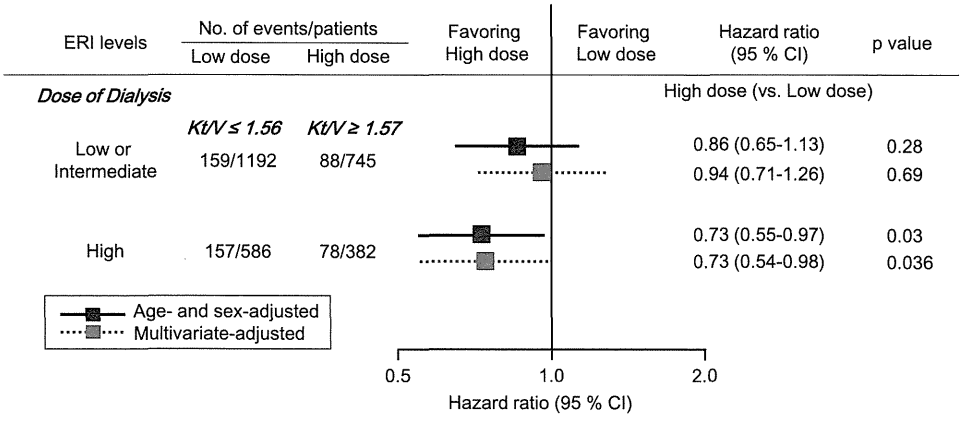

Fig. 3 Effects of high dose of dialysis on all-cause death according to the erythropoietin resistance index levels. For the multivariate-adjusted model, adjustments were made for age, sex, dialysis duration, predialysis systolic blood pressure, antihypertensive agent use, diabetes, history of cardiovascular disease, serum albumin, serum calcium, serum phosphorus, serum total cholesterol, log-transformed serum c-reactive protein, log-transformed serum ferritin, and body mass index. *ERI* erythropoietin resistance index, *ESA* erythropoiesis-stimulating agent, *CI* confidence interval

In conclusion, the present findings have clearly demonstrated that the highest ERI group was associated with increased risks of mortality and cardiovascular events. Therefore, this emphasizes the importance of ESA responsiveness as a significant prognostic factor in HD patients. Additionally, a higher dose of dialysis might be effective for improving the prognosis of HD patients with hyporesponsiveness to ESA, but this finding needs to be interpreted cautiously. Further clinical research might be warranted to elucidate the effect of a high-dose dialysis on the hyporesponsiveness to ESA.

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Conflict of interest The authors declare that they have no relevant financial interests.

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ORIGINAL RESEARCH

Phosphate Binders Prevent Phosphate-Induced Cellular Senescence of Vascular Smooth Muscle Cells and Vascular Calcification in a Modified, Adenine-Based Uremic Rat Model

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Abstract Clinical and experimental studies have reported that phosphate overload plays a central role in the pathogenesis of vascular calcification in chronic kidney disease. However, it remains undetermined whether phosphate induces cellular senescence during vascular calcification. We established a modified uremic rat model induced by a diet containing 0.3 % adenine that showed more slowly progressive kidney failure, more robust vascular calcification, and longer survival than the conventional model (0.75 % adenine). To determine the effect of phosphate on senescence of vascular smooth muscle cells (VSMCs) and the protective effect of phosphate binders, rats were divided into four groups: (1) normal control rats; (2) rats fed with the modified adenine-based diet (CKD); (3) CKD rats treated with 6 % lanthanum carbonate (CKD-LaC); and (4) CKD rats treated with 6 % calcium carbonate (CKD-CaC). After 8 weeks, CKD rats showed circumferential arterial medial calcification, which was inhibited in CKD-LaC and CKD-CaC rats. CKD rats showed increased protein expression of senescence-associated β-galactosidase, bone-related proteins, p16 and p21, and increased oxidative stress levels in the calcified area, which were inhibited by both phosphate binders. However, serum levels of oxidative stress and inflammatory markers, serum fibroblast growth factor 23, and aortic calcium content in CKD-CaC rats were higher than those in CKD-LaC rats. In conclusion, phosphate induces cellular senescence of VSMCs in the modified uremic rat model, and phosphate binders can prevent both cellular senescence and calcification of VSMCs via phosphate unloading. Our modified adenine-based uremic rat model is useful for evaluating uremia-related complications, including vascular calcification.

Keywords Calcium carbonate · Cellular senescence · Chronic kidney disease · Phosphate · Phosphate binder · Vascular smooth muscle cell · Vascular calcification

Introduction

Cellular senescence, also called stress-induced premature senescence, is a state of irreversible cell-cycle arrest of mitotic cells [1] and leads to a reduced capacity to respond to various stimuli. Senescent cells exhibit a flattened and enlarged morphology and express a different series of genes such as p16, p21, p53, and retinoblastoma protein [2]. Recent studies have reported that cellular senescence is involved in various organ dysfunctions in chronic kidney disease (CKD) [3, 4]. Cellular senescence has also been shown to be related to vascular calcification (VC) that contributes to the high cardiovascular mortality in CKD: in vitro studies have reported that replicative senescence of vascular smooth muscle cells (VSMCs) promotes VC [5]. Although phosphate (Pi) is one the most powerful inducers

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of VC [6], it remains undetermined whether Pi overload-related cellular senescence of VSMC is involved in the VC associated with CKD.

Pi binders are useful to counteract Pi overload in CKD. Clinical studies have already confirmed that Ca-containing Pi binders promote VC more than non-Ca-containing Pi binders [7, 8]. Experimental studies have shown that Ca overload accelerates VC by several known mechanisms [1]. However, it remains unknown whether calcium (Ca)-containing Pi binders and non-Ca-containing Pi binders have differential effects on cellular senescence of VSMC, oxidative stress, and inflammation in CKD.

Developing animal models that exhibit extensive and robust VC is another important issue for research in vascular biology [9]. Various types of animal models with VC have been proposed [10]. The adenine-fed renal failure model is one of the most frequently used rat models for its relatively easy induction [11]. Although adenine-fed uremic rat models develop a series of renal failure-related phenotypes including arterial medial calcification, the original model using 0.75 % adenine has several vital limitations: relatively low prevalence and degree of VC, severe and rapid malnutrition, and high fatality in 4–6 weeks [12–14]. Accordingly, a modified adenine-fed uremic rat model with a high probability of developing extensive arterial medial calcification and a longer survival rate is required.

In the preset study, we established a new uremic rat model that develops robust and extensive arterial medial calcification with a higher probability than the conventional (0.75 %) adenine-fed uremic rat model by feeding a modified adenine-based diet. In addition, we investigated the role of Pi overload in CKD focusing on cellular senescence of VSMC and VC, and compared the therapeutic potential of Pi binders (lanthanum carbonate, LaC, and calcium carbonate, CaC) for Pi-related changes in this new uremic rat model.

Materials and Methods

Animal Care and Study Protocols

Male Sprague–Dawley rats (10-week old) were purchased from Kyudo Co. Ltd (Saga, Japan) and fed a standard diet for 7 days before being used in each protocol. All rats had free access to food and water. Both the standard diet and synthetic diet were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan).

In protocol 1 (survival study), the survival rate over 8 weeks (until day 56) was compared between the conventional and new uremic rat models fed adenine-containing diets; (1) CKD-HA, uremic rats fed a conventional high adenine-based diet (0.75 % adenine, 1.0 % Ca, 1.2 % Pi, 19 % grain-based protein); and (2) CKD-LA, uremic rats fed a modified low adenine-based diet (0.3 % adenine, 1.0 % Ca, 1.2 % Pi, 20 % lactose, 19 % casein-based protein) (Supplemental Fig. 1a).

In protocol 2 (model comparison study), to compare the biochemical parameters and VC between CKD-HA and CKD-LA, rats were raised for 2, 4, and 6 weeks in the CKD-HA group, and for 2, 4, 6, and 8 weeks in the CKD-LA group ($n = 8$ rats for each time point in each group; 56 total rats). Blood, 24-h urine, and aorta were obtained at each time period (Supplemental Fig. 1b).

In protocol 3 (treatment study), 32 rats were randomly divided into four groups (n = 8 for each group) and raised under each specific diet as follows for 8 weeks: (1) CNT, rats fed standard diet; (2) CKD, rats fed modified adenine-based diet; (3) CKD-LaC, CKD rats treated with 6 % LaC, and (4) CKD-CaC, CKD rats treated with 6 % CaC (Supplemental Fig. 1c). The content of each diet is shown in Supplemental Table 1. In the present study, we used LaC as a non-Ca-containing Pi binder, because sevelamer chloride, another non-Ca-containing Pi binder, was shown to have pleiotropic effects including anti-inflammatory action that may ameliorate the progression of VC [15, 16].

1 day before being killed, rats were housed in metabolic cages for 24 h and urine was collected for each protocol. Urine was centrifuged at $1500\times g$ for 15 min and the supernatant stored at −30 °C. Rats were killed under sevoflurane anesthesia, and blood and aorta were collected. The abdominal aorta was immersed in formalin for histological analysis; the remainder was stored at −80 °C. Serum and urinary levels of albumin, urea nitrogen, creatinine (Cr), Ca, Pi, and magnesium were measured using an automated analyzer (Auto Analyzer HITACHI 7020; Hitachi High-Technologies Corporation, Tokyo, Japan). The following biochemical parameters were determined by commercially available rat ELISA kits: serum intact fibroblast 23 (Kainos Laboratories Inc., Tokyo, Japan), urinary8-hydroxy-2'-deoxyguanosine (8-OHdG; JaICA, Shizuoka, Japan), and serum tumor necrosis factor-alpha (TNF-α; R&D Systems, Minneapolis, MN, USA). All kits were used according to the manufacturers' instructions, and their qualities were within analytical levels.

Examination of Arterial Calcification and Renal Fibrosis

Four-micrometer sections from paraffin-embedded aorta were deparaffinized and processed for von Kossa staining. Semi-quantitative determination of the degree and prevalence of VC was performed using the four-level scale developed from previous studies [17]: no calcification (calcified area was not detected), mild calcification (calcified area was 1–33 % of the aortic ring), moderate

calcification (calcified area was 34–66 % of the aortic ring), severe calcification (calcified area was 67–100 % of the aortic ring).

To quantitatively evaluate the degree of VC, frozen aortic tissue was weighed and hydrolyzed in 1 mL of 6 mol/L hydrochloric acid for 24 h. The Ca and Pi content of the supernatant was determined using commercially available kits (Calcium E-test and Phospha C-test; Wako, Osaka, Japan) and normalized by wet tissue weight (μg/mg wet tissue weight).

Two-micrometer sections from paraffin-embedded kidney were deparaffinized and processed for Periodic-Acid-Schiff staining and Sirius-Red staining using the standard method for evaluating renal structure and fibrosis. Representative histological images were captured using light microscopy on an Eclipse E800 microscope (Nikon, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed as previously described [18]. Briefly, 4-μm sections from paraffin-embedded aorta were deparaffinized, rehydrated, and prepared for antigen retrieval. Antigen retrieval was performed by microwave for 15 min in citrate buffer (pH 6) for p16, p21, senescence-associated β-galactosidase (SA-β-gal), runt-related gene 2 (Runx2), and osteocalcin. For antigen retrieval for 8-OHdG, sections were treated with RNase solution at 37 °C for 1 h, proteinase K solution (10 μg/mL) for 15 min, hydrochloric acid (4 mol/L) for 20 min, and Trizma base (50 mM) for 5 min at room temperature. p16, p21, and SA-β-gal were used as markers for cellular senescence, Runx2 and osteocalcin as makers for osteoblastic transdifferentiation, and 8-OHdG as the marker for oxidative stress [18, 19]. After inactivation of intrinsic peroxidase by incubation in 0.3 % hydrogen peroxidase, sections were treated with 5 % skim milk for 30 min at room temperature and incubated in a humidified chamber for 1 h at 37 °C with the following primary antibodies: mouse monoclonal anti-β-galactosidase antibody (1:200; Promega Corp., Madison, WI, USA), mouse monoclonal anti-p16 antibody (1:100; sc-1661; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), mouse monoclonal anti-p21 antibody (1:50; sc-6246, Santa Cruz Biotechnologies), rabbit polyclonal anti-Runx2 antibody (1:50; sc-10758, Santa Cruz Biotechnologies), goat polyclonal anti-osteocalcin antibody (1:25; sc-18319, Santa Cruz Biotechnologies), and mouse monoclonal anti-8-OHdG antibody (1:200, Japan Institute for the Control of Aging, Nikken Seil Co. Ltd., Shizuoka, Japan). After washing three times in PBS/Tween-20, sections were incubated with horseradish peroxidase (HRP)-coupled secondary antibody (Nichirei Corporation, Tokyo, Japan) for 30 min at room temperature. HRP was visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide.

Real-Time PCR

Total RNA was extracted from rat tissue frozen in liquid nitrogen by the guanidinium thiocyanate phenol–chloroform method, according to the manufacturer's instructions using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and used to prepare complementary DNA by reverse transcription using a PrimeScript™ RT reagent kit (Perfect Real Time; Takara Bio Inc., Otsu, Japan). Real-time quantitative PCR was performed using SYBR Premix Ex Taq™ (Takara Bio Inc.), Applied Biosystems 7500 Real-time PCR systems (Applied Biosystems, CA, USA), and the following primers purchased from Takara Bio Inc.: rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH), RA015380; rat TNF-α, RA043092; rat alkaline phosphatase, RA041418; rat Runx2, RA067967; rat nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4), RA064409; rat p16, RA047733; and rat p21, RA063054. The cycling conditions were 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C for denaturation and 40 s of annealing at 60 °C. The specificity of the PCR products was confirmed by analysis of the melting curves and additionally by agarose gel electrophoresis. All measurements were performed in duplicate, and mRNA fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal reference.

Statistical Analyses

Statistical analyses were performed using JMP version 10.0 (SAS Institute, Tokyo, Japan). Data are presented as mean ± SD unless otherwise specified. Kaplan–Meier method and log-rank test were used to compare the survival rate between CKD-HA and CKD-LA. Differences between the two groups were analyzed using one-way ANOVA followed by Dunnett's test for serial, parametric data. Differences among groups were analyzed by Cochran-Armitage tests for ordinal data for protocol 2 and by one-way ANOVA followed by Tukey's multiple tests for protocol 3. A two-tailed p value of <0.05 was considered statistically significant.

Results

Modified Uremic Rat Had Longer Survival, Slower Progression of Uremia, and Less Malnutrition than the Conventional Model (Protocol 1 and Protocol 2)

Figure 1 shows the survival curve described by the Kaplan–Meier method in both the groups. Only two of 16 rats survived after 8 weeks in CKD-HA group, while 12 out of

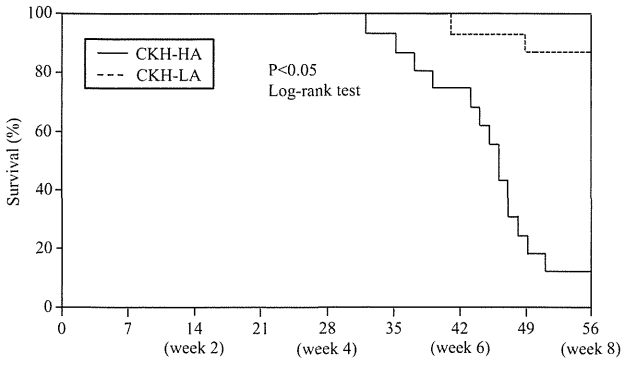

Fig. 1 Survival curve in two adenine-fed rats (protocol 1, survival study). CKD-HA: uremic rats fed a conventional high adenine diet (adenine 0.75 %, Ca 1.0 %, Pi 1.2 %, 19 % grain-based protein); and CKD-LA: uremic rats fed a modified low adenine diet (adenine 0.3 %, Ca 1.0 %, Pi 1.2 %, 20 % lactose, 19 % casein-based protein). *Ca* calcium, *CKD* chronic kidney disease, *Pi* phosphate

16 rats survived after 8 weeks in CKD-LA group; the survival rate in CKD LA group was significantly longer than that in CKD-HA group (log-rank test, $P < 0.05$).

Next, we compared biochemical parameters and vascular pathology of CKD-LA rats at week 8 with those of CKD-HA rats at week 6. Tables 1 and 2 summarize the physical and biochemical parameters obtained from urine and serum every 2 weeks in both groups. Figure 2 shows representative photomicrographs of kidney specimens stained with Periodic-Acid-Schiff and Sirius-Red method obtained every 2 weeks in both the groups. Both CKD-HA rats and CKD-LA rats showed progressive increases in blood urea nitrogen, serum creatinine levels, and urine volume and progressive decreases in body weight, food intake, and serum albumin levels, followed by hyperphosphaturia and hyperphosphatemia. However, the progression of these parameters was slower in CKD-LA than in CKD-HA. Periodic-Acid-Schiff staining and Sirius-Red staining of the kidney showed that fibrotic areas, dilated tubules, and deposition of adenine crystals in the renal cortex increased in both the groups in a time-dependent manner (Fig. 2). The progression of renal injury was also slower in CKD-LA than in CKD-HA.

Serum levels of urea and creatinine in CKD-LA rats at week 8 were almost the same as those in CKD-HA at week 6. Histologically, renal injury in CKD-LA rats at week 8 was similar to that in CKD-HA rats at week 6. Serum Pi levels and urinary Pi excretion in CKD-LA rats at week 8 were higher than those in CKD-HA rats at week 6. Serum albumin level and body weight in CKD-LA rats at week 8 were also higher than in CKD-HA rats at week 6.

Modified Adenine-Based Uremic Rats Induced More Robust and Extensive Arterial Medial Calcification than Conventional Adenine-Based Rats (Protocol 2)

Figure 3a and b shows semi-quantitative analysis of aorta stained using the von Kossa method revealing the prevalence and degree of VC at each time point in the two groups. CKD-HA rats developed VC at week 4 and week 6, and CKD-LA rats developed VC at week 6 and week 8 (Fig. 3b). The degree and prevalence of arterial medial calcification in CKD-LA rats at week 8 was significantly more severe than in CKD-HA rats at week 6 with less variation. The Ca and Pi content in CKD-LA rats at week 8

Table 1 Physical and biochemical parameters in CKD-HA rats (protocol 2, model comparison study)

	Baseline	Week 2	Week 4	Week 6
Body weight (g)	410 ± 23	330 ± 45[a]	305 ± 41[a]	267 ± 70[a]
Food intake (g/day)	23.4 ± 3.4	11.4 ± 2.2[a]	9.8 ± 3.3[a]	7.6 ± 3.1[a]
Urine volume (mL/day)	23.0 ± 9.8	27.5 ± 10.7	28.1 ± 7.1	36.3 ± 9.2[a]
Serum albumin (g/dL)	4.2 ± 0.3	3.5 ± 0.3[a]	3.2 ± 0.6[a]	2.8 ± 0.6[a]
Blood urea nitrogen (mg/dL)	15.9 ± 1.2	26.9 ± 12.7[a]	59.8 ± 18.9[a]	106.5 ± 72.6[a]
Serum creatinine (mg/dL)	0.24 ± 0.8	1.3 ± 0.8[a]	2.4 ± 1.7[a]	3.5 ± 0.8[a]
Corrected serum Ca (mg/dL)	9.8 ± 0.3	9.4 ± 0.6	9.1 ± 0.6	9.7 ± 0.6
Serum Pi (mg/dL)	8.1 ± 0.6	11.2 ± 1.1[a]	15.6 ± 3.1[a]	22.3 ± 5.1[a]
Urinary protein excretion (mg/day)	12.5 ± 5.7	6.1 ± 2.4[a]	8.0 ± 3.9	8.4 ± 1.6
Urinary Ca excretion (mg/day)	0.59 ± 0.27	0.38 ± 0.33	0.27 ± 0.19	0.24 ± 0.23[a]
Urinary Pi excretion (mg/day)	2.8 ± 2.5	23.8 ± 15.2[a]	16.5 ± 4.0[a]	15.3 ± 5.9[a]

Data are mean ± SD. Dunnett's test was used to compare data at each week

CKD-HA uremic rats fed a conventional high adenine diet (0.75 % adenine, 1.0 % Ca, 1.2 % Pi, 19 % grain-based protein), *Ca* calcium, *Pi* phosphate

[a] $P < 0.05$ (vs baseline)

Table 2 Physical and biochemical parameters in CKD-LA rats (protocol 2, model comparison study)

	Baseline	Week 2	Week 4	Week 6	Week 8
Body weight (g)	407.1 ± 5.4	413.9 ± 12.4	407.6 ± 15.8	383.2 ± 24.6[a]	345.5 ± 29.4[a]
Food intake (g/day)	23.4 ± 3.4	16.0 ± 4.9[a]	12.9 ± 5.4[a]	11.2 ± 5.2[a]	10.8 ± 4.7[a]
Urine volume (mL/day)	23.0 ± 9.8	68.8 ± 14.2[a]	77.6 ± 13.9[a]	67.2 ± 15.8[a]	65.2 ± 22.6[a]
Serum albumin (g/dL)	4.2 ± 0.3	4.1 ± 0.3	3.8 ± 0.6	3.5 ± 0.8[a]	3.2 ± 0.6[a]
Blood urea nitrogen (mg/dL)	15.9 ± 1.2	38.5 ± 4.2	54.4 ± 24.9[a]	82.5 ± 25.7[a]	101.0 ± 29.1[a]
Serum creatinine (mg/dL)	0.24 ± 0.3	1.1 ± 0.3[a]	1.8 ± 0.7[a]	2.4 ± 0.9[a]	3.3 ± 0.5[a]
Corrected serum Ca (mg/dL)	9.8 ± 0.3	9.9 ± 0.6	9.4 ± 0.8	9.2 ± 1.4	8.5 ± 1.1[a]
Serum Pi (mg/dL)	8.1 ± 0.6	8.2 ± 0.8	14.5 ± 1.4[a]	21.4 ± 3.1[a]	28.3 ± 2.8[a]
Urinary protein excretion (mg/day)	12.5 ± 5.7	12.4 ± 5.5	10.7 ± 3.2	11.2 ± 1.8	10.1 ± 2.9[a]
Urinary Ca excretion (mg/day)	0.59 ± 0.27	1.0 ± 0.3	1.5 ± 0.9	3.0 ± 1.2[a]	3.9 ± 1.8[a]
Urinary Pi excretion (mg/day)	2.8 ± 2.5	76.7 ± 7.1[a]	90.4 ± 12.1[a]	80.7 ± 16.1[a]	69.0 ± 25.2[a]

Data are mean ± SD. Dunnett's test was used to compare data at each week

CKD-LA uremic rats fed a modified low adenine diet (0.3 % adenine, 1.0 % Ca, 1.2 % Pi, 20 % lactose, 19 % casein-based protein), *Ca* calcium, *Pi* phosphate

[a] $P < 0.05$ (vs baseline)

was greater than those in CKD-HA rats at week 6 (Fig. 3c). Arterial medial calcification in CKD-LA rats at week 8 spread from the ascending aorta to the bilateral femoral arteries (data not shown).

Phosphate Binders Prevented Phosphate-Induced Mineral Derangement (Protocol 3)

Serum and urinary biochemical parameters in each group are shown in Table 3. Serum creatinine and urea nitrogen was significantly higher in CKD, CKD-LaC, and CKD-CaC rats than that in CNT rats ($P < 0.05$), while no significant difference was observed among the other three CKD groups. Serum Pi, intact FGF23 levels, and urinary Pi excretion were significantly higher in CKD rats than in CNT rats ($P < 0.05$); treatment with LaC or CaC significantly decreased these parameters ($P < 0.05$). The urinary Ca level in CKD-CaC rats was significantly higher than those in the other three groups ($P < 0.05$). The levels of urinary protein were comparable among the four groups.

Pi Binders Ameliorated Pi-Induced Aortic Calcification in Uremia

As shown in Fig. 4, both Ca and Pi contents in the aorta of CKD rats were significantly higher than in CNT rats, while treatment with LaC or CaC suppressed both Ca and Pi in the aorta induced by high-Pi loading. However, Ca and Pi contents in the aorta in CKD-CaC rats were significantly greater than those in CKD-LaC rats.

Pi Binders Prevented Pi-Induced Osteoinductive Signaling and Senescence of VSMCs

Figure 5 shows the relative mRNA expression in aortas from each group. The mRNA expressions of Runx2, alkaline phosphatase, Nox4, TNF-α, p16, and p21 in CKD rats were increased compared with that in CNT rats. Treatment with LaC and CaC equally abolished all these Pi-related changes. There were no significant differences in relative mRNA expression of Runx2, alkaline phosphatase, Nox4, TNF-α, p16, and p21 between CKD-LaC and CKD-CaC rats, although mRNA expression of osteoblastic markers (Runx2 and alkaline phosphatase) tended to be higher in CKD-CaC than in CKD-LaC rats.

Pi binders Prevented Pi-Induced Oxidative Stress, Cellular Senescence, and Calcification of VSMCs

To determine whether senescence-related proteins were associated with VC induced by Pi overload, we performed immunohistochemistry for Runx2, osteocalcin, SA-β-gal, p16, p21, and 8-OHdG in the abdominal aortas from each group (Fig. 6). No CNT rats were positive for Runx2, osteocalcin, SA-β-gal, p16, and p21. Both Runx2 and osteocalcin were positively stained in and around the calcified area of the CKD aorta. 8-OHdG was accentuated in both the calcified area and non-calcified area in CKD rats. SA-β-gal, p16, and p21 were also positively stained in and around the calcified area of the aorta in CKD rats. Localizations of SA-β-gal, p16, and p21 were closely associated with that of Runx2,

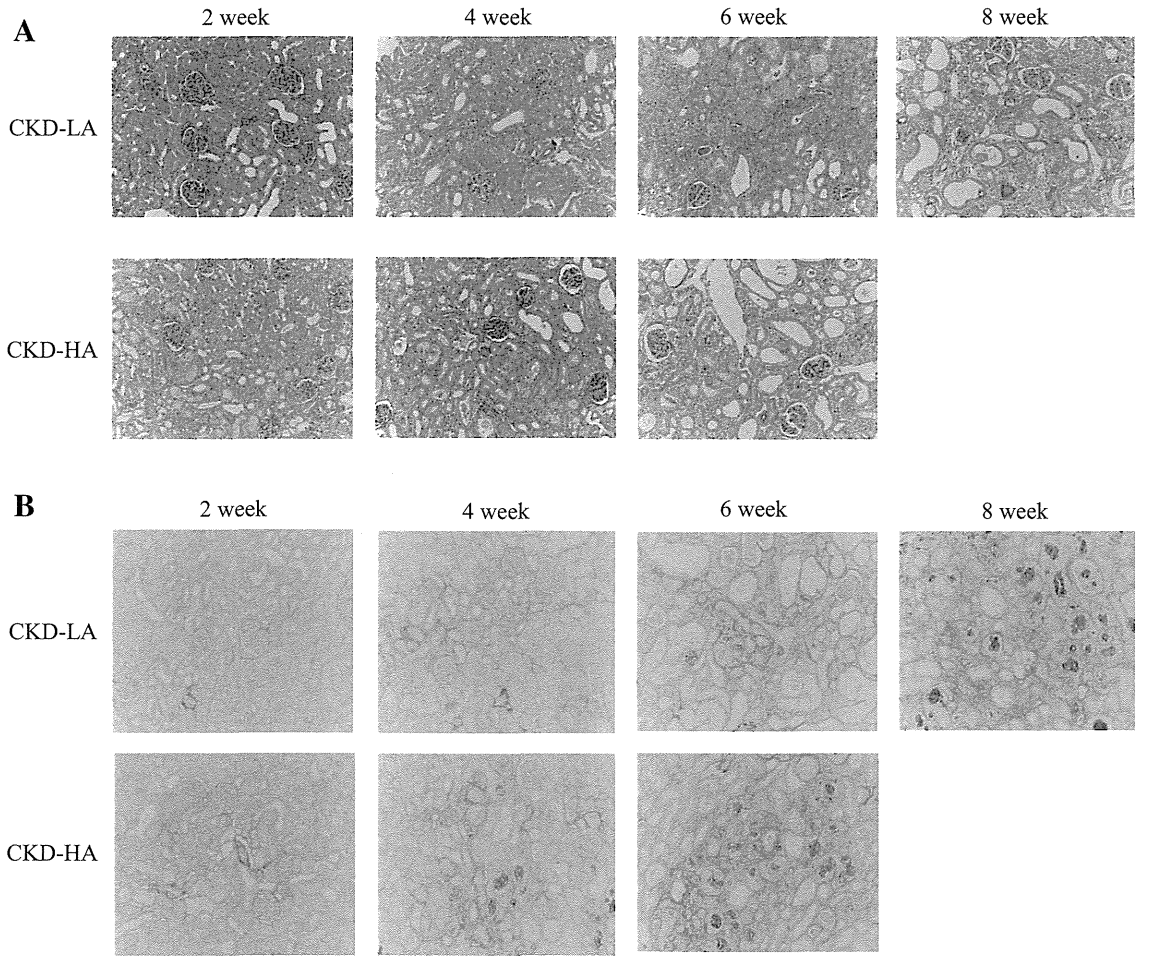

Fig. 2 Kidney histology in two adenine-fed rats (protocol 2, model comparison study). Representative photomicrographs of kidney specimens stained with **a** Periodic-Acid-Schiff and **b** Sirius-Red at each week in two rat models. CKD-HA; uremic rats fed a conventional high adenine diet (adenine 0.75 %, Ca 1.0 %, Pi 1.2 %, 19 % grain-based protein); and CKD-LA: uremic rats fed a modified low adenine diet (adenine 0.3 %, Ca 1.0 %, Pi 1.2 %, 20 % lactose, 19 % casein-based protein). *Ca* calcium, *CKD* chronic kidney disease, *Pi* phosphate

osteocalcin, and 8-OHdG in CKD. Treatment with Pi binders suppressed the expressions of p16, p21, and SA-β-gal (cellular senescence-related proteins), Runx2 and osteocalcin (osteoblastic lineage proteins), and 8-OHdG, indicating that senescence-associated proteins, osteoblastic transdifferentiation, and oxidative stress were induced by Pi overload in CKD.

Pi Binders had Differential Effects on Systemic Inflammation and Oxidative Stress

To compare the effects of the two Pi binders on systemic inflammation and oxidative stress, we measured serum TNF-α levels and the urinary 8-OHdG/Cr ratio in CKD-LaC and CKD-CaC rats. Both serum and urinary markers in CKD-CaC rats were significantly higher than those in CKD-LaC rats (Fig. 7).

Discussion

Recent studies have reported that cellular senescence plays an important role in the pathogenesis of organ dysfunction in CKD [2–5]. However, Pi loading-induced cellular senescence and its effect on VC in VSMC has not been fully investigated in CKD. The present study demonstrated several novel findings. First, a new adenine-based uremic rat model showed slowly progressive renal failure, more extensive and robust arterial medial calcification, and longer survival compared with the conventional, adenine-based uremic rat model. Second, Pi overload-induced cellular senescence of VSMC and VC in the modified adenine-based uremic rat model. Third, CaC and LaC treatment inhibited senescence and transdifferentiation of VSMCs into osteoblastic lineage cells and almost completely prevented VC. Fourth, CKD rats treated by CaC

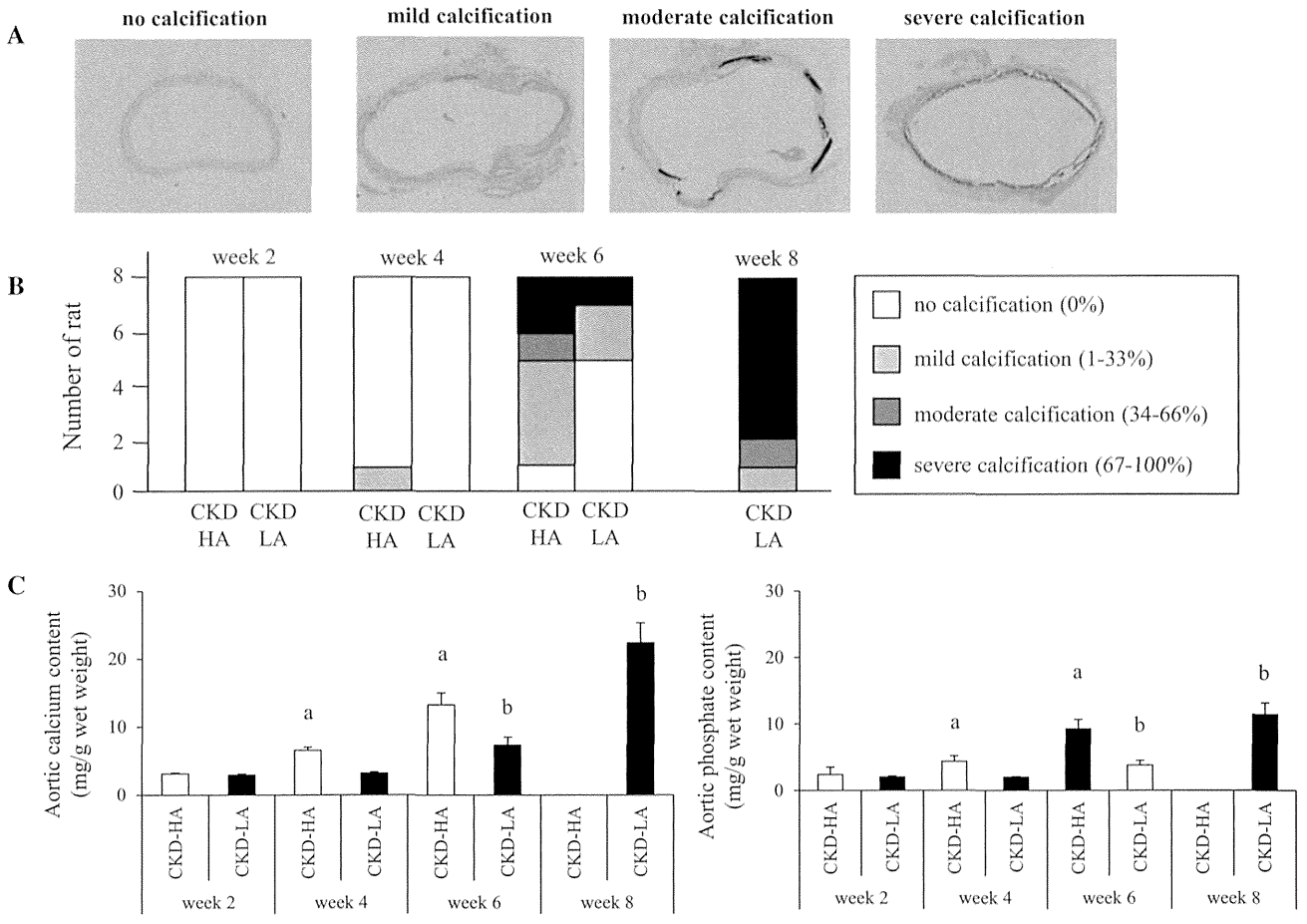

Fig. 3 Prevalence and extent of VC in two adenine-fed rats (protocol 2, model comparison study). **a** Representative photomicrographs of abdominal aorta stained using the von Kossa method and semi-quantification of the degree of VC. No calcification: calcified area is absent; mild calcification: calcified area covers 1–33 % of the aortic ring; moderate calcification: calcified area covers 34–66 % of the aortic ring; and severe calcification: calcified area covers 67–100 % of the aortic ring (original magnification, ×40) **b** Prevalence and degree of VC at every 2 weeks for both the groups. **c** Ca and Pi content in the aorta at each time period in each group. CKD: uremic rats fed a conventional high adenine diet (adenine 0.75 %, Ca 1.0 %, Pi 1.2 %, 19 % grain-based protein) (CKD-HA); and CKD-LA: uremic rats fed a modified low adenine diet (adenine 0.3 %, Ca 1.0 %, Pi 1.2 %, 20 % lactose, 19 % casein-based protein), *Ca* calcium, *CKD* chronic kidney disease, *Pi* phosphate, *VC* vascular calcification. Data are expressed as mean ± SEM. A two-tailed $P < 0.05$ was considered statistically significant. [a]$P < 0.05$ vs. CKD-HA at week 2, [b]$P < 0.05$ vs. CKD-LA at week 2

had greater aortic Ca deposition and increased systemic inflammation and oxidative stress than CKD rats treated by LaC.

It has been a challenge to create a uremic rat model that consistently develops extensive and robust arterial medial calcification without genetic manipulation or a low protein diet [9–14]. In the present study, to overcome the limitations of the conventional adenine rat model, we established a new synthetic diet that modified the conventional adenine-based diet in three ways and compared the modified and conventional adenine-based uremic rat models. First, we used a 0.3 % adenine-based diet to slow progression of CKD and to avoid rapid malnutrition, because a 0.75 % adenine-based diet induces severe and rapid progression of renal failure and malnutrition, leading to high mortality in 4–6 weeks [12–14]. Second, we selected a casein-based diet as the protein source because a casein-based diet can promote Ca and Pi absorption from the gastrointestinal tract [20]. Third, we added 20 % lactose to the diet to further enhance Ca and Pi absorption from the gastrointestinal tract [21]. Both conventional and modified adenine-fed rats developed similar levels of serum creatinine, but the progression of uremia was relatively slower and the serum Pi level was higher in the new model than in the conventional model. The new model survived for 8 weeks and developed robust VC. This modified synthetic diet provided a rat model with extensive and robust arterial medial calcification and a high probability of developing VC after 8 weeks, without using a low protein diet, under a relatively long lifespan. From the animal care viewpoint

Table 3 Biochemical parameters and body weight after 8 weeks (protocol 3, treatment study)

	CNT ($n = 8$)	CKD ($n = 8$)	CKD-LaC ($n = 8$)	CKD-CaC ($n = 8$)
Serum				
Albumin (g/dL)	4.2 ± 0.3	3.3 ± 0.6[a]	4.2 ± 0.3[b]	3.9 ± 0.3[b]
Urea nitrogen (mg/dL)	23.2 ± 2.5	101.0 ± 29.1[a]	126.5 ± 41.9[a,b]	140.2 ± 10.1[a,b]
Creatinine (mg/dL)	0.3 ± 0.3	3.3 ± 0.6[a]	3.5 ± 0.8[a]	3.6 ± 0.6[a]
Ca (mg/dL)	10.0 ± 0.3	8.5 ± 1.1[a]	11.9 ± 0.6[b]	14.4 ± 0.8[a,b]
Pi (mg/dL)	8.4 ± 0.6	28.3 ± 2.8[a]	8.3 ± 0.8[b]	8.4 ± 1.1[b]
Intact FGF23 (pg/mL)	430 ± 90	135,234 ± 85,810[a]	20,052 ± 20,949[a,b]	40,954 ± 27,350[a,b]
Urine				
Protein excretion (mg/day)	12.5 ± 4.2	10.4 ± 1.7	12.0 ± 4.0	7.5 ± 1.7
Ca excretion (mg/day)	1.6 ± 0.8	2.1 ± 0.6	1.9 ± 1.4	6.4 ± 2.3[a,b,c]
Pi excretion (mg/day)	1.9 ± 2.3	69.0 ± 25.1[a]	6.4 ± 2.8[a,b]	0.9 ± 1.7[b]
Mg excretion (mg/day)	5.4 ± 3.4	3.7 ± 1.4	3.6 ± 1.7	3.4 ± 1.4

Data are mean ± SD

CNT normal control rats fed a standard diet (1.0 % Ca, 1.2 % Pi, 19 % grain-based protein), *CKD* uremic rats fed a modified adenine-based diet (0.3 % adenine, 1.0 % Ca, 1.2 % Pi, 19 % casein-based protein, 20 % lactose), *CKD-LaC* CKD rats treated with 6 % lanthanum carbonate, *CKD-CaC* CKD rats treated with 6 % calcium carbonate, *Ca* calcium, *FGF23* fibroblast growth factor 23, *Mg* magnesium, *Pi* phosphate

[a] $P < 0.05$ versus CNT

[b] $P < 0.05$ versus CKD

[c] $P < 0.05$ versus CKD-LaC

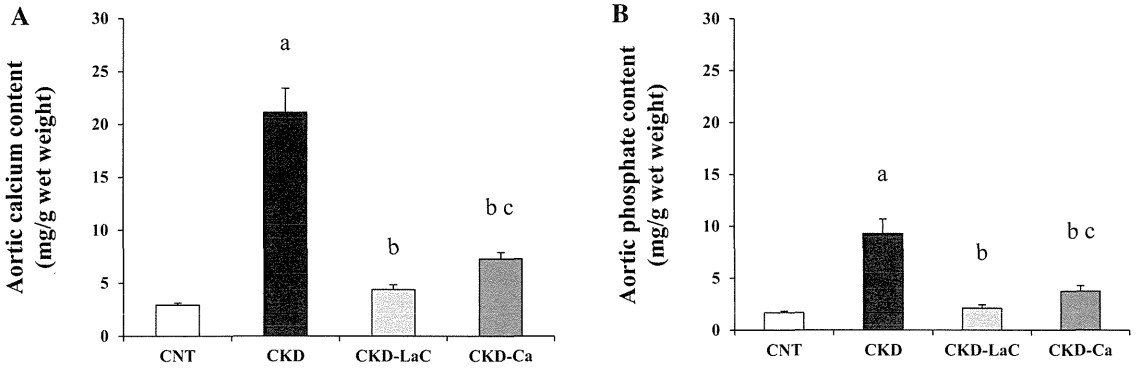

Fig. 4 Effects of phosphate overload on biochemical parameters and VC in chronic kidney disease (protocol 3, treatment study). Quantification of Ca content (**a**) and Pi content (**b**) in aorta. CNT: normal rats fed a standard diet, *CKD* uremic rats fed a modified adenine-containing diet, CKD-LaC CKD: rats treated with 6 % lanthanum carbonate, *CKD-CaC* CKD rats treated with 6 % calcium carbonate. Data are expressed as mean ±SEM. One-way ANOVA followed by Tukey's post hoc tests was performed. *Ca* calcium, *CKD* chronic kidney disease, *Pi* phosphate, *VC* vascular calcification. Data are expressed as mean ± SEM. A two-tailed $P < 0.05$ was considered statistically significant. [a]$P < 0.05$ vs. CNT, [b]$P < 0.05$ vs. CKD

and in accordance with the "three Rs" (reduction, refinement, and replacement), this model is also useful, because a rat model with longer survival and robust calcification can reduce the number of rats used for the study of VC. Collectively, the features of the present modified rat model are suitable for researchers to investigate the complex mechanisms of arterial medial calcification in uremia.

The impact of cellular senescence on Pi-induced VC is an important subject. In vitro studies have reported that senescence of VSMCs is involved in phenotypic changes of VSMCs into osteoblastic lineage cells, contributing to the increase in calcification [5, 22]. Furthermore, cellular senescence of VSMCs is also involved in arterial intimal calcification in atherosclerotic plaques [23]. A recent experimental study reported that indoxyl sulfate, a uremic toxin, increased protein expression of senescence-associated proteins and VC in the aorta of a 5/6 nephrectomized rat model, which were prevented by administration of AST120, an absorber of indol in the gastrointestinal tract [24]. In the present study, senescence-related proteins p16 and p21 and

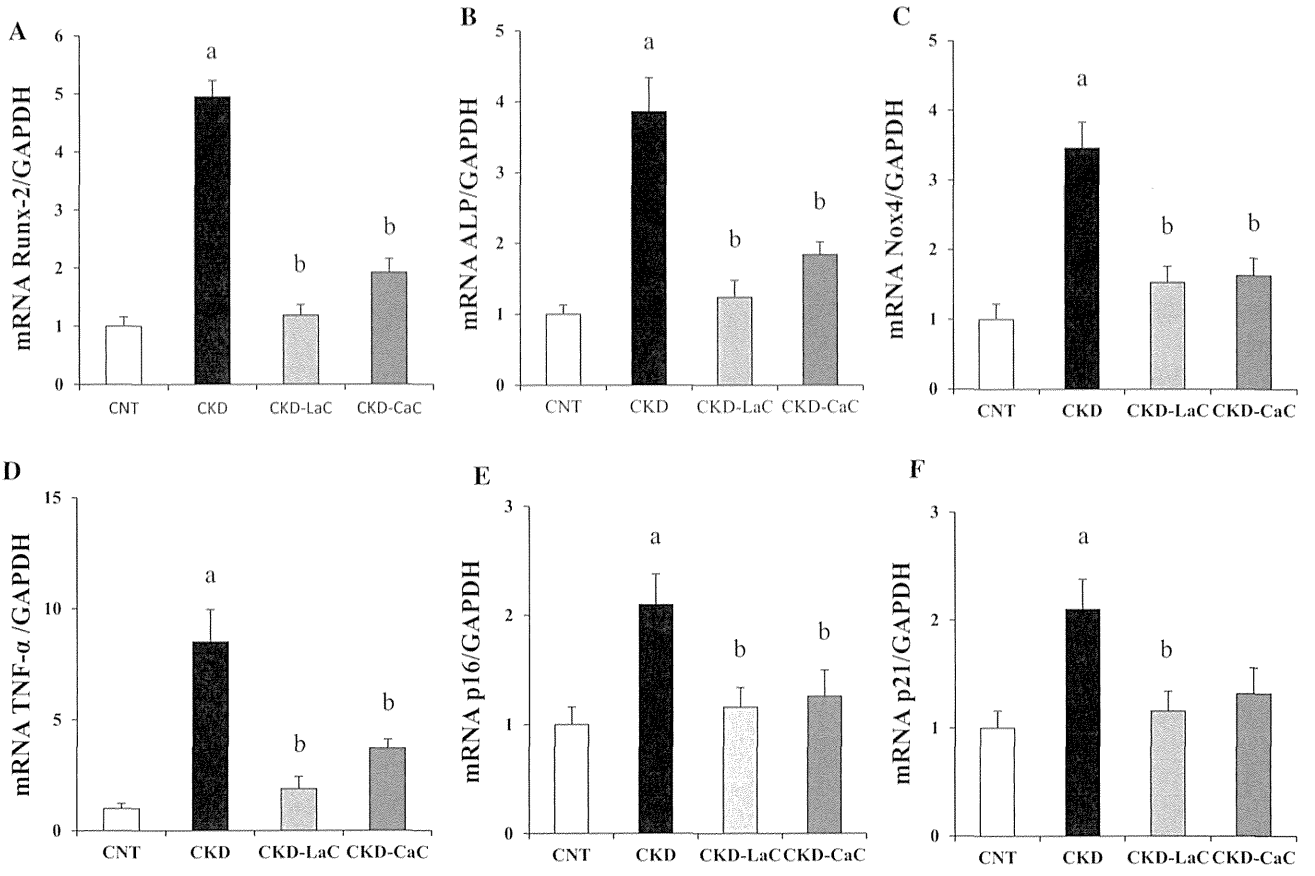

Fig. 5 Effects of phosphate overload on the phenotype of vascular smooth muscle cells (protocol 3, treatment study). The mRNA expression of **a** Runx2, **b** ALP, **c** TNF-α, **d** Nox4, **e** p16, and **f** p21 in the rat aorta. The mRNA expression was corrected to the level of GAPDH. CNT: normal rats fed a standard diet; CKD: uremic rats fed a modified adenine-containing diet; CKD-LaC: CKD rats treated with 6 % lanthanum carbonate; CKD-CaC: CKD rats treated with 6 % calcium carbonate. *ALP* alkaline phosphatase, *BMP-2* bone morphogenetic protein-2, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *Nox4* nicotinamide adenine dinucleotide phosphate oxidase 4, *Runx2* runt-related gene 2, *TNF-α* tumor necrosis factor-alpha. Data are expressed as mean ±SEM. One-way ANOVA followed by Tukey's post hoc tests was performed. Data are expressed as mean ± SEM. A two-tailed $P < 0.05$ was considered statistically significant. $^{a}P < 0.05$ vs. CNT, $^{b}P < 0.05$ vs. CKD

SA-β-gal increased in and around the calcified area. Both Pi binders prevented the expression of senescence-associated markers in the aorta by inhibiting Pi overload. Recently, Takemura et al. reported in their in vitro study that Pi loading increased SA-β-gal and p21 expression in VSMCs and calcification, which were prevented by resveratrol, an inducer of sirt1 [25]. They also reported that down-regulation of p21 by siRNA decreased calcification, suggesting that p21 plays a critical role in the pathogenesis of cellular senescence of VSMCs and in VC. These results suggest that senescence-associated proteins, including p16 and p21, which are up-regulated by Pi overload, are important in the pathology of VC, and regulation of cell senescence may be a promising target for the prevention of Pi-induced VC.

The mechanistic role of Pi overload in the senescence of VSMCs should be elucidated. Recent experimental studies have shown that oxidative stress is involved in transdifferentiation of VSMCs and results in VC, and that anti-oxidative treatment prevents VC. Pi overload induces reactive oxygen species in mitochondria via NADPH oxidase [19, 26]. In the present study, both oxidative stress and senescence markers were co-localized at the calcified area and were ameliorated by both Pi binders. Because oxidative stress is a strong inducer of VSMC senescence [27], these results collectively indicate that Pi-induced oxidative stress might promote cellular senescence of VSMCs, resulting in progression of VC.

Pi overload is an important and potent inducer of VC in CKD, and activates multiple steps of VC [6]. In the present study, treatment with two different Pi binders significantly decreased urinary Pi excretion and serum Pi levels in uremic rats, leading to the prevention of VC, although uremic levels were comparable among CKD, CKD-LaC, and CKD-CaC. Furthermore, Pi binders blunted the increased expression of Runx2 and osteocalcin observed in the aorta of CKD rats. These results are consistent with previous studies that reported a high-Pi diet induces

phenotypic changes of VSMCs into osteoblastic lineage cells and results in VC in uremic rats; these effects are prevented by adequate Pi binder use [28, 29].

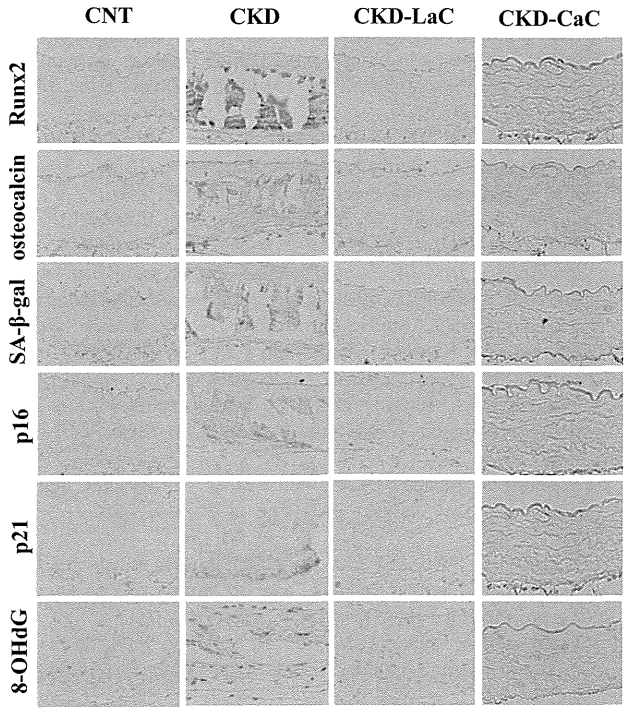

Fig. 6 Histological analysis of the effects of Pi overload on cellular senescence of vascular smooth muscle in aorta (protocol 3, treatment study). Von Kossa staining and immunohistochemistry for Runx2, osteocalcin, SA-β-gal, p16, p21, and 8-OHdG of abdominal aorta for each group (original magnification, ×200). CNT: normal rats fed a standard diet; CKD: uremic rats fed a modified adenine-containing diet; CKD-LaC: CKD rats treated with 6 % lanthanum carbonate; and CKD-CaC: CKD rats treated with 6 % calcium carbonate. *Ca* calcium, *CKD* chronic kidney disease, *8-OHdG* 8-hydroxy-2'-deoxyguanosine, *Pi* phosphate, *Runx2* runt-related gene 2, *SA-β-gal* senescence-associated β-galactosidase

Consequently, the present study showed that this newly developed rat model with a low adenine-based diet is useful to determine whether certain interventions can prevent VC.

Clinical studies have shown that Ca-based Pi binders promote VC more than non-Ca-based Pi binders in CKD patients [7, 8]. Recent experimental studies have shown that Ca loading promotes VC by activating various pathways [6] and promotes the formation of calciprotein particles, aggregates of Ca, Pi, α-fetuin, and other proteins [30]. Because calciprotein particles are shown to accelerate inflammation in macrophages, lymphocytes, and VSMCs [31–33], it has become important to avoid their formation. In the present study, aortic Ca content, systemic oxidative stress level, and inflammatory response in CKD-CaC rats were higher than in CKD-LaC rats. These results were consistent with the previous studies [34, 35]. However, there were no significant differences between two different Pi binders in the phenotypic change of VSMCs determined by real-time PCR and immunohistochemistry, although mRNA of bone-related markers in the aorta tented to be higher in CKD-CaC than in CKD-LaC. One of the explanations is that systemic oxidative stress and inflammation decreased soluble inhibitors of VC-like fetuin in CKD-CaC rats, because soluble inhibitors are negatively regulated by inflammation [30]. In addition, the serum FGF23 level in CKD-CaC rats was higher than that in CKD-LaC rats, although the serum Pi level and urinary Pi excretion level were comparable between the two groups. Because recent studies showed that dietary Ca loading induces FGF23 production and FGF23 affects VSMCs in the absence of klotho [36–38], it is possible that Ca overload in CKD-CaC can promote VC independent of osteogenic transdifferentiation of VSMCs. Hence, further studies are needed to clarify the precise mechanisms of how a Ca-based Pi binder exacerbates VC.

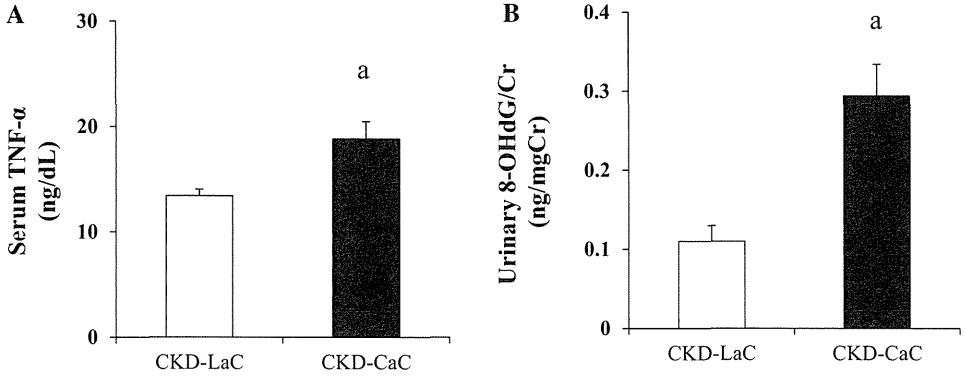

Fig. 7 Different effects of two phosphate binders on oxidative stress and inflammation markers (protocol 3, treatment study). **a** Serum TNF-α level and **b** urinary 8-OHdG/Cr ratio. Serum TNF-α was used as a marker of inflammation, and urinary 8-OHdG/Cr ratio was used as the marker of systemic oxidative stress level. CKD-CaC: uremic rats treated with 6 % calcium carbonate; CKD-LaC: uremic rats treated with 6 % lanthanum carbonate. *Cr* creatinine, *8-OHdG* 8-hydroxy-2'-deoxyguanosine, *TNF-α* tumor necrosis factor-alpha. Data are expressed as mean ± SEM. A two-tailed $P < 0.05$ was considered statistically significant. [a]$P < 0.05$ versus CKD-LaC

There are several limitations in the present study. First, we selected LaC, not sevelamer hydrochloride, as a non-Ca-containing Pi binder. Clinically, sevelamer is widely used and avoids the Ca-loading seen with CaC. Because sevelamer is shown to have anti-inflammatory effects [39–41], we may have observed off-target effects on the serum TNF-α level, urinary 8-OHdG level, and aortic Ca content if we had used sevelamer in the present study. Second, the ability of 6 % LaC to lower urinary Pi level was similar to that of 6 % CaC. Clinically, LaC is shown to be more effective than CaC in binding intestinal Pi [42–44], but in the present study, LaC and CaC were comparable in reducing urinary Pi excretion, indicating that their Pi-binding capacities were almost equivalent. Animal studies have also shown that the same dose of LaC is as effective as CaC [45]. These results indicate that Pi-binding capacity can vary depending on the situation where a given Pi binder is used. Thus, further studies are needed to clarify the difference between CaC and LaC in the Pi-binding capacity and their differential effects on VC and inflammation within the uremic milieu.

In conclusion, Pi overload-induced cellular senescence of VSMCs and arterial medial calcification in uremic rats was inhibited by Pi binder treatment in a novel rat model that showed extensive and robust arterial medial calcification. These results suggest that cellular senescence can play a role in the pathogenesis of Pi overload-related, arterial medial calcification in CKD, and that Pi binders can counteract the cellular senescence of VSMCs, leading to amelioration of VC. Furthermore, our new rat model enables researchers to investigate uremia-related sequela, including VC, because this modified uremic rat model has a longer survival period and develops more consistent and robust phenotypes.

Conflict of interest Shunsuke Yamada, Narihito Tatsumoto, Masanori Tokumoto, Hideko Noguchi, Hiroaki Ooboshi, Takanari Kitazono, and Kazuhiko Tsuruya have nothing to disclose.

Human and Animal Rights and Informed Consent All experimental protocols were reviewed and approved by the Committee on Ethics of Animal Experimentation at Kyushu University Faculty of Medicine (A25-073-1).

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Angiotensin I-Converting Enzyme Gene Polymorphism Enhances the Effect of Hypercholesterolemia on the Risk of Coronary Heart Disease in a General Japanese Population: The Hisayama Study

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Aim: The angiotensin I-converting enzyme (ACE) insertion (I)/deletion (D) polymorphism has been reported to be implicated in susceptibility to coronary heart disease (CHD). However, this association remains inconclusive. The purpose of this study was to clarify the association between the I/D polymorphism of the ACE gene and the development of CHD in a Japanese general population and investigate whether the effects of traditional risk factors on the risk of CHD are heterogeneous among ACE genotypes.

Methods: The subjects included 2,125 community-dwelling individuals 40 years of age or older without cardiovascular disease for whom genetic information was available. All patients were prospectively followed for 19 years, and the association between the ACE polymorphism and the incidence of CHD was examined based on the interactions with traditional risk factors, such as hypercholesterolemia, hypertension, diabetes and smoking.

Results: A total of 161 CHD events occurred during the follow-up period. The age- and sex-adjusted incidence of CHD was not significantly different among the genotypes (5.8, 5.2, and 6.9 per 1,000 person-years for genotypes II, ID and DD, respectively). In a stratified analysis, however, the ACE DD genotype was found to significantly accelerate the risk of developing CHD by hypercholesterolemia (hazard ratio [HR]=4.50, 95% confidence interval=2.02-10.04 for hypercholesterolemia with the DD genotype; HR=1.48, 95% CI=1.04-2.12 for hypercholesterolemia with the ID+II genotype; P for interaction=0.01), even after adjusting for other confounding factors, whereas no such associations were observed for hypertension, diabetes or smoking.

Conclusions: The current findings suggest that the ACE DD genotype enhances the effect of hypercholesterolemia on the development of CHD in the general Japanese population.

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Key words: ACE polymorphism, Coronary heart disease, Cohort study, Hypercholesterolemia

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Introduction

Coronary heart disease (CHD) is a major cause of morbidity and mortality in industrialized countries[1]. Accumulated evidence has identified a number of risk factors, including hypercholesterolemia, hypertension, diabetes and smoking, for the development of

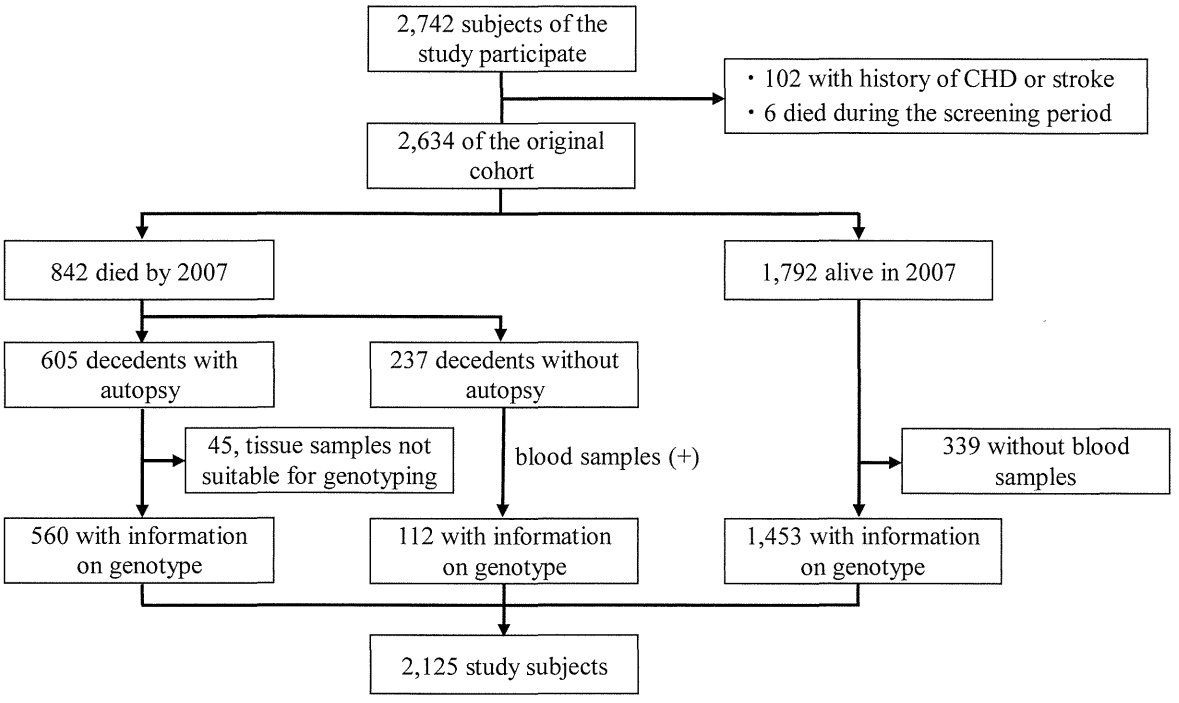

Fig. 1. Flow diagram of the selection of the study subjects.

CHD[2-4]. The renin-angiotensin system has also been acknowledged to be involved in the pathogenetic mechanisms of CHD. The plasma activity of angiotensin-I converting enzyme (ACE) regulates the production of angiotensin II, which has been shown to promote inflammation, reactive oxygen species generation, cell proliferation/apoptosis, fibrosis and oxidized lipid production[5].

The ACE gene contains a polymorphism characterized by either the insertion (I) or deletion (D) of a 287-base pair *Alu* repetitive sequence in intron 16. The D allele of this gene site has been reported to be associated with an increased plasma ACE activity[6]. Since being first reported by Cambien *et al.*[7], this polymorphism has received much attention as a susceptibility gene for CHD[8]. On the other hand, contrary evidence suggesting that there is no association between this polymorphism and the risk of CHD has also been reported[9, 10]. In addition, recent findings of observational studies and clinical trials have raised the possibility that the ACE gene polymorphism modifies the effects of cardiovascular risk factors and their treatment on the risk of CHD[11-15].

In order to clarify these issues, we established a community-based prospective cohort study to explore both environmental and genetic risk factors for cardiovascular disease in Japan. The aim of this study was to elucidate the association between the I/D polymorphism of the ACE gene and the development of CHD in a general Japanese population and investigate whether the influence of traditional risk factors on the risk of CHD is heterogeneous among ACE genotypes.

Methods

Study Protocol

The Hisayama Study is a prospective population-based cohort study of cerebro- and cardiovascular diseases established in 1961 in the town of Hisayama, a suburban community adjacent to Fukuoka City, a metropolitan area on Kyushu Island in Japan. Full community surveys of the health status of the residents have been repeated since that time[16]. In 1988, a screening survey was performed in the town for the present study. A detailed description of this survey has been published previously[17]. Briefly, a total of 2,742 residents 40 years of age or older (80.9% of the total population in that age group) consented to participate in the examination and underwent comprehensive assessments. After excluding 102 subjects with a history of CHD or stroke and six subjects who died during the screening period, a total of 2,634 subjects were registered as the original cohort (**Fig. 1**). In this population, 842 subjects died by 2007, of whom 605 (71.9%) underwent autopsies. In the autopsy cases, tissue samples of the main organs, such as the brain, heart,

lungs, liver, spleen, gastrointestinal tract and kidneys, were formalin fixed, paraffin embedded and stored until 2000, after which they were fresh frozen. Among these cases, 45 patients had tissue samples not suitable for genotyping; thus, genotyping was performed in the remaining 560 cases. Among the deceased subjects who did not undergo an autopsy, 112 provided blood samples before their death. In the surviving subjects, blood samples were gathered from 1,453 participants. Hence, suitable paraffin-embedded tissues, fresh-frozen tissues or blood samples were available for a total 2,125 subjects (866 men and 1,259 women, 80.7% of the original cohort), who were then selected for ACE gene I/D genotyping and enrolled in the present analysis.

Follow-up Survey

The subjects were followed prospectively for 19 years, from December 1988 to November 2007, using repeated health examinations. The health status of subjects who did not undergo regular examinations or who moved out of the town was checked yearly by mail or telephone. We also established a daily monitoring system incorporating the study team and local physicians or members of the town's Health and Welfare office. When CHD was diagnosed or suspected, physicians on the study team examined the subject and evaluated his/her detailed clinical information. The clinical diagnosis of CHD was made based on the patient's history, physical examination findings and ancillary laboratory examination data. Furthermore, when a subject died, an autopsy was performed at the Department of Pathology at Kyushu University. There was no true loss to follow-up during the follow-up period (autopsy rate: 71.9%).

Definition of Coronary Heart Disease

The criteria for a diagnosis of CHD included first-ever acute myocardial infarction, silent myocardial infarction, sudden cardiac death within one hour after the onset of acute illness or coronary artery disease followed by coronary artery bypass grafting or angioplasty[18-20]. Acute myocardial infarction was diagnosed in subjects who met at least two of the following criteria: (1) typical symptoms, including prolonged severe anterior chest pain; (2) abnormal cardiac enzymes more than twice the upper limit of the normal range; (3) evolving diagnostic electrocardiographic changes; and (4) morphological changes, including local asynergy of cardiac wall motion on echocardiography, persistent perfusion defects on cardiac scintigraphy and/or myocardial necrosis or scars ≥ 1 cm long accompanied by coronary atherosclerosis at autopsy. Silent myocardial infarction was defined as myocardial scarring without historical indications of clinical symptoms or abnormal changes in cardiac enzymes. The clinical diagnosis was corrected according to the autopsy findings when necessary.

Definition of the ACE Polymorphism

Extraction of DNA from the blood samples and fresh-frozen tissue samples was performed as described previously, respectively[21, 22]. The ACE I/D genotype was determined using the polymerase chain reaction method, as described by Evans *et al.*[23]. For paraffin-embedded tissues, DNA was extracted using an automatic nucleic acid isolation system (NA-2000; Kurabo Inc., Osaka, Japan), and the ACE I/D genotype was determined using the double polymerase chain reaction method[24]. The accuracy of these genotyping methods was demonstrated in our previous study[24].

Risk Factor Measurement

At baseline, each participant completed a self-administered questionnaire covering their medical history, treatment for hypertension or diabetes, alcohol intake and smoking and exercise habits. The questionnaires were reviewed by trained interviewers at the time of screening. Smoking and alcohol intake were classified as currently habitual or not. Subjects engaging in sports or other forms of exertion ≥ 3 times a week during their leisure time made up the regular exercise group. Blood pressure was measured three times using a standard mercury sphygmomanometer in the sitting position after at least five minutes of rest. The mean of the three measurements was used for the analysis. Hypertension was defined as a blood pressure of ≥ 140/90 mmHg and/or the current use of antihypertensive agents. Body height and weight were measured in light clothing without shoes, and the body mass index (kg/m^2) was calculated. Obesity was defined as a body mass index of ≥ 25 kg/m^2. Electrocardiogram abnormalities included left ventricular hypertrophy (Minnesota Code, 3-1), ST depression (4-1, 2, 3) or atrial fibrillation (8-3). The blood samples were collected from the antecubital vein after an overnight fast to determine the lipid and blood glucose levels. The serum total cholesterol concentrations were determined enzymatically. Hypercholesterolemia was defined as a serum cholesterol level of ≥ 5.69 mmol/L (220 mg/dL). The plasma glucose levels were measured based on the glucose oxidase method. Diabetes was defined according to the criteria recommended by the American Diabetes Association, in addition to a medical history of diabetes, using 75 g oral glucose tolerance tests in 1,992 subjects (93.7%)[25]

Table 1. Clinical characteristics of the study population according to the ACE genotype in 1988

Variable	ACE genotype			*p* value for trend
	II (n=918)	ID (n=927)	DD (n=280)	
Age, y	59 ± 11	60 ± 12	60 ± 11	0.06
Men, %	40.3	41.8	38.2	0.80
Systolic blood pressure, mmHg	133 ± 21	134 ± 22	135 ± 21	0.44
Diastolic blood pressure, mmHg	78 ± 12	77 ± 11	78 ± 11	0.62
Antihypertensive medication, %	13.1	14.7	14.8	0.22
Hypertension, %	39.2	41.4	42.6	0.24
Diabetes, %	9.8	12.1	13.1	0.06
Serum total cholesterol, mmol/L	5.33 ± 1.09	5.37 ± 1.05	5.39 ± 1.10	0.32
Hypercholesterolemia, %	36.2	36.1	33.4	0.42
Body mass index, kg/m^2	23.0 ± 3.2	22.7 ± 3.1	22.8 ± 3.1	0.37
Obesity, %	24.2	22.6	22.5	0.54
Electrocardiogram abnormalities, %	17.0	15.6	17.8	0.93
Smoking habits, %	24.9	23.2	25.6	0.63
Alcohol intake, %	30.0	29.7	31.3	0.70
Regular exercise, %	9.4	11.4	13.7	0.03

ACE, angiotensin I-converting enzyme; HDL, high-density lipoprotein.
The values are presented as the mean ± standard deviation or percentage.

and the fasting and postprandial glucose concentrations in the 133 remaining subjects.

Statistical Analysis

The SAS computer package version 9.3 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. The age- and sex-adjusted mean values of the possible risk factors were calculated and tested according to the analysis of covariance method, and trends in these parameters across the ACE polymorphisms were tested using a multiple regression analysis. The frequency of each risk factor was adjusted for age and sex using the direct method, and trends were examined using a logistic regression analysis. The incidence of CHD was calculated according to the person-year method and adjusted for age and sex using the direct method with 10-year age groups. Differences in the incidence of CHD between the subjects with and without the ACE genotype were analyzed by means of a Cox proportional hazards regression analysis after adjusting for age and sex. Cumulative incidence curves for CHD were estimated according to the Kaplan-Meier method, and differences in incidence were assessed using the log-lank test. Age- and sex-adjusted or multivariable-adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) were also estimated using a Cox proportional hazards model. The interactions in the associations between subgroups were tested by adding a multiplicative interaction term to the relevant Cox model. Two-tailed p values of <0.05 were considered to be statistically significant in all analyses.

Ethics Approval

This study was conducted with the approval of the Kyushu University Institutional Review Board for Clinical Research. Written informed consent for medical research was obtained from each participant or their family members if deceased.

Results

The frequency of each ACE genotype was 43.2% for II, 43.6% for ID and 13.2% for DD; these genotype frequencies were in agreement with the Hardy-Weinberg equilibrium (chi-square, 2.14; *df*, 2; p= 0.66) and are similar to those found in previous studies of Japanese populations[26, 27]. **Table 1** shows the baseline clinical and demographic characteristics of the study subjects according to the ACE genotypes of II, ID and DD. The frequency of regular exercise was higher in the subjects with the DD genotype than in those with the ID or II genotype. There was no evidence of differences in the mean values or frequencies of other risk factors across the ACE genotypes.

During the 19-year follow-up period, 161 first-

Table 2. Age- and sex-adjusted incidence and adjusted HR for coronary heart disease according to the ACE genotype in the 2,125 subjects during the 19-year follow-up period

ACE genotype	Person-years at risk	No. of events	Age- and sex-adjusted incidence (per 1,000 PYs)	Age- and sex-adjusted		Multivariable-adjusted[a]		ACE genotype and interaction	Multivariable-adjusted[a]	
				HR (95% CI)	*p* value	HR (95% CI)	*p* value		HR (95% CI)	*p* value
II	14,890	68	5.8	1.00 (reference)		1.00 (reference)		II	1.00 (reference)	
ID	14,963	66	5.2	0.85 (0.61-1.19)	0.35	0.85 (0.60-1.20)	0.36	ID	0.69 (0.44-1.08)	0.10
DD	4,507	27	6.9	1.18 (0.76-1.85)	0.46	1.18 (0.75-1.85)	0.47	DD	0.60 (0.29-1.23)	0.16
								INT (ID x HC)	1.60 (0.80-3.19)	0.19
								INT (DD x HC)	3.33 (1.30-8.56)	0.01
II + ID	29,853	134	5.5	1.00 (reference)		1.00 (reference)		II + ID	1.00 (reference)	
DD	4,507	27	6.9	1.29 (0.85-1.95)	0.23	1.28 (0.85-1.95)	0.24	DD	0.72 (0.36-1.44)	0.36
								INT (DD x HC)	2.63 (1.10-6.32)	0.03
II	14,890	68	5.8	1.00 (reference)		1.00 (reference)		II	1.00 (reference)	
DD + ID	19,470	93	5.6	0.93 (0.68-1.27)	0.63	0.93 (0.67-1.28)	0.64	DD + ID	0.70 (0.46-1.07)	0.10
								INT (DD + ID x HC)	1.88 (0.99-3.58)	0.06
I	44,743	202	5.6	1.00 (reference)		1.00 (reference)		I	1.00 (reference)	
D	23,977	120	5.8	1.03 (0.82-1.29)	0.80	1.03 (0.82-1.30)	0.79	D	0.75 (0.54-1.03)	0.07
								INT (D x HC)	1.89 (1.20-2.99)	0.006

ACE, angiotensin I-converting enzyme; PYs, person-years; HR, hazard ratio; CI, confidence interval; HC, hypercholesterolemia; INT, interaction term
In the analysis of the I/D allele, the number of subjects and events was counted twice.
a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise.

ever CHD events (98 men and 63 women) were noted. As shown in **Table 2**, the age- and sex-adjusted incidence of CHD (per 1,000 person-years) was 5.8, 5.2 and 6.9 for the ACE genotypes II, ID and DD, respectively. There were no significant differences in the age- and sex-adjusted HRs for the development of CHD among the ACE genotypes. These associations remained substantially unchanged following adjustment for age, sex, ACE genotype, hypercholesterolemia, hypertension, diabetes, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise. The same was true for the analysis of a genetic inheritance pattern of the recessive or dominant genotype as well as the analysis of the I/D allele. In addition, we investigated the interactions between the cardiovascular risk factors and the ACE genotype with respect to the development of CHD. The interaction terms between hypercholesterolemia and the ACE genotypes, except for the dominant genotype, were significant (**Table 2**). On the other hand, there was no evidence of a significant interaction regarding the extent of the effect of other cardiovascular risk factors, namely age, sex, hypertension, diabetes, obesity, smoking, alcohol intake and regular exercise, between the two ACE genotype subgroups (all *p* values for interaction >0.11).

Next, we compared the influence of the cardiovascular risk factors on the development of CHD among the subgroups of the ACE genotype (II + ID and DD), as individuals with the DD genotype have been reported to exhibit an increased plasma ACE activity[6]. When the current subjects were divided into four groups according to the ACE genotype and hypercholesterolemia, the cumulative incidence of CHD was found to be significantly higher in the subjects with the DD genotype and hypercholesterolemia than in those with the II + ID genotypes and non-hypercholesterolemia (**Fig. 2**). As shown in **Fig. 3**, after adjusting for the aforementioned confounding factors, hypercholesterolemia was found to be significantly associated with a 1.53-fold (95% CI 1.06-2.20) higher risk of incident CHD in the subjects with the II + ID genotypes, whereas this risk factor exerted much greater influence in the subjects with the DD genotype (HR 4.02, 95% CI 1.79-9.02; *p* for interaction = 0.03). When the serum total cholesterol levels were used as a continuous variable in the analysis, every 1 mmol/L increment in the serum total cholesterol level was identified to be associated with a 1.16-fold (95% CI, 0.98-1.36) higher risk of CHD in the subjects with the II + ID genotypes and a 1.78-fold (95% CI, 1.29-2.45) higher risk in those with the DD genotype (*p* for heterogeneity = 0.02). Similar findings were observed in the analysis of the I/D allele (**Supplementary Fig. 1**), whereas no significant interactions were observed in the analysis of the ID + DD/II genotypes (**Supplementary Fig. 2**).

We also performed a sensitivity analysis using the data for sudden cardiac death within 24, rather than one, hours after the onset of acute illness. Conse-