Table 3 Hazard ratios for low tertile 1 vs tertiles 2–3 of plasma sex hormone levels for all-cause mortality in men and women

	Unadjusted	Model 1	Model 2
Men (n = 117)			
HR of low testosterone for mortality	3.83 (1.74–8.40)**	3.71 (1.54–8.04)**	3.27 (1.24–12.91)*
Excluding first-year deaths (n = 108)	3.81 (1.53–6.93)**	3.49 (1.14–7.39)**	3.08 (1.11–13.62)*
Excluding deaths from cancer (n = 113)	4.18 (1.77–9.86)**	4.03 (1.70–9.58)**	5.02 (1.51–15.41)*
Women (N = 97)			
HR of low DHEA-S for mortality	3.77 (1.77–8.07)**	3.86 (1.79–8.32)**	4.42 (1.51–12.90)*
Excluding first-year deaths (n = 95)	3.38 (1.55–7.37)**	3.43 (1.56–9.54)**	3.58 (1.12–11.46)*
Excluding deaths from cancer (n = 92)	3.82 (1.69–8.60)**	3.55 (1.54–8.19)**	3.92 (1.28–11.98)*

*P < 0.05; **P < 0.01 vs reference group (tertile 2–3). Values are expressed as HR (95% CI). Model 1, adjusted for age; Model 2, adjusted for age, nutritional parameters, functional parameters and prevalent disease. DHEA-S, dehydroepiandrosterone sulfate; HR, hazards ratio.

calculated free testosterone were not measured, because a direct assay of bioavailable testosterone or an assay of sex hormone binding globulin, which is necessary for free testosterone calculation, is not available in Japan. However, because most of the above-mentioned previous reports have shown an association of total testosterone with mortality, the fundamental findings might not have differed if active forms of testosterone had been analyzed.

In conclusion, a low testosterone level in men and a low DHEA-S level in women are associated with increased mortality risk, independent of multiple risk factors and several pre-existing health conditions in disabled elderly. To our knowledge, the present study is the first that showed testosterone as a predictor of mortality in Asian men. Also, this is the first study that investigated frail or disabled older persons receiving care at facilities. Our results imply the clinical importance of measuring plasma androgen levels even in disabled elderly to estimate their prognosis.

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Relationship between interleukin-6 and cerebral deep white matter and periventricular hyperintensity in elderly women

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Aim: We evaluated the relationships between serum levels of high-sensitivity C-reactive protein (hsCRP) and interleukin (IL)-6 with the severity of leukoaraiosis.

Methods: One hundred and thirty-seven elderly women who attended the Center for Comprehensive Care on Memory Disorders at Kyorin University Hospital were enrolled in this study. Leukoaraiosis was assessed by periventricular hyperintensity (PVH) score and deep white matter hyperintensity (DWMH) score.

Results: Serum log IL-6 level correlated with PVH and DWMH scores, but hsCRP did not. By multinomial logistic analysis, IL-6 was significantly related to DWMH score, independent of age and systolic blood pressure.

Conclusion: IL-6 is presumably an important marker of leukoaraiosis, as is the case with silent cerebral infarction. **Geriatr Gerontol Int 2011; 11: 328–332.**

Keywords: interleukin-6, leukoaraiosis, white matter hyperintensity.

Introduction

Leukoaraiosis, an isointense lesion on T_1-weighted images and hyperintense lesion on T_2-weighted images of magnetic resonance imaging (MRI), is considered to be a type of ischemic change in the brain on the basis of decreased blood flow in the area of leukoaraiosis.[1] In addition, leukoaraiosis is likely to have a relationship with vascular risk factors such as hypertension and diabetes.[2] On the other hand, the severity of leukoaraiosis also has a relationship with symptoms of the geriatric syndromes such as dementia, gait disturbance and functional disability.[3–5] Hence, leukoaraiosis is regarded as a significant brain lesion linking vascular risk factors and the occurrence of geriatric syndromes. Previous research on leukoaraiosis showed that women tended to have more white matter lesions than men,[6] and progression of deep white matter hyperintensity (DWMH) lesion was grater in women than men.[7] Furthermore, Gouw *et al.* showed that leukoaraiosis tended to develop greater in women than men and lacunes were vice versa.[8] Recently, many studies have focused on the relationships between brain ischemia and inflammation. Above all, Hoshi *et al.* demonstrated that serum high-sensitivity C-reactive protein (hsCRP) and interleukin (IL)-6 levels correlated with silent brain infarction.[9] They suggested an involvement of inflammation in cerebral infarction. However, few studies have examined the relationships between inflammatory markers and other cerebral ischemic changes such as leukoaraiosis.Therefore, we investigated whether serum levels of hsCRP and IL-6 have a relationship with leukoaraiosis in elderly women.

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Methods

Patients

One hundred and thirty-seven women who attended the Center for Comprehensive Care on Memory Disorders at Kyorin University Hospital were included in this study. This study was approved by the Ethics Committee of Kyorin University School of Medicine. Accordingly, written informed consent was obtained from all patients.

MRI

Magnetic resonance imaging (MRI) was performed on 1.5-T scanners (Toshiba Medical Systems, Tochigi, Japan). T_1-weighted images (repetition time [TR] = 496 msec, echo time [TE] = 12 msec), T_2-weighted images (TR = 4280 msec, TE = 105 msec) and fluid attenuated inversion recovery-weighted images (TR = 8000 msec, TE = 105 msec, 5 mm slice thickness) were obtained in the axial planes.

Periventricular hyperintensity and DWMH Score

Leukoaraiosis was classified as periventricular hyperintensity (PVH) adjacent to the lateral ventricle, and DWMH located in the deep white matter apart from the lateral ventricles. PVH was evaluated in six regions in three slices. Each region was rated as five grades (0–4) according to the systematic quantification method developed by Junque *et al.*[3] The sum of all grades in the six regions was defined as the PVH score (range 0–40).[4] DWMH was evaluated in the frontal, temporal, parietal and occipital lobes and in the basal ganglia in both hemispheres. Each lesion was rated as three grades according to the diameter, as described by de Groot *et al.*[5] The sum of all grades in five regions in both hemispheres was defined as the DWMH score.[4]

Laboratory tests

Blood samples were obtained in the morning after an overnight fast. Serum levels of hsCRP and IL-6 were measured using nephelometry and enzyme-linked immunosorbent assay, respectively. The intra-assay coefficients of variation for the measurements of hsCRP and IL-6 were 1.3% and 2.9%, respectively.

Statistical analysis

Because the distribution of hsCRP and IL-6 levels appeared to be left-skewed, they were normalized by logarithmic transformation. We used Spearman's ρ to investigate correlations between parameters and PVH score or DWMH score. Also, to test independently the effect of the inflammatory markers associated with the severity of leukoaraiosis, multinomial logistic regression analysis was performed with the grade of PVH (tertiles of PVH score) or DWMH (tertiles of DWMH score) as the dependent variable; and hsCRP or IL-6, together with age and systolic blood pressure (SBP) as independent variables. $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS ver. 17.0.

Results

The characteristics of the study subjects are shown in Table 1. They were non-obese normolipidemic elderly persons, however, SBP was elevated. The distribution of PVH score and DWMH score of these subjects were 1–24 and 0–209, respectively. In Spearman's correlation coefficient, IL-6 correlated with PVH score ($\rho = 0.340$, $P \leq 0.05$) and DWMH score ($\rho = 0.299$, $P \leq 0.05$) (Fig. 1), whereas hsCRP showed no relation to PVH score or DWMH score (Table 2). PVH score and DWMH score also correlated with age and SBP. When log IL-6 and log hsCRP were grouped by tertile (see legend to Fig. 2), it was found that the average PVH score and DWMH score were higher in the highest tertile of IL-6 level than in the lowest tertile according to the Kruskal–Wallis test (Fig. 2a,b). On the other hand, this increment was not found in hsCRP (Fig. 2c,d).

Because leukoaraiosis can be observed on MRI even in normal elderly persons,[10] and hypertension is known to be a risk factor for leukoaraiosis,[11] we performed multinomial logistic regression analysis using PVH or DWMH severity (tertiles of PVH and DWMH score) as the dependent variable, and age, SBP and inflammatory

Table 1 Clinical characteristics of study subjects (women, $n = 137$)

Age (years)	76 ± 7
BMI (kg/m^2)	20.8 ± 3.3
SBP (mmHg)	142 ± 26
DBP (mmHg)	80 ± 14
PVH score (points)	8.2 ± 4.0
DWMH score (points)	61.4 ± 51.0
Total cholesterol (mmol/L)	5.38 ± 0.91
HDL cholesterol (mmol/L)	1.50 ± 0.36
LDL cholesterol (mmol/L)	3.23 ± 0.65
Triglyceride (mmol/L)	1.08 ± 0.46
Log IL-6 (ng/L)	0.35 ± 0.46
Log hsCRP (μg/L)	2.58 ± 0.58

All parameters are expressed as mean ± standard deviation. IL-6 and CRP are shown as log transformed. BMI, body mass index; DBP, diastolic blood pressure; DWMH, deep white matter hyperintensity; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDL, low-density lipoprotein; PVH, periventricular hyperintensity; SBP, systolic blood pressure.

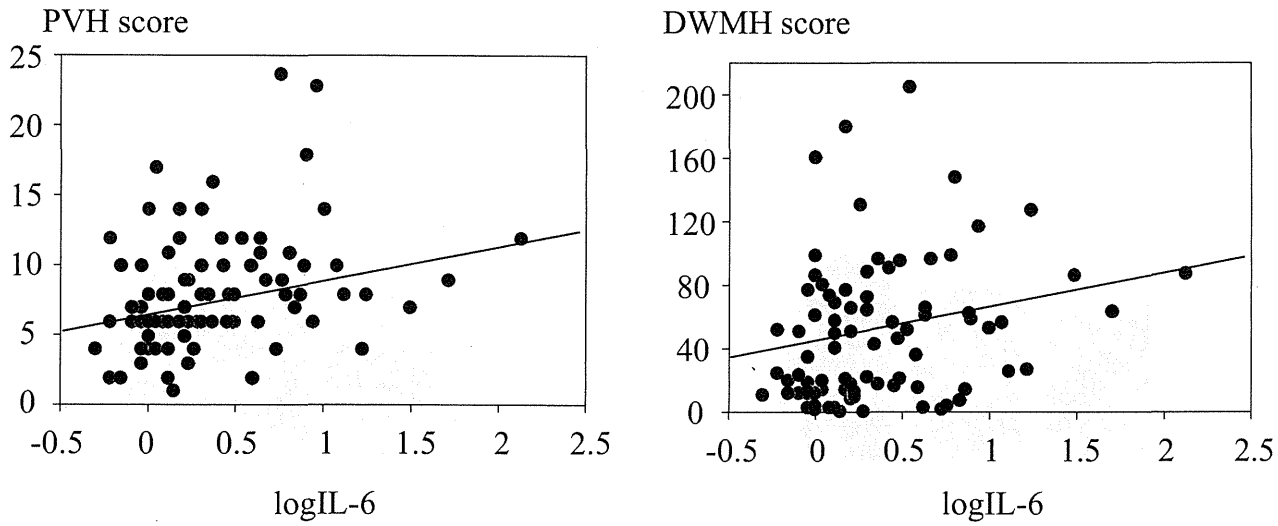

Figure 1 Relations between periventricular hyperintensity (PVH) score and log interleukin (IL)-6 (left panel; $\rho = 0.340$, $P \leq 0.05$, $n = 137$), and deep white matter hyperintensity (DWMH) score and log IL-6 (right panel; $\rho = 0.299$, $P \leq 0.05$, $n = 137$).

Table 2 Spearman's correlation coefficient between leukoaraiosis and parameters

	PVH score		DWMH score	
	ρ	*P*	ρ	*P*
Age	0.411	<0.001	0.271	0.002
BMI	−0.156	0.085	−0.124	0.179
SBP	0.215	0.014	0.232	0.009
Total cholesterol	−0.128	0.192	−0.149	0.134
HDL cholesterol	−0.053	0.595	−0.205	0.041
LDL cholesterol	−0.093	0.349	−0.025	0.802
Triglyceride	−0.014	0.885	0.080	0.421
Smoke	0.337	0.005	0.443	0.000
Log IL-6	0.340	0.002	0.299	0.006
Log hsCRP	−0.018	0.867	0.019	0.855

BMI, body mass index; DBP, diastolic blood pressure; DWMH, deep white matter hyperintensity; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDL, low-density lipoprotein; PVH, periventricular hyperintensity; SBP, systolic blood pressure.

markers as independent variables. As shown in Table 3, it was confirmed that the level of IL-6 was significantly associated with the progression of PVH grade (from lowest to middle and middle to highest) and DWMH score (from middle to highest). However, this trend was not found in hsCRP.

Discussion

In this study, we showed relationships between IL-6 and PVH score and IL-6 and DWMH score. It is assumed that IL-6 has an association with cerebral ischemic changes such as leukoaraiosis as well as silent brain infarction.[9] Additionally, PVH and DWMH were correlated with IL-6, but not with hsCRP. With respect to this point, Schmidt *et al.* suggested that CRP is a marker of active carotid atherosclerosis, but not of a small vessel disease-related brain lesion.[12] On the other hand, it is envisaged that elevated hsCRP levels generally reflect large vessel atherosclerosis. Because leukoaraiosis is regarded as one of the brain changes caused by small vessel disease, our results support the idea of Schmidt *et al.*

Interleukin-6 is one of the principal acute-phase reactants, playing a significant role in the activation of the coagulation–fibrinolysis system. On the other hand, leukoaraiosis has been associated with a hypercoagulable condition. Endothelium-derived adhesion molecules have been reported to be elevated in patients with great leukoaraiosis or lacunar infarcts. Leukocyte-mediated injury of the small vessels and ensuing upregulation of endothelial adhesion molecules are implicated in the pathogenesis of leukoaraiosis.[13]

The Rotterdam Scan Study showed that higher hsCRP levels were associated with presence and progression of leukoaraiosis after adjustment for cardiovascular risk factors and carotid atherosclerosis.[14] The subjects in the Rotterdam Scan Study were a population-based cohort ($n = 1033$), while the subjects in the present study were outpatients in the memory clinic ($n = 137$). In this respect, the difference in characteristics and numbers of the subjects may have given rise to the different results in terms of hsCRP in the present study and the Rotterdam Scan Study.

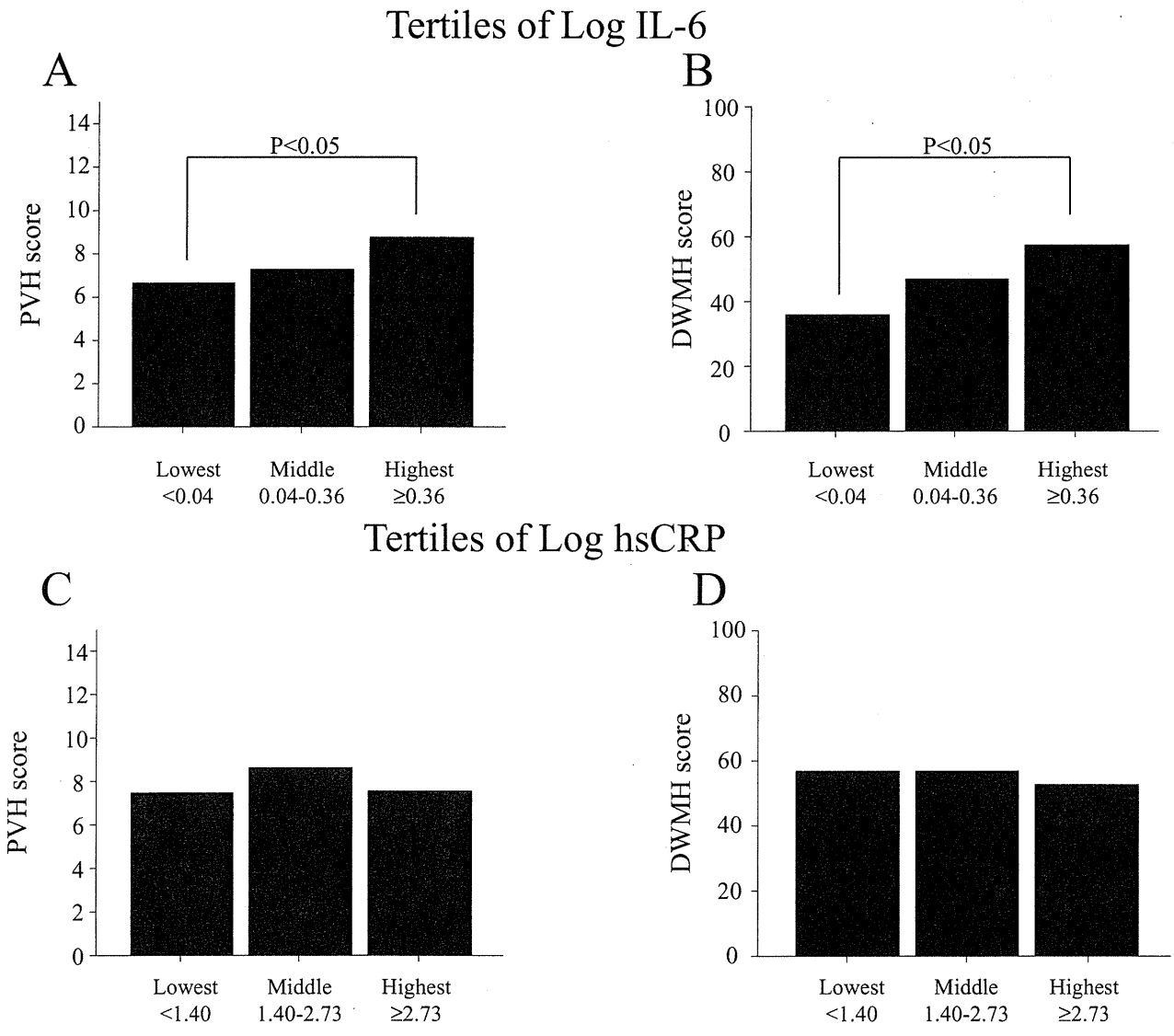

Figure 2 Average of periventricular hyperintensity (PVH) score and deep white matter hyperintensity (DWMH) score by tertile of interleukin (IL)-6 (a,b) and tertile of high-sensitivity C-reactive protein (hsCRP) (c,d). Log IL-6 tertile; lowest, <0.04 pg/mL, $n = 55$, 73.4 ± 7 1 years old (y/o); middle, 0.04–0.36 pg/mL, $n = 38$, 76.9 ± 6.8 y/o; highest, ≥0.36 pg/mL, $n = 44$, 79.5 ± 5.3 y/o. Log hsCRP; lowest, <1.40 ng/mL, $n = 44$, 73.9 ± 7.0 y/o; middle, 1.40–2.73 ng/mL, $n = 46$, 77.6 ± 7.1 y/o; highest, ≥2 73 ng/mL, $n = 41$, 77.8 ± 6.3 y/o.

In the Framingham Heart Study, no association was found between hsCRP and leukoaraiosis on MRI.[15] In the Cardiovascular Health Study, hsCRP level was modestly associated with semi-quantified leukoaraiosis volume, but the effect attenuated after excluding prevalent cerebrovascular and coronary disease cases.[13] In addition, Wright *et al.* was not able to find an association between hsCRP and leukoaraiosis volume.[16] Together, the relationships between leukoaraiosis and hsCRP varied depending upon different reports. This may come from the difference in study subjects and analytical methods. Further investigation is necessary to hold more definite opinion about which inflammatory biomarker represents the presence and development of leukoaraiosis.

Several lines of evidence suggest a relationship between IL-6 and symptoms of the geriatric syndromes, unique features of common health problems associated with poor morbidity in elderly people, such as dementia,[17] functional disability[18] and frailty.[19] On the other hand, the severity of leukoaraiosis also has a relationship with symptoms of geriatric syndromes such as dementia, falls, gait disturbance and functional disability.[3–5] Therefore, IL-6 may be an important biomarker linking the severity of leukoaraiosis to the geriatric syndromes. Because the present study is

Table 3 Associations between inflammation markers and the severity of leukoaraiosis according to tertiles (PVH score or DWMH score) adjusting for age and systolic blood pressure (logistic regression analysis)

	Log hsCRP, μg/L Odds ratio (95% CI)	Log IL-6, ng/L Odds ratio (95% CI)
PVH grade (tertiles)		
Lowest to middle	1.84 (0.78–4.31)	5.80 (1.43–23.60)
Middle to highest	0.39 (0.12–1.32)	4.39 (1.02–18.85)
DWMH grade (tertiles)		
Lowest to middle	0.81 (0.333–1.99)	3.18 (0.78–12.95)
Middle to highest	1.25 (0.48–3.29)	7.85 (1.69–36.38)

Grade of leukoaraiosis according to tertiles of PVH score or DWMH score. CI, confidence interval; DWMH, deep white matter hyperintensity; IL-6, interleukin-6; hsCRP, high-sensitivity C-reactive protein; PVH, periventricular hyperintensity.

cross-sectional, a longitudinal study would corroborate the associations of IL-6 with leukoaraiosis, and IL-6 with the geriatric syndromes.

In conclusion, we demonstrated that IL-6 level is significantly associated with the severity of PVH and DWMH lesions. The results of the present study, together with the previous studies, suggest that IL-6 is an important marker of the progression of cerebral ischemic disease, linking to the presence of geriatric syndromes.

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Association of polypharmacy with fall risk among geriatric outpatients

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Aim: To investigate the association of fall risk with comorbidities and medications in geriatric outpatients in a cross-sectional design.

Methods: A total of 262 outpatients (84 men and 178 women, mean age 76.2 ± 6.8 years) were evaluated. Physical examination, clinical histories and medication profile were obtained from each patient. History of falls in the past year, 22-item fall risk index, 13-point simple screening test for fall, and time interval of one-leg standing test were examined as markers of fall risk.

Results: On univariate analysis, older age, female sex, hypertension, osteoporosis, history of stroke, number of comorbidities, use of antihypertensives, aspirin, bisphosphonates, hypnotics and number of prescribed drugs were significantly associated with either of four indices. On multiple regression analysis, the number of drugs was associated with all of the four indices, independent of other factors associated in the univariate analysis. The association of number of drugs with fall risk indices was stepwise.

Conclusion: In geriatric outpatients, polypharmacy rather than number of comorbidities was associated with fall risk. Prospective and intervention studies are needed to clarify the causal relationship between polypharmacy, comorbidities and fall risk. **Geriatr Gerontol Int 2011; 11: 438–444.**

Keywords: elderly, fall, polypharmacy, risk factors.

Introduction

Falls occur in more than 10% per year of community-dwelling elderly people,[1–3] and approximately 10% of falls lead to bone fracture. Also, falls are reported to be the third leading cause of a bedridden state among the elderly.[4] Previous studies assessed the risk factors of falls in community-dwelling elderly,[5–7] and history of falls, physical ability and living environment were found to be predictors of fall risk. However, these studies have not sufficiently assessed medical comorbidities and therapeutic drugs as risk factors of falls, although many elderly subjects have chronic illness such as hypertension, diabetes, cardiovascular diseases, osteoporosis and insomnia. Falls in patients on medications are more complicated, because some drugs such as aspirin could cause serious bleeding when they have injurious falls, and others such as antihypertensives[8] and hypoglycemic agents[9,10] could cause falls. Therefore, it is important to evaluate the association between fall risk and medical comorbidities or therapeutic drugs. Multiple drug use or polypharmacy is frequently seen in elderly patients because most of them have multiple chronic diseases to be treated. Moreover, inappropriate drug use is frequently seen in patients with polypharmacy.[11]

In Japan, a 22-item fall risk index questionnaire covering physical, cognitive, emotional and social aspects of

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functioning and environmental factors was established.[7] Also, by evaluating the validity of this questionnaire in community-dwelling older people, a simple screening test consisting of five items and total of 13 points was constructed.[2] Using these questionnaires and one-leg standing test[12] as indices of fall risk, we investigated the association of fall risk with comorbidities and medications in geriatric outpatients.

Methods

Patients

A total of 262 consecutive outpatients aged 65 years or older were enrolled who were referred for the treatment of chronic diseases such as hypertension, dyslipidemia, diabetes and osteoporosis every 2–4 weeks at a geriatric clinic located in Tokyo, Japan. All the patients were able to walk independently and were in stable conditions. Patients who had acute illness or overt dementia were excluded. Anthropometric and medical information were obtained including past history of stroke, myocardial infarction and malignancy. All the medical information including diagnoses and the prescribed drugs were obtained from the medical chart recorded by their physicians in charge. The patients whose prescriptions were changed within 1 month before enrollment were excluded. Accordingly, the included subjects had been taking the same drugs for at least 1 month before enrollment.

Ethical consideration

This study was approved by the Institutional Review Board of the Research Institute of Aging Science. We obtained written consent from all participants and/or their guardians.

Four indices of fall tendency

On the day of the enrollment, all patients were examined for four indices to investigate the fall risk: (i) history of fall in the past year (no or yes); (ii) a 22-item portable fall risk index questionnaire developed by the working group of the Ministry of Health, Labor and Welfare (see Appendix I);[7] (iii) 13-point simple screening test to assess the risk of fall which was also developed by the same working group (see Appendix II);[2] and (iv) duration time of open-eye one-leg standing test.

Table 1 Characteristics of study subjects

Age			76.2 ± 6.8 years old
Male	32.1%	(n = 84)	75.3 ± 6.6 years old
Female	67.9%	(n = 178)	76.6 ± 6.8 years old
Comorbidities			
Hypertension	64.1%	(n = 168)	
Dyslipidemia	47.7%	(n = 125)	
Diabetes	18.7%	(n = 49)	
Osteoporosis	24.0%	(n = 63)	
History of stroke	6.5%	(n = 17)	
History of myocardial infarction	3.4%	(n = 9)	
History of cancer	5.3%	(n = 14)	
Number of comorbidities	1.90 ± 1.09		
Drug use			
Antihypertensive use	57.6%	(n = 151)	
Calcium channel blockers	39.3%	(n = 103)	
Angiotensin-II receptors blockers	34.7%	(n = 91)	
Beta-blocker	6.9%	(n = 18)	
Angiotensin converting enzyme inhibitors	5.7%	(n = 15)	
Diuretics	5.0%	(n = 13)	
Statins	24.4%	(n = 64)	
Sulfonylureas	6.5%	(n = 17)	
Aspirin	20.6%	(n = 54)	
Vitamin D	4.6%	(n = 12)	
Bisphosphonates	6.5%	(n = 17)	
H_2-blockers	9.9%	(n = 26)	
Proton pump inhibitors	6.5%	(n = 17)	
Hypnotics	18.3%	(n = 48)	
Number of drugs	3.4 ± 2.8		

Values are expressed as mean ± standard deviation.

Experience of falls in the past year is an established and powerful tool for assessing fall risk,[2] and was reported by the patient and/or his or her family members. Duration time of one-leg standing test, which can be carried out in a narrow limited space of the outpatient office, was measured using the leg with the eyes open, until the raised leg was put down on the floor. We examined both right and left legs once for each, and the longer of the two measurements was used for statistical analysis.[12]

Data analysis and statistical methods

Values are expressed as means ± standard deviation. In order to analyze the relationship between each fall risk index and comorbidities or drugs, variables were compared using Student's *t*-test or the χ^2-test as appropriate. The correlations between the two continuous variables were analyzed using Pearson's *r* coefficient. In multivariate analysis, logistic regression analysis was performed for history of falls and multiple regression analysis for the remaining three indices, to determine the association of fall risk with the variables. Differences between the groups of number of drugs and three indices of fall tendency were analyzed using one-factor ANOVA followed by Tukey–Kramer test. Data were analyzed using JMP version 8.0.1.

Results

The characteristics of the study subjects are shown in Table 1. Calcium channel blockers, angiotensin-II receptor blockers (ARB), statins and aspirins were prescribed in more than 20% of all the patients. Calcium channel blockers prescribed in this study were all long-acting agents, and aspirin dosage prescribed were all 100 mg. Less than 10 patients received insulin therapy, took non-steroidal anti-inflammatory drugs or anticoagulants. No patients were taking neuroleptics, nor antiparkinsonian drugs. Patients prescribed five drugs or more were 36.3%.

On univariate analyses, the number of drugs was the only factor which was significantly associated with history of falls in the past year (no/yes 3.2 ± 2.6/4.0 ± 3.1 drugs, $P < 0.05$). Older age, female, hypertension, osteoporosis, history of stroke, the number of comorbidities, use of ARB, aspirin, bisphosphonates, hypnotics and number of prescribed drugs were significantly associated with either one of three indices of fall risk (Table 2). Number of drugs was significantly correlated with three scores excluding the

Table 2 Univariate analysis of association between risk factor variables and three fall indices: fall-predicting score, simple screening test, one-leg standing test

		Fall risk index (points)	Simple screening test (points)	One-leg standing test (seconds)
Age		0.23***	0.23***	−0.46***
Female	No/Yes	7.0 ± 3.1/8.4 ± 4.0**	3.8 ± 3.3/4.7 ± 3.6*	19.7 ± 11.7/16.2 ± 11.7*
Hypertension	No/Yes	7.2 ± 3.6/8.4 ± 3.8*	3.7 ± 3.3/4.8 ± 3.5*	18.9 ± 11.1/16.2 ± 12.1
Osteoporosis	No/Yes	7.6 ± 3.7/8.9 ± 4.0*	4.3 ± 3.6/4.8 ± 3.1	17.9 ± 11.7/15.6 ± 11.9
History of stroke	No/Yes	7.8 ± 3.7/9.7 ± 4.1*	4.3 ± 3.4/5.6 ± 4.1	17.9 ± 11.8/8.5 ± 8.7**
Number of comorbidies		0.27***	0.17*	−0.24***
Antihypertensives	No/Yes	7.3 ± 3.6/8.5 ± 3.8*	3.7 ± 3.3/4.9 ± 3.5*	18.8 ± 11.4/15.9 ± 12.0
Angiotensin-II receptor blockers	No/Yes	7.6 ± 3.7/8.7 ± 3.8*	3.9 ± 3.4/5.2 ± 3.5**	17.6 ± 11.5/16.3 ± 12.2
Calcium channel blockers	No/Yes	7.6 ± 3.7/8.5 ± 3.7	4.1 ± 3.5/4.8 ± 3.5	18.8 ± 11.6/14.3 ± 11.6**
Aspirin	No/Yes	7.7 ± 3.8/8.9 ± 3.8*	4.1 ± 3.5/5.5 ± 3.7*	18.0 ± 11.8/13.5 ± 11.5*
Bisphosphonates	No/Yes	7.8 ± 3.8/9.9 ± 2.5*	4.3 ± 3.5/6.5 ± 2.7*	17.3 ± 11.8/14.9 ± 11.7
Hypnotics	No/Yes	7.6 ± 3.6/9.7 ± 4.1***	4.2 ± 3.6/5.2 ± 3.1	17.6 ± 11.9/15.2 ± 11.3
Number of drugs		0.30***†	0.27***†	−0.35***

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$, compared to "No" by simple Student's *t*-test. For age, number of comorbidities and number of drugs, Pearson's correlation coefficient between each indices of fall tendency are shown. †For analysis of number of drugs, a questionnaire asking "whether taking five or more drugs" were excluded for analysis. Therefore, fall risk index was analyzed by a total of 21 items, and a simple screening test by a total of 11 points. For other risk factor variables shown in the table, mean ± standard deviations are expressed. Other risk factor variables not shown in this table showed no statistically significant relationship with either one of three indices.

[Table 2 amended after online publication date September 27, 2011]

question on polypharmacy. Number of comorbidities was significantly associated with age ($r = 0.32$, $P < 0.0001$) and with the number of drugs ($r = 0.62$, $P < 0.0001$).

Next, on multivariate analyses, the questionnaire asking "whether taking five or more drugs" were excluded from the fall risk index and the simple screening test. Therefore, the fall risk index was analyzed by a total of 21 items and the simple screening test by a total of 11 points in this analysis. To evaluate the association of four fall risk indices with comorbidities and drugs, all the variables that were significantly associated in either one of four univariate analyses were entered into the model. As shown in Table 3, the number of drugs was the only factor which was significantly associated with all four indices, independent of age, sex and other variables. Because each disease variable or drug variable might have affected the number of comorbidities or the number of drugs in this analysis, we just compared the number of comorbidities and the number of drugs to exclude the double count in next analysis. As shown in Table 4, the number of drugs was significantly associated with all of the four fall risk indices independent of age, sex and the number of comorbidities, while the number of comorbidies was inversely associated with history of falls and simple screening test. As shown in Figure 1, the association of the number of drugs with

Table 3 Multivariate analysis of association between risk factor variables and four fall indices: history of falls in a year, fall risk index, simple screening test, one leg standing test

		History of fall in a year (No = 0/Yes = 1) Odds ratio (95% CI)	Fall risk index (21 items)† β	Simple screening test (11 points)† β	One-leg standing test (s) β
Age		1.00 (0.96–1.05)	0.073	0.127	–0.370***
Female	(No = 0/Yes = 1)	2.36 (1.12–5.00)*	0.199**	0.197**	–0.149*
Hypertension	(No = 0/Yes = 1)	1.87 (0.61–5.76)	0.166	0.218*	–0.110
Osteoporosis	(No = 0/Yes = 1)	0.67 (0.28–1.60)	0.093	0.027	0.023
History of stroke	(No = 0/Yes = 1)	1.43 (0.38–5.45)	0.080	0.032	–0.083
Number of comorbities		0.60 (0.38–0.95)*	–0.062	–0.237*	–0.024
Antihypertensives					
	(No = 0/Yes = 1)	0.52 (0.18–1.54)	–0.141	–0.158	0.142
Aspirin	(No = 0/Yes = 1)	1.59 (0.72–3.50)	0.053	0.046	0.002
Bisphosphonates	(No = 0/Yes = 1)	2.27 (0.73–7.07)	0.055	0.105	0.033
Hypnotics	(No = 0/Yes = 1)	0.84 (0.33–2.15)	0.094	–0.018	0.084
Number of drugs		1.24 (1.07–1.45)*	0.247**	0.335***	–0.250**

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Logistic regression analysis was performed for history of fall in a year, and multiple regression analysis for the remaining three. The risk factor variables used in these multivariate analyses were those associated in either of the four univariate analysis significantly. †The questionnaire asking "whether taking five or more drugs" were excluded from the scores in this analysis. Therefore, fall risk index were analyzed by a total of 21 items and simple screening test by a total of 11 points. CI, confidence interval; β, standardized regression coefficient.
[Table 3 amended after online publication date September 27, 2011]

Table 4 Multivariate analysis of association between number of comorbidities and drugs with four fall indices: history of falls in a year, fall risk index, simple screening test, one-leg standing test

	History of fall in a year (No = 0/Yes = 1) Odds ratio (95% CI)	Fall-risk index (21 items)† β	Simple screening test (11 points)† β	One-leg standing test (s) β
Age	1.00 (0.96–1.05)	0.101	0.115	–0.376***
Female (No = 0/Yes = 1)	1.73 (0.90–3.34)	0.207**	0.191**	–0.110
Number of comorbidities	0.63 (0.45–0.89)*	0.073	–0.137	–0.034
Number of drugs	1.23 (1.08–1.41)*	0.223*	0.316***	–0.233**

*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Logistic regression analysis was performed for history of fall in a year, and multiple regression analysis for the remaining three. †The questionnaire asking "whether taking five or more drugs" were excluded from the scores in this analysis. Therefore, fall risk index was analyzed by a total of 21 items and simple screening test by a total of 11 points. CI, confidence interval; β, standardized regression coefficient.

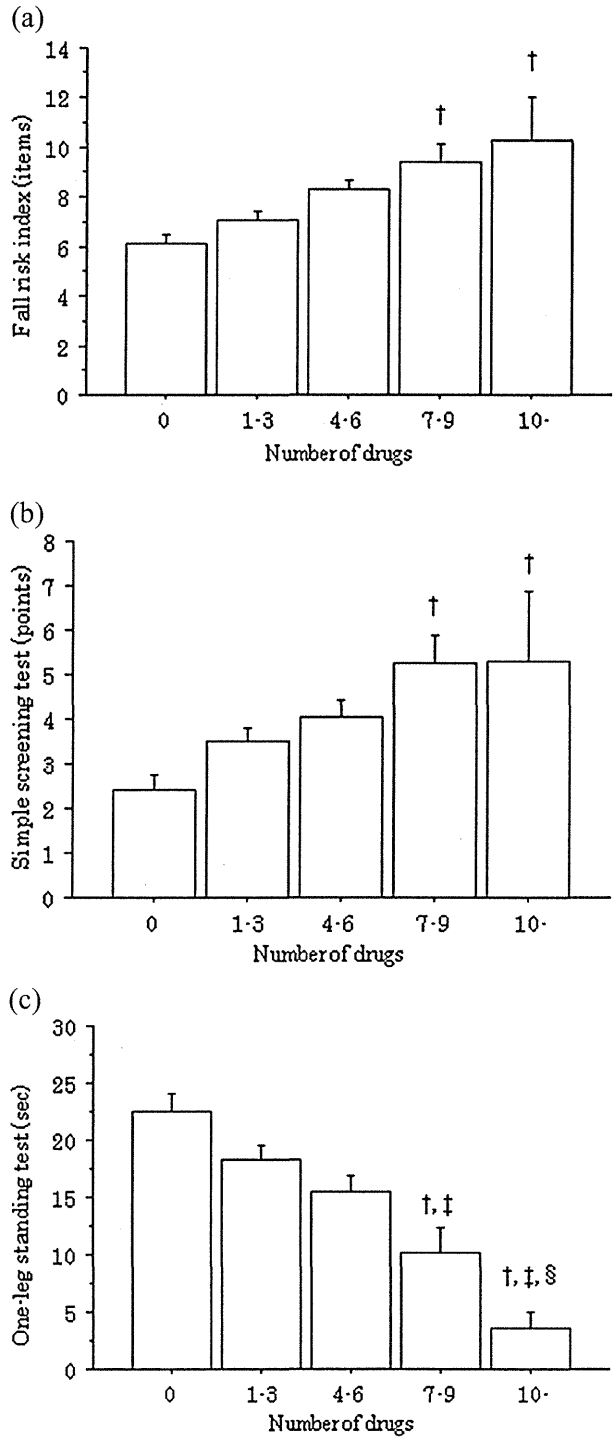

Figure 1 Averages of fall risk according to the number of drugs. (a) Fall risk index excluding the questionnaire concerning polypharmacy. (b) Simple screening test excluding the questionnaire concerning polypharmacy. (c) Duration time of one-leg standing test. The differences between the number of the drugs were compared through ANOVA, $P < 0.0001$ for (a), $P < 0.005$ for (b), $P < 0.0001$ for (c). For post-hoc analysis, $^{\dagger}P < 0.05$ vs 0 drug; $^{\ddagger}P < 0.05$ vs 1–3 drugs; $^{\S}P < 0.05$ vs 4–6 drugs. Values are expressed as mean ± standard error.

fall predicting score, simple screening test and duration time of one-leg standing test was stepwise.

Discussion

Epidemiological studies have assessed the risk of falls in community-dwelling people, but not in geriatric outpatients, who are likely to fall and need special consideration for the treatment of their illness. This cross-sectional study investigated the association between comorbidities, medications and fall risks in Japanese elderly outpatients and found that all four indices were significantly associated with the number of drugs. Because polypharmacy is frequently seen in patients with multiple comorbidities, this study compared the impact of the number of drugs with that of the number of comorbidities on fall risk, and found the significance of polypharmacy as fall risk in elderly outpatients.

In the present study, the number of comorbidities was inversely associated with the history of fall in the past year and with an 11-point simple screening test in the multivariate analysis. The reason is unclear; however, there are some speculations about this. None of the patients with four or more comorbidities ($n = 19$, 79.4 ± 5.2 years old) had history of fall in the past year. This accounts for the lower points of the simple screening test in these patients, because the history of fall consists of 5 points out of a total of 11 points in the simple screening test. So the question is why they had lower frequency of falling experiences, although they are at higher risk of falls according to fall risk index and one-leg standing test (9.6 ± 3.8 items and 8.6 ± 9.4 s, respectively). These patients may take care not to fall in their daily lives because of their consciousness of fall risk or frailty, or maybe due to elevated vigilance of caregivers and their constant physical assistances. They might have simply forgotten their fall experiences due to subclinical cognitive impairment, although demented patients were not included in this study. It is also possible that the patients who had more comorbidities and had fallen did not meet our inclusion criteria because of their recent injurious falls or their severe conditions.

Several medications and comorbidities have been reported as risks of fall.[6,7,13–19] Among these, diabetes,[9,10] imsonia,[13] hypnotics[13–15] and antihypertensive use[8] were not significantly associated with fall risk in our study. Only 20 patients (40.8% of diabetic patients) were prescribed hypoglycemic agents such as sulfonylurea ($n = 17$) or insulin ($n = 3$) in this study. Because hypoglycemia is considered to be the main cause of accidental falls in diabetic patients, relatively less prescription of hypoglycemic agents might have affected our result. The patients who were prescribed hypnotics tended to be at higher risk of falls in univariate analysis, which did show statistical significance. Also, antihypertensives such as diuretics are reported to increase the fall risk.[8] No

association between these drugs and fall risk in our study might be due to the small sample size. Other drugs such as major tranquillizers,[14] antidepressants[17,18] and antiparkinsonians[19] might increase fall risk; however, very few patients used these drugs in this study.

There are some other limitations. First, the causal relationship of the associations observed in this study is unknown because of the cross-sectional design. Polypharmacy has been regarded as a risk in several aspects in elderly patients. Previous studies have shown that adverse drug events were seen more frequently in the polypharmacy patients during their stay in the geriatric inpatient ward,[20] and polypharmacy was one of the important predictors for postdischarge mortality in elderly patients after emergent hospitalization.[21] Because patients with multiple diseases and in severer conditions are likely to take more medications, we used the number of comorbidities in analysis as fall risk variables. However, it is still unclear whether polypharmacy is a risk of falls independent of severity of each comorbidity. Interventional studies to reduce the number of drugs are needed to clarify the causal relationship between polypharmacy and fall risk. Second, this study did not evaluate the fall itself. The validity of four indices used in this study is well established as fall risk markers. However, prospective studies which evaluate the incidence of fall should be carried out in the future. Third, although the included subjects were receiving the same prescriptions for more than 1 month, the exact duration of each drug use or polypharmacy was not assessed in this study. Consequently, the long-term adverse effects over months or years seen in elderly patients should be more precisely investigated.

In summary, this study demonstrated that geriatric outpatients with polypharmacy were at higher risk of falls, consistent with the previous studies conducted in community-dwelling elderly. Our finding may add new information on pharmacotherapy in elderly patients with chronic diseases. Prospective studies and intervention studies examining the effect of drug reduction are needed in the future.

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Appendix I. 22 items of fall-predicting score (questionnaire)

Q1. Have you fallen during the last 12 months?	Yes, 1; No, 0.
Q2. Have you tripped during the last 12 months?	Yes, 1; No, 0.
Q3. Can you climb stairs without help?	Yes, 0; No, 1.
Q4. Do you feel your walking speed has declined recently?	Yes, 1; No, 0.
Q5. Can you cross a road within the green signal interval?	Yes, 0; No, 1.
Q6. Can you walk 1 km without stopping?	Yes, 0; No, 1.
Q7. Can you stand on one foot for about five seconds?	Yes, 0; No, 1.
Q8. Do you use a stick when you walk?	Yes, 1; No, 0.
Q9. Can you squeeze a towel tightly?	Yes, 0; No, 1.
Q10. Do you feel dizzy at times?	Yes, 1; No, 0.
Q11. Is your back bent?	Yes, 1; No, 0.
Q12. Do you have knee pain?	Yes, 1; No, 0.
Q13. Do you have a problem with your vision?	Yes, 1; No, 0.
Q14. Do you have a hearing problem?	Yes, 1; No, 0.
Q15. Do you think you are forgetful?	Yes, 1; No, 0.
Q16. Do you feel anxious about falling when you walk?	Yes, 1; No, 0.
Q17. Do you take five or more prescribed medicines?	Yes, 1; No, 0.
Q18. Do you feel unsafe because your home is dark?	Yes, 1; No, 0.
Q19. Are there any obstacles in your house?	Yes, 1; No, 0.
Q20. Is there any difference in level within your home?	Yes, 1; No, 0.
Q21. Do you have to use stairs in daily living?	Yes, 1; No, 0.
Q22. Do you have to walk on a steep slope around your house?	Yes, 1; No, 0.

Appendix II. Simple screening test for risk of falls

Q1. Have you fallen during the last 12 months?	Yes, 5 points; No, 0.
Q2. Do you feel your walking speed has declined recently?	Yes, 2 points; No, 0.
Q3. Do you use a cane when you walk?	Yes, 2 points; No, 0.
Q4. Is your back bent?	Yes, 2 points; No, 0.
Q5. Do you take five or more prescribed medicines?	Yes, 2 points; No, 0.

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OBSTRUCTIVE SLEEP APNEA EXACERBATES ENDOTHELIAL DYSFUNCTION IN PEOPLE WITH METABOLIC SYNDROME

To the Editor: The process of aging can be found in a variety of organs, frequently overlapping in the metabolic, cardiopulmonary, and nervous systems. A recent study showed that visceral fat accumulation is associated with metabolic risk factor clustering in older adults.[1] Obstructive sleep apnea (OSA) and metabolic syndrome (MetS) are well known as risk factors for cardiovascular disease and comorbid disorders in obese and older adults,[2] but whether OSA affects vascular endothelial dysfunction, a surrogate marker of cardiovascular disease,[3] in people with MetS has not been determined. Flow-mediated dilation (FMD) of the brachial artery, an indicator of endothelial vasomotor function, was therefore examined in people with MetS with or without OSA.

Forty-nine consecutive overweight subjects (body mass index $\geq 25.0\,kg/m^2$, aged 35–69) who were referred for medical examinations were enrolled and categorized into three groups; with MetS but not OSA (MetS group, n = 21), with MetS and OSA (MetS+OSA group, n = 14), and with no metabolic risk factors but overweight (control group, n = 14). MetS was defined using the International Diabetes Federation criteria and OSA using polysomnography. Participants who had some risk factors but did not meet the criteria for MetS and those who declined to undergo polysomnography were excluded. Blood sampling and measurement of FMD were performed early in the morning after an overnight fast. FMD was measured using ultrasound as percentage change in brachial artery diameter as previously described.[4]

The MetS and MetS+OSA groups had significantly lower plasma high-density lipoprotein cholesterol (HDL-C) (41.9 ± 9.4 and 40.7 ± 5.9 vs 57.9 ± 12.5 mg/dL, $P < .001$) and higher triglycerides (192.2 ± 57.7 and 157.3 ± 52.4 vs 104.1 ± 34.4 mg/dL, $P = .008$) and glycosylated hemoglobin ($5.71 \pm 0.87\%$ and $5.81 \pm 0.90\%$ vs $4.80 \pm 0.38\%$, $P = .001$) than the control group. Although the apnea-hypopnea index was 34.0 ± 13.6 events per hour in MetS+OSA group, in contrast to 3.1 ± 1.6 events in the MetS group ($P < .001$), there were no significant differences between the MetS and MetS+OSA groups in terms of cardiovascular risk factors, including age, body mass index, waist circumference, blood pressure, low-density lipoprotein cholesterol (LDL-C), and homeostasis model assessment of insulin resistance (data not shown).

The control group had a significantly lower increase in percentage of FMD (%FMD) than the other two groups. Moreover, %FMD in the MetS and OSA group was significantly lower than that in the MetS group (Figure 1), whereas nitroglycerine-induced endothelium-independent dilation was comparable between the groups ($15.0 \pm 4.2\%$ control, $13.5 \pm 3.2\%$ MetS, $11.5 \pm 3.5\%$ MetS+OSA). On multiple regression analysis, OSA (yes = 1, no = 0) was significantly related to %FMD, independent of age, waist circumference, systolic blood pressure, LDL-C, HDL-C, triglycerides, fasting plasma glucose, and smoking ($\beta = -0.324$, $P = .04$). The results of other multiple regression models were similar (data not shown).

It has been shown that continuous positive airway pressure treatment improves endothelial vasomotor function with no influence on metabolic risk factors,[5,6] indicating that vascular endothelial dysfunction in people with OSA is attributable to OSA-induced hypoxia. These findings imply that OSA is an additional risk factor in people with MetS. Consistent with the present results, it has been reported that OSA is independently associated with carotid intima-media thickness and pulse wave velocity, other

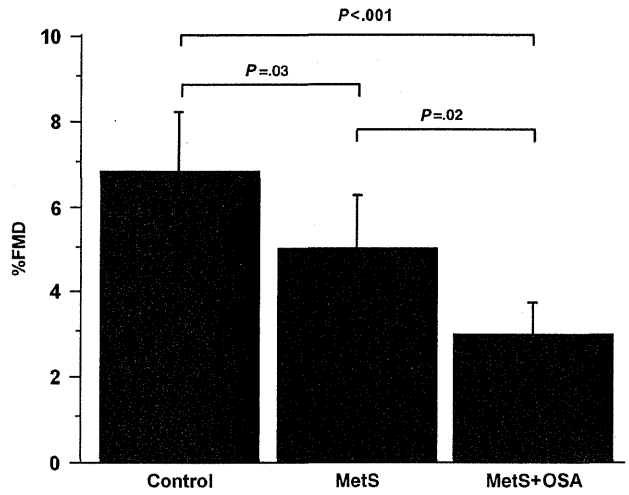

Figure 1. Increase in percentage of flow-mediated diameter (%FMD) of the brachial artery in control overweight subjects (control, n = 14), patients with metabolic syndrome (MetS, n = 21), and patients with MetS and obstructive sleep apnea (MetS+OSA, n = 14). Data are shown as means ± standard deviations.

markers of atherosclerosis, in people with MetS.[7] In conclusion, the results of the current study suggest that OSA exacerbates endothelial dysfunction in people with MetS, possibly leading to greater risk of cardiovascular disease.

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COMMENTS/RESPONSES

RELEVANT OUTCOMES IN INTERVENTION TRIALS FOR SARCOPENIA

To the Editor: We read with interest the paper by Brass and Sietsema on drug development to treat sarcopenia.[1] The authors raise important points to consider when designing clinical trials addressing sarcopenia-related outcomes.

As they state, a universally accepted definition for sarcopenia needs to be established. The difficulty encountered in doing so is a direct result of the complexity of the problem. The European Working Group for Sarcopenia in Older Persons (EWGSOP) has recently developed and published a practical clinical definition and consensus diagnostic criteria for age-related sarcopenia[2] that several international scientific societies, namely the European Geriatric Medicine Society (EUGMS), the European Society for Clinical Nutrition and Metabolism (ESPEN), the International Association of Gerontology and Geriatrics—European Region, and the International Association of Nutrition and Aging, have endorsed. In line with Brass and Sietsema's suggestion, the EWGSOP advocates a definition that allows chronic disease, besides aging per se, to contribute to sarcopenia.

For the diagnosis of sarcopenia, EWGSOP recommends using the presence of low muscle mass and reduced muscle function (strength or performance) and variously applies these characteristics to further define such conceptual stages as presarcopenia, sarcopenia, and severe sarcopenia. EWGSOP also reviewed a wide range of tools that can be used to measure the specific variables of muscle mass, muscle strength (e.g., hand grip), and physical performance (e.g., gait speed). The report summarizes currently available data defining sarcopenia cutoff points according to age and sex; suggests an algorithm for sarcopenia case finding in older individuals based on measurements of gait speed, grip strength, and muscle mass; and presents a list of suggested primary and secondary outcome domains for research.

In their review, Brass and Sietsema emphasize the standards that trials should meet to establish efficacy. They point out that efficacy should be measured according to meaningful clinically relevant end points and that surrogate markers of benefit will not be sufficient to validate Food and Drug Administration (FDA) approval. This is a complex issue for sarcopenia, because it fulfills criteria for a geriatric syndrome and is thus characterized by a complex interplay

Sirtuin 1 Retards Hyperphosphatemia-Induced Calcification of Vascular Smooth Muscle Cells

Aya Takemura, Katsuya Iijima, Hidetaka Ota, Bo-Kyung Son, Yuki Ito, Sumito Ogawa, Masato Eto, Masahiro Akishita, Yasuyoshi Ouchi

Objective—Arterial calcification is associated with cardiovascular disease as a complication of advanced atherosclerosis. Aged vascular cells manifest some morphological features of a senescent phenotype. Recent studies have demonstrated that mammalian sirtuin 1 (SIRT1), a histone deacetylase, is an exciting target for cardiovascular disease management. Here, we investigated the role of SIRT1 in a calcification model of vascular smooth muscle cells (SMCs).

Methods and Results—In adenine-induced renal failure rats with hyperphosphatemia, massive calcification was induced in the aortic media. Senescence-associated β-galactosidase (SAβ-gal) activity, a marker of cellular senescence, in medial SMCs was significantly increased, and its induction was positively associated with the degree of calcification. In cultured SMCs, inorganic phosphate (Pi) stimulation dose-dependently increased SAβ-gal-positive cells, and Pi-induced senescence was associated with downregulation of SIRT1 expression, leading to p21 activation. The activation via SIRT1 downregulation was blunted by inhibition of Pi cotransporter. Activation of SIRT1 by resveratrol significantly reduced the senescence-associated calcification. Conversely, SIRT1 knockdown by small interfering RNA accelerated the Pi-induced SMC senescence and subsequent calcification. In addition, SIRT1 knockdown induced phenotypic change from a differentiated state to osteoblast-like cells. The senescence-related SMC calcification was completely prevented by p21 knockdown. In addition to Pi-induced premature senescence, SMCs with replicative senescence were also more sensitive to Pi-induced calcification compared with young SMCs, and this finding was attributable to augmented p21 expression.

Conclusion—SIRT1 plays an essential role in preventing hyperphosphatemia-induced arterial calcification via inhibition of osteoblastic transdifferentiation. In addition, Pi-induced SMC calcification may be associated with both premature and replicative cellular senescence. (***Arterioscler Thromb Vasc Biol.* 2011;31:2054-2062.)**

Key Words: cellular senescence ■ hyperphosphatemia ■ longevity gene SIRT1 ■ vascular calcification ■ vascular smooth muscle cell

Atherosclerotic vascular damage associated with aging manifests several features, namely atherosis, sclerosis, and calcific change, finally leading to cardiovascular events. These pathological changes result in arterial wall thickening (localized morphological changes) and arterial stiffening (functional changes).[1] Arterial calcification makes the management of hemodynamics more difficult in the elderly, because ectopic calcium deposition in the aorta and arteries contributes to vessel wall stiffening and loss of elastic recoil.[2] These pathological conditions result in unstable hemodynamic consequences, finally leading to a decline in end-organ perfusion and subsequent ischemic events. Recently, several reports have demonstrated that aortic calcification detectable on chest X-ray examination is a strong predictor of future cardiovascular events beyond traditional risk factors.[3]

Arterial calcification is anatomically separated into two types, intimal and medial calcification.[4] Intimal calcification, which is seen as patchy scattered deposits only occurring within atherosclerotic plaques, is shown to be associated with plaque vulnerability.[5] On the other hand, medial calcification, which is frequently seen in the elderly and in diabetes and chronic renal failure, is observed as continuous linear deposits along the internal elastic lamina.[6] Advanced atherosclerosis with both types of calcified lesions is the consequence of overlapping pathological mechanisms.

Ectopic calcification in the vasculature has been shown to result from passive precipitation of calcium with aging and osteoporosis, the so-called calcium shift theory, as a previous hypothesis.[7] However, accumulating recent evidence has shown it to be attributable to an active "cell-mediated process" resembling osteogenesis in bone rather than passive mineral precipitation in vascular smooth muscle cells (SMCs).[8,9]

Silent information regulator-2 (Sir2), an NAD^+-dependent HDAC, is highly conserved in organisms ranging from Archaea

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to humans.[10] In yeast, Sir2 has been shown to play critical roles in DNA repair, stress resistance, and longevity. Mammalian sirtuin 1 (SIRT1), the closest homolog of Sir2, regulates the cell cycle, apoptosis, and metabolism by interacting with a number of molecules, including p53, promyelocytic leukemia protein, Foxo, Ku70, and peroxisome proliferator-activated receptor-γ.[11] A previous study has shown that SIRT1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts.[12] In addition, we have recently demonstrated that SIRT1 inhibits oxidative stress-induced premature senescence in vascular endothelial cells.[13] However, the detailed mechanism of how SIRT1 affects vascular SMC senescence and arterial calcification remains unclear.

In this study, we hypothesized that SIRT1 plays an important role in preventing arterial calcification due to renal failure, in association with modulation of cellular senescence. Here, we demonstrated the protective potential of SIRT1 against hyperphosphatemia-induced premature and replicative senescence and subsequent calcification in SMCs.

Methods

Aortic Calcification in Renal Failure Rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.[14] All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Detailed methods are described in the supplemental materials, available online at http://atvb.ahajournals.org.

Induction of SMC Calcification

Primary human aortic SMCs (HASMCs) were treated with a pathological concentration of inorganic phosphate (Pi) up to 3.2 mmol/L in culture medium as previously described.[29] To quantitatively measure Pi-induced calcification, two distinct experiments were performed as previously described[14]: (1) intracellular calcium deposition as determined by *o*-cresolphthalein complexone method, and (2) visualization of mineralization as determined by von Kossa staining, Detailed methods are described in the supplemental materials.

Senescence-Associated β-Galactosidase Staining

To assess senescent changes in the phenotype of cultured HASMCs or aortic medial cells of rats, staining for senescence-associated β-galactosidase (SAβ-gal), a well-established biomarker of cellular senescence, was performed. Detailed methods are described in the supplemental materials.

Knockdown of SIRT1 or p21 by Small Interfering RNA

HASMCs were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1, $p21^{WAF1/CIP1}$, or both. Detailed methods are described in the supplemental materials.

Real-Time Polymerase Chain Reaction Analysis: Osteoblastic Markers

To examine whether Pi stimulation induces change to an osteoblastic phenotype, the expression of Runx-2/Cbfa-1 and alkaline phosphatase, which are well known to be representative osteoblastic markers, was checked using real time-polymerase chain reaction analysis. In addition, the effect of knockdown of SIRT1, p21, or both by siRNA on the osteoblastic phenotypic change in HASMCs was examined. Primer sequences are shown in Supplemental Figure I.

Results

Association of Senescent Vascular Cells With Aortic Medial Calcification in Renal Failure Rats

The adenine-fed rats had severe renal failure, with a huge increase in serum creatinine (3.0±0.9 mg/dL in renal failure rats versus 0.3±0.0 mg/dL in control rats), similar to a previous report.[14] The renal failure rats showed an approximately 2.0-fold increase in serum phosphorus (18.9±4.7 mg/dL) compared with control rats (9.8±0.9 mg/dL). Histological assessment using von Kossa staining showed that the aorta in renal failure rats had extensive linear calcification, which was localized in the aortic media, resembling the typical Mönckeberg's pattern (Figure 1A). Numerous SAβ-gal positive cells were found in the aortic media of renal failure rats, whereas the aortic wall in control rats did not contain senescent cells (Figure 1B). The senescent cells were mainly localized to the calcified area and its surrounding area, which was defined as the area not stained black by von Kossa staining. Quantitative assessment showed that the number of senescent cells with high SAβ-gal activity was positively correlated with the calcified area in the aortic media (Figure 1C).

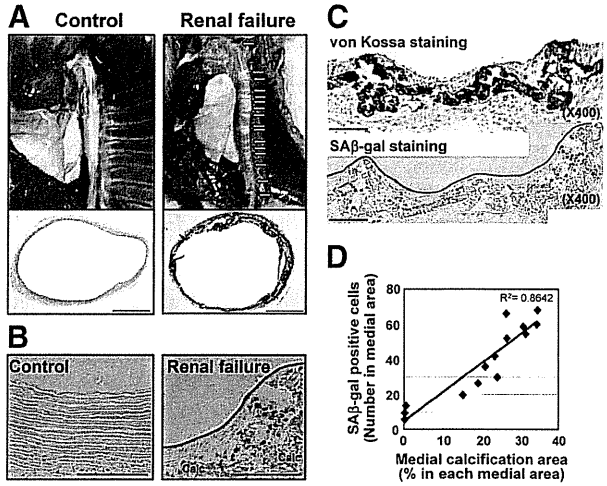

Figure 1. Presence of senescent vascular cells colocalized with calcification in aortic media of renal failure rats. A, Rats with severe renal failure had massive calcification throughout the aorta (right) compared with control rats (left) (n=5). Yellow arrows indicate calcified area. Morphological assessment by von Kossa staining showed extensive calcification in the aortic media of renal failure rats. Scale bar=500 μm. B, Senescent vascular cells (senescence-associated β-galactosidase [SAβ-gal]-positive: blue) were significantly detected throughout the calcified area (Calc) in renal failure rats, whereas these senescent cells were not present in control rats. Scale bar=100 μm. C, Localized association between calcification and senescent cells is shown in renal failure rats. SAβ-gal-positive cells were frequently found in areas with marked calcification. D, The association of the number of SAβ-gal-positive cells with the calcified area in each photograph was evaluated. The senescent cell number was linearly correlated with the area of calcification in the aortic media of renal failure rats (calcified area in media: percentage).

Pi Induces Cellular Senescence in Cultured SMCs

On the basis of our results obtained from animal experiments, we hypothesized that senescent SMCs in the aortic media are strongly associated with the development of arterial calcification. Therefore, the effect of excessive Pi stimulation (2.6 mmol/L) on cellular senescence in cultured SMCs was examined. SAβ-gal-positive senescent HASMCs were significantly induced by not only angiotensin II (Ang II) but also Pi

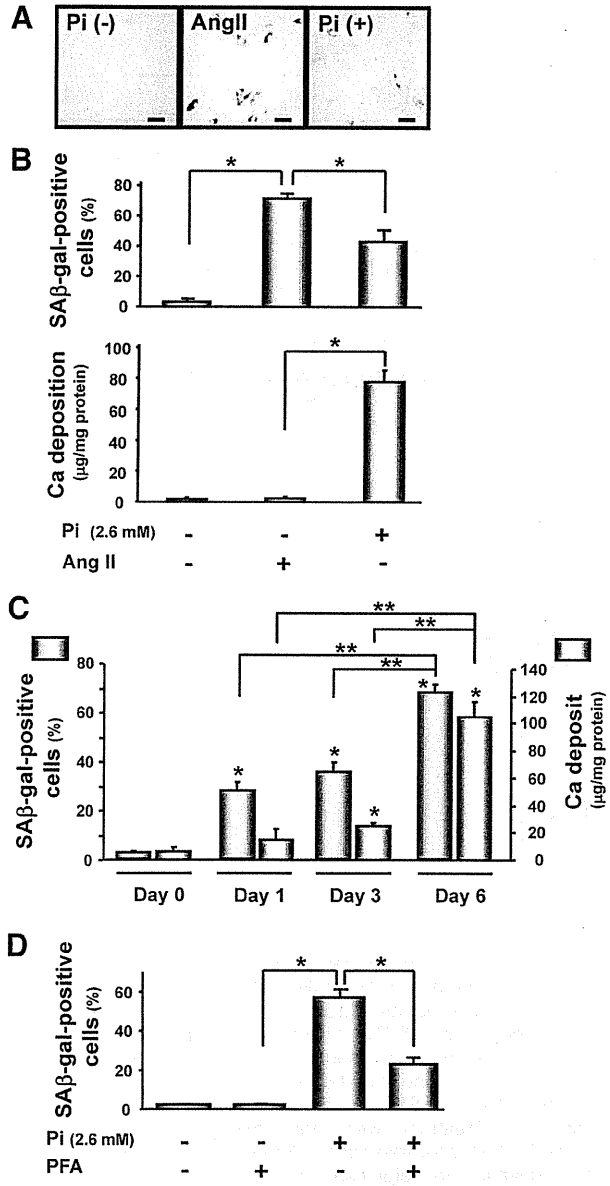

Figure 2. Inorganic phosphate (Pi) stimulation induces cellular senescence in vascular smooth muscle cells (SMCs) via its cotransporter. A, The effect of Pi on senescent transition in human aortic SMCs (HASMCs) was examined. Representative photographs showed that senescence-associated β-galactosidase (SAβ-gal) activity (blue) in cells was significantly induced by not only angiotensin II (Ang II: 10 pmol/L, as a positive control) but also Pi stimulation (2.6 mmol/L). B, The number of senescent cells was significantly increased by not only Ang II but also Pi. Calcium deposition was significantly increased by Pi; however, calcification in HASMCs was not induced by Ang II alone in the absence of Pi. C, Senescent cells were significantly increased by Pi stimulation even on day 1; however, a statistically significant increase in calcium deposition was found from day 3 and later. D, Inhibition of the phosphate cotransporter Na-dependent phosphate cotransporter by the inhibitor phosphonoformic acid (PFA) (100 μmol/L) reduced SAβ-gal activity, which was increased by Pi (2.6 mmol/L) in HASMCs. Each experiment was performed al least 3 times.

stimulation (Figure 2A). Notably, Pi stimulation increased calcium deposition; however, Ang II alone did not (Figure 2B). It suggests that high-dose Pi condition, but not stress by Ang II alone, is indispensable to induce SMC calcification. These findings also suggest that intracellular Pi influx at least is essential to induce this SMC calcification model.

In addition, to determine how many days after the initiation of Pi stimulation the cells showed a senescent phenotype and subsequent calcification, the time-dependent effects of Pi stimulation on both SAβ-gal activity and calcium deposition were examined. As shown in Figure 2C, SAβ-gal-positive cells were significantly increased by Pi stimulation even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Cotreatment with phosphonoformic acid, an inhibitor of Na-dependent phosphate cotransporter (NPC), showed significant inhibition of Pi-induced senescence (Figure 2D). Our previous report showed that treatment with PFA completely inhibited Pi-induced SMC calcification,[15] suggesting the importance of increased intracellular influx of phosphate in Pi-induced SMC senescence.

Downregulation of SIRT1 by Pi

Treatment of HASMCs with Pi caused downregulation of SIRT1 expression in a time-dependent manner (Figure 3A). The decline was dependent on Pi concentration (data not shown). An increase in acetylation of both substrates of SIRT1, histone-3 and p53 (a nonhistone substrate), was found according to the decline in SIRT1 deacetylase activity. In addition, expression of p21, a downstream molecule of p53, was significantly induced by Pi as well. Quantitative assessment showed that an increase in these expression levels of acetylated (Ac)-p53 and p21 on day 3 and day 6 was statistically significant compared with the pretreatment levels, suggesting that downregulation of SIRT1 activity may mediate the subsequent increase in Ac-p53 and p21 expression.

To address whether SIRT1 downregulation-related SMC senescence and calcification are reversible or not, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, the continuation of Pi up to day 10 was associated with SIRT1 downregulation and subsequent upregulation of Ac-p53 and p21, leading to induction of senescence-related calcification. However, the slight increase in senescent cells was not statistically significant, although calcification was significantly induced. Of note, the Pi-induced downregulation of SIRT1 was almost completely reversed by withdrawal (termination) of Pi stimulation (exchange of Pi from 2.6 mmol/L to 1.4 mmol/L as a normal level on day 6) as shown in Figure 3B. According to the restoration of SIRT1, levels of both Ac-p53 and p21 were also decreased without more progression. In addition, termination of Pi showed no progression of senescence-related calcification; however, preexisting senescent cells and calcification on day 6 continued without regression.

Next, NPC inhibition by PFA completely blunted Pi-induced SIRT1 downregulation and subsequent activation of its downstream p53/p21 pathway (Figure 3C).

Regulation of SIRT1 Modulates Pi-Induced SMC Senescence and Calcification

The effects of modulation of SIRT1 activity on Pi-induced cellular senescence were investigated. First, sirtinol, a chem-

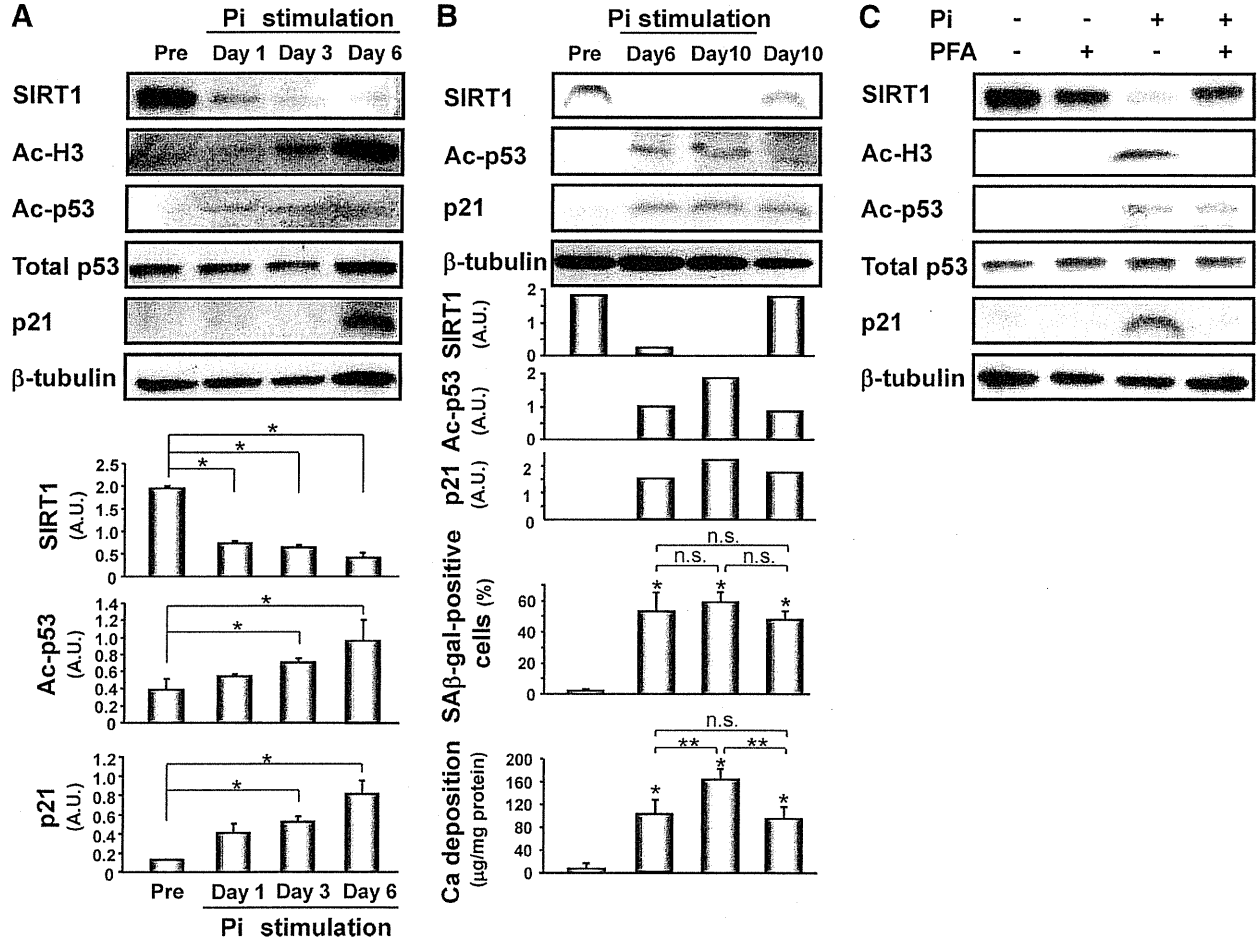

Figure 3. Inorganic phosphate (Pi) stimulation leads to sirtuin 1 (SIRT1) downregulation and subsequent p21 activation. A, The effect of Pi on SIRT1 expression and its downstream pathway was examined. Treatment of human aortic SMCs (HASMCs) with Pi (2.6 mmol/L) showed downregulation of SIRT1 expression, leading to an increase in acetylation of its substrates (acetylated [Ac]-H3 and Ac-p53) and p21 expression. Bottom: Quantitative analysis showed that Pi gradually induced not only SIRT1 downregulation but also upregulation of Ac-p53 and p21. B, To address whether SIRT1 downregulation-related senescence and subsequent calcification are reversible, the effects of continuation or termination of high-dose Pi were examined. As shown in 4th lane from left, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with restoration of SIRT1, whereas continuation (up to day 10, 3rd lane from left) of Pi stimulation showed further progression of calcification. C, Treatment with phosphonoformic acid (PFA), a Na-dependent phosphate cotransporter inhibitor, completely reversed Pi-induced SIRT1 downregulation. A decline in Ac-H3 and Ac-p53 reflected the restoration of SIRT1 deacetylase activity. Pi-induced p21 activation was significantly inhibited by inhibition of Pi transport.

ical inhibitor of SIRT1, induced an increase in SAβ-gal-positive cells even under a normal Pi (1.4 mmol/L), and the increased number of senescent cells induced by Pi was significantly augmented by sirtinol (Figure 4A). Sirtinol dose-dependently augmented Pi-induced calcification, although no augmentation was found under a normal Pi (Figure 4B and 4C). Conversely, treatment with resveratrol, an activator of SIRT1, significantly reduced both Pi-induced senescent transition and calcification in a dose-dependent manner (Figure 4D to 4F).

Second, complete knockdown of SIRT1 by siRNA caused a significant increase in acetylation of both substrates (histone-3 and p53) and p21 expression (Figure 5A). Similarly to sirtinol, SIRT1 inhibition by siRNA also augmented not only senescent transition (Figure 5A, bottom) but also calcium deposition (Figure 5C, top).

Although stimulation with Ang II alone could increase the number of SAβ-gal-positive cells, it did not increase calcium deposition. To understand the mechanism of these discrepant phenomena, the effect of Ang II alone on osteoblastic phenotypic change was examined. Ang II alone did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation (Figure 5B).

To understand the detailed mechanism by which SIRT1 modulates senescence-related calcification, the effect of SIRT1 on phenotypic change in HASMCs was examined. Pi inhibited the expression of caldesmon, a differentiated SMC lineage marker, and complete knockdown of SIRT1 augmented the Pi-induced partial downregulation of caldesmon (Figure 5C, middle). In contrast, real-time polymerase chain reaction analysis showed that Pi induced the expression of two representative osteoblastic markers, Runx-2/Cbfa-1 and alkaline phosphatase (Figure 5C, bottom) with statistical significance. In addition, complete knockdown of SIRT1 using siRNA significantly accelerated the Pi-induced os-