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ORIGINAL ARTICLE

Lower physical activity, but not excessive calorie intake, is associated with metabolic syndrome in elderly with type 2 diabetes mellitus: The Japanese elderly diabetes intervention trial

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Aim: A decline in physical activity has been shown to be associated with metabolic syndrome (MetS), leading to cardiovascular events. However, this is difficult to manage well in the elderly with multiple atherosclerotic risk factors. In this study, we investigated the correlation between physical activity and clinical parameters in the presence and absence of MetS in Japanese elderly subjects with type 2 diabetes mellitus (T2DM). In addition, we determined which factor, calorie intake or physical activity, mainly contributes to the prevalence of MetS.

Methods: Cross-sectional analysis of 846 consecutive Japanese elderly (408 men and 438 women, mean age 68.7 years) was carried out at the time of enrolment (2000–2002) in the Japanese Elderly Diabetes Intervention Trial. Their level of physical activity was evaluated using the Baecke questionnaire, consisting of three components: work, sports and leisure. Total activity score (TAS) as the sum of each activity score was divided into four quartiles (Q1 to Q4).

Results: After adjustment for age and sex, there was a positive association of TAS with high-density lipoprotein cholesterol, although no significant correlation between other lipid parameters and TAS was found. In addition, fasting plasma glucose, insulin level and physical measurements, such as waist circumference, waist/hip ratio and body mass index,

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were inversely associated with TAS. Although no correlation between TAS and cognitive function Mini-Mental State Examination was found, TAS was positively associated with instrumental ADL and negatively associated with geriatric depression score (GDS), suggesting that a decline in physical activity in the elderly is associated with depressed mood rather than a decline of cognitive function. Total calorie intake appeared to increase according to TAS; however, this did not reach statistical significance. In a subanalysis comparing the presence and absence of MetS, the TAS grade in the MetS group was significantly lower than that in the non-MetS group, although there was no significant difference in total calorie intake between the groups.

Conclusion: These results showed that lower physical activity, but not excessive calorie intake, is independently associated with the prevalence of MetS in the elderly with T2DM. In our routine work, encouraging physical activity might contribute to preventing MetS and subsequent atherosclerotic disease in the elderly, rather than strict management of abnormal laboratory parameters using multiple drugs. **Geriatr Gerontol Int 2012; 12 (Suppl. 1): 68–76.**

Keywords: depression, elderly, excessive calorie intake, Japanese Elderly Diabetes Intervention Trial study, metabolic syndrome, physical activity, work activity.

Background

Type 2 diabetes mellitus (T2DM) is an age-related disease with an estimated prevalence in Japan of more than 5% of the population.[1] The setting of treatment goals in medical care, especially in elderly patients, has been believed to be difficult because of several factors. In concrete terms, the purpose of treatment is not only to simply improve glucose intolerance, but also to maintain a higher quality of life (QOL) and prolong healthy longevity in parallel with prevention of diabetic complications. Several prospective intervention studies have recently shown some evidence that intensive glycemic control effectively slows the onset and progression of diabetic vascular complications associated with T2DM.[2,3] However, these epidemiological investigations did not consider the various associations with physical activity in elderly diabetic patients.

Physical activity promotes health and longevity.[4] Excess bodyweight and a sedentary lifestyle are well-established risk factors for not only T2DM, but also cardiovascular disease (CVD). Randomized trials have shown that a combination of weight loss and increased physical activity can reduce the incidence of T2DM and CVD.[5–7] In developed countries, 80% of all deaths from CVD occur in people aged 65 years and older.[8] The Framingham Heart Study has shown an inverse association between physical activity and CVD mortality risk, even in 285 elderly individuals.[9] However, this did not reach statistical significance, possibly as a result of the limited number of events. However, the precise mechanisms whereby physical activity lowers CVD risk are not well understood. In addition, it is possible that a decline in physical activity might lead to several undesirable conditions, including cognitive decline, in the elderly.

Metabolic syndrome (MetS) is loosely defined as a cluster of CVD risk factors, including disturbed insulin and glucose metabolism, hypertension, abdominal obesity and dyslipidemia. A low level of physical activity is believed to be an important determinant of this cluster of metabolic risk factors. Thus far, little is known about the association between physical activity and MetS in Japanese elderly patients with T2DM. To clarify which factors are mainly associated with the prevalence of metabolic syndrome (MetS) in the elderly with T2DM, we carried out a large-scale prospective study, the Japanese Elderly Diabetes Intervention Trial (J-EDIT), which was started in 2001.[10] To address how elderly patients with T2DM should be treated, a randomized controlled intervention study in Japanese elderly patients with diabetes has been carried out.

In the J-EDIT study, we investigated the correlation between physical activity and MetS in the elderly with T2DM. In particular, we focused on the association of oral calorie intake with physical inactivity in the presence or absence of MetS.

Methods

Study population

Participants were enrolled in the J-EDIT, which is a recently completed trial of intensive or standard treatment for diabetes in the primary prevention of CVD in the elderly. J-EDIT included 1173 diabetic patients who were aged 65 years or older (mean age 71.8 ± 4.6 years) and whose serum glycated hemoglobin A1c (HbA1c)

level was >7.4% from 39 institutes and hospitals (Tokyo University Hospital, Kobe University Hospital, Nagoya University Hospital and Tokyo Metropolitan Geriatric Hospital etc.) in Japan. Patients with chronic renal failure (serum creatinine > 1.5 mg/dL), severe heart failure or symptomatic cerebral infarction were also excluded from the present study. Written informed consent was obtained from all patients.

From these patients enrolled in the J-EDIT, we selected 846 patients with T2DM (mean age 71.9 ± 4.6 years, 408 men (mean age 71.5 ± 4.5 years) and 438 women (mean age 72.2 ± 4.7 years) in whom complete data on baseline physical activity (Baecke questionnaire) and nutritional survey were obtained at entry. We excluded patients who had difficulties in communicating, dementia or serious deterioration of activities of daily life from the present study.

Physical activity assessed by Baecke questionnaire

At enrolment in the present study, physical activity was evaluated by a self-administered validated Baecke physical activity questionnaire, as previously reported.[11] Baecke physical activity score is classified into three domains: work activity, sports activity and non-sporting leisure activity. These three components consisted of items on the frequency, duration, and pace of walking and bicycling during the previous week, the average amount of time spent weekly on hobbies and gardening, and the average amount of time spent monthly on odd jobs and sports. Types of odd jobs, sports and hobbies (e.g. dancing or fishing) were also assessed. Many previous reports have confirmed the reliability of this score in many individuals, suggesting that it might be a useful monitoring tool for assessing the association of multiple domains of physical activity with MetS in elderly patients with T2DM, with acceptable reliability and validity. In analyses, total activity score (TAS; maximum 15 points) was divided into four quartiles (Q1 to Q4) as follows; Q1: <5.7, Q2: ≥5.7 and <7.7, Q3: ≥7.7 and <10.5, Q4: ≥10.5.

Comprehensive geriatric assessment

To perform comprehensive geriatric assessment (CGA), we carried out several evaluations. Mini-Mental State Examination (MMSE) was used to assess cognitive function.[10] Geriatric Depression Scale (GDS) was used to assess depression status. We also checked basic activities of daily life (bADL) and instrumental activities of daily life (iADL), as determined by the Tokyo Metropolitan Institute of Gerontology (TMIG) index of competence.[12]

Physical measurements

Height, weight, waist circumference and hip circumference were measured at enrolment. Body mass index (BMI) and waist-to-hip ratio (W/H ratio) were calculated using these parameters.

Nutritional assessment of dietary calorie intake

Calorie intake was assessed using a self-reported questionnaire that has been previously shown to be valid and reliable.[13] Nutritional habit was evaluated every trimester through 7-day food records. Each energy intake, such as protein, carbohydrate and fat, and total calorie intake were calculated in all patients.

Laboratory measurements and blood pressure

Blood samples were obtained at the time of enrolment and stored in vapor-phase liquid nitrogen (–170°C). Glycemic metabolism, such as fasting plasma glucose and HbA1c; lipid parameters, such as total, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG); and renal function, such as serum creatinine, were measured. Blood pressure (BP) was measured in the non-dominant arm after 5 min of sitting quietly in accordance with the current recommendations for clinic blood pressure of each hospital.

Metabolic risk factor criteria

In the present study, MetS was defined according to the criteria proposed by the Japanese Society of Internal Medicine (JSIM), the International Diabetes Federation (IDF) and the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III).[13–16]

Statistical analysis

Analyses of covariance (ANCOVA) was used to assess independent associations between our two indices of habitual physical activity (daily step count and daily duration of activity at an intensity >3 MetS) and the presence or absence of MetS and five individual diagnostic criteria (BMI, TG, HDL-C, systolic BP and/or diastolic BP, and glucose and/or HbA1c), after controlling for age and sex. We divided the patients arbitrarily into four quartiles of physical activity (Q1: lowest group to Q4: highest group). In addition, pre-existing illness (such as cerebrovascular disease, ischemic heart disease, diabetes and retinopathy) at baseline was also considered. The χ^2-test for linear trends was used to analyze independent associations between habitual physical activity and the metabolic syndrome in adjusted models. Data are presented as mean ± standard deviation (SD), with all statistical comparisons made at the 0.05 level of significance.

Results

Comparison of parameters according to physical activity

Cross-sectional analysis of 846 consecutive Japanese elderly (408 men and 438 women; mean age 68.7 years) was carried out at the time of enrolment (2000–2002) in the J-EDIT study, a randomized, double-blind, recently completed trial of intensive or standard treatment for the prevention of CVD in elderly diabetics.

An index of physical activity was calculated using the Baecke score, including three components (work, sports and leisure). TAS was divided into four quartiles (Q1 to Q4). The baseline characteristics of patients according to their TAS grade are shown in Table 1. Regarding lipid parameters, HDL-C was positively associated with TAS, although there was no significant correlation between other lipid parameters and TAS. A negative correlation of fasting plasma glucose and plasma insulin level with TAS was found. Regarding the association between BP and TAS, no significant tendency was found.

There was a negative association between TAS and physical measurements, such as BMI, waist circumference, hip circumference and W/H ratio. In particular, high significance was observed especially in the young elderly (data not shown). However, there was no significant association with each component of TAS.

Comparison of CGA according to physical activity

Regarding comprehensive geriatric assessment (CGA), TAS was positively associated with TMIG index as instrumental ADL and negatively associated with geriatric depression score (GDS; Table 1). In contrast, there was no significant correlation between TAS and cognitive function, as determined by MMSE. These results suggest that a decline in physical activity in the elderly is associated with a depressive tendency rather than cognitive dysfunction.

Comparison of calorie intake according to physical activity

Next, we measured oral calorie intake. The calorie intake from protein and lipid were positively associated with TAS; however, there was no correlation with calorie intake from carbohydrate (Table 1). Total calorie intake tended to increase according to TAS grade, but the tendency did not reach statistical significance. Next, the total calorie intake was compared according to TAS grade in each group, divided by sex and age (Fig. 1). There was no significant difference between total calorie intake and TAS in all subgroups.

There was no significant difference between both sexes (Fig. 2a). TAS in the old elderly was significantly lower than that in the young elderly in both sexes. Comparing pre-existing illness, there was a correlation between TAS grade and cerebrovascular disease, but not coronary heart disease or diabetic retinopathy (Fig. 2b).

Impact of lower physical activity, but not excessive calorie intake, in elderly with MetS

It is well known that there is a correlation between a sedentary lifestyle and obesity. Even in the elderly, it is possible that the prevalence of MetS is associated with not only excessive calorie intake, but also their behavior. Therefore, next, we examined which factor, excessive calorie intake or physical inactivity, mainly contributes to the prevalence of MetS. First, we divided all the patients into two groups, MetS and non-MetS, using the definition of MetS of the Japanese Society of Internal Medicine (JSIM).

First, calorie intake from several types of food was compared between MetS and non-MetS (Table 2). Calorie intake from protein and fat in MetS was higher than that in non-MetS. However, for carbohydrate-derived and total calorie intake, no significant difference was found between both groups. Furthermore, in addition to the JSIM criteria, we divided the patients into two groups, MetS and non-MetS, using other clinical definitions, IDF and NCEP-ATP III. Even with each definition, TAS grade in the MetS group was lower than that in the non-MetS group (Fig. 3a). Interestingly, there was no significant difference in total calorie intake between both groups. Among the three components of TAS, work activity showed a more significant correlation with the prevalence of MetS than the other components, sports or leisure activity (Fig. 3b).

Discussion

The present study analyzed the possible association between lower physical activity and prevalence of MetS in Japanese elderly patients with T2DM who were enrolled in the J-EDIT study. The present study had two aims: (i) to evaluate the association between TAS as total physical activity and clinical parameters in the diabetic elderly; and (ii) to determine which factor, total calorie intake or physical activity, mainly contributes to the presence of MetS in the elderly with T2DM. In the present study, physical activity was assessed by the Baecke questionnaire,[11] because this is an easy, fast and valid tool for the assessment of physical activity in epidemiological studies concerning elderly populations.

The present study showed several results, as follows: TAS grade as total physical activity level in the young elderly was higher than that in the old elderly. No significant difference in TAS was found between both sexes. The presence of cerebrovascular disease in the

Table 1 Baseline characteristics: Comparison of each parameter according to four quartiles of total physical activity score

Parameters	All	TAS category				*P*-value
		Quartile 1	Quartile 2	Quartile 3	Quartile 4	
n (male/female)	846 (408/438)	130 (69/61)	295 (134/161)	104 (64/40)	317 (141/176)	0.0101
Age (years)	71.9 ± 4.6	73.5 ± 4.8	72.1 ± 4.6	72.0 ± 4.6	71.0 ± 4.4	<.0001
HbA1c (%)	8.5 ± 1.3	8.4 ± 1.2	8.4 ± 1.3	8.4 ± 1.3	8.5 ± 1.3	0.4467
FBS (mg/dL)	166 ± 49	170 ± 48	171 ± 52	164 ± 40	162 ± 49	0.0423
FIRI (mg/dL)	10.1 ± 10.1	12.4 ± 13.6	10.6 ± 11.0	8.3 ± 5.5	9.5 ± 8.8	0.0206
TC (mg/dL)	203 ± 35	198 ± 33	205 ± 37	203 ± 34	203 ± 35	0.6871
TG (mg/dL)	133 ± 96	146 ± 80	129 ± 67	152 ± 190	125 ± 76	0.105
HDL (mg/dL)	56 ± 18	51 ± 15	57 ± 18	55 ± 20	59 ± 18	0.0012
LDL (mg/dL)	121 ± 31	117 ± 32	124 ± 32	120 ± 27	120 ± 31	0.7604
sCre (mg/dL)	0.89 ± 0.97	1.00 ± 0.96	0.85 ± 0.41	0.83 ± 0.26	0.91 ± 1.41	0.9329
SBP (mmHg)	137 ± 16	138 ± 18	138 ± 15	136 ± 16	136 ± 16	0.3507
DBP (mmHg)	75 ± 10	75 ± 11	76 ± 10	74 ± 10	75 ± 9	0.28
Pulse pressure (mmHg)	62 ± 14	62 ± 16	62 ± 13	62 ± 15	61 ± 13	0.7543
BMI (kg/m2)	23.8 ± 3.5	24.8 ± 4.1	23.9 ± 3.4	23.4 ± 3.3	23.6 ± 3.4	0.0017
Waist circumference (cm)	84.0 ± 10.2	86.9 ± 11.1	84.3 ± 9.3	83.9 ± 10.5	82.5 ± 10.2	0.0003
Hip circumference (cm)	94.0 ± 7.9	95.5 ± 8.6	93.8 ± 7.6	94.3 ± 7.3	93.4 ± 7.9	0.0553
W/H circumference ratio	0.89 ± 0.07	0.91 ± 0.07	0.91 ± 0.07	0.89 ± 0.07	0.88 ± 0.07	0.0011
MMSE	28.0 ± 2.7	26.8 ± 3.9	28.0 ± 2.3	27.9 ± 2.4	28.5 ± 2.2	0.1815
GDS-15	4.1 ± 3.2	5.6 ± 3.3	4.4 ± 3.2	4.3 ± 3.1	3.2 ± 2.9	<.0001
TMIG index	11.6 ± 2.2	9.9 ± 3.5	11.6 ± 1.8	11.7 ± 1.9	12.2 ± 1.5	<.0001
Protein intake (g/day)	66.7 ± 19.3	60.4 ± 19.1	67.4 ± 17.9	67.9 ± 18.9	68.3 ± 20.3	0.0023
Fat intake (g/day)	50.0 ± 18.0	46.0 ± 21.2	48.8 ± 16.7	49.2 ± 17.3	51.3 ± 18.7	0.0237
Carbohydrate intake (g/day)	244.7 ± 54.4	240.2 ± 65.1	246.0 ± 52.5	246.0 ± 52.5	244.7 ± 49.0	0.476
Total calorie intake (kcal/day)	1735.5 ± 417.4	1661.6 ± 489.0	1747.5 ± 397.4	1747.4 ± 407.1	1751.4 ± 405.4	0.0697

BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood glucose; FIRI, fasting insulin resistance index; GDS, Geriatric Depression Scale; HbA1c, glycated hemoglobin A1c; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LTS, leisure-time activity score; MMSE, Mini-Mental State Examination; PP, pulse pressure; SBP, systolic blood pressure; sCr, serum creatinine; SS, sports score; TAS, total activity score; TC, total cholesterol; TG, triglyceride; TMIG, Tokyo Metropolitan Institute of Gerontology; W/H, waist-to-hip; WAS, work activity score. MMSE, Barthel index, TMIG index, and GDS-15 are on a scale of 0 to 30, 0 to 20, 0 to 13, and 0 to 15, respectively.

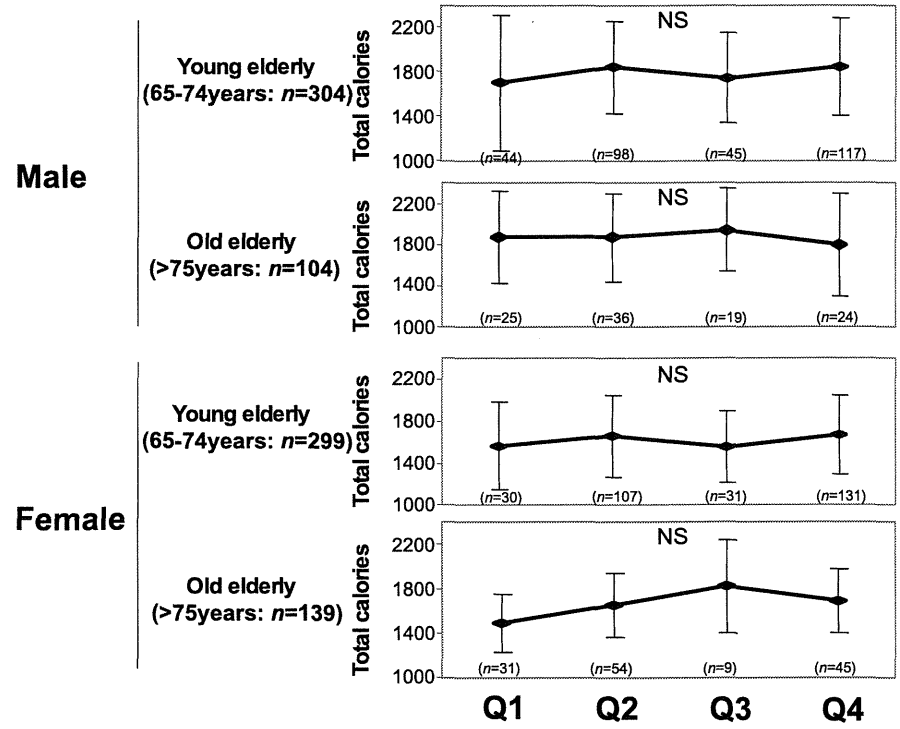

Figure 1 Total calorie intake according to total physical activity score at baseline. NS, not significant.

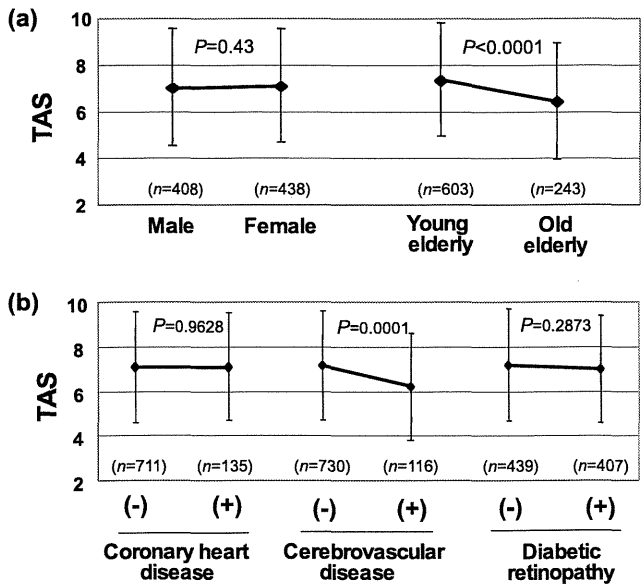

Figure 2 Total physical activity score according to sex, age and pre-existing illness at baseline. TAS, total activity score.

elderly was associated with a decline in TAS; however, the presence of ischemic heart disease or retinopathy did not contribute to the decline in TAS. Although no significant difference in total calorie intake from dietary food was found between MetS and non-MetS, TAS grade in the MetS group was significantly lower than that in the non-MetS group. Based on these results, we could conclude that a decline in physical activity, but not excessive calorie intake, is independently associated with the prevalence of MetS in the elderly with T2DM.

An inverse association between physical inactivity and MetS has been shown in several cohorts; however, there have been very few studies specifically in the elderly. In addition, one advantage of the J-EDIT study is that it obtained information on both physical activity and calorie intake. Many epidemiological observational investigations have shown a consistent inverse association between physical activity and the risk of new cardiovascular events.[17] Even in the elderly, it has been shown that maintaining a higher level of physical activity confers a reduction in coronary heart disease.[18,19] Therefore, we should encourage physical activity in elderly patients in our routine work. The protective potential of physical activity against cardiovascular events might be related to its beneficial effects on not only physical parameters (i.e. bodyweight, BP and other metabolic parameters), but also improvement of depressed mood.

In our analyses regarding CGA, TAS was strongly associated with depression scale. In contrast, no association was found between physical activity and cognitive function (MMSE score). This suggests that impairment of psychological health, especially depression and anxiety, is mainly dependent on physical activity in elderly individuals if cognitive function is not impaired. It appears that, besides traditional risk factors, depressive symptoms are associated with increased risk of CVD, leading to a worse prognosis. Recent reports

Table 2 Dietary calorie intake according to presence or absence of metabolic syndrome at baseline

	Male			Female		
	MetS (–)	MetS (+)		MetS (–)	MetS (+)	
Age (years)	70.9 ± 4.2	71.9 ± 4.8	NS	71.7 ± 4.5	73.0 ± 4.6	NS
Total calorie intake (kcal/day)	1814 ± 410	1856 ± 479	NS	1639 ± 367	1664 ± 328	NS
Protein (g/day)	70 ± 19	69 ± 20	NS	65 ± 20	63 ± 16	NS
Fat (g/day)	53 ± 18	54 ± 22	NS	48 ± 17	49 ± 15	NS
Carbohydrate (g/day)	253 ± 56	257 ± 65	NS	235 ± 49	237 ± 38	NS
Protein-to-energy ratio (%)	15.3 ± 2.1	149 ± 2.3	NS	15.6 ± 2.2	15.2 ± 2.0	NS
Fat-to-energy ratio (%)	25.7 ± 4.8	25.8 ± 5.4	NS	25.8 ± 4.6	26.0 ± 4.6	NS
Carbohydrate-to energy Ratio (%)	59.0 ± 6.0	59.2 ± 6.6	NS	58.6 ± 6.0	58.8 ± 5.5	NS

MetS, metabolic syndrome; NS, not significant.

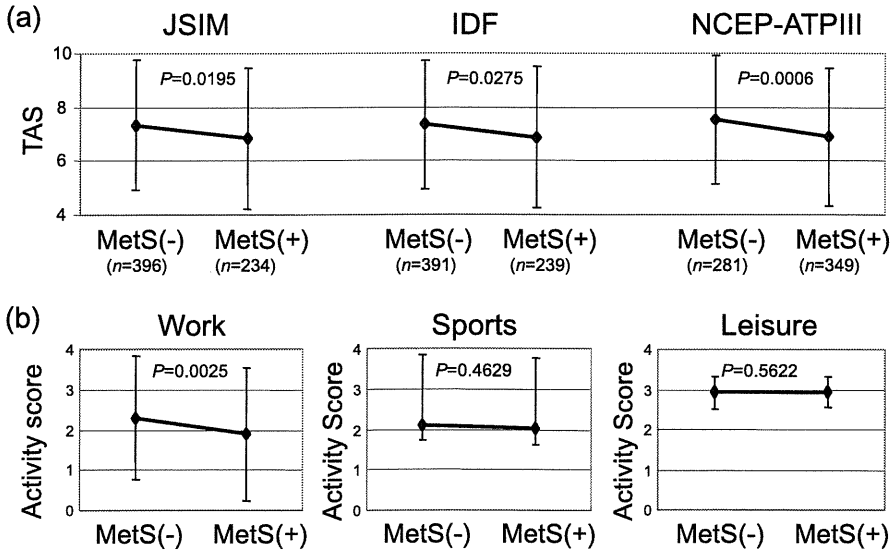

Figure 3 Decline in total physical activity score in elderly patients with metabolic syndrome (MetS). Comparison of total activity score according to each definition and impact of work activity. IDF, International Diabetes Federation; JSIM, Japanese Society of Internal Medicine; NCEP-ATPIII, National Cholesterol Education Program Adult Treatment Panel III.

showed that depression not only leads to a poor outcome in patients with established CVD, but also increases the risk of CVD in apparently healthy persons.[20–22] In addition, prospective studies have shown that depressed persons develop a more sedentary lifestyle.[23,24] Therefore, in the elderly in particular, it is clear that a depressed mood readily leads to a decline in physical activity, resulting in a sedentary lifestyle and eventually MetS. Patients with lower TAS as a result of a markedly depressed mood might have a higher incidence of future CVD.

How can we prevent and treat MetS in the elderly? The proportion of elderly Japanese persons with BMI ≥ 25 kg/m^2 (the insulin resistance threshold proposed by the World Health Organization Western Pacific Region) has risen progressively to a current level of 31% in men and 30% in women.[25] Diseases associated with inactivity are now an important global public health problem, with 11.7% of deaths in developed countries being linked to obesity and MetS.[26,27] Therefore, this is becoming even more significant, considering the problem of the high prevalence of physical inactivity, obesity and MetS. In fact, relatively few investigations have assessed the crucial correlation of physical activity with MetS in elderly patients. In middle age, numerous clinical trials have shown the importance of lifestyle modification, including moderate-intensity physical activity for at least for 30 min a day, most days of the week, and a bodyweight loss of 5–7%, to improve the individual components of MetS. However, the question of whether medical staff should have a similar therapeutic strategy even in elderly patients has been raised. Therefore, in the present study, we focused on the correlation between TAS and the presence of MetS, which is characterized by central obesity, dyslipidemia, hyperglycemia and hypertension.[28] Of note, our analyses showed evidence that inadequate physical activity, but not excessive calorie intake, might be a major reason for MetS in the elderly. In particular, among the three components in physical activity, we found a significant correlation of "work activity", but not sports or leisure activity, with MetS.

Recently, many clinical studies have been carried out to determine the influence of primary care counseling on the level of physical activity and the maintenance of changes in behavior regarding physical activity. Most of the studies have shown that recommending physical activity can achieve an increase in weekly energy expenditure, even in the elderly.[29-31] In the present study, our obtained data might imply that the patients might not have been aware of the importance of physical activity. Therefore, we emphasize the advice that a higher physical activity level in elderly patients is indispensable to maintain healthy condition.

Several limitations of the present study warrant consideration. Physical activity and several of the risk factors were assessed by self-reporting. It is possible that more precise assessment of these factors might have shown a different contribution of these variables to the reduction in CVD risk. In addition, the present data were obtained by cross-sectional analysis at enrolment in the J-EDIT study. Therefore, the present study does not allow any assessment of the cause–effect relationship for the associations found. Further investigation to elucidate how lower physical activity in elderly T2DM patients finally affects the outcome of cardiovascular events by longitudinal follow up is necessary.

The present study showed that lower physical activity, but not excessive calorie intake, is independently associated with the prevalence of MetS in the elderly with T2DM. In our routine work, encouraging physical activity in the elderly might contribute to the prevention of MetS and subsequent atherosclerotic disease, rather than strict management of abnormal laboratory parameters using multiple drugs.

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Conflict of interest

There is no conflict of interest. The J-EDIT Study Group has not cleared any potential conflicts.

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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Polypharmacy as a risk for fall occurrence in geriatric outpatients

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Objective: To investigate the predictors of falls, such as comorbidity and medication, in geriatric outpatients in a longitudinal observational study.

Methods: A total of 172 outpatients (45 men and 126 women, mean age 76.9 ± 7.0 years) were evaluated. Physical examination, clinical history and medication profile were obtained from each patient at baseline. These patients were followed for up to 2 years and falls were self-reported to their physicians. The factors associated with falls were analyzed statistically.

Results: A total of 32 patients experienced falls within 2 years. On univariate analysis, older age, osteoporosis, number of comorbid conditions and number of drugs were significantly associated with falls within 2 years. On multiple logistic regression analysis, the number of drugs was associated with falls, independent of age, sex, number of comorbid conditions and other factors that were significantly associated in univariate analysis. A receiver–operator curve evaluating the optimal cut-off value for the number of drugs showed that taking five or more drugs was a significant risk.

Conclusion: In geriatric outpatients, polypharmacy is associated with falls. Intervention studies are needed to clarify the causal relationship between polypharmacy, comorbidity and falls. **Geriatr Gerontol Int 2012; 12: 425–430.**

Keywords: bone/musculo-skeletal, elderly, falls, geriatric medicine, internal medicine, polypharmacy.

Introduction

Previous studies have assessed the risk factors for falls in community-dwelling elderly,[1–3] but not in geriatric outpatients, and history of falls, physical ability and living environment were found to be predictors of falls. Outpatients have different characteristics from community-dwelling elderly, and previous studies have not assessed whether medical comorbidity and therapeutic drugs might be risk factors for falls. Falls in patients on medication are complicated, because some drugs, such as aspirin, can cause serious bleeding when they have injurious falls, and others, such as antihypertensive[4] and hypoglycemic[5,6] agents, can cause falls.

Previously, we reported that polypharmacy was associated with the tendency for falls using four indices of fall tendency in a cross-sectional setting in geriatric outpatients,[7] though that study did not evaluate fall occurrences, and also not in a longitudinal manner. Therefore, we aimed at investigating whether polypharmacy was predictive of fall occurrences in a prospective fashion. For this purpose, we followed geriatric outpatients for up to 2 years, and assessed whether polypharmacy is a risk for fall occurrence, together with other risks.

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The validity of two novel indices of fall tendency, the 22 items fall risk index[8] and the 13 points simple screening test,[3] which were used in our previous study, have been confirmed in community-dwelling elderly, but not in geriatric outpatients. Therefore, in the present investigation, the association of these two indices with falls was also evaluated to confirm their validity in geriatric outpatients in a longitudinal study.

Methods

Patients

From 2006 to 2007, a total of 190 consecutive patients aged 65 years or older who were receiving treatment for chronic diseases, such as hypertension, dyslipidemia, diabetes and osteoporosis, who were seen every 2–4 weeks at the outpatient clinic of the Research Institute of Aging Science, Tokyo, were enrolled. All the patients were able to walk independently and their condition was stable. Patients who had acute illness or overt dementia were excluded. Anthropometric and medical information including past history of stroke, myocardial infarction, malignancy and prescribed drugs was obtained from each patient at baseline from the medical chart recorded by the physician in charge. However, 18 patients were excluded, because they were lost to follow up soon after enrolment and the medical information was not fully obtained. All prescribed drugs had not been changed in the included patients for at least 2 months before enrolment. The patients were followed up for 2 years.

Occurrence of falls

During the follow-up period, the patients and their family members responded to the annual questionnaire asking about the occurrence of falls within the past year. The questionnaire was repeated for 2 years.

Indices of fall tendency

After enrolment, the patients were examined for two indices to investigate the fall tendency. These were (i) a questionnaire of the 22 items portable fall risk index;[8] and (ii) the 13 points simple screening test to assess the fall tendency.[3]

Ethical consideration

The present 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.

Data analysis and statistical methods

Values are expressed as mean ± standard deviation. In order to analyze the relationship between falls and comorbidity or drugs, variables were compared using Student's *t*-test or χ^2-test as appropriate. Significant factors found in univariate analysis were included in multivariate logistic regression analysis to determine the association of falls with other variables. Receiver–operating curve (ROC) analysis was carried out to identify the optimal cut-off value of the number of drugs for predicting falls within 2 years. The value with the highest sum of sensitivity and specificity was used as the optimal cut-off value. Logistic regression analysis was carried out to assess the validity of the two indices of fall tendency, adjusted by age and sex. *P*-values <0.05 were considered statistically significant. Data were analyzed using JMP version 8.0.1 (SAS Institute, Cary, North Carolina, USA).

Results

Baseline medical information and two indices of fall tendency were evaluated in 172 patients (Table 1). Drugs prescribed in less than 5% of the patients are not shown. Because only patients who were in a stable condition and were able to walk independently were included, patients with Parkinson's disease, severe paresis or painful arthralgia were not included. Calcium channel blockers prescribed in the present study were all long-acting agents, and the prescribed aspirin dosage was 100 mg in all cases. Only a few patients were receiving insulin therapy, sulfonylureas, angiotensin converting enzyme inhibitors, β-blockers, α-blockers, non-steroidal anti-inflammatory drugs or anticoagulants. No patients were taking neuroleptics or antiparkinsonian drugs.

After 1 year, all patients, except for one who died of congestive heart failure, were followed up ($n = 171$, follow-up rate 99.4%). Falls occurred in 22 patients. Only a higher age was associated with falls within 1 year on univariate analysis (non-fallers: 76.4 ± 6.8 years, fallers: 81.0 ± 6.9 years, $P = 0.004$).

After another year (2 years after enrolment), one patient had died of lung cancer, and five patients were lost to follow up. A total of 165 patients were evaluated (follow-up rate 95.9%), and 10 patients had fallen during the second year; thus a total of 32 patients had fallen within 2 years. As shown in Table 2, higher age, osteoporosis, number of comorbid conditions and number of drugs were significant factors associated with falls. To determine the association of falls with these significant factors, multivariate logistic regression analysis was carried out, and as shown in Table 2, the number of drugs was the only factor that was significantly associated with falls within 2 years.

As polypharmacy was assumed to be a risk for falls within 2 years, the cut-off of the number of the drugs was analyzed. Figure 1 shows the ROC curves to define the optimal cut-off point in relation to falls within

Table 1 Characteristics and univariate analysis of association with fallers and non-fallers within 2 years and risk factors

Total		Non-fallers ($n = 133$)	Fallers ($n = 32$)	P-value (Fallers vs. Non-fallers)
Age (years)	77.0 ± 7.0	76.3 ± 6.9	80.0 ± 6.9	0.007
Body mass index (kg/cm^2)	22.7 ± 3.2	22.7 ± 3.3	22.7 ± 3.1	0.98
No. comorbid conditions	1.9 ± 1.1	1.8 ± 1.1	2.3 ± 0.9	0.009
No. drugs	3.2 ± 2.8	2.8 ± 2.7	4.9 ± 2.5	<0.0001
Female ($n = 122$)	–	72.9%	78.1%	0.66
Hypertension ($n = 106$)	–	62.4%	71.8%	0.41
Dyslipidemia ($n = 76$)	–	47.3%	40.6%	0.56
Diabetes ($n = 23$)	–	12.8%	18.8%	0.40
Osteoporosis ($n = 59$)	–	30.8%	56.3%	0.01
History of stroke ($n = 6$)	–	2.3%	9.4%	0.09
History of myocardial infarction ($n = 3$)	–	0.8%	6.3%	0.10
History of cancer ($n = 8$)	–	5.3%	3.1%	0.99
Calcium channel blocker ($n = 59$)	–	33.3%	46.9%	0.16
Angiotensin II receptor blocker ($n = 56$)	–	33.3%	37.5%	0.68
Statin ($n = 40$)	–	23.5%	28.1%	0.65
Aspirin ($n = 31$)	–	19.0%	24.1%	0.61
Bisphosphonate ($n = 9$)	–	4.6%	9.4%	0.38
H2-blocker ($n = 9$)	–	3.8%	12.1%	0.80
Proton pump inhibitor ($n = 11$)	–	5.3%	12.1%	0.23
Hypnotic ($n = 31$)	–	16.7%	28.1%	0.14

Values are expressed as mean ± SD ($n = 165$).

Table 2 Logistic regression analysis of association of falls within 2 years with age, sex, other significant factors found in univariate analysis, and polypharmacy

	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (/1 year)	1.08 (1.03–1.13)†	1.06 (0.99–1.13)	1.06 (0.99–1.13)
Sex (male = 0, female = 1)	1.39 (0.56–3.48)	0.98 (0.29–3.23)	0.75 (0.23–2.38)
Osteoporosis ($n = 0$, Y = 1)	3.12 (1.43–6.84)†	2.76 (0.92–7.38)	3.02 (0.96–6.15)
No. comorbid conditions (/disease)	1.63 (1.14–2.32)*	0.90 (0.55–1.47)	0.99 (0.62–1.56)
No. drugs (/drug)	1.29 (1.12–1.48)‡	1.30 (1.08–1.57)*	–
Five or more drugs ($n = 0$, Y = 1)	5.04 (2.25–11.3)‡	–	4.50 (1.66–12.2)†

*$P < 0.05$, †$P < 0.005$, ‡$P < 0.0005$. CI, confidence interval.

2 years: the area under the ROC was 0.731, and the optimal cut-off value of the number of drugs was five (sensitivity 0.576, specificity 0.788). Logistic regression analysis showed that taking five or more drugs was significantly associated with an increased risk of falls (odds ratio 4.5, 95% CI 1.7–12.2) after adjustment for age, sex, osteoporosis and number of comorbid conditions (Table 2).

Also, the association between falls and two indices of fall tendency was evaluated to confirm the validity of each index in geriatric outpatients. As both indices included the questionnaire asking whether patients were "taking five or more drugs," the number of drugs was excluded from this analysis because of duplication in the statistical model. As shown in Table 3, the 22 items fall risk index showed a tendency towards an association with falls within 2 years, odds ratio 1.12 (95% CI 1.00–1.26; $P = 0.05$), whereas the 13 points screening test was significantly associated with falls after adjustment for age, sex and other factors significantly associated in the univariate analysis. Therefore, these indices are considered to be good predictors of falls in geriatric outpatients, as has been shown in community-dwelling elderly subjects.

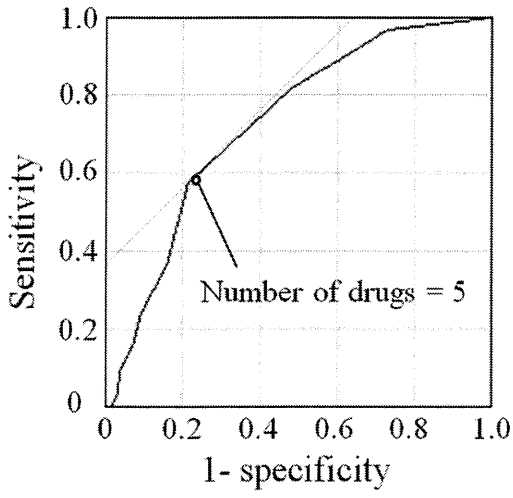

Figure 1 Receiver–operating curves to define optimal cut-off value of number of drugs at baseline in relation to falls within 2 years. Area under the curve was 0.731, optimal cut-off value of the number of drugs was five (sensitivity = 57.6%, specificity = 78.8%).

Discussion

The risk of falls has been assessed in community-dwelling elderly, and history of falls, physical ability and living environment were found to be predictors of falls. Also, in nursing home residents, cognitive function, gait disturbance and urinary incontinence are reported to be risk factors for falls,[9,10] and length of stay, disease condition, surgical procedures and some specific drugs are reported to be risk factors in hospital inpatients.[11,12]

Nevertheless, the risks in geriatric outpatients have not been sufficiently assessed, although assessment of fall risk in geriatric outpatients is important; their medical conditions or drugs might cause falls, and drugs, such as antiplatelet agents or anticoagulants, might cause critical bleeding after a fall. Also, physicians could prevent falls in their patients by giving advice during regular consultations, if risk factors are identified.

In our previous cross-sectional study assessing geriatric outpatients, polypharmacy was significantly correlated with indices of fall tendency, and the present follow-up study of geriatric outpatients showed the impact of polypharmacy on falls within 2 years. Statistical analyses showed that polypharmacy was a risk factor for falls, independent of age, sex and comorbidity.

Besides polypharmacy, several medications and comorbid conditions have been reported as risks for falls.[13–22] Among these, diabetes,[5,6] insomnia,[13] hypnotics,[13–15] antiarrhythmics[22] and antihypertensive agents[14] were not significantly associated with fall risk in the present study. Just 11 patients (45.9% of diabetic patients) were prescribed hypoglycemic agents, such as a sulfonylurea ($n = 8$) or insulin ($n = 3$), and the relatively low rate of prescription of hypoglycemic agents might have affected our result. Neither hypnotics nor antihypertensives were associated with falls. This result might be a result of the small sample size. Anti-arrhythmics were taken by just three patients (digoxin: $n = 2$, class IA anti-arrhythmic drug: $n = 1$). Other drugs, such as major tranquillizers,[14] antidepressants[17,18] and antiparkinsonian agents,[19,22] might increase fall risk; however, no patient used these drugs in the present study. In the present study, most of the patients were in a stable condition throughout the 2 years, though their drugs were changed gradually according to their medical conditions during the observation period. We only used the number of drugs at baseline for statistical analysis; however, the number of drugs increased from 3.2 ± 2.8 to 3.9 ± 3.0 during the 2 years. There were 17 patients whose number of drugs had been decreased, 70 patients not changed and 78 patients increased. The number of drugs after 2 years was also associated with falls ($P < 0.0005$). The optimal cut-off point for the number of drugs was again five (area under ROC curve 0.780, sensitivity 0.576, specificity 0.788). Furthermore, the changes in number of drugs were also associated with falls ($P < 0.05$), and the optimal cut-off point for the change in number of drugs was +1 (area under ROC curve 0.649, sensitivity 0.727, specificity 0.409).

Table 3 Logistic regression analysis of association between 2-year fall occurrences with two indices of fall tendency; 22 items fall risk index and 13 points simple screening test

	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age (/year)	1.08 (1.03–1.15)**	1.06 (0.99–1.13)	1.06 (1.00–1.13)
Sex (male = 0, female = 1)	1.39 (0.56–3.48)	0.75 (0.23–2.43)	0.79 (0.24–2.56)
Osteoporosis ($n = 0$, Y = 1)	3.12 (1.43–6.84)**	2.56 (0.96–6.82)	2.61 (0.98–6.95)
No. comorbid conditions (/disease)	1.63 (1.14–2.32)*	1.24 (0.83–1.86)	1.32 (0.88–1.97)
Fall risk index (/item)	1.23 (1.11–1.37)***	1.12 (1.00–1.26)	–
Simple screening test (/point)	1.19 (1.06–1.33)**	–	1.14 (1.01–1.29)*

*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. CI, confidence interval.

Consequently, polypharmacy, especially taking five or more drugs, should be considered a risk for falls.

There were several limitations of the present study. First, the falls were self-reported by the patients. Although all the patients had no overt dementia, they might have forgotten the incident of falling. We attempted to count the total fall occurrences in each patient; however, we could not differentiate the repeated falls in the second year from the fall occurrence in the first year. In fact, we asked 22 patients who reported falls in the first year about fall occurrence during the second year, but they did not accurately recall whether they experienced falls in the first or second year. Second, five patients were lost to follow up at 2 years for unknown reasons. The follow-up ratio was acceptable, although some of the patients might have fallen, have been no longer able to come to the clinic and moved to nursing homes. This might have slightly influenced the result. Also, the cause of falls in polypharmacy patients is not explained. Potentially inappropriate medications, which could cause adverse drug reactions, are usually seen in patients with polypharmacy, and falls might be the consequence of adverse drug reactions, such as dizziness, instability and light-headedness. Pathophysiological assessments and drug-reducing interventions are expected to elucidate the causal relationship.

Additionally, we showed that the 22-item fall risk index and its simple screening test were useful to predict falls in geriatric outpatients. Although both indices have been validated in community-dwelling elderly people, the present finding also showed their association with fall risk among geriatric outpatients. The difference of statistical significance between fall risk index and simple screening test might be a result of small sample size or the difference in the contribution of each item to total scores between the two indices. "Taking five or more drugs" accounts for only one item out of the 22-item fall risk index; in contrast, the same questionnaire accounts two points in the 13-point simple screening test. Because polypharmacy was a strong risk factor of falls in elderly outpatients in the present study, the proportion of polypharmacy in the scores might have caused the discrepancy. Taken together, it is likely that 13-point screening test was more suitable to our subjects who were taking several medicines.

In summary, the present study showed that geriatric outpatients with polypharmacy were at a high risk of falls, especially those receiving five or more drugs. Our finding might add new information for pharmacotherapy and geriatric research in elderly patients with chronic diseases. Intervention studies examining the effect of drug reduction for the prevention of falls are required in the future.

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Disclosure statement

The authors declare no conflict of interest.

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Original article

Thrombomodulin, a novel molecule regulating inorganic phosphate-induced vascular smooth muscle cell calcification

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ABSTRACT

Hyperphosphatemia has emerged as a cardiovascular risk factor that stimulates calcification in vessels. We explored molecules that were induced by inorganic phosphate (Pi) at an early stage in vascular smooth muscle cells (VSMC). In the present study, we examined the role of thrombomodulin (TM) in Pi-induced VSMC calcification based on the results of DNA microarray analysis. Both mRNA and protein expression of TM were markedly augmented in Pi-induced calcification. Conversely, knockdown of TM by siRNA significantly inhibited calcification, in addition to Pi-induced apoptosis which plays critical roles in VSMC calcification. We further found that TM suppressed both of mRNA and protein expression of growth arrest-specific gene 6 (Gas6), a key molecule regulating apoptosis. Recombinant extracellular epidermal growth factor (EGF)-repeat domain of TM exaggerated calcification and this effect was abrogated by a neutralizing antibody for EGF receptor, suggesting that the cleaved and secreted form of TM may activate EGF receptor. We also found that downregulation of Gas6 by TM/EGF receptor axis was mediated by ERK in VSMC calcification. In the aorta of adenine-fed rat, a typical medial calcification model with hyperphosphatemia, we found that TM expression was increased. Furthermore, in human calcified aorta, increased TM expression was also observed. These results indicate that TM is a novel molecule that promotes apoptosis and vascular calcification by regulation of Gas6, presumably via EGF receptor/ERK axis.

1. Introduction

Vascular calcification is a significant feature of vascular pathology, since this lesion is associated with cardiovascular disease [1–3]. It has been recognized that inorganic phosphate (Pi) is an important inducer of vascular smooth muscle cell (VSMC) calcification, which is morphologically similar to that observed in calcified human heart valves and the aortic media [4]. Pi transport via type III sodium-phosphate cotransporter (Pit-1) is a critical step in the initiation of calcification [5]. However, few studies have explored the target molecules of Pi in calcification of VSMC.

To identify molecules that play an initial and central role in Pi-induced calcification, we employed DNA microarray analysis. Among the identified genes, we focused on thrombomodulin (TM), which was increased 1.4 fold at 24 h after treatment with Pi, because TM has been found to have diverse effects on cellular proliferation [6], cell–cell adhesion [7], and inflammation [8], all of which are important steps in the pathogenesis of atherosclerosis.

TM is a cell membrane-bound glycoprotein that functions as a thrombin cofactor in the activation of protein C. Normally, expression of TM in the arterial wall is limited to the endothelium and is not expressed in the medial smooth muscle. Interestingly, in human atherosclerotic lesions, TM is expressed in VSMC of the media and intima [9,10], suggesting its pathological roles. TM consists of five distinct domains: the NH2-terminal domain, epidermal growth factor (EGF)-like domain, serine/threonine-rich region, transmembrane domain, and cytoplasmic tail [9]. In particular, the EGF-like domain consists of six tandem EGF-like motifs that are homologous to domain III of the human EGF precursor [11]. In VSMC, the EGF-like domain of TM has been reported to exert proatherosclerotic effects [6].

ERK is a well-known downstream signaling molecule of TM [6,12]. It has been reported that TM prolongs thrombin-induced ERK phosphorylation and nuclear retention in vascular endothelial cells [12]. In VSMC, recombinant TM containing the EGF-like domain stimulates ERK phosphorylation, which increases cell proliferation [6]. The ERK

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pathway is also known to play a critical role in osteoblast differentiation and mineralization [13–15]. Furthermore, in calcifying valvular interstitial cell cultures, prolonged elevation of phosphorylated ERK (pERK) was found, and blocking pERK resulted in a marked decrease in nodule number, nodule size, and total calcified area. Although the involvement of ERK activation in the process of VSMC calcification has been shown in previous reports [16,17], the upstream and downstream signaling of ERK is unclear.

Regarding the molecular mechanisms of vascular calcification, we have recently reported that downregulation of the growth arrest-specific gene 6 (Gas6)-mediated survival pathway plays a pivotal role in Pi-induced apoptosis and subsequent calcification [18,19]. In the present study, we demonstrated that TM is a novel molecule regulating vascular calcification. Pi induces TM expression and cleaves the extracellular domain of TM, which stimulates the EGFR/ERK axis. Then, ERK inhibits Gas6 expression, leading to cell death and the development of calcification.

2. Materials and methods

2.1. Cell culture and materials

Human aortic smooth muscle cells (HASMC) were purchased from Clonetics Corp. (San Diego, CA) HASMC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HASMC were used up to passage 8 for the experiments. MEK inhibitors (PD98059 and U0126) and a matrix matalloproteinase (MMP) inhibitor (GM6001) were purchased from Calbiochem (La Jolla, CA, USA). Lavendustin A was from TOCRIS (Ellisville, MO, USA), and EGF receptor (EGFR) neutralizing antibody was from Millipore (Temecula, CA, USA). Recombinant human TM peptide containing 6 EGF-like domains (rhTME1-6) was prepared as described previously [20].

2.2. Calcifying medium and quantification of calcification

HASMC were maintained in growth medium (DMEM containing 15% FBS and 100 U/mL of penicillin and 100 mg/mL of streptomycin; final P_i concentration = 1.4 mmol/L). Pi(−) means the concentration of Pi (1.4 mmol/L) in growth medium without additional Pi stimulation. To induce VSMC calcification, we added Pi, a mixed solution of 1 mol/L Na_2HPO_4 and 1 mol/L NaH_2PO_4 whose pH was adjusted to 7.4 to serum-supplemented DMEM to a final concentration of 2.6 mmol/L (Pi(+)). Ca deposition was evaluated by the o-cresolphthalein complexone method (C-Test; WAKO). von Kossa staining was performed as follows. After treatment with 5% silver nitrate (Wako) solution for 1 h, the samples were exposed to strong light to visualize calcium deposits, and then 5% sodium thiosulfate solution was added.

2.3. High-density oligonucleotide microarray analysis

Total RNA was prepared using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA extracted from VSMC with or without 2.6 mmol/L Pi treatment for 24 h was amplified up to approximately 100 Ag cRNA and hybridized to a high-density oligonucleotide microarray (GeneChip Rat Genome U34A; Affymetrix, Santa Clara, CA, USA) as described previously [21].

2.4. Small interfering RNA

Four small interfering RNAs (siRNA) were designed to target human TM (Dharmacon). The sequences of TM siRNAs were 5′-GGACGUGG AUGACUGCAUA-3′, 5′-GUCAUUUCCUUGC UACUGA-3′, 5′-GCACUCAA UGCUCAAUG-3′, and 5′-GCAUUCGGGCUUGCUCAUA-3′. Those of Pit-1 were 5′-CCAUGGUGGCAAUGACGUA-3′, 5′-GAAUGUGAACUUCGGGCAA-3′, 5′-CCAAGAAGCGAAUUCGAAU-3′, and 5′-CCUAAUGGUUUGCGAGCU U-3′. Those of Gas6 siRNAs were 5′-GUGACGAGGGCUUUGCGUA-3′ and 5′-GGAGAAGGCUUGCCGAGAU-3′. Regarding the experiments using siRNAs, we used all four siRNAs targeting TM or Pit-1, and two siRNAs targeting Gas6 at the same time. Non-targeting control siRNA was synthesized using standard templates (Dharmacon). The efficiency of siRNA was validated by immunoblotting and real-time PCR of the cell lysates and total RNA at 6 days. To evaluate the effect of TM on VSMC calcification, siRNAs were transfected using transfection reagent (Upstate Biotechnology) when HASMC had reached 70~80% confluence, and were then transfected each time the medium was changed every 2 days. On day 6, apoptosis and Ca deposition were measured.

2.5. Aortic calcification in renal failure rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described [22]. All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Twelve-week-old male Wistar rats (Nippon Clea Inc., Japan) were pair-fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus; Nippon Clea Inc.) in the control group or CE-2 chow containing 0.75% adenine (Sigma) in the renal failure group for 4 weeks. Then, the diet was returned to normal chow for an additional 4 weeks. After induction of renal failure for 8 weeks in total, the rats were sacrificed to collect samples. After perfusion with saline at a constant, nonpulsatile pressure of 100 mm Hg, the aorta was immediately embedded in OCT compound frozen section and sequentially cut into cross-sections with 5-μm thickness from each part of the aorta. To detect calcification in the aortic wall, each cross-section was subjected to von Kossa staining to demonstrate mineralization.

2.6. Calcified aortic specimens of human

Human aortic specimens were obtained from four autopsy cases (3 cases from patients who have atherosclerosis and chronic kidney disease (CKD); 82- and 84-year-old men and 90-year old woman and 1 case from patient without atherosclerosis and CKD; 33-year-old man). This protocol was approved by the Institutional Review Board of Tokyo Metropolitan Geriatric Hospital.

2.7. Statistical analysis

All values are presented as mean ± SEM. Statistical comparisons were conducted by ANOVA, followed by Fisher's test. A value of $p<0.05$ was considered statistically significant.

An expanded Methods section is available in the online Supplemental Methods.

3. Results

3.1. Expression and localization of TM in Pi-induced VSMC calcification

To confirm the results obtained by DNA microarray analysis (Table S1), we examined the expression of TM in the process of Pi-induced calcification. Both mRNA and protein expression of TM were markedly increased by treatment with Pi for 6 days in a time-dependent manner (Figs. 1A, B). The increased expression of TM was also observed by immunofluorescent staining at 6 days (Fig. 1C). We found that this increase was limited in the cytoplasmic fractions, not in the nuclear fraction of Pi-treated HASMC by immunoblotting (Fig. S1A). Furthermore, Pi-induced TM upregulation was blocked by Pit-1 siRNA, suggesting that the increase in TM expression by Pi was mediated by Pit-1, type III sodium-phosphate cotransporter (Fig. 1D). We also examined whether TM transcription activity was increased by Pi. When we

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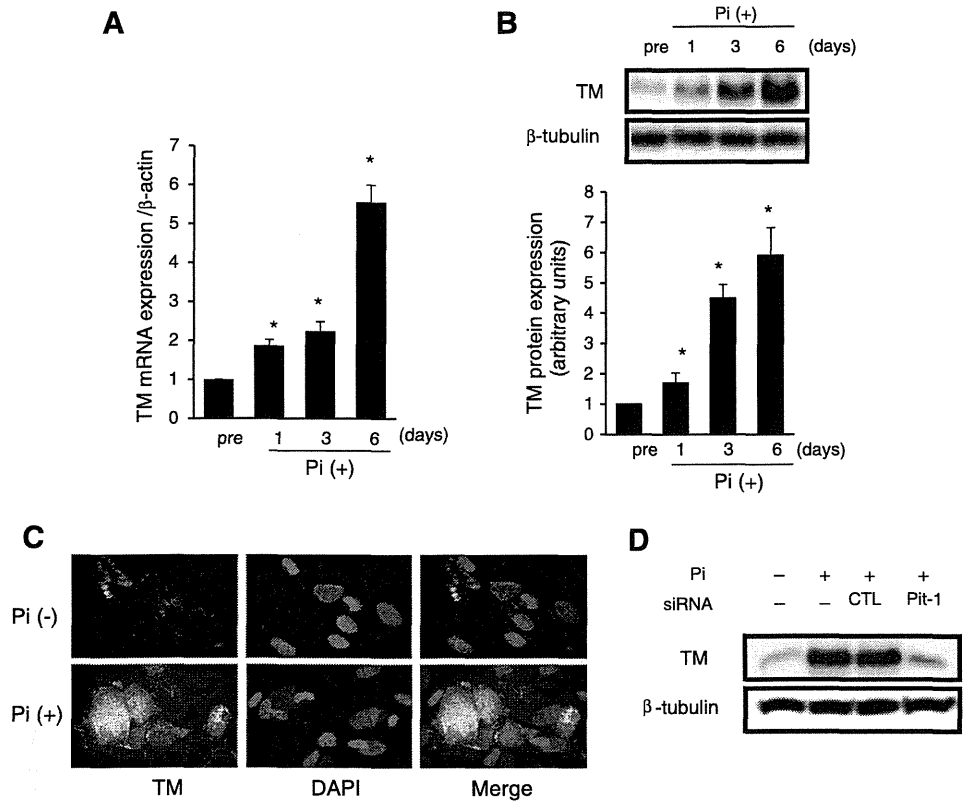

Fig. 1. Expression of thrombomodulin in Pi-induced VSMC calcification. Expression of thrombomodulin (TM) mRNA (A) and protein (B) in the presence of 2.6 mmol/L Pi (Pi(+)) on the indicated days (n=3). (C) The increase in TM expression with Pi treatment (2.6 mmol/L) at 6 days was also detected by immunofluorescent staining (green). Nuclei were counterstained with DAPI (blue). (D) HASMC were transfected with 100 nmol/L Pit-1 siRNA or nonspecific (CTL) siRNA in the presence of 2.6 mmol/L Pi. On day 6, cell lysates were harvested. All values are presented as mean ± SEM. *p<0.05. The analysis was conducted by ANOVA followed by Fisher's test.

transfected the TM-luc construct into HASMC, Pi increased luciferase activity by 2~2.5 fold (Fig. S1B).

3.2. TM augments VSMC apoptosis via downregulation of Gas6.

Apoptosis is a crucial and initiating event in Pi-induced VSMC calcification [23]. Furthermore, it has been suggested that TM expressed in VSMC exerts an atherogenic effect on neighboring VSMC after its release from dead cells [6]. To investigate the relation between TM and apoptosis, we performed double immunostaining of TUNEL and TM in Pi-treated VSMC. TM expression was co-localized with TUNEL-positive cells, indicating that Pi-induced TM expression is associated with apoptosis (Fig. 2A).

To further examine the effect of TM on apoptosis with quantitative analysis, we performed knockdown of TM using siRNA. On day 6, the increased expression of both TM mRNA and protein was markedly suppressed by siRNA (Figs. 2B, C). Under this condition, Pi-induced apoptosis and calcium deposition were also significantly inhibited by TM siRNA (Figs. 2D, E).

Our previous study suggested that the Gas6-mediated survival pathway is downregulated by Pi, leading to apoptosis and calcification [18]. To clarify whether TM affects Gas6 expression in the process of Pi-induced apoptosis, we examined the expression of Gas6 in HASMC when TM expression was knocked down by siRNA. As expected, both protein and mRNA expression of Gas6 were clearly restored by TMsiRNA (Figs. 3A, B). Furthermore, Gas6 transcriptional activity was significantly increased by TM siRNA, suggesting that the induction of Gas6 expression by TM siRNA was attributable to restoration of transcription activity (Fig. 3C). We further found that Akt phosphorylation, a downstream signal of Gas6, was also restored by TM siRNA, whereas Akt expression was not changed (Fig. S2A). Thus, TM regulates Gas6-mediated survival pathway and induces apoptosis during VSMC calcification.

3.3. Pi increases TM secretion, and rhTM augments Pi-induced calcification via EGFR

We next investigated the molecular mechanism of Gas6 regulation by TM. Since the EGF-like domain of TM has been reported to exert atherogenic effects on VSMC [6], we hypothesized that the cleaved and secreted form of TM exerts the above mentioned effects. In fact, TM secretion in the culture medium was increased by Pi (Fig. 4A). Given the functional role of the EGF-like extracellular domain of TM, we examined whether EGFR was activated by the TM secretion. EGFR phosphorylation (pEGFR) was increased by Pi in a time-dependent manner for 6 days (Fig. 4B). Conversely, the increased expression of pEGFR was significantly decreased by TM siRNA (Fig. 4C).

Addition of rhTME1-6 or EGF significantly augmented Pi-induced VSMC calcification (Fig. 4D). These stimulatory effects of rhTME1-6 or EGF on calcification were significantly inhibited by an EGFR neutralizing antibody (Fig. 4D). Furthermore, lavendustin-A, a protein tyrosine kinase inhibitor specific for EGFR, abrogated both rhTME1-6- and EGF-stimulated VSMC calcification (Fig. S3).

3.4. ERK is a downstream signal regulated by TM/EGFR axis

Since ERK is a well-known downstream signal of TM, we examined the role of ERK in Pi-induced VSMC calcification. When TM was knocked down by siRNA, Pi-induced ERK phosphorylation was inhibited, whereas ERK expression was not affected (Fig. 5A). Immunofluorescent

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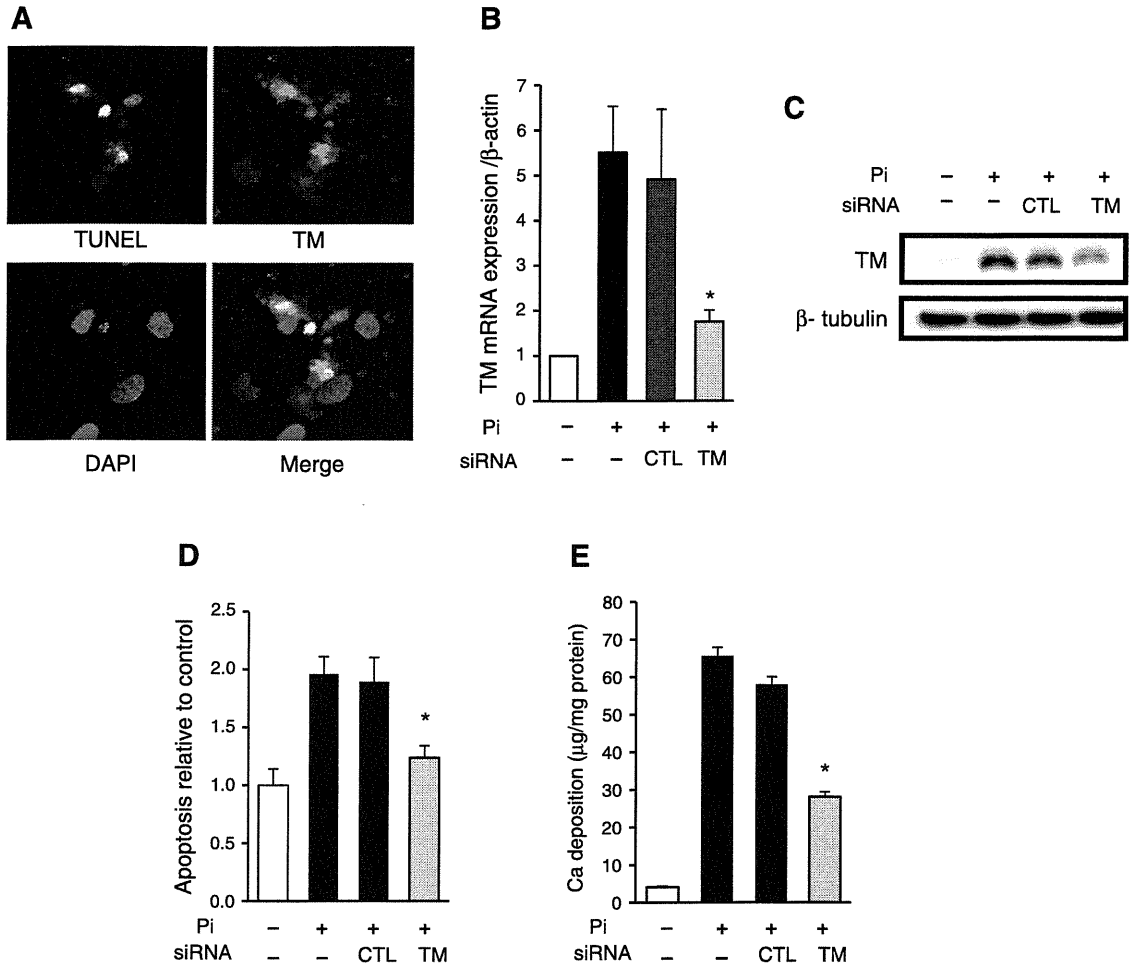

Fig. 2. Effect of TM knockdown on Pi-induced apoptosis and calcification. (A) After 2.6 mmol/L Pi treatment for 6 days, TUNEL-positive cells (green) and TM (red) expression were evaluated by immunofluorescent staining. Nuclei were counterstained with DAPI (blue). (B) and (C) HASMC were transfected with 100 nmol/L TM siRNA and nonspecific (CTL) siRNA in the presence of 2.6 mmol/L Pi. On day 6, total RNA and protein were extracted to confirm the efficiency of TM knockdown. Under the same condition, apoptosis (D, n = 3) and Ca deposition (E, n = 5) were measured. All values are presented as mean ± SEM. *p<0.05 vs. Pi(+), CTL siRNA. The analysis was conducted by ANOVA followed by Fisher's test.

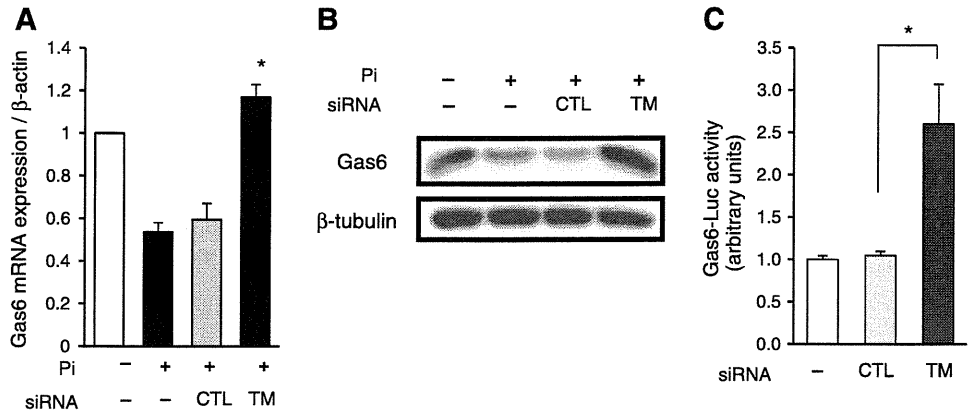

Fig. 3. Effect of TM on Gas6 expression. HASMC were transfected with 100 nmol/L TM siRNA and nonspecific (CTL) siRNA in the presence of 2.6 mmol/L Pi. On day 6, total RNA was extracted to determine Gas6 expression (A), and cell lysates were harvested for Gas6 and β-tubulin expression (B). (C) HASMC were transiently transfected with 0.8 μg pGL3Gas6(1827) construct. Twenty four hours after transfection, 100 nmol/L of TM siRNA, or nonspecific (CTL) siRNA was added. The cells then were treated with 2.6 mmol/L Pi, 6 h after siRNA transfection, and were incubated for an additional 24 h. Relative promoter activities are expressed as mean ± SEM (n = 4). Three separate transfection experiments showed similar results. *p<0.05 vs. Pi(+), CTL siRNA. The analysis was conducted by ANOVA followed by Fisher's test.

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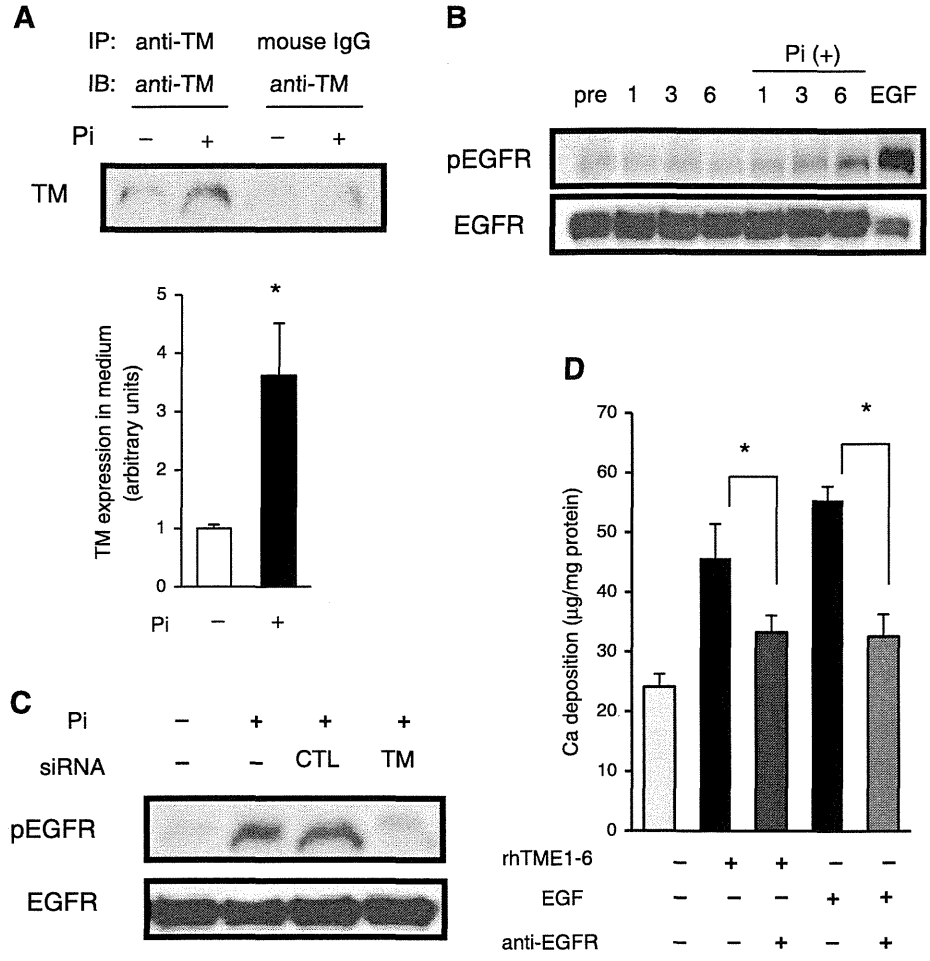

Fig. 4. Effect of TM on VSMC calcification is mediated by EGFR. (A) TM secretion into the medium was increased in the presence of 2.6 mmol/L Pi. (B) Expression of pEGFR in the absence or in the presence of Pi on the indicated days. Positive control is EGF (50 ng/mL) treatment for 6 days. (C) HASMC were transfected with 100 nmol/L TM siRNA or nonspecific (CTL) siRNA in the presence of 2.6 mmol/L Pi. On day 6, cell lysates were harvested for immunoblotting with pEGFR and EGFR. (D) HASMC were cultured with or without rTME1-6 (50 μg/mL), EGF (50 ng/mL), or anti-EGFR neutralizing antibody (10 μg/mL) in the presence of Pi ($n=5$). All values are presented as mean ± SEM. *$p<0.05$. The analysis was conducted by ANOVA followed by Fisher's test.

staining showed that VSMC highly expressing TM shows the higher phosphorylation of ERK (Fig. 5B). We confirmed that the expression of TM was not changed by treatment with MEK inhibitors (U0126 and PD98059) (Fig. S4). Other MAP kinases such as p38 and JNK were not activated by Pi (Fig. S5). U0126 significantly suppressed Pi-induced VSMC apoptosis as well as calcification (Figs. 5C, D). Similar effects were also obtained with PD98059 (data not shown). As shown in Fig. 5E, ERK inhibition by U0126 and PD98059 restored both mRNA and protein expression of Gas6. Furthermore, Gas6 transcriptional activity was significantly increased by U0126 (Fig. 5F) and PD98059 (data not shown). Akt phosphorylation was also increased by MEK inhibitors (Fig. S2A). Addition of rhTME1-6 further increased ERK phosphorylation in the presence of Pi, while the expression of Gas6 was decreased by rhTME1-6 (Fig. 5G). rhTME1-6-mediated regulation of ERK and Gas6 was abrogated by the EGFR neutralizing antibody (Fig. 5G).

3.5. Increased TM expression in a rat model of VSMC calcification and human atherosclerotic lesions with calcification

Then, we examined the expression of TM in the aorta of adenine-fed rat, a typical medial calcification model with renal failure and hyperphosphatemia [22]. TM expression was increased adjacent to the calcified area of the aorta in 0.75% adenine-fed rats, compared with that of control rats (Fig. 6A). The protein expression of TM was significantly increased in calcified aorta of adenine-fed rats (Fig. 6B).

Next, to check the clinical relevance of TM in vascular calcification, we examined the expression of TM in the atherosclerotic aorta of an 84-year-old man with CKD and coronary heart disease. von Kossa staining and TUNEL assay were performed to detect calcification and apoptosis, respectively. As shown in Fig. 6C, TUNEL-positive cells were found in the aortic sections adjacent to calcified areas. Staining for smooth muscle α-actin suggested that TUNEL-positive cells were VSMC (Fig. S6). No von Kossa-positive cells or TUNEL-positive cells were found in the aorta of a 33-year-old healthy man (data not shown). To examine whether TM expression was co-localized with apoptotic cells, we performed double staining of TM and TUNEL. In the calcified aorta, TM expression was localized close to TUNEL-positive cells (Fig. 6C, lower panels). Furthermore, the expression of pERK was increased in similar regions of the calcified aorta and co-localized with TM (Fig. 6D). We obtained comparable results using calcified aortas from an 82-year-old man and a 90-year-old woman (data not shown). These results suggest that TM/ERK expression is increased in human calcified aorta.

4. Discussion

In the present study, we demonstrated that TM is a novel molecule regulating vascular calcification, and found that TM downregulates the Gas6 survival pathway, leading to VSMC calcification. The EGFR/ERK

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