and blood flow were similar between the normoalbuminuric patients with and without renal insufficiency. These results indicate that diabetic patients with normoalbuminuric renal insufficiency may have a lower risk of systemic arterial damage.

Among the study limitations of the present research, we note, firstly, that administration of ACEI [17] and ARB [18] can decrease urinary albumin excretion and slow the decline in the GFR among type 2 diabetic patients. Therefore, we cannot rule out the possibility that those medications may alter the natural history of diabetic nephropathy and peripheral circulation in lower-leg arteries. Secondly, our data were obtained in a Japanese population, and therefore it remains to be established whether these results can be generalized to other ethnicities. Additionally, we used cross-sectional study design. Further prospective study is necessary to clarify the impact of nephropathy on insufficient blood flow in lower-leg arteries among diabetic patients.

In conclusion, we have demonstrated that impaired blood flow in lower-leg arteries caused by higher arterial stiffness and greater vascular resistance associates with eGFR in albuminuric type 2 diabetic patients with normal ABI. Our findings are the first to clarify that diabetic nephropathy may increase the risk of lower-limb ischemia even though the patient has no apparent PAOD.

Conflict of interest

The authors state that they have no conflict of interest.

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Coronary artery calcification, arterial stiffness and renal insufficiency associate with serum levels of tumor necrosis factor-alpha in Japanese type 2 diabetic patients

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ABSTRACT

Although the kidneys are the major source of proinflammatory cytokines, association of tumor necrosis factor-alpha (TNF-α) with severity of atherosclerosis or kidney function in diabetic patients is unclear. Two hundred type 2 diabetic patients and 30 age-matched nondiabetic subjects consecutively admitted to our hospital were enrolled. The Agatston coronary artery calcium score (CACS), a quantitative marker of coronary atherosclerosis, was obtained using multidetector-row computed tomography. Arterial stiffness was assessed by brachial-ankle pulse wave velocity (baPWV). Diabetic patients had higher log(CACS + 1) ($p = 0.0089$), baPWV ($p = 0.0293$), frequency of elevated urinary albumin excretion (UAE) ($p < 0.0001$) and TNF-α ($p = 0.0029$) and similar estimated glomerular filtration rate (eGFR) compared to nondiabetic subjects. When diabetic patients were grouped into four subgroups with or without elevated UAE and renal insufficiency (UAE of ≥30 or <30 mg/24 h and eGFR of <60 or ≥60 ml/min per 1.73 m^2), patients with micro- and macroalbuminuric renal insufficiency showed the highest log(CACS + 1) ($p < 0.0001$), baPWV ($p = 0.0068$) and TNF-α ($p < 0.0001$) of these groups. Log(CACS + 1) ($p = 0.0008$) and baPWV ($p = 0.0006$) positively and eGFR ($p < 0.0001$) negatively correlated with TNF-α in diabetic patients. We find that coronary artery calcification, arterial stiffness, and renal insufficiency associate with circulating levels of TNF-α in type 2 diabetic patients.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in type 2 diabetic patients. Elevated urinary albumin excretion (UAE) [1,2] and a decrease in glomerular filtration rate [3,4] both contribute to the development of end-stage renal disease and CVD events in diabetic patients. Current diabetes guidelines recommend screening for elevated UEA as the earliest clinical evidence of nephropathy [5] as well as screening for a decline in estimated glomerular filtration rate (eGFR) as calculated by the modification of diet in renal disease (MDRD) formula to detect chronic kidney disease attributed to diabetes [6]. Vascular inflammation contributes to the pathogenesis of both micro- and macrovascular complications of diabetes through the actions of proinflammatory cytokines [7,8]. Tumor necrosis factor-alpha (TNF-α)

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can be produced by adipose tissues [9], kidneys [10] and atherosclerotic lesions in the arterial walls [11]. In the kidneys, advanced glycation end-products (AGEs) [12], angiotensin II [13] and oxidized LDL [14] can stimulate TNF-α synthesis from the renal cells and initiate local effects of renal damage. The activities of this cytokine are not limited to the renal injury; intra-arterial TNF-α administration can cause vascular inflammation and impair endothelial function [15]. Circulating levels of TNF-α are elevated in patients with metabolic syndrome [16], regarded as a prediabetic state, and in obese [17] or nephropathic [18] patients with type 2 diabetes. Such patients are therefore considered at high risk of CVD events.

Diabetic nephropathy is a major contributor to the development of atherosclerosis including vascular calcification [19] and arterial stiffness [20]. Vascular calcification is a manifestation of atherosclerosis that begins early in the atherosclerotic process [21]. A number of studies have documented the usefulness of electron-beam computed tomography (EBCT) [22] and multidetector-row computed tomography (MDCT) [23] for detection and evaluation of the extent of calcium deposits in the coronary arteries among asymptomatic patients before the onset of life-threatening cardiac events. Autopsy [22] and angiographic studies [24] of the coronary arteries reveal that the amount of calcification correlates with the burden of atherosclerosis. Diabetic patients show twofold prevalence of extensive coronary artery calcification compared with nondiabetic subjects [25]. Aortic stiffness is greater in diabetic patients than in nondiabetic subjects [26]. Large artery stiffness leads to increased systolic blood pressure and ventricular mass and hampers coronary filling in diastole [27], leading to increased CVD risk. Although coronary artery calcification and large artery stiffness are powerful predictors of CVD events [24,28], the association of TNF-α with these risk factors has not been fully elucidated in diabetic patients.

In the present study, we investigated the association of circulating levels of TNF-α with the severity of atherosclerosis or renal insufficiency in type 2 diabetic patients.

2. Materials and methods

Two hundred type 2 diabetic patients and 30 age-matched nondiabetic subjects ranging in age from 50 to 69 years who had been consecutively admitted to our hospital between November 2004 and October 2007 were recruited for the study. All patients were admitted for strict glycemic control or assessment of diabetic complications; no patients had clinical history of cerebrovascular disease, coronary arterial disease, or peripheral artery occlusive disease. Patients who abused alcohol or showed liver cirrhosis, severe nephropathy (serum creatinine > 176.8 μmol/l), malignant neoplasm, acute illness or urinary tract infections were excluded from the study. Patients were considered to have cerebrovascular disease if they had a history of sudden focal neurological deficit. Coronary arterial disease was diagnosed if the patients had a history of myocardial infarction or showed abnormal electrocardiographic findings. Peripheral arterial occlusive disease was diagnosed if the patient had an abnormal ankle-brachial index (ABI) of <0.9 at rest [29]. Presence of pyuria or hematuria was diagnosed by microscopic examination and counting of the number of white blood cells or red blood cells per high-power field in the last voided urine of a 24-h collection. Although administration of angiotensin converting enzyme inhibitor (ACEI) and angiotensin II receptor blocker (ARB) can act to prevent angiotensin II actions, decrease UAE, slow the decline in the glomerular filtration rate and reduce circulating levels of TNF-α due to anti-inflammatory effects [30–32], all patients with hypertension (>140/90 mmHg) received antihypertensive agents for the management of high blood pressure. Although hydroxymethylglutaryl coenzyme A reductase inhibitor (statins) can reduce serum levels of TNF-α [33], the medication was used for the treatment of dyslipidemia in patients. Nondiabetic subjects were admitted for hormonal evaluation of adrenal mass and subjects with normal function were included in the study. The study was approved by the ethics committee of our institution, and informed consent was obtained from all subjects before the examinations, which were done during their stay in the hospital.

Blood samples were drawn before breakfast in the morning after a 12-h overnight fast. Blood pressure was measured by a mercury sphygmomanometer with the patient in the sitting position after 5 min of rest. Three readings separated by 2 min were taken and the average was used for the analysis. An automatic device (BP-203RPE; Colin, Komaki, Japan) was used to measure ABI and brachial-ankle pulse wave velocity (baPWV), as an index of stiffness in elastic and muscular arteries [34]. A trained ophthalmologist carried out fundus ophthalmoscopies and defined diabetic patients as either without retinopathy, having simple retinopathy or proliferative retinopathy. Diabetic patients were classified by the measurement of UAE in 24-h urine collection as having normoalbuminuria, microalbuminuria, or macroalbuminuria when at least two of three specimens were at diagnostic thresholds of less than 30, 30–300, or greater than 300 mg/24 h, respectively [5]. The Japanese ethnic factor for the MDRD equation has been reported to be 0.881 [35]. Therefore, eGFR was calculated by the MDRD formula as follows: eGFR (ml/min per 1.73 m^2) = $0.881 \times 186.3 \times age^{-0.203} \times SCr^{-1.154}$ (if female × 0.742), where SCr is serum creatinine (mg/dl). Renal insufficiency was defined as eGFR of <60 ml/min per 1.73 m^2 [36]. Patients with diabetes were screened for distal symmetric polyneuropathy using a 128-Hz tuning fork applied to the bony prominence at the dorsalis surface of both great toes, just proximal to the nail bed [37]. When the tuning fork was placed on the foot for 10 s, if the patients required >10 s to detect the vibration, vibration perception was regarded as compromised. Each subject was also classified as a current smoker or nonsmoker. Nonsmokers were defined as not using tobacco for at least the previous 3 years. Serum TNF-α concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-α kit, R&D Systems, Minneapolis, MN).

Twenty-four contiguous slices of 2.5-mm thickness of the proximal coronary arteries were obtained during a single breath hold using a sixteen multidetector-row computed tomography (16-MDCT) scanner (LightSpeed Ultrafast 16, GE Healthcare, Milwaukee, WI). These scans are electrocardiographically triggered at 70% of the R–R interval, near the end of diastole and before atrial contraction, to minimize the effect of

cardiac motion. Coronary calcium score was analyzed on an Advantage Windows version 4.2 workstation (GE Healthcare, Milwaukee, WI) with scoring software (Smartscore version 3.5). The Agatston coronary artery calcium score (CACS), including both intimal and medial calcification in left main, left anterior descending, circumflex, and right coronary arteries, were obtained as a quantitative marker of calcium burden in the coronary artery [38]. From comparison between 16-MDCT and EBCT, 16-MDCT can detect coronary calcification and may be an alternative to EBCT [23]. The tests were almost equivalent in coronary artery calcium scoring: 16-MDCT score = 7.7 + 1.015 × EBCT score ($r^2 = 0.955$).

Statistical evaluation was carried out on SPSS software version 11.0 for Windows (SPSS Inc., Chicago, IL). Normality of distribution of each variable was assessed with the Kolmogorov–Smirnov test. Comparison between the two groups was done using the unpaired Student's t-test. A multiple comparison of significant differences among the four groups was carried out by one-way analysis of variance followed by Scheffe's F-test. The χ^2-test for 2 × 2 or Bonferroni test for 2 × 4 contingency tables was used to compare the frequencies between two groups or among four groups. Pearson's correlation coefficient was applied to assess the relation between normally distributed variables. Since distribution of CACS was highly skewed, common log-transformed CACS [log(CACS + 1)] was used for linear regression analysis. Stepwise multiple regression analyses were performed to evaluate the association of log(CACS + 1) or baPWV with 12 possible risk factors for atherosclerosis, four factors for microangiopathy and TNF-α in diabetic patients. The F-value was set at 4.0 at each step. Values are expressed as the means ± S.D. p values <0.05 were considered to be statistically significant.

3. Results

3.1. All subjects

Clinical characteristics of all subjects are summarized in Table 1. There were no significant differences between the groups for prevalence of male gender, age, body mass index (BMI), total cholesterol (TC), frequency of smoking habit and ABI. However, compared with nondiabetic subjects, diabetic patients had higher fasting plasma glucose (FPG) ($p < 0.0001$), hemoglobin A1c (HbA1c) ($p < 0.0001$), triglycerides (TGs) ($p = 0.0203$) and systolic blood pressure (sBP) ($p = 0.0186$) and lower HDL cholesterol (HDL-C) ($p = 0.0011$) and diastolic blood pressure (dBP) ($p = 0.0432$). Diabetic patients had higher frequency of elevated UAE ($p < 0.0001$), baPWV ($p = 0.0293$), log(CACS + 1) ($p = 0.0089$) and TNF-α ($p = 0.0029$) than in the nondiabetic subjects, whereas eGFR and serum levels of calcium (Ca), inorganic phosphate (IP) and alkaline phosphatase (ALP) were similar in the two groups. To clarify the associations among severity of atherosclerosis and kidney function in diabetic patients,

Table 1 – Clinical characteristics in diabetic patients and age-matched nondiabetic subjects

	Nondiabetic subjects	Diabetic patients	p-Value
Number	30	200	–
Male gender (%)	13 (43.3)	112 (56.0)	0.2703
Age (year)	58.9 ± 5.4	60.3 ± 5.8	0.2040
BMI (kg/m^2)	23.4 ± 3.5	24.2 ± 3.6	0.2709
Duration of diabetes (year)	–	11.5 ± 8.2	–
Treatment (D/OHD/I)	–	16/85/99	–
FPG (mmol/l)	5.50 ± 0.62	7.96 ± 2.39	<0.0001
HbA1c (%)	5.2 ± 0.4	8.4 ± 1.7	<0.0001
TC (mmol/l)	5.39 ± 0.68	5.29 ± 0.90	0.5632
HDL-C (mmol/l)	1.55 ± 0.43	1.31 ± 0.37	0.0011
TGs (mmol/l)	1.25 ± 0.53	1.60 ± 0.79	0.0203
Statins (%)	–	52 (26.0)	–
Blood pressure (mmHg)			
Systolic	129 ± 17	136 ± 15	0.0186
Diastolic	81 ± 11	77 ± 10	0.0432
ACEI and/or ARB (%)	–	70 (35.0)	–
Smokers (%)	6 (20.0)	60 (30.0)	0.3614
Retinopathy (%)	–	92 (46.0)	–
Micro- and macroalbuminuria (%)	0 (0)	82 (41.0)	<0.0001
eGFR (ml/min per 1.73 m^2)	71.4 ± 7.8	70.3 ± 14.4	0.6776
Neuropathy (%)	–	91 (45.5)	–
ABI	1.15 ± 0.07	1.15 ± 0.07	0.7132
Brachial-ankle PWV (cm/s)	1479 ± 223	1585 ± 250	0.0293
Log(CACS + 1)	0.80 ± 0.98	1.36 ± 1.09	0.0089
Ca (mmol/l)	2.29 ± 0.08	2.27 ± 0.10	0.1343
IP (mmol/l)	1.14 ± 0.15	1.12 ± 0.16	0.4419
ALP (IU/l)	230 ± 65	235 ± 63	0.6802
TNF-α (pg/ml)	1.05 ± 0.40	1.35 ± 0.51	0.0029

Data are expressed as n (%) or mean ± S.D. D, diet, OHD, oral hypoglycemic drugs, I, insulin.

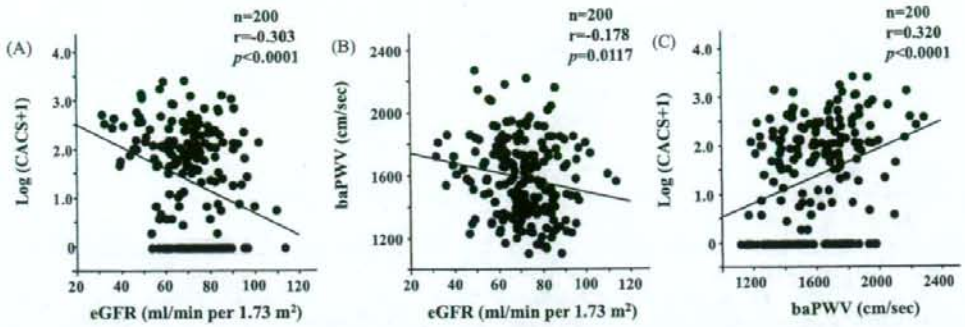

Fig. 1 – Simple linear regression analyses among estimated glomerular filtration rate (eGFR), coronary artery calcium score (CACS) and brachial-ankle pulse wave velocity (baPWV) in type 2 diabetic patients. (A) eGFR vs. log(CACS + 1); (B) eGFR vs. baPWV; (C) baPWV vs. log(CACS + 1).

simple linear regression analyses, as shown in Fig. 1, were performed. Log(CACS + 1), baPWV and eGFR were negatively [eGFR vs. log(CACS + 1), $p < 0.0001$; eGFR vs. baPWV, $p = 0.0117$] or positively [baPWV vs. log(CACS + 1), $p < 0.0001$] correlated with each other.

3.2. *Nephropathy*

To clarify the association of kidney disease with the severity of atherosclerosis, patients were classified into four subgroups with or without elevated UEA and renal insufficiency. The

Table 2 – Diabetic patients classified into four subgroups with or without elevated urinary albumin excretion (UAE) and renal insufficiency according to the levels of UAE of ≥30 or <30 mg/24 h and estimated glomerular filtration rate (eGFR) of <60 or ≥60 ml/min per 1.73 m²

	Normoalbuminuria		Micro- and macroalbuminuria	
	Without renal insufficiency	With renal insufficiency	Without renal insufficiency	With renal insufficiency
eGFR (ml/min per 1.73 m²)	75.3 ± 9.8	54.2 ± 6.3	76.7 ± 11.2	47.6 ± 8.0
Number	100	18	57	25
Male gender (%)	52 (52.0)	10 (55.6)	32 (56.1)	18 (72.0)
Age (year)	59.7 ± 5.8	63.3 ± 5.1	59.6 ± 5.4	62.6 ± 6.5
BMI (kg/m²)	24.1 ± 3.8	23.6 ± 3.3	24.4 ± 3.6	24.7 ± 3.4
Duration of diabetes (years)	10.3 ± 7.9	11.1 ± 7.9	12.0 ± 8.0	15.9 ± 8.7[a]
Treatment (diet/OHD/insulin)	10/49/41	1/9/8	3/20/34	2/7/16
FPG (mmol/l)	7.96 ± 2.66	7.47 ± 1.91	7.98 ± 2.18	8.30 ± 2.03
HbA1c (%)	8.3 ± 1.8	8.5 ± 1.4	8.6 ± 1.6	8.0 ± 1.6
TC (mmol/l)	5.18 ± 0.86	5.63 ± 0.84	5.24 ± 0.95	5.61 ± 0.94
HDL-C (mmol/l)	1.35 ± 0.42	1.37 ± 0.40	1.29 ± 0.27	1.15 ± 0.29
TGs (mmol/l)	1.45 ± 0.79	1.81 ± 1.09	1.60 ± 0.69	2.06 ± 0.56[b]
Statins (%)	24 (24.0)	6 (33.3)	14 (24.6)	8 (32.0)
Blood pressure (mmHg)				
Systolic	133 ± 15	131 ± 11	140 ± 16[a]	142 ± 12[a]
Diastolic	76 ± 10	74 ± 11	79 ± 9	77 ± 9
ACEI and/or ARB (%)	24 (24.0)	6 (33.3)	25 (43.9)	15 (60.0)[b]
Smokers (%)	32 (32.0)	3 (16.7)	18 (31.6)	7 (28.0)
Retinopathy (%)	28 (28.0)	7 (38.9)	38 (66.7)[b]	19 (76.0)[b]
Neuropathy (%)	34 (34.0)	10 (55.6)	30 (52.6)	17 (68.0)[a]
ABI	1.15 ± 0.07	1.14 ± 0.05	1.14 ± 0.07	1.14 ± 0.08
Brachial-ankle PWV (cm/s)	1520 ± 236	1611 ± 245	1637 ± 244[a]	1712 ± 261[b]
Log(CACS + 1)	1.03 ± 0.99	1.61 ± 1.18	1.51 ± 1.13	2.15 ± 0.82[c]
Ca (mmol/l)	2.27 ± 0.11	2.26 ± 0.08	2.27 ± 0.09	2.23 ± 0.11
IP (mmol/l)	1.12 ± 0.17	1.13 ± 0.16	1.10 ± 0.16	1.12 ± 0.14
ALP (IU/l)	235 ± 60	218 ± 70	240 ± 68	241 ± 57

Data are expressed as *n* (%) or means ± S.D. D, diet; OHD, oral hypoglycemic drugs; I, insulin.
[a] $p < 0.05$ vs. normoalbuminuric patients without renal insufficiency.
[b] $p < 0.01$ vs. normoalbuminuric patients without renal insufficiency.
[c] $p < 0.001$ vs. normoalbuminuric patients without renal insufficiency.

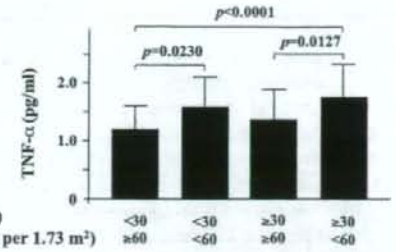

Fig. 2 – Serum levels of tumor necrosis factor-alpha (TNF-α) in patients with type 2 diabetes grouped into four subgroups with or without elevated urinary albumin excretion (UAE) and renal insufficiency according to the levels of UAE of ≥30 or <30 mg/24 h and estimated glomerular filtration rate (eGFR) of <60 or ≥60 ml/min per 1.73 m².

clinical characteristics are shown in Table 2. There were no significant differences among these groups for prevalence of male gender, age, BMI, FPG, HbA1c, TC, HDL-C, frequency of patients taking statins, dBP, prevalence of smoking habit, ABI, Ca, IP and ALP. Patients with micro- and macroalbuminuric renal insufficiency had the longest duration of diabetes ($p = 0.0225$), highest TGs ($p = 0.0061$), sBP ($p = 0.0364$), frequency of patients taking ACEI and/or ARB ($p < 0.01$), retinopathy ($p < 0.01$) and neuropathy ($p < 0.05$). Furthermore, these patients showed the highest baPWV ($p = 0.0068$) and log(CACS + 1) ($p < 0.0001$) among the groups. Although 18 of 200 (9%) patients showed renal insufficiency, they had normoalbuminuria and similar log(CACS + 1) and baPWV compared to normoalbuminuric patients without renal insufficiency.

3.3. TNF-α

Serum concentrations of TNF-α in each group are shown in Fig. 2. Both normoalbuminuric ($p = 0.0230$) and micro- and macroalbuminuric ($p < 0.0001$) patients with renal insufficiency had higher TNF-α than in normoalbuminuric patients without renal insufficiency. To clarify the association of the severity of atherosclerosis or kidney function with serum levels of TNF-α, simple linear regression analyses were performed as shown in Fig. 3. Log(CACS + 1) ($p = 0.0008$) (Fig. 3A) and baPWV ($p = 0.0006$) (Fig. 3B) positively and eGFR ($p < 0.0001$) (Fig. 3C) negatively correlated with TNF-α among these patients.

3.4. Risk factors

Stepwise multiple regression analyses were performed to examine the association of log(CACS + 1) or baPWV with 12 possible risk factors for atherosclerosis (age, male sex, BMI, duration of diabetes, FBS, HbA1c, sBP, dBP, TC, HDL-C, TGs and smoking habit), four factors for microangiopathy (retinopathy, micro- and macroalbuminuria, eGFR and neuropathy) and TNF-α. Age ($\beta = 0.034$; $F = 7.193$), smoking habit ($\beta = 0.410$; $F = 6.791$), retinopathy ($\beta = 0.575$; $F = 15.670$), eGFR ($\beta = -0.014$; $F = 6.768$) and TNF-α ($\beta = 0.323$; $F = 4.985$) for log(CACS + 1) ($r^2 = 0.214$, $p < 0.0001$), and age ($\beta = 10.791$; $F = 18.338$), sBP ($\beta = 6.593$; $F = 38.377$), retinopathy ($\beta = 79.888$; $F = 6.620$) and TNF-α ($\beta = 67.305$; $F = 5.111$) for baPWV ($r^2 = 0.326$, $p < 0.0001$) were identified as significant independent variables, respectively.

4. Discussion

In this study, we have demonstrated for the first time that severity of coronary artery calcification, arterial stiffness and renal insufficiency associate with circulating levels of TNF-α in type 2 diabetic patients. Our findings support the notion that TNF-α is a key molecule in the development of micro- and macrovascular complications and that use of anti-inflammatory agents may therefore act to reduce CVD events in diabetic patients. However, 9% of our patients with normoalbuminuric renal insufficiency showed characteristically distinct atherosclerotic features from those of patients with micro- and macroalbuminuric renal insufficiency, even though both groups had higher circulating levels of TNF-α than in normoalbuminuric patients without renal insufficiency.

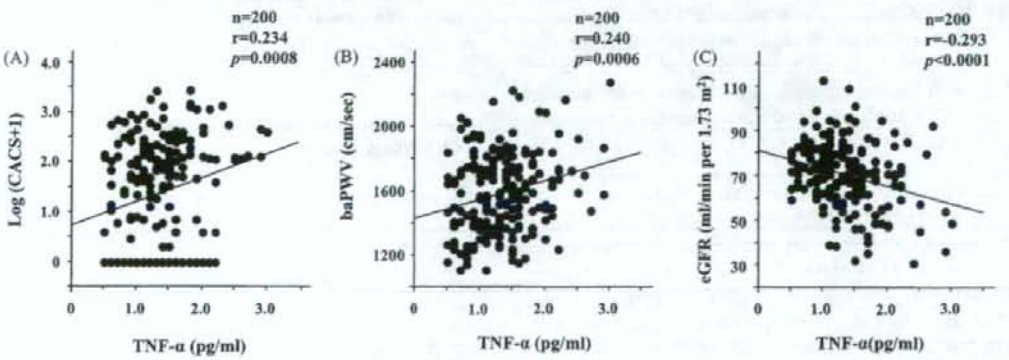

Fig. 3 – Simple linear regression analyses between serum levels of tumor necrosis factor-alpha (TNF-α) with coronary artery calcium score (CACS) (A), brachial-ankle pulse wave velocity (baPWV) (B) or estimated glomerular filtration rate (eGFR) (C) in type 2 diabetic patients.

Our data reveal that when diabetic patients are subgrouped with or without elevated UAE and renal insufficiency, patients with micro- and macroalbuminuric renal insufficiency show the highest coronary artery calcification, arterial stiffness and serum levels of TNF-α among the groups. Although these factors except for TNF-α have been reported previously, our study demonstrates that age [39], smoking habit [40], retinopathy [41], kidney function [19] and TNF-α are independent variables for coronary artery calcification, and age [42], hypertension [43], retinopathy [44] and TNF-α are independent variables for arterial stiffness, respectively. Kidney is a major source of TNF-α synthesis [10], but there are several extrarenal sources of this molecule, including adipose tissue [9] and atherosclerotic lesions [11]. These results indicate that TNF-α is a predictor of both coronary artery calcification and arterial stiffness and is independent of reduced kidney function for coronary artery calcification in type 2 diabetic patients.

Although we did not investigate the primary source of TNF-α, a previous report noted that renal production of this cytokine is elevated in patients with type 2 diabetes [45]. In the kidneys, TNF-α is produced mainly by infiltrating macrophages [46], as well as by intrinsic renal cells including endothelial, mesangial, glomerular and tubular epithelial cells [10]. Various atherogenic factors such as AGEs [12], angiotensin II [13] and oxidized LDL [14] can stimulate TNF-α synthesis from these cells and initiate local effects of renal damage. Endothelial cells in the intimal layer actively regulate vascular tone and permeability and the balance between coagulation and fibrolysis [47]. Thus, the increased permeability for albumin through the vascular wall in the kidneys reflects systemic endothelial injury that leads to increased risk of CVD events.

Nephropathy in patients with type 2 diabetes is more heterogeneous than in type 1 diabetic patients. Type 2 diabetic patients often develop renal dysfunction in the absence of increased albuminuria, which conditions initially develop during the prediabetic state secondary to age, hypertension and other factors [48]. In the United States, 30% of newly diagnosed type 2 diabetic patients were found to exhibit renal insufficiency without retinopathy and albuminuria [49]. A longitudinal study demonstrates that risk for progression of renal failure or death in diabetic patients with normoalbuminuric renal insufficiency is lower than in patients with albuminuric renal insufficiency [50]. In the present study, 9% of the diabetic patients had renal insufficiency without elevated UAE. Although these patients had higher serum levels of TNF-α compared to normoalbuminuric patients without renal insufficiency, the severity of atherosclerosis was similar in the two groups. This inconsistency between abnormal TNF-α production and the severity of atherosclerosis in patients with normoalbuminuric renal insufficiency has not been clarified.

Cytotoxic activities of inflammatory cytokines are not limited to renal damage. Intra-arterial TNF-α administration causes vascular inflammation and endothelial dysfunction [15], which is an early stage of the atherosclerotic process. Diabetic patients have a greater amount of coronary calcium deposition than in nondiabetic subjects [25] and excessive coronary calcification associates with insulin resistance [51]. Coronary artery calcium is present only in atherosclerotic lesions and can be quantified safely with ultrafast computed tomography. The resulting calcium score represents the presence and extent of atherosclerosis and is useful in predicting CVD events [24]. Vascular calcification is an active and regulated process related to various inflammatory cells and cytokines. Macrophages can contribute to the development of vascular calcification through TNF-α production, as a regulator of bone formation [52]. TNF-α changes the phenotype of vascular smooth muscle cells and induces osteoblastic differentiation [53]. Diabetic patients have a higher aortic stiffness than in nondiabetic subjects [26]. Pulse wave velocity, which depends on arterial radius and wall thickness [54], is widely used to assess arterial distensibility. Functional or structural alterations of vessel wall properties can alter arterial compliance and increase blood pressure. TNF-α can impair endothelial function and lead to functional stiffness in the vessel wall due to reduced nitric oxide bioavailability [55]. Vascular smooth muscle cell migration into the intima followed by proliferation and matrix deposition is a central feature of early atherosclerosis [56]. Thus, both an increase in intima-media thickness [57] and vascular calcification [52,53] caused by excessive TNF-α production in the diabetic state are involved in the pathogenesis of structural rigidity of the vessel wall.

Because the present study is a cross-sectional design, further prospective study is required to clarify the role of abnormal TNF-α secretion in the development of micro- and macrovascular complications in patients with type 2 diabetes. In addition, our data were obtained in a Japanese population, and it remains to be established whether these results can be generalized. Furthermore, the inconsistency between abnormal TNF-α production and the severity of atherosclerosis in patients with normoalbuminuric renal insufficiency has not been clarified. Finally, because the administration of ACEI, ARB and statins can affect UAE, glomerular filtration rate and circulating levels of TNF-α, the influences of these medications on our data are unclear.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Journal of Pineal Research

Consumption of vegetables alters morning urinary 6-sulfatoxymelatonin concentration

Abstract: Melatonin, which is contained in certain vegetables, may have an influence on circulatory melatonin concentrations. This study examined the effects of the consumption of vegetables on 6-sulfatoxymelatonin concentrations in morning urine. Ninety-four healthy women aged 24–55 were recruited through a city public health center in Japan. The women randomly allocated to the intervention group were requested to consume high amounts of six selected vegetables, with a target of 350 g/day for 65 days, while those in the control group were asked to avoid the same six vegetables during the same period. First-void morning urine was collected before and at the end of the intervention period, and creatinine-adjusted 6-sulfatoxymelatonin concentrations were measured. At the end of the intervention period, daily mean intake of melatonin from the six vegetables was 1288.0 ng in the intervention group and 5.3 ng in the control group. In the intervention group, the mean concentration of 6-sulfatoxymelatonin changed from 48.1 [95% confidence interval (CI): 40.4–57.2] ng/mg creatinine to 49.6 (95% CI: 42.8–57.3) ng/mg creatinine across the intervention period. In the control group, the mean concentration of 6-sulfatoxymelatonin changed from 55.5 (95% CI: 48.7–63.2) ng/mg creatinine to 50.8 (95% CI: 44.0–58.7) ng/mg creatinine across the intervention period. A comparison of the two groups with regard to the changes in the 6-sulfatoxymelatonin concentrations across the intervention period showed a significant difference ($P = 0.03$). The results indicate that increased consumption of vegetables raises circulatory melatonin concentrations.

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Key words: 6-sulfatoxymelatonin, intervention study, Japanese, vegetables, women

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Introduction

Human melatonin has been studied with regard to its beneficial role in protecting against cancers [1–3]. Several in vitro studies demonstrated the oncostatic effect of melatonin on different tumor cells, such as cells from breast cancer, colon cancer, melanoma, ovarian carcinoma, prostate cancer, neuroblastoma, pituitary tumor, larynx carcinoma, oral carcinoma, bladder carcinoma and erythroleukemia [4–6]. Studies in animals reported the anticarcinogenesis effect of orally ingested melatonin on tumors in mammary and liver [7, 8]. Antioxidant effects of melatonin and its metabolites such as N^1-acetyl-5-methoxykynuramine (AMK) and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) were also examined in laboratory studies [9–13]; free radicals play an important role in cancer cell proliferation, and may influence on age-related diseases [14].

Compared with those, the epidemiologic studies of melatonin in relation to the risk of cancer are still scarce. Thus far, two epidemiologic studies were conducted prospectively to assess the association of melatonin concentrations with breast cancer risk; one study found a lower risk of breast cancer with higher melatonin concentrations measured in the first morning urine [15], and the other study found no evidence that melatonin concentrations in 24-hr urine samples associated with the risk for breast cancer [16]. Melatonin exists in edible plants and vegetables [17–19]. Beneficial effects of vegetable intake in protecting against cancer and cardiovascular disease have been suggested by epidemiologic evidence [20, 21]. Although several mechanisms for these beneficial effects have been already hypothesized and studied [21, 22], melatonin contained in the vegetables might be another possible agent to support the positive effects. In our previous cross-sectional study among Japanese females, urinary melatonin concentrations increased significantly with increased vegetable intake in the regular diet [23]. We hypothesized that the consumed vegetables may be one of the sources of melatonin in humans; no previous study had examined this hypothesis.

The aim of the present randomized-controlled study was to examine the effects of the consumption of vegetables containing high amounts of melatonin, on 6-sulfatoxymelatonin concentrations in first-void morning urine as a reflection of the nocturnal melatonin concentrations [24–26]. Hattori et al. [17] investigated the presence of melatonin in vegetables and identified that certain vegetables commonly consumed in Japan contained certain amounts of melatonin; however, the melatonin contents of all commercially available vegetables have not yet been

identified. Hence, our focus is on the effects of specific vegetables already identified to be containing known amounts of melatonin.

Materials and methods

Study participants

Study subjects were recruited through a city public health center in Gifu prefecture, Japan. Female volunteers who had no hypertension, who did not use medications for a sleep disorders, depression, or mental disorders, who were not pregnant, and who were not currently engaged in night-shift work were invited to participate in this study. Ninety-eight healthy women aged 24–55 who agreed to participate and completed the informed consent form were registered. The subjects were stratified into three age groups: 24–35 yr, 36–45 yr, and 46 yr and older. They were randomly assigned to either the intervention group or the control group. Forty-nine subjects were assigned to the intervention group, in which the subjects were provided with and requested to consume selected vegetables, and 49 subjects were assigned to the control group, in which the subjects were requested to avoid these vegetables. Before the intervention started, one subject withdrew from the intervention group. During the intervention period, two subjects in the intervention group and one subject in the control group dropped out of the study. Overall, 46 subjects in the intervention group and 48 subjects in the control group completed the study.

Data collection and intervention

Before entering the intervention period, all the subjects were asked to fill out a self-administered questionnaire to provide general information about their demographic characteristics and other information such as parity status, past disease history, medication use within the previous 6 months, and sleeping habits. Physical activity levels were measured before and at the end of the intervention period by using a validated questionnaire, and these data were translated into metabolic equivalents per week [27, 28]. A validated food frequency questionnaire was administered to estimate the nutrient intake in the regular diet [29]. All subjects were required to fill out a 3-day dietary record, which was performed and included detailed instructions, before and at the end of the intervention period. In the record, the subjects were asked to provide the names and quantities of all the foods, beverages, and snacks which they consumed. Other than following the requirements of the study, the subjects were advised to maintain their regular diet and lifestyle.

The intervention period was from October 4, 2005 through December 7, 2005, a period of 65 days. For intervention agents, we selected the following six vegetables which, according to Hattori et al. [17], are relatively high in melatonin of the vegetables whose melatonin concentrations are available, and are commonly consumed in Japan: sweet corn, *goya* (bitter gourd), *kaiware* (Japanese radish sprout), *shungiku* (garland chrysanthemum), *shimeji* mushroom, and *shiitake* mushroom. The subjects who were assigned to the intervention group were provided these vegetables in 10 weekly deliveries to their residences throughout the intervention period. The exact contents of each delivery were based on the availability of the vegetables in the local market, but each package contained a sufficient amount of all six vegetables to provide a total of 350 g/day. The subjects in the intervention group were given a target of consuming 350 g of the delivered vegetables per day throughout the study period. Using a preformatted journal, the subjects reported the contents of the deliveries and the amounts consumed. In the same journal, the subjects recorded their nightly bedtimes. On the other hand, the subjects assigned to the control group were required to avoid the above six vegetables during the intervention period. When consumption of one of the six vegetables was inevitable, they were to record the consumed amount. They also recorded their nightly bedtimes. The subjects in both groups were required to avoid the following food items and products as they have been reported to contain large amounts of melatonin [17]: barley, *awa* (foxtail millet), *hie* (Japanese barnyard millet), brown rice, *gokokumai* (rice with five kinds of grains), banana, *aojiru* (green juice), and kale.

First-void morning urine was collected from all the subjects on 2 consecutive days starting 2 days before the intervention started and the last 2 consecutive days of the intervention period. The subjects were requested to go to bed around midnight or earlier on the nights before urine collection. The times that the urine was collected were recorded by the subjects. The urine samples were immediately frozen and stored at −80°C until being assayed. Urinary 6-sulfatoxymelatonin was measured radioimmunologically with kits purchased from IBL Laboratories (Hamburg, Germany). The sensitivity was 1.0 ng/mL, and the interassay coefficient of variation was 11.3%. To adjust for a variation in the dilution of urine, 6-sulfatoxymelatonin concentrations were expressed as urine 6-sulfatoxymelatonin/urine creatinine [26, 30].

Data analysis

For the analysis of 6-sulfatoxymelatonin concentrations, we used the mean of the concentrations during each 2-day period. The 6-sulfatoxymelatonin concentrations and nutrient intake were logarithmically transformed for statistical analysis to approximately normalize their distributions. Geometric means and 95% confidence interval (CI) of 6-sulfatoxymelatonin concentrations were calculated. To compare the difference in the concentrations of urinary 6-sulfatoxymelatonin before and at the end of the intervention, a paired t-test was conducted separately for each group. To compare the two groups in terms of the change in the 6-sulfatoxymelatonin concentrations across the intervention period, a two sample t-test was conducted. All P-values are two sided.

To assess the difference in the consumption of the six vegetables as well as other food items and nutrients across the intervention period, the information from the 3-day dietary record was analyzed. One dietitian interpreted all the records and estimated the intake of each nutrient, and

then the other dietitian confirmed the estimations. Based on the 3-day record, the intake of melatonin from the six vegetables was estimated by multiplying the consumed amounts in grams by the following melatonin concentrations in the vegetables: 1366 pg/g tissue for sweet corn, 13,353 pg/g tissue for bitter gourd, 657 pg/g tissue for Japanese radish sprout, 417 pg/g for garland chrysanthemum, 300 pg/g for *shimeji* mushroom and 387 pg/g for *shiitake* mushroom (partially reported by Hattori et al.) [17]. The intake of nutrients other than melatonin was calculated according to the Standard Tables of Food Composition in Japan, 5th edition, published by the Science and Technology Agency of Japan. The total amounts, in gram units, of the six vegetables consumed by the subjects throughout the study period were estimated from the journal entries. The melatonin intake was then estimated using the method described above. This study was approved by the institutional review board at Gifu University Graduate School of Medicine. All the statistical analyses were performed with SAS (SAS Institute Inc., Gary, NC, USA).

Results

The baseline characteristics of the subjects by study group are summarized in Table 1. The change in the daily mean intake of the six vegetables before and at the end of the intervention period was obtained by using the data from the 3-day food records (Table 2). It was estimated that the subjects in both groups consumed similar amounts of the six vegetables before the intervention period. During the intervention period, the daily mean intake of the six vegetables in grams was almost 150 times higher among the subjects in the intervention group than among those in the control group. In line with the intake of the six vegetables, the estimated intake of melatonin sourcing from these vegetables increased in the intervention group and decreased in the control group. Similarly, it was estimated from the journals kept by the subject during the intervention period that the daily mean intake of melatonin from the six vegetables for the entire period was 1231 ng among the subjects in the intervention group whereas the equivalent value was 1.2 ng among the subjects in the control group. Among the subjects in the intervention group, sweet corn was the most consumed agent in grams [mean consumption for the entire period: 5263 g, standard error of mean (SEM): 204 g], and the bitter gourd was the second-most consumed agent (mean consumption for the entire period: 5104 g, SEM: 266 g). The total mean amount of sweet corn consumed by the control subjects during the intervention period was 10 g (SEM: 2 g), and that of bitter gourd was 3 g (SEM: 2 g). The estimated intake of other nutrients is also presented in Table 2. Among the subjects in the intervention group, the intake of crude fiber, vitamin A, vitamin C, and vitamin E increased significantly at the end of the intervention period. Between the groups, the difference in change for each of the above nutrients across the intervention period was significant. In both groups, caffeine intake increased significantly at the end of the intervention period.

The primary measures of this study, the concentrations of 6-sulfatoxymelatonin in first-void morning urine before and at the end of the intervention period, are shown in Table 3. At the baseline, the concentrations of urinary 6-sulfatoxymelatonin were higher among the subjects in the control group (55.5, 95% CI: 48.7, 63.2 ng/mg creatinine) than among the subjects in the intervention group (48.1, 95% CI: 40.4, 57.2 ng/mg creatinine), although the difference was not statistically significant. At the end of the intervention period, the mean concentration of 6-sulfatoxymelatonin significantly decreased by 8.5% ($P = 0.03$) in the control group, whereas the concentration increased slightly and non-significantly by 3.1% in the intervention group. A comparison of the net change in baseline-end values of 6-sulfatoxymelatonin between the two groups showed a significant difference across the intervention period ($P = 0.03$).

Table 1. Baseline characteristics of women in the melatonin intervention and control groups

	Melatonin-contained vegetables supplemented group (n = 46)	Control group (n = 48)
Age, years [mean ± standard error of mean (SEM)]	38.6 ± 0.9	40.3 ± 1.0
Height, cm (mean ± SEM)	157.5 ± 1.0	156.2 ± 0.6
Weight, kg (mean ± SEM)	51.7 ± 0.9	50.4 ± 0.9
Body mass index, kg/m^2 (mean ± SEM)	20.9 ± 0.4	20.7 ± 0.3
Married, no. (%)	41 (89.1)	44 (91.7)
Parous, no. (%)	36 (78.3)	45 (93.8)
Smoking status		
Current smoker, no. (%)	0 (0.0)	2 (4.2)
Past smoker, no. (%)	3 (6.5)	5 (10.4)
Ever used aspirin within 6 months, no. (%)	4 (8.7)	2 (4.2)
Reported 0 alcohol use in regular diet, no. (%)	9 (19.6)	20 (41.7)
Average bedtime (mean ± SEM)		
Weekday	23:19 ± 0:07	23:16 ± 0:08
Weekend	23:32 ± 0:08	23:36 ± 0:09
Average wake time (mean ± SEM)		
Weekday	6:06 ± 0:04	5:50 ± 0:05
Weekend	7:08 ± 0:09	6:54 ± 0:08

Table 2. Daily intake of melatonin-contained vegetables and other nutrients at the baseline and at the end of study period, in the melatonin intervention and control groups

	Melatonin-contained vegetables supplemented (n = 46)				Control (n = 48)			
	Baseline		End of intervention		Baseline		End of intervention	
	Mean	Standard error of mean (SEM)	Mean	SEM	Mean	SEM	Mean	SEM
From the six vegetables								
Sweet corn (g)	2.5	1.0	65.7	7.1	2.6	0.8	0.4	0.2
Bitter gourd (g)	8.7	2.9	84.5	8.6	9.3	4.1	0.3	0.3
Japanese radish sprout (g)	0.3	0.2	39.0	4.8	0.7	0.5	0.1	0.1
Garland chrysanthemum (g)	1.2	0.6	48.3	5.5	0.3	0.3	0.7	0.7
Shimeji mushroom (g)	3.0	0.7	36.7	4.7	3.7	1.0	0.1	0.1
Shiitake mushroom (g)	2.3	0.6	37.4	5.9	2.1	0.7	0.4	0.2
Total six vegetables intake (g)	18.1	3.2	311.6	24.2	18.8	4.6	2.1	0.9
Melatonin intake from the six vegetables (ng)	124.9	38.4	1288.0	118.1	132.8	54.7	5.3	3.8
All vegetable intake (g)	270.4	18.8	412.1	24.8	301.5	22.2	248.8	15.6
Nutrients intake from the total diet								
Energy (kcal)	1878	44	1892	54	1874	64	1846	62
Protein (g)	67.5	1.7	69.2	2.5	67.4	2.3	67.0	2.3
Fat (g)	59.4	2.1	59.9	2.8	57.9	2.9	55.8	2.9
Cholesterol (mg)	284	14	291	21	259	21	310	19
Carbohydrate (g)	257	7	264	7	263	10	259	11
Crude fiber (g)	14.7	0.6	21.1	1.2	16.2	0.8	14.5	0.7
Vitamin A (μg)	463	30	688	55	609	65	463	31
Vitamin B1 (mg)	1.00	0.06	1.10	0.05	1.10	0.11	1.11	0.13
Vitamin B2 (mg)	1.50	0.15	1.57	0.08	1.48	0.20	1.50	0.16
Vitamin C (mg)	160.5	29.1	213.3	25.9	153.2	22.1	179.5	36.3
Vitamin D (μg)	8.74	0.97	8.87	1.20	10.13	1.86	6.86	0.94
Vitamin E (mg)	7.4	0.3	12.7	3.3	7.9	0.4	7.2	0.4
Calcium (mg)	576	36	595	32	604	33	522	31
Iron (mg)	8.5	0.4	9.0	0.5	8.9	0.5	8.4	0.4
Folate (μg)	331.19	16.40	523.67	29.04	338.29	17.83	346.41	17.51
Salt (g)	10.8	0.8	11.3	0.9	10.2	0.5	9.6	0.4
Alcohol (g)	4.2	0.8	3.7	0.8	2.8	0.7	3.1	1.0
Caffeine (g)	0.18	0.02	0.25	0.04	0.18	0.02	0.21	0.02
Mean bedtime before the urine collection days (2 days average)	23:22	0:05	23:28	0:07	23:26	0:06	23:19	0:07
Mean time of urine collection (2 days average)	6:07	0:06	6:25	0:08	5:50	0:05	6:18	0:07
Level of physical activities (metabolic equivalent/wk)	44.7	6.5	52.2	6.5	44.0	5.9	55.1	6.8

Table 3. Change in creatinine-adjusted urinary 6-sulfatoxymelatonin concentration by food intake group

	Melatonin-contained vegetables supplemented group (n = 46)			Control group (n = 48)			Difference in change between the groups
	Initial	Final	P^a	Initial	Final	P^a	P^b
Geometric mean[c] (ng/mg creatinine)	48.1	49.6	0.39	55.5	50.8	0.03	0.03
95% Confidence interval[c]	(40.4, 57.2)	(42.8, 57.3)		(48.7, 63.2)	(44.0, 58.7)		

[a]The paired *t*-test.
[b]The two-sample *t*-test.
[c]Obtained by taking antilog of log-transformed values.

Discussion

Increasing the consumption of vegetables that are high in melatonin for more than 2 months raised morning 6-sulfatoxymelatonin concentrations in urine compared with suppressing the intake of such vegetables. The finding supports the results of our previous cross-sectional study, which implied an association between vegetable intake and melatonin concentrations in humans [23]. Several previous studies administered melatonin orally, in which the amount varied from 0.1 to 80 mg at a time, and reported the increased concentration of serum, plasma, or urinary

melatonin [31–35]. The amount of melatonin from the six vegetables in the current study was quite small compared with the amounts administered in these studies. However, it was reported that infusion of 10 μg of melatonin still increased serum melatonin concentrations about 40 times at 5 min among healthy male volunteers [36]. Had we used edible plants with higher levels of melatonin such as certain medical herbs [37], the results may have been more dramatic. Moreover, Reiter et al. have showed that with rats, blood melatonin levels increased substantially after they were fasted for 24 hr and then given exclusively a high melatonin-contained food [38]. Fasting or restricting vegetables to washout their possible effect before the intervention was not practiced among the participants of the current study. Still, our focus was on consumption of common vegetables for certain period of time in regular diets.

Although we randomly divided the subjects into two study groups, the initial mean value of urinary 6-sulfatoxymelatonin concentrations was somewhat higher among the subjects in the control group than among the subjects in the intervention group. Several baseline factors and characteristics appeared to be distributed unequally by study group, and this might have been associated with the observed difference in 6-sulfatoxymelatonin concentrations at the baseline. Total vegetable consumption was one such factor. At the baseline, it was estimated from the 3-day diet record that the daily consumption of the six vegetables was slightly different between the groups, and the total vegetable intake was larger in the control group than in the intervention group. Therefore, melatonin sourcing from total vegetable consumption may have contributed to the elevated urinary 6-sulfatoxymelatonin concentrations in the control group at the baseline. As information on melatonin contents of many other vegetables was unavailable, we were not able to estimate the degree of the effect of total vegetable intake on the urinary 6-sulfatoxymelatonin concentration.

Other such factors were regular alcohol consumption and parity status. The prevalence of non-alcohol drinkers was about two times higher among the subjects in the control group than that among the subjects in the intervention group. We observed that the baseline 6-sulfatoxymelatonin concentrations were negatively associated with baseline alcohol intake by using the linear regression model ($P = 0.02$). Inverse associations of human urinary melatonin concentrations with both temporal and regular alcohol intake were previously reported in several studies [39–41], but not in all [42, 43]. Likewise the proportion of parous women was significantly higher in the control group than in the intervention group. A few previous studies assessed the relationship between the parity status and melatonin concentration and some of them reported high melatonin concentrations among parous women [16, 44, 45]. Other potential factors [39, 44, 46–49], such as the influence of bedtime, body mass index, smoking status, concentration of physical activity, aspirin use or coffee consumption, on baseline urinary 6-sulfatoxymelatonin concentrations may be eliminated as the values of these characteristics were not significantly different between the study groups at the baseline.

The current study showed that urinary 6-sulfatoxymelatonin concentrations were reduced in the control group. Besides the change in the consumption of the vegetables, seasonal changes during the study period (end of summer to early winter) or changes in the length of the days may have influenced on the 6-sulfatoxymelatonin concentrations in both groups, which may partially explain the decrease in melatonin concentrations at the end of the period. Using raw data from our previous cross-sectional study [23], we observed that each additional hour of daylight was associated with a 16.4% higher 6-sulfatoxymelatonin concentration in morning urine. Also, it was reported in a previously conducted nested case–control study of breast cancer that among 353 female control subjects, concentrations of 6-sulfatoxymelatonin in urine samples collected in winter was 11.4% lower than the concentrations in the samples collected in fall [16].

In summary, the daily consumption of a high amount of selected vegetables increased 6-sulfatoxymelatonin concentrations in first-void morning urine in approximately a 2-month period. To our knowledge, this is the first intervention study of its kind. Although the relationship between urinary melatonin concentrations and the risk for cancer is not yet clearly established in humans, the results of the current study raise the possibility that vegetable intake in the regular diet has an influence on melatonin concentrations, which may lead the beneficial effect in terms of reduction and prevention of cancer and cardiovascular disease.

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Original Article

Self-reported Diabetes Mellitus and Risk of Mortality from All Causes, Cardiovascular Disease, and Cancer in Takayama: A Population-based Prospective Cohort Study in Japan

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ABSTRACT

Background: Diabetes mellitus has been reported to be a major risk factor for cardiovascular disease (CVD), and higher risk of CVD among women than that among men has been observed in many studies. Further, the association of diabetes with increasing risk of cancer has also been reported. Well-designed studies conducted among men and women in the general Japanese population remain scarce.

Methods: Our cohort consisted of 13355 men and 15724 women residing in Takayama, Japan, in 1992. At the baseline, the subjects reported diabetes in a questionnaire. Any deaths occurring in the cohort until 1999 were noted by using data from the Office of the National Vital Statistics. The risk of mortality was separately assessed for men and women by using a Cox proportional hazard model after adjusting for age; smoking status; body mass index (BMI); physical activity; years of education; history of hypertension; and intake of total energy, vegetables, fat, and alcohol.

Results: Diabetes significantly increased the risk of mortality from all causes [hazard ratio (HR): 1.35, 95% confidence interval (CI): 1.11-1.64] and from coronary heart disease (CHD) (HR: 2.96, 95% CI: 1.59-5.50) among men, and that from all causes (HR: 1.74, 95% CI: 1.34-2.26) and cancer (HR: 1.88, 95% CI: 1.16-3.05) among women. Diabetes was not significantly associated with mortality from CHD among women.

Conclusion: The findings suggest that diabetes increases the risk of mortality from CVD among men and that from cancer among women. The absence of increased risk of mortality from CHD among women may suggest a particular pattern in the Japanese population.

Key words: Diabetes mellitus, Mortality, Cardiovascular disease, Cancer, Cohort study

INTRODUCTION

Diabetes mellitus is a major risk factor of cardiovascular disease (CVD), and epidemiological studies from different areas have reported that people with diabetes are at higher risk of mortality from CVD and from all causes.[1-4] Japan was ranked fifth among the World Health Organization member states in terms of the estimated number of cases of diabetes, and the increased risk of mortality among people with diabetes imposes a significant health burden on the nation. A previous study reported that the relative effect of diabetes on the risks of mortality from CVD and all causes among the Asian population did not differ from those among the Caucasian population.[5] The same study also reported that the CVD risks associated with diabetes were similar in men and women. However, detailed information regarding each cohort from Japan that contributed to the study has not yet been published and was therefore unavailable. Another study in Japan indicated that the increase in the risk of heart disease among diabetic patients was slightly higher in men than in women, although the study was not based on an underlying observational epidemiological study, and comparison was made between the data of diabetic patients and population statistics.[6] One prospective study in Japan reported that the presence of diabetes increased the risk of CVD, and another study conducted on atomic bomb survivors reported an

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association between HbA1c and mortality from CVD.[7,8] In both these studies, the combined risks for men and women were calculated, and the number of participants included was rather limited. Another study has reported an increase in the risk of CVD in diabetic patients,[9] although the participants of this study were not selected from the general population but consisted of patients. Thus, a study on a large cohort of men and women selected from the general Japanese population in order to assess the relationship between diabetes and the risk of mortality from CVD and all causes is desired.

It has also been reported that diabetes is associated with an increased risk of cancer. Moreover, studies conducted in Japan and those conducted abroad have reported a relationship of the risk of cancer or specific cancer in several sites with diabetes and with the HbA1c and fasting plasma glucose levels.[8,10-14] Cancer is the leading cause of death in Japan, and mortality from cancer exceeds that from CVD in both men and women.[15,16] The risk of cancer mortality in diabetic patients is likely to have an impact on the nation's public health.

The aim of the current study is to conduct a prospective cohort study to assess the association between diabetes and mortality from CVD, cancer, and all causes. We examined the male-female difference in the risks of mortality associated with diabetes. Our cohort was community-based and was selected from the general Japanese population, and the risks were assessed after considering the risk factors for CVD and cancer.

METHODS

Study Participants

The data were obtained from the Takayama study in Japan. The details of the Takayama study have been described elsewhere.[17-19] In brief, the study population consisted of men and women who were residents of Takayama City and were 35 years or older in 1992. At the baseline, a self-administered questionnaire was administered to 36990 residents, and a 92.0% response rate was obtained. Of the participants who responded to the questionnaire, those who did not complete more than 45% of it (n = 595, 1.7%) and those who gave unreliable or inconsistent responses (n = 1871, 5.5%) were excluded from the cohort. The final fixed cohort consisted of 31552 subjects, including 14427 men and 17125 women. Physician diagnoses of diabetes and other major diseases, including hypertension, were reported in the questionnaire in response to the question "Have you ever been told by a physician that you have following diseases?" The participants answered this question for each listed disease. Those who reported a history of cancer, myocardial infarction, angina, or stroke were excluded from the cohort. The final cohort for the current study consisted of 29079 subjects, including 13355 men and 15724 women.

Information regarding the baseline characteristics of the study cohort, such as age; height; weight; cigarette smoking; use of medication, including aspirin; and length of education in years, was reported in the questionnaire. Women's health issues, including menopausal status and use of hormone replacement therapy, were also asked in the questionnaire. In addition, a semi-quantitative, validated, food-frequency questionnaire (FFQ) that quantified 169 food items was administered.[17] From the FFQ data, the total daily calorie intake and the intake of each nutrient and food item were estimated according to the Japanese Standard Tables of Food Composition, 5th edition, published by the Science and Technology Agency of Japan. Detailed information on the FFQ, including its validity and reproducibility, has been previously described.[17] The amount of regular physical activity was estimated from the validated questionnaire and expressed in terms of metabolic equivalents per week.[20,21]

Ascertainment of Mortality

Deaths in the cohort that occurred between September 1992 and December 1999 were recorded. After obtaining permission from the Ministry of Internal Affairs and Communication to review the data regarding deaths, each cause of death was confirmed using the data from the Office of the National Vital Statistics. The major endpoint of this study was mortality from all causes, CVD, cancer, and causes other than cancer and CVD. We further analyzed mortality from several diseases that were frequently observed in the cohort. The Statistics and Information Department of the Japanese Ministry of Health and Welfare listed all the causes of deaths, which were coded according to the International Classification of Diseases, 10th Revision (ICD-10). Deaths from cancer were classified as codes C00 through C97, and deaths from CVD, as codes I00 through I99 and Q25 through Q28.

Data Analysis

The age-adjusted mortality rates per 10000 person-years classified according to the status of diabetes were separately calculated for men and women by standardization to the rates among subjects of the Takayama study by sex and by 10-year age category. We compared the characteristics of the participants with and without diabetes by using *t* tests for continuous variables and chi-square tests for categorical variables. The intake of each food and nutrient was logarithmically transformed for statistical testing in order to approximately normalize its distribution. To assess the magnitude of the association of diabetes with mortality from each cause, a Cox proportional hazard model was applied to estimate hazard ratios (HRs) with 95% confidence intervals (CIs). To track the subjects who moved out of the study area, we referred to the city residential registers, and these subjects were counted as censored subjects. Age was included in the model for adjustment since it is a potent risk factor for

diabetes, CVD, and cancer. We also considered a multivariate model with adjustments for other factors that were associated with diabetes: age; smoking status; body mass index (BMI); physical activity; years of education; history of hypertension; total energy intake; and intake of vegetables, fat, and alcohol. The intake of each food and nutrient in the model was adjusted for the total energy intake by using the regression analysis proposed by Willett.[22] All statistical analyses were performed using SAS (SAS Institute Inc., Gary, NC).

RESULTS

At the baseline, 5.9% males and 2.7% females reported that they had diabetes. Table 1 summarizes the baseline characteristics of the participants by the diabetes status in both men and women. The subjects with diabetes were significantly older, and their BMI was slightly but significantly higher than that of non-diabetic subjects. The subjects with diabetes were less physically active, more frequently reported a history of hypertension, and their caloric intake was significantly lower than the subjects without diabetes.

During the follow up, 1163 deaths occurred in men over 91036.7 person-years, and 899 deaths occurred in women over 110123.1 person-years. Standardized mortality rates by the diabetes status and the HRs of mortality among men are shown in Table 2. The risks of mortality from all causes and from coronary heart disease (CHD) were significantly higher among diabetic men than among non-diabetic men. Further analysis of the disease-specific mortality by using multivariate adjustment showed an increased risk of mortality from liver cancer (HR: 4.30, 95% CI: 1.98-9.38) among diabetic men as compared with that among non-diabetic men.

The standardized mortality rates by the status of diabetes and the HRs of mortality among women are shown in Table 3. The risks of mortality from all causes, cancer, and causes other than cancer and CVD were significantly higher among diabetic women than among non-diabetic women. The risks of mortality from CHD and stroke, and the total CVD risk did not differ between diabetic and non-diabetic women. Further analysis of the mortality from several diseases by using multivariate adjustment revealed a higher risk of mortality from colorectal cancer (HR: 4.30, 95% CI: 1.78-10.41) among diabetic women than among non-diabetic women. We repeated the analysis after excluding women who reported the current use of hormone replacement therapy, and the results were essentially the same.

DISCUSSION

The current study suggests that self-reported diabetes

Table 1. Baseline characteristics of 13355 men and 15724 women by diabetes status in Takayama, Japan, 1992-1999

	Men					Women				
	Without Diabetes n = 12561		With Diabetes n = 794		Two-sided *P* value†	Without Diabetes n = 15301		With Diabetes n = 423		Two-sided *P* value†
	Mean (± standard deviation)					Mean (± standard deviation)				
Age (y)	53.7	(12.1)	58.5	(11.0)	<0.01	54.9	(13.0)	63.1	(11.8)	<0.01
Body mass index (kg/m^2)	22.5	(2.8)	23.0	(2.8)	<0.01	22.0	(2.9)	22.4	(3.3)	<0.01
Height (cm)	164.8	(6.8)	163.7	(6.8)	<0.01	152.1	(6.4)	150.1	(6.1)	<0.01
Physical activity (MET/week)	27.3	(41.7)	23.4	(38.3)	0.01	18.9	(29.8)	14.4	(23.1)	<0.01
Total energy intake (kcal/d)	2610	(868)	2504	(872)	<0.01	2115	(778)	1877	(708)	<0.01
Total vegetable intake (g/d)	370.0	(258.6)	408.1	(287.3)	<0.01	393.4	(264.2)	431.9	(288.7)	0.01
Total fat intake (g/d)	61.2	(28.6)	60.7	(27.8)	0.64	55.5	(26.6)	49.3	(25.0)	<0.01
Total alcohol intake (g/d)	42.1	(41.4)	39.5	(43.2)	<0.01	7.8	(16.9)	4.3	(11.8)	<0.01
	No. (%)					No. (%)				
Currently married	11380	(90.6)	713	(89.8)	0.46	11380	(74.4)	251	(59.3)	<0.01
Education 12 years or more	5358	(42.7)	299	(37.7)	0.01	5151	(33.7)	85	(20.1)	<0.01
Cigarette smoking status*										
Never smoker	2051	(16.3)	127	(16.0)	<0.01	11328	(74.0)	296	(70.0)	0.04
Current smoker	6757	(53.8)	375	(47.2)		1800	(11.8)	52	(12.3)	
Former smoker	3394	(27.0)	265	(33.4)		600	(3.9)	14	(3.3)	
History of hypertension	2279	(18.1)	246	(31.0)	<0.01	2605	(17.0)	124	(29.3)	<0.01
Aspirin use within the past 6 months	508	(4.0)	39	(4.9)	0.23	1,043	(6.8)	19	(4.5)	0.06
Post-menopausal women						8680	(56.7)	351	(83.0)	<0.01
Current hormone replacement therapy use						239	(1.6)	10	(2.4)	0.19

* Do not add up to 100% because of missing data
† *t* test for continuous variables and chi-square test for categorical variables