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Elevated Levels of VE-Cadherin–Positive Endothelial Microparticles in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease

Hidenobu Koga, MD,* Seigo Sugiyama, MD, PHD,* Kiyotaka Kugiyama, MD, PHD,† Keisuke Watanabe, MD,* Hironobu Fukushima, MD,* Tomoko Tanaka, MD,* Tomohiro Sakamoto, MD, PHD,* Michihiro Yoshimura, MD, PHD,* Hideaki Jinnouchi, MD, PHD,‡ Hisao Ogawa, MD, PHD*

Kumamoto and Yamanashi, Japan

OBJECTIVES The purpose of this study was to examine whether CD144-EMP (endothelium-derived microparticles) is useful as a specific marker of endothelial cell (EC) dysfunction and to determine whether plasma levels of circulating CD144-EMP predicted coronary artery disease (CAD) in patients with type 2 diabetes mellitus (DM).

BACKGROUND Endothelial cell dysfunction is involved in atherogenesis; however, the quantitative assessment of EC dysfunction has yet to be established clinically. Endothelium-derived microparticles are small, membrane-shed vesicles that are generated from the EC surface in response to cellular dysfunction and/or injury. Diabetes mellitus is known to be associated with EC dysfunction and accelerated atherosclerosis.

METHODS We characterized EMP using anti-CD144 (VE-Cadherin) antibody in various atherosclerosis-related cells and investigated the association between the levels of CD144-positive microparticles and hydrogen-peroxide–induced EC injury and acetylcholine-induced coronary vasomotion. Furthermore, we evaluated plasma CD144-EMP levels in patients with and without DM.

RESULTS We demonstrated that CD144-positive microparticles were derived selectively from human EC. The levels of CD144-EMP reflected the degree of in vitro hydrogen-peroxide–induced EC injury and impairment of in vivo endothelium-dependent coronary vasodilation ($p < 0.01$). Plasma CD144-EMP levels were increased significantly in DM patients compared with patients without DM ($p < 0.001$). In DM patients, the elevated levels of CD144-EMP were the most significant risk factor for CAD relative to all other traditional risk factors (odds ratio [OR] 3.5, 95% confidence interval [CI] 1.8 to 6.9, $p < 0.001$). Notably, plasma CD144-EMP identified a subpopulation of established CAD patients in DM subjects without typical anginal symptoms (OR 10.6, 95% CI 3.9 to 29.5, $p < 0.001$).

CONCLUSIONS The CD144-positive EMP exist in human plasma, and plasma CD144-EMP levels can be a clinically specific and quantitative marker of EC dysfunction and/or injury. Measurement of CD144-EMP, by providing a quantitative assessment of EC dysfunction, may be useful for identifying DM patients with increased risk of CAD. (J Am Coll Cardiol 2005;45: 1622–30) © 2005 by the American College of Cardiology Foundation

Endothelial cells (EC) play an important role in the maintenance of vascular integrity and homeostasis, with EC dysfunction having been implicated in the pathogenesis of both atherosclerosis and plaque instability (1–4). Recent studies have demonstrated that endothelial dysfunction is also a predictor of future coronary events and coronary artery disease (CAD) (3,5,6). Endothelial cell dysfunction may be detected clinically by measuring impairment of endothelium-dependent vasodilation (7,8) or by determining the plasma levels of circulating soluble markers (9,10). These methods, however, are either relatively nonspecific, complicated, or operator-dependent (11). A therapeutic strategy based on the concept of vascular protection would be valuable for preventing cardiovascular events and, accordingly, there is a need to establish a practical and quantitative marker of EC dysfunction and/or injury. In this regard, several recent studies have shown that the EC-plasma membrane sheds small vesicles, termed endothelium-derived microparticles (EMP), in response to either cellular injury/dysfunction or apoptosis. This raises the possibility that the levels of EMP in the plasma may be a clinical surrogate marker of in vivo EC dysfunction and/or injury and CAD (12–17).

Currently the number of patients with type 2 diabetes mellitus (DM) is increasing in the population. Diabetes mellitus is an established risk factor for accelerated atherosclerosis, with EC dysfunction thought to be a key factor involved in the pathogenesis of athero-thrombogenic dia-

From the *Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; †2nd Department of Internal Medicine, Interdisciplinary Graduate School of Medicine and Engineering University of Yamanashi, Yamanashi, Japan; and the ‡Department of Jinnouchi Diabetes Center, Kumamoto, Japan. This study was supported in part by grants-in-aid for C(2)-14570679 from the Ministry of Education, Science, and Culture, Tokyo; 14C-4 from the Ministry of Health, Labour, and Welfare, Tokyo; Smoking Research Foundation, Tokyo; Mochida Memorial Foundation for Medical and Pharmaceutical Research, Tokyo; the Suzuken Memorial Foundation, and Japan Heart Foundation/Pfizer, Japan Inc., Grant for Research on Cardiovascular Disease, Tokyo.

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Abbreviations and Acronyms

ACh = acetylcholine
CAD = coronary artery disease
CI = confidence interval
DM = diabetes mellitus
EC = endothelial cells
EMP = endothelium-derived microparticles
FCS = fetal calf serum
FITC = fluorescein isothiocyanate
HAoSMC = human aortic smooth muscle cells
HCAEC = human coronary artery endothelial cells
HDL = high-density lipoprotein
hs-CRP = high-sensitivity C-reactive protein
LAD = left anterior descending coronary artery
LDL = low-density lipoprotein
LMT = left main trunk coronary artery
OR = odds ratio
PBS = phosphate-buffered saline
sICAM-1 = soluble intercellular adhesion molecule-1

betic complications. Acute coronary syndromes resulting from plaque rupture and erosion are commonly found in patients with DM (18–20), and even after intensive medical management, myocardial infarction and stroke frequently occur in these patients (20,21). Furthermore, silent myocardial ischemia is a common feature in DM patients, and it is a clinically important to detect CAD among DM patients with no symptoms or signs suggestive of CAD (22). In the present study, we investigated whether CD144-EMPs were useful as a specific marker of EC dysfunction and determined whether plasma levels of circulating CD144-EMPs predicted CAD in patients with type-2 DM.

METHODS

Cell culture and blood cell preparations. Human coronary artery endothelial cells (HCAEC) (Lot:3F0175, Cambrex Bio Science Walkersville Inc., Walkersville, Maryland) were cultured in medium-199 containing 10% fetal calf serum (FCS), heparin, and endothelial cell growth supplement (BD Bioscience, San Jose, California), while human aortic smooth muscle cells (HAoSMC) (Cambrex Bio Science Walkersville Inc.) were cultured in Dulbecco's modified Eagle medium-containing 20% FCS and human monocyte-derived macrophages in RPMI-1640 containing 20% heat-inactivated human serum. Peripheral blood mononuclear cells were prepared by density gradient centrifugation (d = 1.077 g/ml) and then plated onto a culture dish in order to separate adherent monocytes. Platelet-rich plasma was separated from citrated peripheral blood by centrifugation at 160 g for 10 min. The platelet-rich plasma was then centrifuged at 15,000 g for 30 min and the platelet-pellet was re-suspended in phosphate-buffered saline (PBS).

Fluorescence microscopy and flow cytometric analysis of CD-144 in EC. Human coronary artery endothelial cells were plated onto a fibronectin-coated glass dish and incubated with bis-benzimide and a fluorescein isothiocyanate (FITC)-conjugated antibody against CD144 (VE-cadherin) (Serotec Ltd., Oxford, United Kingdom). After washing the culture with PBS, we measured expression of CD144 in the cells using fluorescence microscopy. For the flow cytometric analyses, the cultured HCAEC were detached with cold PBS containing ethylenediaminetetraacetic acid and re-suspended in PBS containing 1% FCS and 0.1% sodium azide. The cells were then incubated for 30 min with CD144-FITC or control-IgG-FITC, followed by washing and analysis of CD144 expression by FACScalibur (Becton Dickinson, Franklin Lakes, New Jersey).

Characteristics of atherosclerosis-related cell-derived microparticles. In order to generate atherosclerosis-related cell-derived microparticles, each cell preparation was stimulated with a lethal concentration of hydrogen peroxide (H_2O_2) (15) followed by a further 2 h incubation. The culture medium containing microparticles was then collected and centrifuged at 5,000 g for 10 min to remove crude cell debris and nuclei, with the supernatant then being ultracentrifuged for a further 2 h at 100,000 g (16,23). The pellets of microparticles were then re-suspended in PBS before labeling with either CD144-FITC, CD31-FITC (BD Pharmingen, San Diego, California), or control-IgG-FITC. Furthermore, the isolated human platelet-derived microparticles were incubated with CD31-FITC and CD42b-phycoerythrin (BD Pharmingen) for 30 min. The preparations were analyzed by FACScalibur with CellQuest software (Becton Dickinson), with microparticles being defined as elements with a size <1.5 μm relative to standard beads (4.2 μm).

Assay of endothelial cell death induced by H_2O_2. Human coronary artery endothelial cells were cultured on six-well plates until confluent followed by exposure for 2 h to various concentrations of H_2O_2. The culture medium was collected for analysis of EMP levels using FACScalibur. The remaining adherent HCAECs were then washed with medium-199, and cell viability was measured by the MTS Assay Kit (Promega, Madison, Wisconsin), with the percentage of dead cells calculated as described previously (4).

Clinical study population. The study incorporated enrollment of 232 consecutive Japanese patients with DM who had coronary artery angiography at Kumamoto University Hospital. Fifteen patients with acute phase and unstable conditions including severe valvular disease (n = 3), active infection (n = 1), untreated malignant diseases (n = 2), active autoimmune disease (n = 4), and severe congestive heart failure (n = 5) were excluded. Coronary artery disease was defined as angiographic evidence of stenosis in more than 75% of the major coronary arteries. The study also included 102 nondiabetic patients without angiographic evidence of CAD (<10% stenosis in coronary arteries) or carotid plaques determined by ultrasound (maximum intima-media thickness in the common carotid artery <1.1 mm) as a control group. Informed consent was obtained from all patients before the study, which was carried out in

accordance with the guidelines approved by the ethics committee at our institution.

Measurement of circulating plasma levels of CD144-EMP and sICAM-1. Blood samples were drawn by venipuncture into blue-top vacutainer tubes containing sodium citrate after a 12-h overnight fast, before any mechanical intervention. Blood samples were assayed immediately after venipuncture. Platelet-rich plasma was separated from whole blood by centrifugation at 160 g for 10 min. The PRP was then centrifuged at 6,000 g for 1 min to prepare platelet-poor plasma. For the EMP assay, 50 μl platelet-poor plasma in TruCount tubes (Becton Dickinson) was incubated with CD144-FITC and CD42b-phycoerythrin for 30 min. Then 1 ml of PBS buffer was added and the sample was ready for flow cytometry. Endothelium-derived microparticles were defined as elements with CD144-positivity and CD42b-negativity with a size <1.5 μm (12,13). We calculated the absolute number of EMPs as described previously (24). We also measured plasma levels of soluble intercellular adhesion molecule-1 (sICAM-1), related to endothelial dysfunction and CAD (9,11), by sICAM-1 ELISA (R & D Systems Inc., Minneapolis, Minnesota).

Quantitative coronary angiography and measurement of coronary blood flow. Quantitative coronary angiography and measurement of coronary blood flow were performed in order to assess EC dysfunction as described previously (7,25,26). Quantitative coronary angiography was performed in 31 patients without CAD in the same manner, as described (25). After baseline angiography, graded concentrations of acetylcholine (ACh: 10, 50 μg/min) were infused directly into the left main trunk coronary artery (LMT) through a Judkins catheter for 2 min. The trunk of the left anterior descending coronary artery (LAD) was divided into proximal, mid, and distal segments of equal length. The lumen diameter at the center of each segment was measured quantitatively with the use of a computer-assisted coronary angiographic analysis system (Cardio 500, Kontron Instruments, München, Germany) by two observers blinded to the study protocol. Coronary artery diameter responses to ACh (10, 50 μg/min at LMT) were expressed as percentage change from baseline coronary artery diameters.

Coronary blood flow velocity was measured in 24 of 31 patients by using 0.014-inch wire, equipped with a Doppler crystal at its tip (Flow Wire, Cardiometrics, Mountain View, California), that was advanced through the Judkins catheter and carefully positioned in the straight proximal segment of the LAD as described previously (25). The stable peak flow velocity signals at baseline and during a 2-min infusion of ACh (10 μg/min at LMT) were used for analysis (Flow Map, Cardiometrics). Coronary blood flow was calculated from blood velocity and arterial diameter. The response of coronary blood flow to intracoronary infusion of ACh (10 μg/min at LMT) was expressed as a percentage change from the value of the baseline blood flow just before ACh infusion. After an additional 10 min, intracoronary infusion of isosorbide dinitrate (1 mg) was given. Hemodynamic measurements and coronary angiography were repeated before and at each of the infusions.

Statistical analysis. The statistical analyses were performed with Stat View-V software (SAS Institute, New York, New York). Results were expressed as mean ± SE. High-sensitivity C-reactive protein (hs-CRP), triglycerides, fasting plasma glucose, hemoglobin A_1c, and CD144-EMP levels were expressed as medians and interquartile range. Comparisons of plasma EMP levels in control, DM without CAD, and DM with CAD were determined by one-way analysis of variance. The frequencies for gender, smoking, and hypertension were compared between the two groups by using chi-square analysis. Comparisons between two groups were carried out using the unpaired t test (normal distributed variables: age, body mass index, total cholesterol, LDL cholesterol, HDL cholesterol, and sICAM-1) and the nonparametric Mann-Whitney U test (triglycerides, hs-CRP, fasting plasma glucose, hemoglobin A_1c, and CD144-EMP). Univariate and multivariate logistic regression analyses were performed to determine the relationship between EMP and other factors, using the following factors as categorical covariates defined according to the cutoff points of the American Heart Association (27): age >75years, hypertension systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg or taking an antihypertensive drug, high body mass index >25 kg/m^2, high total cholesterol >240 mg/dl, high LDL >160 mg/dl, hypertriglyceridemia >150 mg/dl, low HDL <40 mg/dl, high fasting plasma glucose ≥126 mg/dl, high hemoglobin A1c (HbA1c) >7.0%, or increased hs-CRP >3.0mg/l. The EMP data were logarithmically transformed (log EMP) to obtain a normal distribution and were then analyzed using linear regression analysis. A p value <0.05 was considered statistically significant.

RESULTS

CD144-positive microparticles are identified as specific microparticles derived from EC. Figure 1A shows intense CD144 (VE-cadherin) immunostaining in HCAEC localized at the cell-cell marginal contacts. No CD144 staining was observed in HAoSMC, monocyte-derived macrophages, lymphocytes, or platelets (data not shown). The FACS analysis demonstrated that HCAEC expressed CD144 with a mean fluorescence intensity of 37.2 ± 4.6 (Fig. 1B). Figure 1C shows that HCAEC-derived microparticles expressed CD144, whereas microparticles derived from HAoSMC, monocyte-derived macrophages, lymphocytes, or platelets did not express this marker (Figs. 1D to 1H). Microparticles from endothelial cells and platelets were positive for CD31 (Figs. 1I and 1J) and CD31 positive-CD42b negative microparticles were generated from isolated human platelets (Fig. 1K). These results demonstrated that only CD144 was a specific marker of EMP.

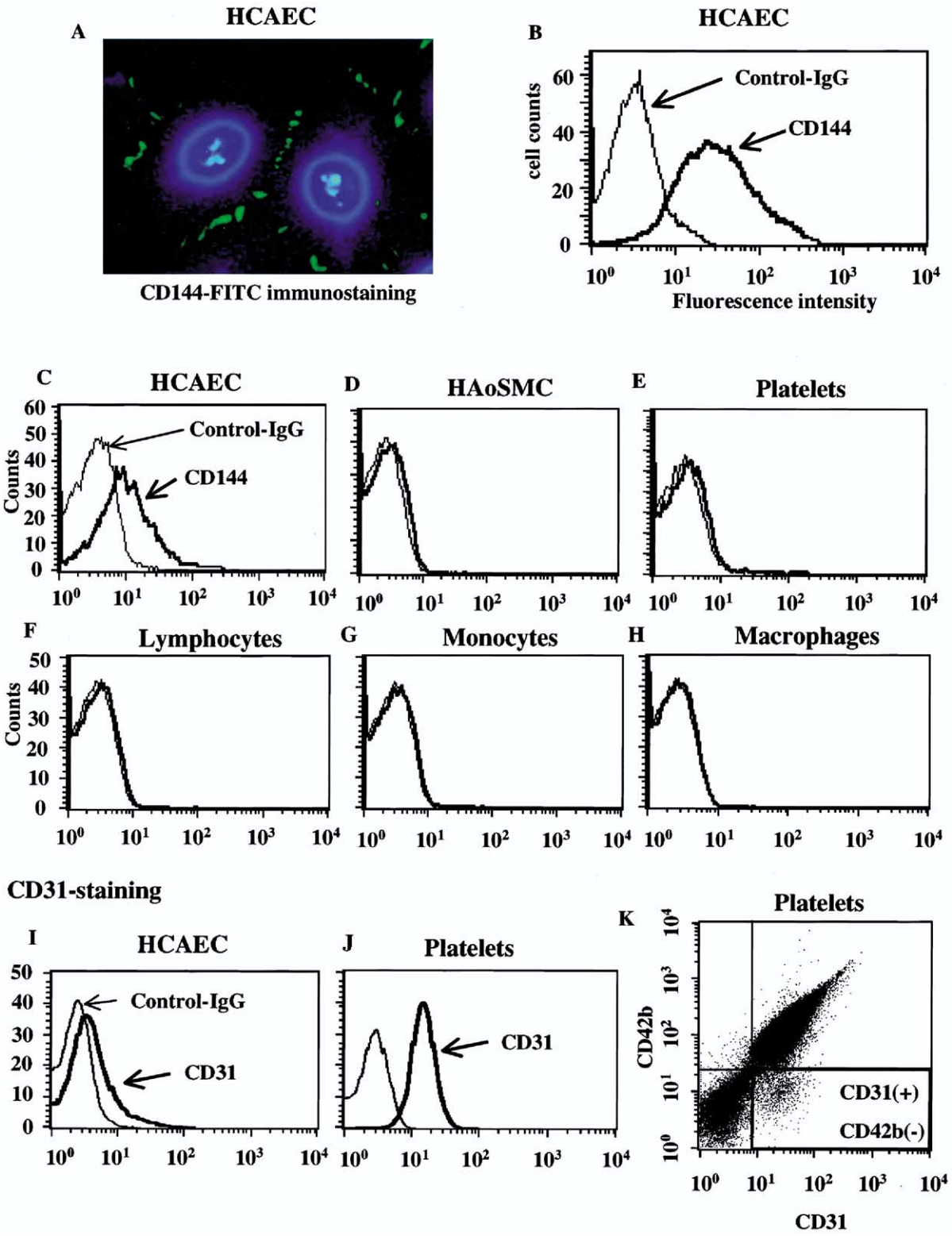

Figure 1. (A) An immunofluorescent photomicrograph showing CD144-expression in human coronary artery endothelial cells (HCAEC). The **green signal** indicates fluorescein isothiocyanate (FITC)-CD144 while the **blue signal** indicates the nucleus (n = 4). **(B)** A histogram analysis of CD144-expression in HCAEC detected by FACScalibur. The **thin line** represents control immunoglobulin G (IgG) and the **thick line** represents CD144 (n = 5). **(C to H)** Histogram analyses of CD144-expression in microparticles derived from a variety of cells involved in atherosclerosis detected using FACScalibur. **(C)** HCAEC; **(D)** HAoSMC (human aorta smooth muscle cell); **(E)** platelets; **(F)** lymphocytes; **(G)** monocytes; **(H)** macrophages. The **thin lines** represent control IgG and the **thick lines** represent CD144 (n = 5). **(I to J)** Histogram analyses of CD31-expression in microparticles derived from HCAEC and platelets. **(K)** Representative dot-plot graphs of microparticles expressing CD31-CD42b derived from human platelets. The **lower right section of graph** shows CD31-positive and CD42b-negative microparticles derived from isolated human platelets.

CD144-EMP levels in culture medium are associated with H_2O_2-induced EC death in vitro. Exogenous H_2O_2 over the concentration range 50 to 800 μmol/l caused injury in HCAEC in a concentration-dependent manner as assessed by MTS assay (Fig. 2A). This concentration range of H_2O_2 also simultaneously increased EMP levels in the culture medium in a concentration-dependent manner (Fig. 2A). The linear regression analysis demonstrated a significant positive correlation between EC injury evaluated by the MTS assay and EMP levels in cultured HCAEC ($r = 0.79$, $p = 0.002$; Fig. 2B).

Plasma CD144-EMP are associated with endothelial dysfunction in human coronary arteries in vivo. Microparticles in human plasma were defined as elements with a size <1.5 μm relative to standard beads (Fig. 3A). A histogram analysis of the distribution of EMPs expressing CD144 in human plasma is shown in Figure 3B. Figures 3C and 3D are representative dot-plot graphs of EMP expressing CD144-CD42b in patients with or without DM. The lower right sections of the graphs indicate EMP in plasma as CD144-positive and CD42b-negative microparticles. We demonstrated that CD144-EMP had a significant correlation with coronary vasomotion of the proximal segment ($r = -0.547$, $p = 0.001$; Fig. 4A) in response to ACh (endothelium-dependent vasodilation) (10 μg/min), whereas sICAM-1 (commonly used soluble plasma marker of EC dysfunction) was not correlated ($r = -0.309$, $p = 0.09$; Fig. 4B). Figure 4C also demonstrates that there is a strong significant inverse correlation between CD144-EMP levels and percentage change of coronary diameter by ACh (50 μg/min) at the proximal segment ($r = -0.763$, $p < 0.001$), whereas sICAM-1 was weakly correlated ($r = -0.407$, $p = 0.02$; Fig. 4D). Furthermore, only CD144-EMP was independently and significantly associated with percentage change of coronary diameter at the proximal segment in response to ACh (50 μg/min) by multiple regression analysis ($r = -0.704$, $p < 0.001$). The plasma CD144-EMP levels also had a significant correlation with coronary vasomotion in response to graded concentrations of ACh (10, 50 μg/min) at the mid and distal segments of the LAD (mid: 10 μg/min, $r = -0.472$, 50 μg/min, $r = -0.661$; distal: 10 μg/min, $r = -0.536$, 50μg/min, $r = -0.507$; $p < 0.01$) as well as proximal segment.

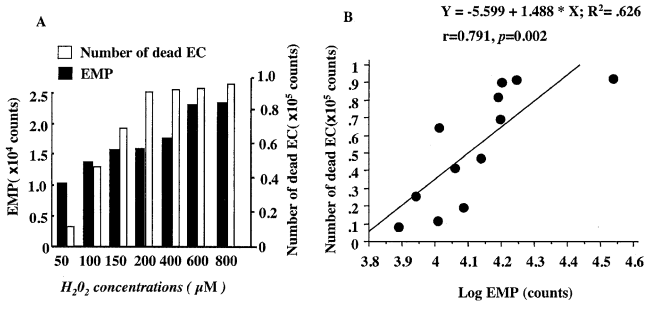

Figure 2. (A) A bar graph showing CD144-EMP (endothelium-derived microparticles) levels in cultured medium of HCAEC and the number of dead HCAEC after exposure for 2 h to various concentrations of H_2O_2. The **solid bars** represent CD144-EMP levels and **open bars** represent the number of dead HCAEC (n = 4) **(B)** A linear-regression graph demonstrating the significant correlation between the number of dead HCAECs and CD144-EMP levels in cultured medium after exposure to various concentrations of H_2O_2 ($r = 0.791$, $p = 0.002$). Abbreviations as in Figure 1.

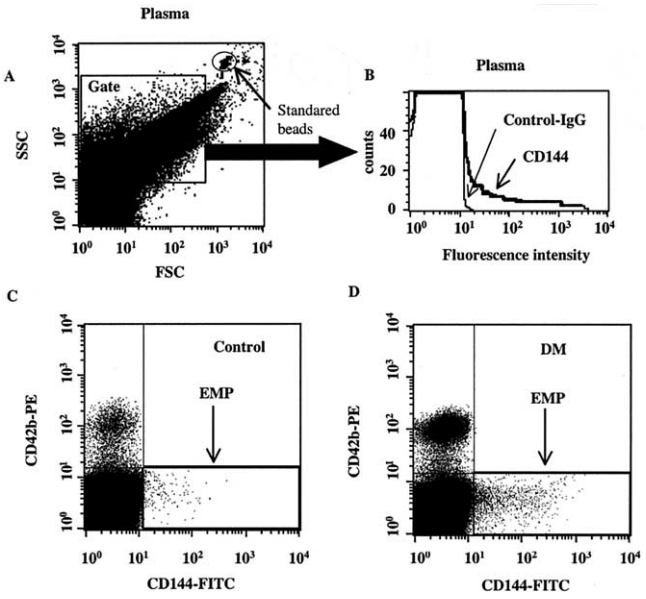

Figure 3. (A) Forward scatter (FSC) and side scatter (SSC) analysis of microparticles in human plasma. The section containing microparticles was gated using a size <1.5 μm in FSC-SSC scatter using standard beads (bead size = 4.2μm). **(B)** A histogram analysis of CD144-expression in the gated microparticles in human plasma. The **thin line** represents control-IgG and the **thick line** represents CD144. (n = 5) **(C to D)** Representative dot-plot graphs of microparticles expressing CD144-CD42b in human plasma in patients with or without DM. The **lower right section of each graph** contains EMP as CD144-positive and CD42b-negative microparticles. DM = diabetes mellitus; other abbreviations as in Figures 1 and 2.

Figures 4E and 4F also demonstrate that plasma levels of CD144-EMP and sICAM-1 have a significant inverse correlation with coronary blood flow in response to ACh (10 μg/min) (CD144-EMP: $r = -0.588$, $p < 0.01$; sICAM-1: $r = -0.457$, $p = 0.02$). Only CD144-EMP was independently and significantly correlated with ACh-induced coronary blood flow by multiple regression analysis ($r = -0.491$, $p = 0.01$). In contrast, levels of CD144-EMP did not have a significant association with endothelium-independent vasodilation by isosorbide dinitrate at the proximal, mid, and distal segments of the LAD (proximal: $r = 0.07$, $p = 0.7$; mid: $r = 0.09$, $p = 0.6$; distal: $r = 0.07$, $p = 0.7$).

Elevated plasma levels of CD144-EMP and sICAM-1 in patients with DM. The baseline clinical characteristics of the patients in the study are summarized in Table 1. The levels of circulating CD144-EMP in patients with DM were significantly greater than in nondiabetic control patients (0.602 [0.476 to 0.783] × 10^6/ml vs. 0.310 [0.204 to 0.405] × 10^6/ml, $p < 0.001$; Fig. 5A). Furthermore, CD144-EMP levels in DM patients with CAD were significantly higher than in those without CAD (0.706 [0.577 to 1.067] × 10^6/ml vs. 0.541 [0.423 to 0.652] × 10^6/ml, $p < 0.001$; Fig. 5B). We also found that circulating

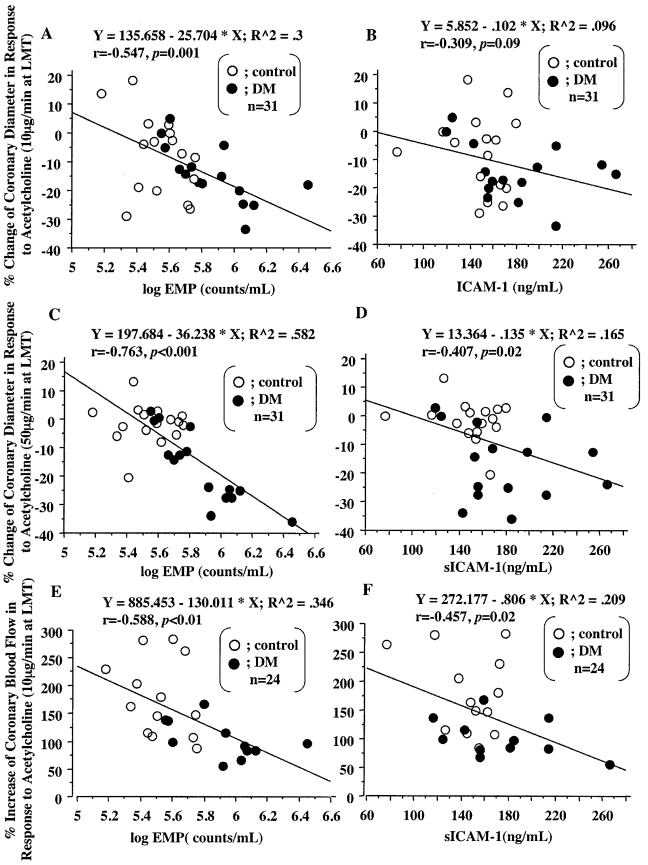

Figure 4. (A to D) Linear regression analysis showing the relationship between plasma levels of CD144-EMP or soluble intercellular adhesion molecule-1 (sICAM-1) and percentage change in coronary artery luminal diameter at the proximal segment of LAD in response to intracoronary acetylcholine (10 μg/min, 50 μg/min at left main trunk coronary artery [LMT]) infusion. **Open circles** are nondiabetic control patients and **filled circles** are patients with DM. **(E to F)** Correlation between plasma levels of CD144-EMP or sICAM-1 and percent increase from baseline values of coronary blood flow in response to intracoronary infusion of acetylcholine (10 μg/min at LMT). **Open circles** are nondiabetic control patients and **filled circles** are patients with DM. Abbreviations as in Figures 1 to 3.

CD144-EMP levels in DM patients without CAD were significantly elevated compared with the control group (0.541 [0.423 to 0.652] $\times$ 10^6 counts/ml vs. 0.310 [0.204 to 0.405] $\times$ 10^6 counts/ml, $p < 0.001$; Fig. 5B). The levels of circulating sICAM-1 in DM patients were elevated significantly compared with the control group (186.6 $\pm$ 3.9 ng/ml vs. 163.6 $\pm$ 5.2 ng/ml, $p = 0.003$), and the plasma levels of sICAM-1 in DM patients with CAD were higher than DM patients without CAD (193.3 $\pm$ 5.9 ng/ml vs. 177.7 $\pm$ 5.1 ng/ml, $p = 0.04$).

Plasma levels of CD144-EMP, but not sICAM-1, predicted presence of CAD in patients with DM. The univariate logistic-regression analyses showed that higher levels of CD144-EMP (>0.78 $\times$ 10^6/ml; 75th percentile of the distribution of EMP in patients with DM), longer duration of DM (>12 years; 75th percentile of the distribution of DM in the present study) (28), high LDL cholesterol, low HDL cholesterol, and hypertension were significant risk factors for the presence of CAD in patients with DM. However, high sICAM-1 levels (>215.0 ng/ml; 75th percentile of the distribution of sICAM-1 in patients with DM) could not detect presence of CAD in patients with DM by the univariate analysis. There was no significant relationship between CAD and other risk factors such as increased levels of fasting glucose and HbA_{1c} (Table 2). The multivariate analysis indicated that an elevated CD144-EMP level was the most significant and independent risk factor for CAD in patients with DM (Table 3).

Notably, the plasma CD144-EMP levels identified a subpopulation of DM patients who had CAD without typical chest symptoms (odds ratio [OR] 10.6, 95% confidence interval [CI] 3.9 to 29.5, $p < 0.001$).

DISCUSSION

This study demonstrated that CD144-positive EMP exists in human plasma and that plasma levels of these microparticles are associated clinically with coronary endothelial dysfunction and/or injury. We also found significantly elevated levels of CD144-EMP in patients with DM compared with nondiabetic control patients. CD144-EMP levels were significantly higher in DM patients with CAD than in those without CAD, with the elevated CD144-EMP levels being found to be a significant independent predictor for the presence of CAD in patients with DM. Of particular interest was our finding that higher CD144-EMP levels were a significant risk factor for the presence of CAD in patients without symptomatic episodes of angina.

Microparticles are small membrane-shed vesicles exhibiting various biological activities that are released from many different cell types into the extracellular space in response to either cellular injury/dysfunction or apoptosis (15,29). Previous reports have defined circulating EMP as particles <1.5 μm in size that reacted positively with several nonspecific EC markers such as CD31 (12,13,17). Recent studies investigating the possible contribution of EMP to human diseases defined these particles arbitrarily as CD31-positive and CD42b-negative microparticles and showed that the circulating CD31(+)-CD42b(−)-EMP was elevated in patients with hypertension and acute coronary syndromes, and the EMP levels correlated significantly with plasma levels of platelet microparticles (12,17). CD31 is expressed abundantly in platelets, and we observed that CD31(+)-CD42b(−) microparticles were also generated from isolated human platelets in vitro (Fig. 1K). We also found CD31(+)-CD42b(−) EMP in plasma contained CD61P-positive microparticles with 30 $\pm$ 5.3% on histogram analysis (n = 5, date not shown). Taken together, these results suggest that the plasma CD31(+)-CD42b(−) EMP detected in these earlier studies may include platelet microparticles and that the levels of these EMP cannot accurately reflect EC dysfunction and/or injury. In our study, we used vascular endothelial cadherin (VE-cadherin; CD144) to identify EMP, as this molecule is expressed specifically by only EC to maintain stability of the EC-

Table 1. Clinical Characteristics of the Subjects

	Control (n = 102)	DM (n = 232)	p Value
Men/women, n	56/46	147/85	0.2
Age, yrs	66.1 ± 1.2	66.6 ± 0.6	0.6
Body mass index, kg/m^2	24.4 ± 0.9	24.0 ± 0.2	0.5
Hypertension, n (%)	45 (44)	144 (62)	<0.01
Current smoking, n (%)	16 (16)	49 (21)	0.3
Total cholesterol, mg/dl	191.3 ± 3.0	201.0 ± 2.6	<0.05
LDL cholesterol, mg/dl	115.5 ± 2.4	122.2 ± 2.2	0.08
HDL cholesterol, mg/dl	58.0 ± 1.7	53.3 ± 1.0	<0.05
Triglycerides, mg/dl (IQR)	97.0 (72.0–129.0)	117.0 (89.0–161.5)	<0.01
High-sensitivity CRP, mg/l (IQR)	0.50 (0.30–1.00)	1.30 (0.50–3.50)	<0.01
Fasting plasma glucose, mg/dl (IQR)	95.5 (89.0–107.0)	126.0 (106.0–159.8)	<0.001
Duration of diabetes, yrs	—	10.0 ± 0.6	—
Hemoglobin A_{1c}, % (IQR)	5.2 (4.9–5.6)	6.7 (6.1–7.7)	<0.001
Medication therapy			
Sulfonylurea, n (%)	—	93 (40)	—
Alpha-glucosidase inhibitor, n (%)	—	50 (22)	—
Insulin, n (%)	—	48 (21)	—

Values are mean ± SE. High-sensitivity CRP, triglycerides, fasting plasma glucose, and hemoglobin A_{1c} levels were expressed as medians (interquartile range).

CRP = C-reactive protein; DM = diabetes mellitus; HDL = high-density lipoprotein; IQR = interquartile range; LDL = low-density lipoprotein.

adherens junction and also suppresses apoptosis of EC (30,31). Using this approach, we demonstrate that CD144-EMPs are endothelial specific microparticles associated with in vitro EC injury in cultured human vascular endothelial cells and CD144-EMP exists in human plasma.

Endothelial dysfunction is a systemic disorder that plays a critical role in the pathogenesis of atherosclerosis and its complications (4,6). In the past, EC function has been assessed by measuring either endothelium-dependent vasodilation or plasma levels of soluble markers. Although these two methods are useful for evaluating the condition of the vasculature, with abnormal results initiating treatment to prevent atherosclerosis and cardiovascular complications (7–10), a quantitative, comparable, and specific marker of EC dysfunction has yet to be established. Brachial artery imaging during reactive hyperemia using high-resolution ultrasound is also used extensively as a noninvasive method to determine endothelial dysfunction, although this method suffers from a lack of standardization as a result of considerable operator dependence of several technical aspects (11). Plasma levels of thrombomodulin, adhesion molecules, and Von Willebrand factor are used commonly as soluble markers of endothelial dysfunction, although neither molecule is derived specifically from dysfunctional endothelium (10). We also observed that there was a significant inverse correlation between circulating levels of CD144-EMP in human plasma and endothelial-dependent vasodilation as well as coronary blood flow response to acetylcholine in coronary artery. Taken together, these results indicate that CD144-EMP can be a specific marker for quantifying endothelial dysfunction and/or injury.

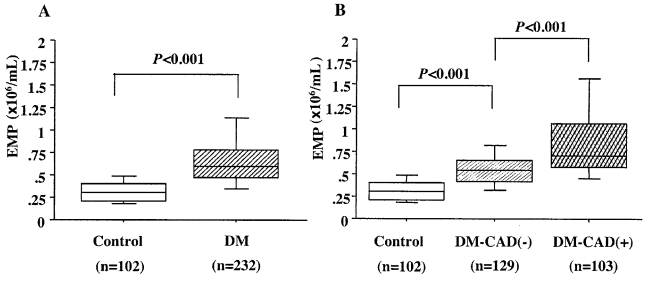

Figure 5. **(A)** Box-and-whisker plot showing plasma CD144-EMP values in patients with and without DM. **(B)** Box-and-whisker plot showing plasma CD144-EMP values in control patients, DM patients with coronary artery disease (CAD), and DM patients without CAD. In these plots, **lines within boxes** represent median values; the **upper and lower lines of the boxes** represent the 25th and 75th percentiles, respectively; and the **upper and lower bars outside the boxes** represent the 90th and 10th percentiles, respectively. Abbreviations as in Figures 1 to 3.

Table 2. Univariate Logistic Regression Analysis of Risk Factors for CAD in Patients With DM

	OR (95% CI)	p Value
Age (>75 yrs)	1.8 (0.9–3.4)	0.07
Current smoking (yes)	1.1 (0.6–2.1)	0.7
Hypertension (yes)	2.0 (1.1–3.4)	**0.01**
Body mass index (high)	0.9 (0.5–1.5)	0.7
Total cholesterol (high)	0.8 (0.4–1.7)	0.6
LDL cholesterol (high)	2.9 (1.3–6.3)	**0.008**
Triglycerides (high)	1.0 (0.6–1.8)	0.9
HDL cholesterol (low)	2.2 (1.1–4.3)	**0.02**
Fasting plasma glucose (high)	0.8 (0.5–1.4)	0.4
Hemoglobin A_{1c} (high)	1.1 (0.7–1.9)	0.6
High-sensitivity CRP (high)	1.8 (0.9–3.6)	0.07
Duration of DM (long >12 yrs)	1.9 (1.1–3.4)	**0.02**
sICAM-1 (high >215.0 ng/ml)	1.4 (0.7–2.5)	0.3
EMP (high >0.78 × 10^6/ml)	4.1 (2.2–7.7)	**<0.001**

Bold indicates significant values.

CAD = coronary artery disease; CI = confidence interval; EMP = endothelial microparticle; OR = odds ratio; sICAM-1 = soluble intercellular adhesion molecule-1; other abbreviations as in Table 1.

Table 3. Multivariate Logistic Regression Analysis of Risk Factors for CAD in Patients With DM

	OR (95% CI)	p Value
EMP ($>0.78 \times 10^6$/ml)	3.5 (1.8–6.9)	**0.0002**
HDL cholesterol (low)	2.4 (1.2–5.1)	0.02
LDL cholesterol (high)	2.3 (1.0–5.5)	0.04
Duration of DM (long >12 yrs)	2.0 (1.1–3.6)	0.02
Hypertension (yes)	1.9 (1.1–3.5)	0.03

Abbreviations as in Tables 1 and 2.

It is well established that endothelial dysfunction is a key feature of type 2 DM and is thought to be a crucial cause of diabetic cardiovascular complications. As myocardial ischemia and myocardial infarction occur frequently in patients with DM without typical chest symptoms (22), it is often difficult to determine clinically whether or not DM patients have CAD (32). In the present study, we found that plasma levels of CD144-EMP were elevated significantly in patients with DM compared with nondiabetic control patients, and that higher levels of CD144-EMP predicted the presence of CAD in patients with DM. Furthermore, plasma CD144-EMP levels appeared to reveal the presence of CAD in DM patients without typical chest pain. On the basis of these findings, we propose that CD144-EMP may be a useful surrogate marker for identifying patients with DM with an increased risk of atherosclerosis. Furthermore, we also found that the plasma levels of CD144-EMP in 120 CAD patients without DM (0.645 [0.534 to 0.817] $\times$ 10^6/ml) were elevated significantly compared with control groups and DM patients without CAD (Koga et al., unpublished observation, November 2004). As a next step, we need to investigate the pathophysiologic roles of EMP in the development of atherosclerosis and to examine in large clinical trials whether CD144-EMP is a prognostic marker for future cardiovascular events.

In conclusion, we demonstrated the presence of CD144-EMP in human plasma, with increased levels being found in patients with DM, especially in those individuals with CAD. Therefore, VE-cadherin positive EMPs may be a useful surrogate marker for evaluating endothelial dysfunction and/or injury. Treatment of atherosclerotic patients that focuses on the integrity and condition of ECs based on assessments of plasma CD144-EMP levels may provide potential therapeutic strategies for preventing cardiovascular complications.

Reprint requests and correspondence: Dr. Seigo Sugiyama, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto City, Kumamoto, Japan 860-8556. E-mail: ssugiyam@kumamoto-u.ac.jp.

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