Increased Plasma Levels of Immunoreactive Endothelin and von Willebrand Factor in NIDDM Patients

TOSHIO MORISE, MD
YOSHIHIRO TAKEUCHI, MD
MITSUHIRO KAWANO, MD
ICHIRO KONI, MD
RYOYU TAKEDA, MD

OBJECTIVES — To elucidate the significance of plasma levels of endothelin (ET) and von Willebrand factor (vWF) as possible markers for endothelial dysfunction in non-insulin-dependent diabetes mellitus (NIDDM).

RESEARCH DESIGN AND METHODS — Plasma levels of ET and vWF were determined in 22 NIDDM patients with or without retinopathy and 10 normal control subjects.

RESULTS — The plasma levels of immunoreactive endothelin (irET) and vWF in NIDDM patients were 0.78 ± 0.06 pmol/l and 218.3 ± 18.4%, respectively, which represented significant ($P < 0.05$, $P < 0.01$, respectively) differences from the values in the control group (0.50 ± 0.06 pmol/l and 139.1 ± 11.1%, respectively, $n = 10$). However, when the diabetic patients were divided into two groups according to the presence or absence of diabetic retinopathy, the plasma levels of irET and vWF in the NIDDM patients with retinopathy were significantly higher (1.01 ± 0.07 pmol/l and 283.0 ± 21.4%, respectively, $n = 12$) compared with the control group and NIDDM patients without retinopathy (0.59 ± 0.06 pmol/l and 164.3 ± 17.0%, respectively). Plasma levels of irET showed a significant ($P < 0.01$) positive correlation with the levels of vWF.

CONCLUSIONS — These data strongly suggest that increased plasma irET reflects the endothelial cell damage in NIDDM.

Angiopathy is the major complication in diabetes mellitus, and endothelial cell damage is suspected to be an important cause of angiopathy. Endothelin (ET)-1 was originally isolated and structurally characterized from the medium of porcine endothelial cells and shows a potent, long-lasting vasoconstriction (1) and stimulation of the proliferation of vascular smooth muscle cells (2). Regarding the relation between angiopathy and plasma ET levels, Takahashi et al. (3) reported increased plasma immunoreactive endothelin (irET) levels in non-insulin-dependent diabetes mellitus (NIDDM) patients, although the mechanism for the increase in irET is still unclear. One possible explanation is that the diabetic endothelial cell damage may be responsible for the increase in plasma irET levels. To investigate this hypothesis in patients with diabetes mellitus, we studied the relation between plasma irET levels and endothelial dysfunction by using von Willebrand factor (vWF) as a possible marker of endothelial cell damage.

RESEARCH DESIGN AND METHODS

Plasma irET and vWF concentrations were studied in 22 patients with NIDDM and 10 normal subjects after informed consent was obtained. Subjects were chosen from the diabetic patients of Kanazawa University Hospital who did not have hypertension (blood pressure <140/90 mmHg), evident renal dysfunction (serum creatinine levels <110 μmol/l and the absence of macroalbuminuria), microalbuminuria (urinary albumin <20 mg/day), or signs of macrovascular disease randomly except for their age, which we tried to match with that of normal control subjects. The diabetic patients were divided into two groups according to the presence or absence of diabetic retinopathy (simple retinopathy without proliferative change), which was assessed by ophthalmoscopic examination by a diabetic ophthalmologist. Each subject fasted overnight and then rested in the supine position for at least 30 min before blood was taken from an antecubital vein. Blood for irET determination was drawn into a chilled siliconized glass tube containing EDTA-2Na and centrifuged at 4°C to separate plasma, which was stored at −70°C until

From the Second Department of Internal Medicine, School of Medicine, Kanazawa University, Kanazawa City, Japan,

Address correspondence and reprint requests to Toshio Morise, MD, Second Department of Internal Medicine, School of Medicine, Kanazawa University, Takara-machi 13–1, Kanazawa City, 920 Japan.

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ET, endothelin; irET, immunoreactive endothelin; NIDDM, non-insulin-dependent diabetes mellitus; vWF, von Willebrand factor.

Table 1—*Clinical characteristics of diabetic patients and control subjects*

Subjects	*n* (M/F)	Age (years)	Duration of diabetes (years)	HbA_{1c} (%)	Average of 1 year HBA_{1c}	Plasma glucose (mmol/l)
Control	5/5	42 ± 3	—			
Diabetic						
Without retinopathy	6/6	43 ± 4	8.5 ± 3.2	7.3 ± 1.2	7.5 ± 2.2	12.2 ± 3.3
With retinopathy	5/5	44 ± 4	9.2 ± 4.4	7.0 ± 0.7	7.2 ± 1.8	11.9 ± 4.2

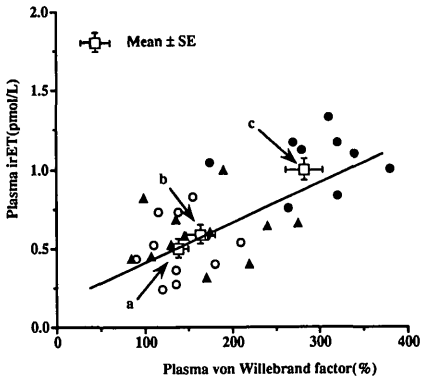

Figure 1—*Correlation between plasma levels of irET and vWF in NIDDM patients. Plasma levels were significantly higher in NIDDM patients with retinopathy (●; c) than those without retinopathy (▲; b) and control subjects (○; a). Plasma irET and vWF levels in control subjects were significantly higher (P < 0.05) than those in diabetic patients with or without retinopathy. y-axis = 0.03 + 0.0024 x-axis, r = 0.66, P < 0.01, n = 32.*

extraction. Plasma irET was determined by enzyme immunoassay after extraction with a Sep-Pak C_{18} cartridge column (Millipore, Bedford, MA) as previously reported (4). In brief, 4 ml acidified plasma was extracted with Sep-Pak C_{18}, and ET was eluted with 80% methyl alcohol. The eluent was evaporated to dryness and dissolved with assay buffer (0.02 mol/l phosphate buffer, pH 7.0, containing 10% Block Ace, 0.4 mol/l NaCl, and 2 mmol/l EDTA. The detection limit of plasma irET was 0.2 pmol/l. The interassay and intraassay variations were 11.5 and 8.5%, respectively. The cross-reactivity of this assay with ET families was as follows: ET-1 (1–21), 100%; ET-2, 160%; ET-3 and human big ET-1 (1–39), <0.5%. Plasma vWF was evaluated by an enzyme-linked immunosorbent assay method reported by Matucci-Cerinic et al. (12). In brief, microtiter plates containing 100 μl/well of a 1:500 dilution of goat antiserum to human vWF in a coating buffer of NaHCO and NaCO (pH 9.6) were incubated overnight at room temperature. A standard curve for vWF measurements was made by using plasma pooled from six healthy volunteers and diluted from 1:40 to 1:5,120. Samples were diluted in phosphate-buffered saline with 1% bovine serum albumin. A 1:500 dilution of monoclonal antiserum to vWF was added for 30 min, and a 1:1,000 dilution of peroxidase conjugated anti-mouse IgG was added, followed by an addition of substrate (CosmoBio, Tokyo). The mean optical densities of unknowns were matched to corresponding dilutions to standards and multiplied by the dilution factor. The results were expressed as percent increase or decrease with respect to control values.

Values are means ± SE. Statistical analysis was performed using the unpaired Student's *t* test or one-way analysis of variance followed by Tukey's method for the multiple comparison of means, with $P < 0.05$ as the level of significance.

RESULTS— Clinical details of the diabetic patients and normal control subjects are summarized in Table 1. There were no significant differences among the three groups. The plasma levels of irET and vWF in the NIDDM patients were 0.78 ± 0.06 pmol/l and 218.3 ± 18.4% ($n = 22$), respectively, which represented significant ($P < 0.05$, $P < 0.01$, respectively) differences from the values in the control group (0.50 ± 0.06 pmol/l and 139.1 ± 11.1%, respectively, $n = 10$). However, when the diabetic patients were divided into two groups according to the presence or absence of diabetic retinopathy, the plasma levels of irET and vWF in NIDDM patients with retinopathy were significantly higher (1.01 ± 0.07 pmol/l and 283.0 ± 21.4%, respectively, $n = 12$) compared with the control group and NIDDM patients without retinopathy (0.59 ± 0.06 pmol/l and 164.3 ± 17.0%, respectively, $n = 10$) (Fig. 1). Between the NIDDM patients without retinopathy and control subjects, there was no significant difference in the plasma levels of irET or vWF. Plasma levels of irET showed a significant ($P < 0.01$) positive correlation with the levels of vWF (Fig. 1).

CONCLUSIONS— This study demonstrated that there is an overall increase in plasma irET in NIDDM, particularly in patients with retinopathy. After the first report by Takahashi et al. (3), increased plasma irET levels have been reported in NIDDM (5,7), in insulin-dependent diabetes mellitus (6), and in both (3). In these reports, the mechanism for the increase in irET was thought to be a reflection of the endothelial cell damage like that in patients with atherosclerosis (7), although no definite evidence was found. The possibility that plasma irET can be a marker of endothelial dysfunction has been reported in patients with Kawasaki disease (mucocutaneous lymph node syndrome), which is characterized by systemic vasculitis. In Kawasaki disease, plasma irET was markedly increased, and this increase was due to endothelial cell damage caused by the vasculitis (4).

We also suspect that increased plasma irET in diabetic patients, presumably derived from endothelial cells, reflects endothelial cell damage and may in turn be related to the complications of diabetes. To investigate this hypothesis in patients with NIDDM, we studied the re-

lation between the plasma irET levels and endothelial dysfunction. A gold standard for endothelial dysfunction is not available, so we used the plasma concentration of vWF as a measure of endothelial function, because in vitro and in vivo data suggest that vWF, a glycoprotein synthesized by endothelium (8), is released when endothelial cells are damaged (9). In NIDDM patients, Stehouwer et al. (11) reported that vWF was associated with an increased risk of new cardiovascular events and can be a useful marker of diabetic complications. However, in diabetes, no study is available in which both irET and vWF were examined and compared. In this study, there was an overall increase in plasma vWF in NIDDM, particularly in patients with retinopathy, and more interestingly, irET showed a significant positive correlation with plasma levels of vWF. These data strongly suggest that increased plasma irET reflects the endothelial cell damage in NIDDM. It is not clear whether an increase in release from endothelial cells or stimulation of synthesis of ET in endothelial cells may be responsible for increased plasma irET and the evaluation of mRNA and/or content of ET should be necessary to investigate the mechanism.

In NIDDM, there is no agreement as to the relative predictive value of glycemic control, blood pressure, and other factors, such as dyslipoproteinemia, for the development of diabetic complications (10). Although our data represent results in a small group of patients with no follow-up, we strongly suggest that plasma irET should be taken into consideration as a sensitive indicator of endothelial damage in patients with NIDDM-like vWF as reported by Stehouwer et al. (11).

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