Elevation of N-(Carboxymethyl)valine Residue in Hemoglobin of Diabetic Patients

Its role in the development of diabetic nephropathy

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OBJECTIVE — Advanced glycation end products (AGEs) are a risk factor for diabetic complications. We have developed an assay method for N-(carboxymethyl)valine (CMV) of the hemoglobin (CMV-Hb), which is an AGE generated from HbA1c. Herein, we describe the clinical utility of CMV-Hb measurement for the diagnosis of diabetic nephropathy.

RESEARCH DESIGN AND METHODS — BALB/c mice were immunized with carboxymethylated Hb and monoclonal antibody against CMV-Hb. This antibody was characterized by a surface plasmon resonance. We developed a latex immunoassay using the antibody and measured CMV-Hb from erythrocytes in type 2 diabetic patients and healthy control subjects (age 64.6 ± 12.0 vs. 61.1 ± 13.2 years, NS; HbA1c 6.9 ± 1.5 vs. 5.2 ± 0.4%, P < 0.0001).

RESULTS — A monoclonal antibody against CMV-Hb β-chain NH2-terminal and an assay method for measurement for CMV-Hb were both developed in our laboratory. CMV-Hb levels were significantly greater in the diabetic patients than in the control subjects (18.2 ± 6.9 vs. 12.7 ± 6.9 pmol CMV/mg Hb, P < 0.0001). No correlation was found between CMV-Hb and HbA1c, or CMV-Hb and glycated albumin. Levels of CMV-Hb increased as the diabetic nephropathy progressed.

CONCLUSIONS — We established an assay method for CMV-Hb and confirmed the presence of CMV-Hb in circulating erythrocytes. CMV-Hb was more prevalent in diabetic patients than in healthy subjects. Furthermore, it was significantly higher in patients with diabetic nephropathy, suggesting that the presence of CMV-Hb may be a valuable marker for the progression of diabetic nephropathy.

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ADVANCED GLYCAZATION END PRODUCTS

Advanced glycation end products (AGEs) are a risk factor for diabetic complications. AGEs are heterogeneous structures that include carboxymethyl lysine (CML) and pentosidine. AGEs have been reported to occupy specific receptors for AGEs (RAGEs) on cells and thereby activate various genes including NF-κB, with the expression of inflammatory cytokines (11), which may either initiate or propagate diabetic complications.

On the basis of these findings, we decided to develop an assay for N-(carboxymethyl)valine (CMV) of the Hb (CMV-Hb). We successfully raised a specific monoclonal antibody against CMV-Hb that was used to develop an assay. CMV-Hb levels were measured in diabetic patients and healthy subjects, and the clinical significance of the results was discussed.

RESEARCH DESIGN AND METHODS

Preparation of CMV-Hb and CM-Hb

CMV was synthesized by reacting valine with glyoxylic acid according to Kihlberg et al. (12). The product was purified by recrystallization from methanol/chloroform, and identified as CMV by nuclear magnetic resonance and mass spectrometry (data not shown).

Carboxymethylated Hb (CM-Hb) was prepared through a modification of the
CMV-Hb in diabetic patients

Measurement of CMV by amino acid analysis

Erythrocytes were hydrolyzed with carboxymethyl-2-aminobutyric acid as an internal standard in 6 N HCl for 20 h at 110°C. The hydrolysates were applied to a cation-exchange column (AG50W-X8, Bio-Rad) and equilibrated with a 200 mmol/l acetic acid–triethylamine (TEA) buffer (pH 2.8). After washing the column, CMV-containing fractions were obtained by eluting the column. The fractions were dried and amino acid analysis was performed by the phenylisothiocyanate method coupled with high-performance liquid chromatography (HPLC) analysis. Separation was achieved by a linear gradient of 90% buffer A (260 mmol/l sodium acetate, 11% acetic acid, 2% TEA, 5% acetonitrile, pH 4.5) and 10% buffer B (60% acetonitrile) to 50% buffer A and 50% buffer B over a 12-min interval with flow rate of 1 ml/min. CMV concentration was calculated by comparing areas to the standard curve of synthetic CMV. The internal standard carboxymethyl-2-aminobutyric acid was used to correct for percentage of recovery of CMV.

Preparation of monoclonal anti–CMV-Hb antibody

The monoclonal antibody was prepared by a method modified from Galfre and Milstein (14). In brief, BALB/c mice were injected with 0.5 mg CM-Hb in 50% Freund’s complete adjuvant. After 2 weeks, the mice were given a booster of 0.5 mg CM-Hb in 50% Freund’s incomplete adjuvant, followed by four additional booster injections every 2 weeks. The titer of CM-Hb in immunized mouse sera was determined by enzyme-linked immunosorbent assay. The splenic lymphocytes from the mice immunized with CM-Hb were fused to myeloma P3U1 cells in the presence of polyethylene glycol. A cell line positive to β-chain NH₂-terminal CMV peptide (CM-Val-His-Leu-Thr-Pro-Glu-Glu) but negative to noncarboxymethylated peptide (Val-His-Leu-Thr-Pro-Glu-Glu), which corresponds to NH₂-terminal of Hb β-chain, was obtained.

Anti–CMV-Hb antibody present in the ascites fluid of BALB/c mice was collected by precipitation with 40% saturated ammonium sulfate. The antibody was dialyzed against phosphate buffer (pH 8.0) and purified by DEAE-cellulose chromatography.

Biosensor analysis of anti–CMV-Hb antibody

The BIACore (Pharmacia Biosensor, Uppsala, Sweden) uses surface plasmon resonance (SPR) technology to study macromolecular interaction (15). The affinity of monoclonal anti–CMV-Hb antibody was determined by using BIACore. Synthetic peptide, Hb, CM-Hb, human serum albumin (HSA), or CM-HSA were immobilized on a Sensor Chip CM5 at a concentration of 1,000 resonance units (RU) (~1 ng/mm² of protein) using standard procedures. Then, 100 μg/ml anti–CMV antibody was injected and allowed to flow over the sensor chip surface at a flow rate of 30 μl/min. The amount of material bound to the sensor chip surface in any experiment was expressed in RUs (16), whereby 1 RU represents 1 pg of banded protein per square millimeter on the sensor chip surface.

Latex immunoassay

We incubated 1 ml of the purified antibody (0.3 g/l) in 0.05 mol/l glycine-NaOH buffer (pH 8.2) with 1 ml of 1% latex suspension at 37°C for 1 h. We added 2 ml 0.05 mol/l glycine-NaOH buffer (pH 8.2) containing 0.5% bovine serum albumin and incubated the mixture at 37°C for 2 h. The latex particles were pelleted by centrifugation and then resuspended in 3.35 ml 0.1 mol/l Tris-HCl buffer (pH 8.2) containing 0.1 mol/l NaCl and 0.1% NaN₃.

In the CMV-Hb assay, an additional buffer (R1) containing 0.1 mol/l Tris-HCl buffer (pH 8.2), 0.1 mol/l NaCl, and 0.1% NaN₃ was used to dilute R2 and to control the stability of agglutination. In the present CMV-Hb assay, 220 μl of R1 and 5 μl of the sample were mixed and preincubated for 4 min at 37°C in the reaction cells. After the addition of 100 μl of R2, changes in absorbance were measured. The blood sample had a CMV-Hb level of 15 pmol CMV/mg Hb, measured by amino acid analysis, and was used as a standard. The samples were prepared by diluting 10 μl whole blood with 10 ml 0.15% SDS solution. The CMV-Hb concentration of the samples was calculated by fitting the standard curve values to a three-dimensional curve made on the 502X (A&G Corp., Tokyo, Japan) and corrected for Hb concentration. The intra-assay and interassay coefficients of variation were 5.66 and 4.10%, respectively.

Measurement of HbA₁c

HbA₁c was measured by an HPLC cation-exchange chromatography system (HA-8121, Kyoto Daichi, Kyoto, Japan). The intra-assay and interassay coefficients of variations were 0.42 and 0.49%, respectively.

Patients and healthy control subjects

After giving their informed consent, 207 patients with type 2 diabetes and 94 healthy control subjects took part in the study. The subjects’ clinical characteristics are listed in Table 1. Type 2 diabetes was defined according to the diagnostic criteria of the American Diabetic Association (17), and further criteria excluded patients with positive anti–islet cell antibody or anti-GAD antibody. The healthy

| Table 1—Characterization of diabetic patients and healthy control subjects |
|-----------------------------|-----------------------------|-----------------------------|
|                          | Healthy control subjects    | Diabetic patients            |
| Age (years)               | 63.1 ± 13.2                 | 64.6 ± 12.0                  |
| Male/female               | 51/43                       | 98/101                      |
| Duration of treatment (years) | —                          | 10.5 ± 8.5                   |
| Treatment category        | —                           | 68/76/64                    |
| HbA₁c (%)                 | 5.2 ± 0.4                   | 6.9 ± 1.5*                  |
| SBP/diDBP (mmHg)          | 115.7 ± 14.6/73.5 ± 13.6   | 134.5 ± 15.9/74.6 ± 9.1     |
| BMI                        | 22.3 ± 1.3                 | 23.4 ± 2.7                  |
| Total protein (μg/l)       | 73 ± 4                      | 69 ± 4                      |
| Serum creatinine (μmol/l)  | 60.9 ± 9                    | 68 ± 42*                    |
| Total cholesterol (mmol/l) | 4.96 ± 0.75                 | 4.76 ± 0.96                 |
| Blood urea nitrogen (mmol/l)| 5.46 ± 1.39               | 6.46 ± 2.53*                |

Data are n or mean ± SD. SBP, systolic blood pressure; diDBP, diastolic blood pressure. *P < 0.001 for diabetic patients compared with control subjects; †P < 0.05 for diabetic patients compared with control subjects.
control group excluded subjects who regularly took oral drugs, had a history of chronic diabetes or ischemic heart disease, or were apoplectic patients or cancer patients.

The samples were stored at –80°C until the CMV-Hb assay was performed. The other laboratory analyses, such as HbA1c, total cholesterol, triglyceride, and serum creatinine, were done immediately after blood collection.

Statistical analysis
Data are expressed as means ± SD. Student’s t tests were used for the statistical evaluation of significant differences between the two groups. Correlation was assessed by linear regression analysis.

RESULTS

Measurement of CMV by HPLC
The chromatograms of synthetic CMV and CM-2-aminobutyric acid (internal standard) are presented in Fig. 1. A peak of the erythrocyte sample and that of synthetic CMV showed the same behavior in several different HPLC elution modes. We concluded that the detected peak in human erythrocytes was CMV. The CMV content in erythrocytes from healthy subjects was ~13 pmol CMV/mg Hb.

Characterization of monoclonal anti–CMV-Hb antibody
We evaluated the reactivity and specificity of the anti-CMV monoclonal antibody by the SPR. Anti–CMV-Hb strongly bound to the carboxymethylated synthetic peptide fragment of the Hb β-chain NH₂-terminal (Val-His-Leu-Thr-Pro-Glu-Glu) fragment. The response was 29,500 RU, with a rate of disappearance (Kₒ) of 5.8 × 10⁻⁹ mol/l. Anti–CMV-Hb did not react with noncarboxymethylated synthetic peptide of the Hb β-chain NH₂-terminal (Val-His-Leu-Thr-Pro-Glu-Glu) (Fig. 2A).

Anti–CMV-Hb antibody also did not react with either the carboxymethylated lysine containing synthetic peptide fragment (Ala-His-Gly-Lys-CM-Val-Leu-Gly-Ala-Phe-Ser) or the 66Lys containing β-chain peptide fragment (Ala-His-Gly-Lys-Val-Leu-Gly-Ala-Phe-Ser) (Fig. 2B).

As shown in Fig. 2C, we calculated the amount of combined antibody on a sensor chip. The antibody bound strongly to CM-Hb; the amount of bound antibody
was 1,230 pg/ng protein. The affinity constant of the antibody for CM-Hb was estimated to be $1.0 \times 10^{8}$ mol/l. On the other hand, noncarboxymethylated Hb, CM-HSA, and HSA showed less reactivity toward anti–CMV-Hb antibody.

**Correlation between amino acid analysis and latex immunoassay**

To test whether the CMV-Hb can be measured by latex immunoassay, we compared CMV-Hb analysis by immunoassay and amino acid analysis. The correlation between latex immunoassay and amino acid analysis was linear ($y = 1.177x - 0.04$, $r = 0.85$, $P < 0.0001$) (Fig. 3).

**CMV-Hb levels in diabetic patients and control subjects**

CMV-Hb levels ranged from 6.8 to 25.8 pmol CMV/mg Hb (12.7 ± 6.9 pmol CMV/mg Hb) in healthy subjects. The CMV-Hb levels in the diabetic patients were higher than those in healthy subjects and ranged from 6.8 to 78.3 pmol CMV/mg Hb (18.2 ± 6.9 pmol CMV/mg Hb) (Fig. 4).

There was no sex difference in either diabetic patients or healthy subjects, and there was no correlation between age and CMV-Hb level.

The CMV-Hb levels in diabetic patients, excluding instances of nephropathy, failed to show a significant correlation with the HbA$_{1c}$ ($r = 0.12$, $P = 0.097$) and glycated albumin ($r = 0.15$, $P = 0.394$) values, which were the glucose control markers. Moreover, no correlation was seen with sorbitol, a polyl pathway intermediate ($r = 0.11$, $P = 0.39$) (data not shown).

No significant difference in CMV-Hb and HbA$_{1c}$ levels were found among the various treatment categories of the diabetic group.

**The CMV-Hb levels in diabetic nephropathy**

The relationship between diabetic nephropathy and CMV-Hb levels was investigated. The stages of diabetic nephropathy were categorized into the following four subgroups: patients with normoalbuminuria ($n = 110$), patients with microalbuminuria ($n = 47$), patients with macroalbuminuria ($n = 52$), and patients with elevated serum creatinine ($n = 27$) (Fig. 4).

As shown in Fig. 4C, the CMV-Hb levels in the elevated serum creatinine group and macroalbuminuria group were significantly higher than those in the normalalbuminuria group, but the difference between the normalalbuminuria group and macroalbuminuria group was not statistically significant. No other significant differences in HbA$_{1c}$ levels were seen in these four groups. Moreover, there was a significant correlation between CMV-Hb levels and serum creatinine levels in the diabetic group ($r = 0.44$, $P < 0.01$) (data not shown).

**CONCLUSIONS**

Carboxymethylated proteins are considered to be a kind of AGE protein. Carboxymethylation usually occurs on the lysine residue of proteins in vivo. We have confirmed the presence of CMV in human Hb and raised a monoclonal antibody reactive toward CMV-Hb. It was much less reactive to noncarboxymethylated Hb or CM-HSA, showing that the antibody was specific to CMV. For convenience of use in a clinical laboratory, we developed a sensitive latex immunoassay using the monoclonal antibody. This assay method enabled us to assess, through the use of an immunoassay, the level of CMV-Hb in clinical samples rapidly and conveniently.

Recently, Cai and Hurst (18) confirmed carboxymethylation of the valyl residue of human Hb—namely, CMV-Hb—in circulating erythrocytes by gas chromatography. Our results for the CMV-Hb value of healthy subjects were similar to their reported values.

We first found that the CMV-Hb levels in a group of diabetic patients were increased compared with those of healthy subjects (Fig. 4). We showed that the CMV-Hb levels in diabetic patients in-
creased as the diabetic nephropathy progressed. We therefore concluded that CMV-Hb may be a marker for diabetic nephropathy progression. However, we did not observe a correlation between the CMV-Hb levels of diabetic retinopathy or diabetic neuropathy.

HbA1c, an early product of Maillard reactions, is formed by glycation of the NH2-terminal valine of Hb β-chain. It is a clinical marker of blood glucose control (19), but it may proceed through further glycation to form AGEs such as CMV. Thus, it is important to assess both HbA1c and CMV in diabetic patients and to investigate the relationship between these two glycation products.

In our study, CMV-Hb increased during the progression of nephritic complications, with the highest CMV-Hb value being observed in patients with increased creatinine. However, we could not find a significant correlation between HbA1c values and the severity of nephropathy. This result suggests that CMV might be a better marker of diabetic nephropathy.

In conclusion, we confirmed the presence of CMV in the Hb of circulating erythrocytes, with higher levels in diabetic patients compared with healthy control subjects. We found an increase in CMV-Hb in patients having elevated serum creatinine, consistent with the hypothesis that CMV-Hb may be a marker of late diabetic complications. Moreover, since it is now believed that the formation of end products such as CMV-Hb from early glycation products such as HbA1c requires conditions of oxidative stress, the measurement of CMV-Hb may be valuable way for estimating glyco-oxidation.

References


